PINEAL - ADRENAL GLAND INTERACTIONS : IN SEARCH OF AN ANTI-STRESSOGENIC ROLE FOR MELATONIN.

A Thesis

Submitted in Fulfilment of the

Requirements for the Degree of

MASTER OF SCIENCE

of

Rhodes University

by

ELIZABETH JOY VAN WYK

January 1993

And I said to the man who stood at the gate of the year:
"Give me a light
that I may tread safely into the unknown."
And he replied :
"Go out into the darkness
and put your hand into the hand of God.
That shall be to you better than light
and safer than a known way."

[M. Louise Haskins (1875 - 1957)]

TABLE OF CONTENTS

CHAPTER FIVE : EFFECT OF GLUTAMATE AND GABA ON PINEAL GLAND BIOCHEMISTRY

LIST OF ABBREVIATIONS

Å	Angstrom
ACTH	Adrenocorticotropic hormone
ALA	5-Aminolaevulinate
AMP	Adenosine monophosphate
AMPA	D-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
APB	L-2-amino-4-phosphonobutyrate
ATP	Adenosine triphosphate
BC	Before Christ
BH_4	Tetrahydrobiopterin
CCO	Carbohydrate craving obesity
cf	Cited from
CGB	Corticosteroid binding globulin
Ci	Curie
CNS	Central nervous system
CoA	Coenzyme A
cpm	Counts per minute
CRH	Corticotropin-regulatory hormone
CSF	Cerebrospinal fluid
DHA	Dihydroalprenolol
DNA	Deoxyribonucleic acid
dpm	Disintegrations per minute
GABA	Gamma-amino butyric acid
HIAA	Hydroxyindole acetic acid
HIOMT	Hydroxyindole-O-Methyltransferase
HPA	Hypothalamic-pituitary-adrenal axis
HT	5-Hydroxytryptamine (serotonin)
HTOH	5-Hydroxytryptophol
HTP	5-Hydroxytryptophan
MEL	Melatonin
MIAA	5-Methoxyindole acetic acid
MT	Methoxytryptamine
MTOH	Methoxytryptophol
mRNA	Messenger RNA
NAc5HT	N-Acetylserotonin
NAD	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NAS	N-Acetylserotonin
NAT	N-Acetyltransferase
NMDA	N-methyl-D-aspartate
RNA	Ribonucleic acid
SAD	Seasonal affective disorder
SCN	Suprachiasmatic nucleus
SEM	Standard error of the mean
TLC	Thin layer chromatography
USA	United States of America

LIST OF TABLES

LIST OF FIGURES

ACKNOWLEDGMENTS

I wish to thank the following people for their contribution to this thesis:

My supervisor, Dr S. Daya for the opportunity of working on this most interesting project, and for his guidance and assistance.

The Foundation for Research and Development and Rhodes University for the financial assistance which made this study possible.

Mr Edward Williams for his ever-willing and expert assistance with sacrificing the rats.

Mr Jason Eason for his assistance with a number of the studies, and especially for his good humour.

Mr Andy Soper for nursing the scintillation counter which was so central to all of these studies, as well as his help with the computer.

The numerous staff members and colleagues in the Biochemistry department who offered help, advice and when all else failed, moral support.

My friend, Avril Guthrie, for her friendship, encouragement and moral support, as well as her assistance with proof-reading and correcting this thesis.

PWT South Africa Pty Ltd for the use of their printer and photostat facilities. I would also like to thank Mrs Barbara James for her help with typing.

And finally I am especially grateful to my family, my Father, Mother, Sylvia, Steven and Wayne for their interest, moral support and encouragement, not to mention their practical assistance with correcting and producing this thesis.

ABSTRACT

The multiple functions of the pineal gland have been collectively interpreted as constituting a general anti-stressogenic role. The adrenal glands play a central role in maintaining homeostasis. The major neuroendocrine consequence of long-term stress is elevated circulating glucocorticoid levels.

In this study, the effect of chronic, oral hydrocortisone treatment on pineal biochemistry was investigated in male Wistar rats of the albino strain. The results show that seven days of oral hydrocortisone treatment endows the pineal gland with the ability to increase melatonin synthesis in organ culture. The increase is accompanied by a rise in NAT activity, cyclic AMP levels and enhanced specific binding to the pineal ß-adrenergic receptors. It appears that hydrocortisone sensitizes the pineal gland to stimulation by ß-adrenergic agonists, thus rendering the pineal more responsive to ß-adrenergic agonists.

Further studies were directed at demonstrating an anti-stressogenic function for the pineal gland by investigating whether the principal pineal indole, melatonin, could protect against the deleterious effects of elevated, circulating hydrocortisone levels. The results show that chronic, oral hydrocortisone treatment significantly increases liver tryptophan pyrrolase activity. The catabolism of tryptophan by tryptophan pyrrolase is an important determinant of tryptophan availability to the brain, and therefore, brain serotonin levels. The findings show that melatonin inhibits basal and hydrocortisone-stimulated liver tryptophan pyrrolase apoenzyme activity in a dose-dependent manner. This inhibition suggests that melatonin may protect against excessive loss of tryptophan from circulation and against deficiencies in the cerebral serotinergic system which are associated with mood and behavioural disorders.

It was shown that another deleterious effect of chronic hydrocortisone treatment is a significant increase in the number of glutamate receptors in the forebrain of male Wistar rats. The increase in receptor number observed in this study is probably due

to an increase in the synthesis of glutamate receptors and is associated with a marked reduction in the affinity of the glutamate receptors for glutamate. For practical reasons, it was not possible to demonstrate an effect of melatonin on either glutamate receptor number or the affinity of glutamate receptors for glutamate in rat forebrain membranes.

In view of the neurotoxic effect of glutamate in the CNS, the functional significance of recently described glutamate receptors in the pineal gland was investigated. The results show that 10^{-4} M glutamate significantly inhibits the isoprenaline-stimulated synthesis of N-acetylserotonin and melatonin in organ culture when the pineal glands were pre-incubated with glutamate for 4 hours prior to stimulation with isoprenaline, and when glutamate and isoprenaline were administered together *in vitro*. GABA, a glutamate metabolite, could not mimic the decrease in isoprenaline-stimulated melatonin, and it is likely that the observed effects were directly attributed to glutamate. Incubation of the pineal gland with 10^{-4} M glutamate in organ culture did not affect HIOMT activity in pineal homogenates, but significantly elevated both basal and isoprenaline-stimulated NAT activity. It was concluded that glutamate only inhibits melatonin synthesis in intact pineal glands and not in pineal homogenates.

The present study has provided further support for an interaction between the pineal and the adrenal glands. There is an ever increasing likelihood that melatonin is an anti-stressogenic hormone and that the pineal gland may have a protective role to play in the pathology of stress-related diseases.

CHAPTER 1

LITERATURE REVIEW

1.1 INTRODUCTION

After centuries of disregard, the pineal gland has, over the last few decades, finally become appreciated as an important, functional neuroendocrine gland. It has overcome a history clouded by unfounded philosophical ideologies, and survived the demand for its profound cytological and physiological evolutions to be dismissed as phylogenetic regression. Far from being a vestigial remnant with enigmatical function, the pineal gland is presently recognized as being an active, functioning and integral component of the neuroendocrine system.

While the full extent of pineal function has not been clearly defined, its many actions have been collectively interpreted as constituting a general function to regulate and stabilize homeostatic equilibrium in close association with changing environmental conditions. The evolutionary changes within the pineal gland should be considered to reflect adaptations to the increasingly sophisticated integrating systems which have evolved in higher animals, rather than functional regression.

In the following review, an attempt has been made to describe anatomical, cytological and phylogenetic aspects of the pineal gland as well as to describe the unique mechanism by which it is regulated. In view of the central role previously ascribed to the adrenal glands in maintaining homeostatic conditions *in vivo*, the interaction between the pineal and adrenal glands is explored with the aim of substantiating the anti-stressogenic role proposed for

the pineal gland.

1.2 HISTORICAL ASPECTS OF PINEAL RESEARCH

The history of pineal research has traditionally been divided into 3 eras, each reflecting the prevailing philosophies and available technology.

1.2.1 The First Period (circa 300 BC - mid 19th century)

The discovery of the human pineal gland is credited to the famous anatomist, Herophilos, (325 - 280 BC). At a time when the problem of localizing the soul was the principle challenge to philosophers and scientists, many were attracted to the cone-shaped, unpaired organ on account of its location on top of the brain. The brain had already been proposed as the seat of the soul and during the course of the following nearly 20 centuries, a myriad of theories were proposed implicating the pineal as a regulator of the flow of the spirit. The dualistic philosophy concerning mind and matter supported by Rene Descartes (1596 - 1650) lead him to devise theories of pineal function that have been hailed as insightful and ingenious given the predominant philosophies of the time. He conceived brain function to be purely physico-mechanical and the body to be merely a machine. According to Descartes, the pineal gland - the seat of the soul - was responsible for directing the "fine, fierce wind of the spirit" along different pores of the ventricular system to stimulate target muscles. The Renaissance (14th - 16th centuries) revived numerous scientific ideologies, but biological research was slow to respond and even after this period of intellectual revival, ancient views about the spiritual significance of the brain were incorporated into physiological studies. With few technological tools at their disposal,

anatomical and physiological data were limited and primitive.

1.2.2 The Second Period (Mid 19th to mid 20th centuries)

By the 18th and 19th centuries, scientists in general were approaching their subjects more systematically with greater emphasis on personal observation and experimentation. The development of increasingly sophisticated tools and techniques enabled a more methodical and thorough investigation of pineal macro- and micro-anatomical structure in non-vertebrate and vertebrate animals. Comparative cytological studies revealed that the pineal gland of lower vertebrates obviously functions as a photosensory organ and was referred to as "the third eye". The gradual loss of the photosensory apparatus in higher vertebrates was interpreted as phylogenetic regression, and the complex, solid organ in mammals was considered to be a rudimentary remnant of the non-mammalian pineal gland having enigmatical function. However, many cytological characteristics including subcellular granules and clear vesicles consistent with a secretory function were observed in the mammalian pineal which indicated a glandular function. In 1898, Heubner described a boy with pinealoma showing signs of premature puberty. This was the first indication of an antigonadotrophic hormone produced by the pineal and following the discovery of the endocrine organs by Claude Bernard (1813 - 1878) and Brown - Sequard (1817 - 1894), the pineal was considered a serious candidate for an endocrine role. Marburg (1930) was the first to suggest a hormonal connection between the pineal gland and the hypothalamus. He believed specific pineal parenchyma produced an antigonadotrophic hormone which normally acted on the hypothalamus to retard sexual development in infants. Hypopinealism or destruction of the specific parenchyma would hence cause premature puberty while hyperpinealism would be responsible for delayed puberty. Bargmann, better known for his valuable and

well-respected review on the microscopic anatomy, histology and cytology of the pineal gland from fish to man published in 1943, also critically surveyed all available endocrinological research on the gland. Based on the findings of Berblinger (1932) and Roussy and Mosinger (1938) which indicated a neural connection between the pineal gland and the hypothalamus, Bargmann proposed that the regulation of hypothalamic function was possibly of a neural nature and not necessarily hormonal. The pineal, the hypothalamus and the pituitary gland together represented a functional system regulating gonadal growth and function. According to Berblinger, the pineal and the pituitary exerted antagonistic effects on the vegetative centres in the hypothalamus, the former exercising an inhibitory influence while the latter would stimulate the same centres.

1.2.3 The Third Period (Mid 19th century onwards)

There is no clear division between the second and third periods of pineal research. The tremendous multi-disciplinary approach facilitated by the technological advancement following World War II has lead to a burgeoning of information on every aspect of pineal anatomy, histology, cytology, biochemistry, physiology and pharmacology. International communication and co-operation has been stimulated through numerous international congresses and publications devoted to pineal research. Although many scientists outside the immediate field are reluctant to accept the diminutive organ as a *bona fide* endocrine gland on account of its historical vestigal associations, the pineal gland has secured its place in the neuroendocrine system and its principle metabolite currently recognized as the pineal hormone, melatonin, has been used commercially. The full extent of the function of the pineal gland is yet to be established, but many have proposed that there are no organs within the body that escape its influence and it has been

dubbed "the regulator of all regulators."

In spite of the tremendous advances in our understanding of the pineal gland over the past four decades, there still remain many grey areas which are the target of ongoing research. Current aims of research are focused on gaining a more complete understanding of the pineal system in order to establish whether it may contribute to the cause or relief of many problematic diseases of our time including cancer, heart and other stress-related diseases, depressive disorders, Parkinson's and Alzheimer's diseases.

1.3 PINEAL ANATOMY

1.3.1 Embryology and Location

Embryologically, the pineal gland or epiphysis cerebri develops from an evagination of the region of the neural tube which becomes the diencephalon [Ariëns Kappers, 1971]. The size, shape and location of the gland varies in different species. In the rat, it is superficially located between the cerebral hemispheres, in front of the cerebellum where it is covered by the confluence of the superior sagittal and transverse sinuses (Figures 1.1 and 1.2). The cone-shaped organ remains attached to the brain via the pineal stalk, which is formed by the fusion and forward projection of the walls of the original epiphyseal evagination, but it loses direct neural contact with the brain. The stalk, which consists of pinealocytes, pinealoblasts and fibrocytes, is generally divided into three distinct sections: a proximal part, a midpart and a distal part. The proximal part is connected to the brain between the posterior and habenular commissures. The midpart is often extremely thin and connects the proximal and distal parts. The latter is the longest of the three sections, its terminal being

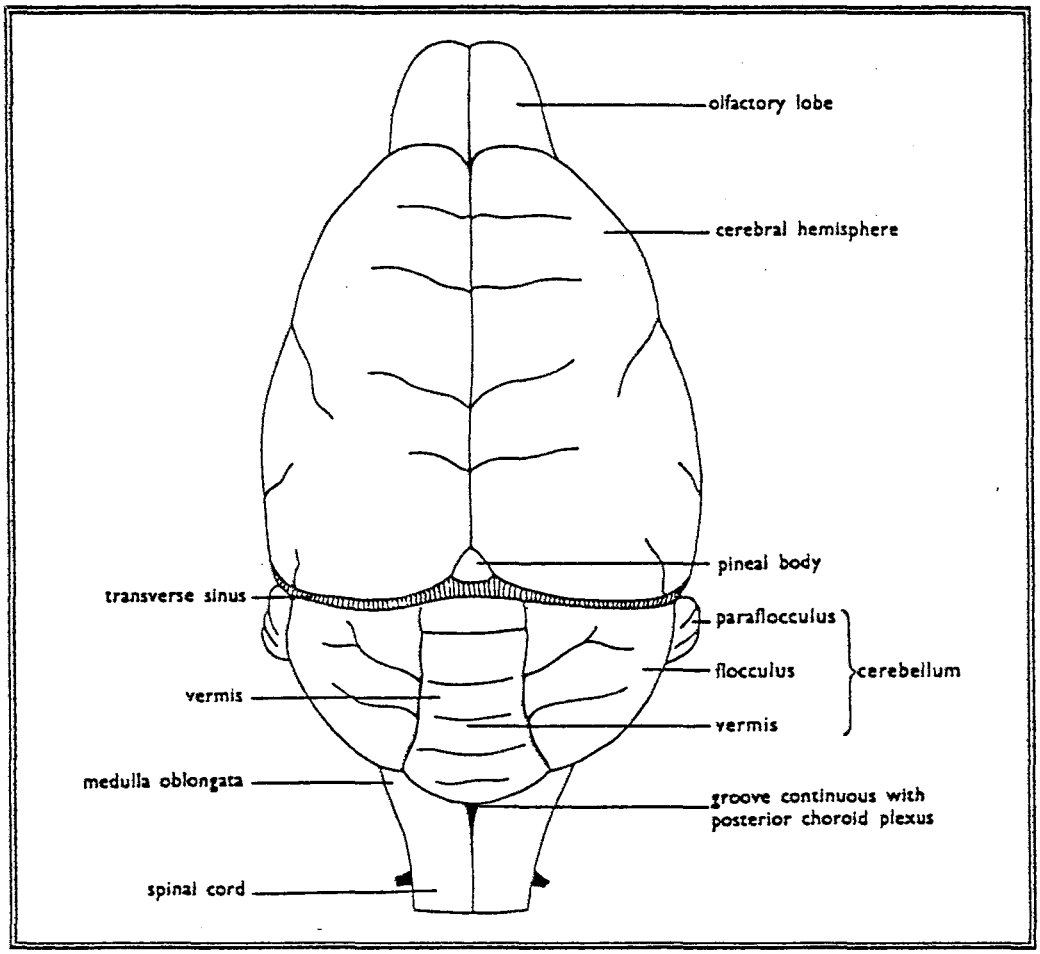

FIGURE 1.1 : Dorsal view of the Rat Brain [Daya, 1982]

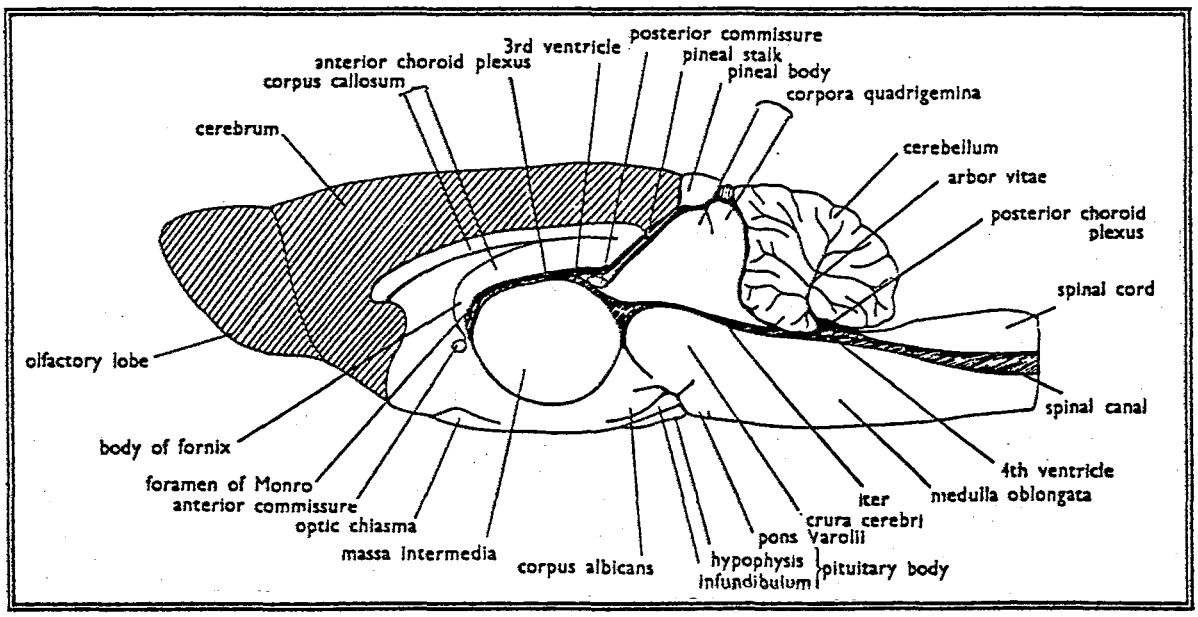

FIGURE 1.2 : Medial Sagittal View of the Rat Brain

[Daya, 1982]

connected to the pineal gland.

1.3.2 Blood Supply

Like other circumventricular organs, the pineal gland is located outside the blood brain barrier. It has been shown in the rat to have one of the richest blood systems in the body, exceeding that of the other endocrine organs, being surpassed only by that of the kidney [Goldman and Wurtman, 1964].

The blood supply to the pineal gland is provided by up to four branches of the posterior cerebral artery which penetrates the pineal capsule and forms a dense capillary network in the pineal parenchyma [Hodde, 1979]. Venous blood is collected by twelve to sixteen superficial veins which drain into the great cerebral vein and thereafter via the superior sagittal sinus into the systemic venous circulation.

The high rate of blood flow through the pineal indicates that hormones in the blood could quickly be detected by the gland and secretions from the pineal could equally rapidly be transported to the systemic circulation.

1.3.3 Innervation

The innervation of the pineal gland has attracted much attention and has been proven to be of utmost importance for its function. Two types of pineal innervation have been distinguished: an afferent or pinealofugal system which has shown evolutionary regression and gradually been surpassed by a complex efferent or pinealopetal nerve system. The latter is predominantly derived from the sympathetic nervous system, although parasympathetic innervation has also been described in some animals.

1.3.3.1 Pinealofugal Innervation

In vertebrates in which the pineal gland functions as a photosensory organ, photic stimuli perceived by the gland are transported to the brain via sensory neurons in the pineal tract [Ariëns Kappers, 1971]. The nerve fibres have been found to terminate in the pretectal area, the area of the subcommissural organ and in the deep mesencephalic periventricular grey nuclei [Ueck, 1979]. There is evidence to suggest that the neurotransmitter involved is acetylcholine [Wake *et al*, 1974]. The exact function of this nervous communication is unknown.

This type of innervation which is predominant in fish and amphibians, is also present to a limited extent in reptiles and birds, but is totally absent in mammals [Ariëns Kappers, 1971].

1.3.3.2 Sympathetic Innervation

Phylogenetically, the sympathetic innervation starts developing in fish and develops progressively in amphibians, reptiles and birds as the pinealofugal innervation disappears [Ariëns Kappers, 1971]. It is of vital importance in mammals where it is responsible for the profound effects of light on the pineal gland (Figure 1.3). There are two major components of this system: the retino-hypothalamic projection and the suprachiasmatic nucleus.

The retinal-hypothalamic pathway is an unmyelinated tract in the optic nerve which originates in the ganglion layer of the retina [Hendrickson *et al*, 1972]. The axons of ganglion cells pass through the optic nerve and project directly from the optic chiasma to make synaptic contact with neuronal parikarya in the ipsilateral and contralateral suprachiasmatic nuclei of the anterior hypothalamus. The retino-hypothalamic projection is distinct from

the primary optic tract and the inferior accessory optic tract.

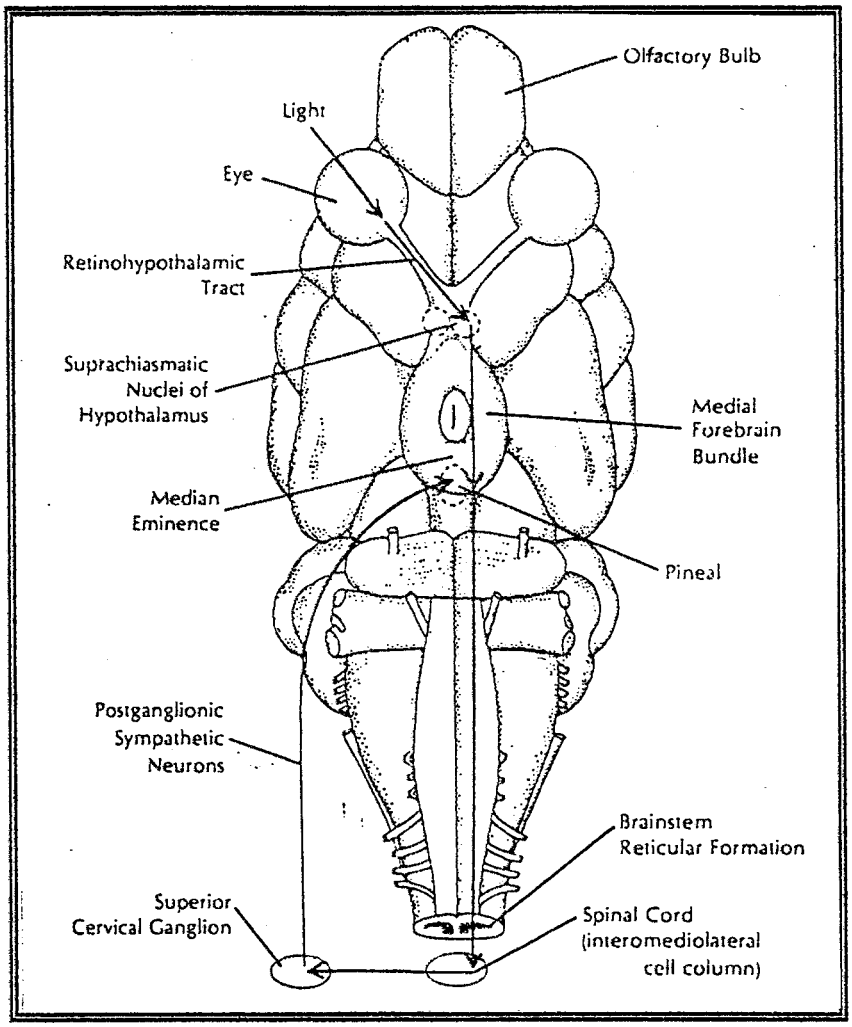

FIGURE 1.3 : Sympathetic Neural Pathway to the Pineal Gland

[Daya, 1982]

Axonal projections from the suprachiasmatic nucleus follow a multi-synaptic pathway through the brainstem, eventually terminating on neurons in the intermediolateral cell columns of the upper thoracic cord. The axons of the preganglionic sympathetic neurons leave the spinal cord in the ventral roots passing in the direction of the head in the sympathetic trunk to synapse with postganglionic

fibres in the superior cervical ganglia. The postganglionic fibres enter the cranial cavity via the internal carotid plexus and form discrete nerves called the nervi conarii which penetrate the pineal capsule at the apex of the gland. The nerves ramify among the pinealocytes and eventually terminate in the perivascular spaces of the pineal parenchyma [Young and Silman, 1982; Reiter, 1988; Klein, 1978]. The dense network of nerve fibres known as a plexus, is characterized not by the formation of classical synapses, but rather by the frequent appearance of swollen areas in the neural processes described as varicosities. These varicosities contain dense-cored vesicles within which neurotransmitters such as norepinephrine, dopamine, serotonin, GABA, histamine and octopamine are present [Klein, 1978]. Norepinephrine is the principal neurotransmitter involved in pineal function [Wolfe, 1965].

1.3.3.3 Parasympathetic Innervation

In some mammals, parasympathetic innervation has been observed in the pineal gland. Preganglionic fibres arising in the superior salivatory nucleus in the medulla oblongata, leave the brainstem with the facial nerve. The fibres run with the greater petrosal nerve and reach the pineal gland together with the sympathetic fibres in the nervi conarii synapsing with ganglionic neurons along the nerves or within the pineal [cf. Young and Silman, 1982]. The function of the parasympathetic innervation is unknown.

Electrophysiological investigations have provided evidence of nervous stimuli reaching the pineal gland from parts of the limbic system via the medullary striae, the habenular nuclei and the pineal stalk [Dafny, 1977; Ariëns Kappers, 1971]. This is significant in that it indicates that stimuli of limbic origin may exert an influence on pineal function. The limbic system is

associated with olfaction, autonomic functions and certain aspects of emotion and behavior. It has been proven that olfaction does exert an influence on pineal function.

Extrahypothalamic neurosecretory fibres have also been observed in the pineal of some mammals. While the function of these fibres is unknown, they are considered to be associated with peptidergic compounds demonstrated in the pineal [Ariëns Kappers, 1971].

1.4 PINEAL CYTOLOGY

Cytologically, the pineal gland of vertebrates has undergone profound evolutionary changes which should be considered when attempting to define the functional significance of the gland. The primary anlage of all vertebrate pineal glands is derived from the embryonic ectoderm, but the cellular differentiation in each vertebrate class is distinct.

1.4.1 The cellular structure of the submammalian pineal gland

Three cell types have been distinguished in the pineal gland of the lower vertebrates (fish and amphibians): photoreceptor cells, ependymal supportive or interstitial cells and sensory nerve cells [Ariëns Kappers, 1971; Oksche *et al*, 1987].

1.4.1.1 Photoreceptor cells

The photoreceptor or primary sensory cells are not neurons, but have a similar structure to the cone cells of the lateral eye. They display distinct structural and functional compartmentalization in four basic cellular regions: the outer segment, the inner segment, the cell soma and the basal process or

synaptic pedicle [Ariëns Kappers, 1971; Collin and Oksche, 1981].

The complex outer segment is characterized by a single ciliary structure and numerous successive membraneous evaginations which undergo constant degeneration and renewal. It is known as the photic pole because of its sensitivity to light.

The distal section of the inner segment contains aggregates of mitochondria to provide energy for the transduction process in the outer segment. It is connected to the proximal section through a neck region which is transversed by parallel arrays of microtubules. The proximal section contains a second concentration of mitochondria, rough endoplasmic reticulum, polysomes, lysosomes, glycogen particles and Golgi bodies which elaborate clear vesicles and dense cored vesicles.

The cell soma is the largest of the four segments and contains the nucleus.

The basal process or pedicle is a projection from the cell body toward the basal membrane which separates the perivascular spaces from the parenchyma, were it establishes synaptic contact with sensory neurons. Microfilaments and tubules run longitudinally through the central part of the cellular extensions. The cytoplasm contains rough and smooth endoplasmic reticulum, few free ribosomes, single mitochondria, synaptic ribbons and vesicles.

In higher vertebrates, the outer segment becomes transformed into a rudimentary, frequently club-shaped structure. The photoreceptive capacity of the cells is lost and they are referred to as *rudimentary photoreceptor* cells [Collin, 1971]. This cell type gradually replaces the photoreceptor cells in reptiles and birds [cf. Ariëns Kappers, 1971]. The basal processes may ramify

into smaller branches which terminate in close association with the basal membrane or, in some cases, penetrate through the membrane and project into the perivascular spaces. Synaptic contact with sensory nerve cells is diminished and the latter are quantitatively reduced. Synaptic ribbons disappear and are replaced by vesicle-crowned ribbons which occur in all regions of the cytoplasm [cf. Collin and Oksche, 1981]. They are electron-dense rodlets surrounded by a single layer of small clear vesicles and are often found close to the cell membrane.

1.4.1.2 Ependymal Supportive or Interstitial Cells

These are highly variable cells located between the photoreceptor cells. They exhibit extensive microvilli which form tight and intermediate junctions and occasionally desmosomes with each other and the photosensory cells. Their cytoplasm contains abundant smooth endoplasmic reticulum, free ribosomes, mitochondria, lysosome-like organelles, dense core granules and Golgi complexes which produce coated vesicles. Glycogen particles may be scattered in various amounts throughout the cytoplasm. Microtubules are few or missing, but microfilaments course through the cytoplasm. Pinocytotic vesicles have been observed in the microvilli and near the apical cell membrane. Myeloid bodies are found closely associated with the nuclear envelope and endoplasmic reticulum [cf. Collin and Oksche, 1981].

1.4.1.3 Sensory Nerve Cells

Multipolar, bipolar and unipolar sensory neurons have been observed in the pineal gland [cf. Ariëns Kappers, 1971]. They form somato-somatic, axo-somatic, somato-dendritic and axo-dendritic ribbon synapses with the basal pedicle of the photoreceptor cells. An increase in the density of the post-synaptic membrane has been

linked with active neurotransmission. The axons of these sensory nerve cells constitute the pineal tract which conveys the photic stimuli to the brain [cf. Ariëns Kappers, 1971; cf. Collin and Oksche, 1981].

1.4.2 The cellular structure of the mammalian pineal gland

The mammalian pineal gland generally contains two categories of cells principally derived from the photoreceptor and supportive cell lines described in submammalian pineal glands. They are pinealocytes and interstitial or glial cells [Pévet, 1981].

1.4.2.1 Pinealocytes

Pinealocytes are the predominant cells in the mammalian pineal gland and are variously referred to as pinealocytes of population I, chief cells, parenchymal cells and pinealocytes *sensu stricto* or true pinealocytes. They are differentiated from the second category of cells by the presence of granular vesicles [Pévet, 1981]. Pinealocytes are derived from the photoreceptor cells by further progress of the rudimentation process characterized by the complete disappearance of the photoreceptor pole and secondary neurosensory cells [Collin, 1971]. They belong to the APUD cell series.

The pinealocytes are devoid of the distinct cellular compartmentalization observed in their phylogenetic predecessors and exhibit a functional rearrangement of cell organelles. They are shaped like unipolar or multipolar neurons having large cell bodies with a varying number of processes emerging from the cell soma, similar to the basal processes described previously. The spherical or oval nucleus is located in the cell body and contains the chromatin material irregularly arranged in a combination of

helices, granular masses and mesh structures in the peripheral area. The cytoplasm contains smooth and granular endoplasmic reticulum, free ribosomes, mitochondria, glycogen granules, lipid droplets and various membraneous structures of unknown function. The processes are rich in microfilaments coursing longitudinally through the cytoplasm and like the rudimentary photoreceptor cells, contain clear vesicles, vesicle-crowned ribbons and granular vesicles.

The granular vesicles which characterize the true pinealocytes arise from juxtanuclear Golgi saccules and migrate to the endings of the processes to release their content into the perivascular space. They are considered to be of importance to the functioning of the pineal gland and their numbers appear to depend on different physiological conditions. Their numbers undergo a dramatic increase following administration of gonadotrophic hormone [Karasek and Marek, 1978] and following cold stress [Matsushima and Morisawa, 1978].

Vesicle-crowned rodlets, once considered to be phylogenetic relics of the ribbon synapses [Collin, 1971], are now regarded as functionally significant because of the numerical changes they exhibit under various physiological conditions [Karasek and Vollrath, 1989]. These organelles have been shown to increase in number after cold [Krstic, 1973] and immobilization [Milne *et al*, 1968] stress.

Depending on the degree of evolution and the systematic position, a given pineal gland may contain representatives of all the cells in the photoreceptor cell line or only a limited set dominated by one particular cell type [Collin and Oksche, 1981]. Only one single population of true pinealocytes have been described in the pineal gland of the rat.

1.4.2.2 Interstitial Cells

The second category of cells is derived from the ependymal supportive cells and comprise interstitial or glial cells. This group of cells, also referred to as pinealocytes of population II, are categorized by the absence of granular vesicles and their location close to the perivascular spaces [Pévet, 1981].

1.5 PINEAL MELATONIN BIOCHEMISTRY

A major interest in pineal biochemistry was stimulated by McCord and Allen in 1917 when they reported that pineal extracts contained a substance which lightened the skin of amphibians. The active blanching agent was isolated by Lerner *et al* [1958] and identified as N-acetyl-5-methoxytryptamine. It was termed melatonin because of its ability to cause aggregation of melanin granules in melanophores. This initial discovery led to the identification of other indoles in the pineal gland and the unravelling of their biosynthetic pathway (Figure 1.4).

1.5.1 Indole Metabolism in the Pineal Gland

Indole metabolism in pinealocytes commences with the uptake of the essential amino acid precursor, tryptophan, from the bloodstream. A small amount of the tryptophan is utilized in the synthesis of pineal proteins, but the major portion is converted to 5-hydroxytryptophan. This initial hydroxylation is catalysed by tryptophan hydroxylase [Lovenberg *et al*, 1967, 1968; Jequier *et al*, 1969] in the presence of oxygen, ferrous iron and a reduced pteridine cofactor [Snyder and Axelrod, 1964a; Lovenberg *et al*, 1962]. Tryptophan hydroxylase has a low affinity for its substrate, tryptophan, and it has been suggested that the

availability of tryptophan is a rate determining factor in the pathway.

5-Hydroxytryptophan is decarboxylated by a pyridoxal dependent aromatic-L-amino acid decarboxylase [Snyder and Axelrod, 1964a] to form 5-hydroxytryptamine or serotonin [Lovenberg *et al*, 1962; Snyder and Axelrod, 1964a]. This enzyme is more concentrated in the pineal gland than in any other organ in the body [Snyder and Axelrod, 1964b], and pineal serotonin concentrations are significantly higher than those found in any other tissues examined [Giarman and Day, 1959]. Serotonin can undergo a three way transformation:

(1) A portion is methoxylated by hydroxyindole-O-methyltransferase (HIOMT) to form 5-methoxytryptamine, a biologically active pineal hormone. S-adenosylmethionine serves as the methyl donor.

(2) Serotonin is also oxidized by monoamine oxidase to 5-hydroxyindole acetaldehyde [Axelrod *et al*, 1969]. This unstable intermediate follows one of two metabolic fates. It may be converted by aldehyde dehydrogenase to 5-hydroxyindole acetic acid [Wurtman and Larin, 1968; Lerner and Case, 1960] which is then O-methylated by HIOMT to form 5-methoxyindole acetic acid [Wurtman and Axelrod, 1967]. Alternatively, 5-hydroxyindole acetaldehyde may be converted to 5-hydroxytryptophol by alcohol dehydrogenase [McIsaac and Page, 1959] which is then methylated by HIOMT to form 5-methoxytryptophol [Wurtman and Axelrod, 1967].

(3) The major pathway of serotonin metabolism is its conversion to N-acetylserotonin by the enzyme N-acetyltransferase. This enzyme constitutes an important rate-determining step in the synthesis of melatonin [Klein and Weller, 1970]. The acetyl

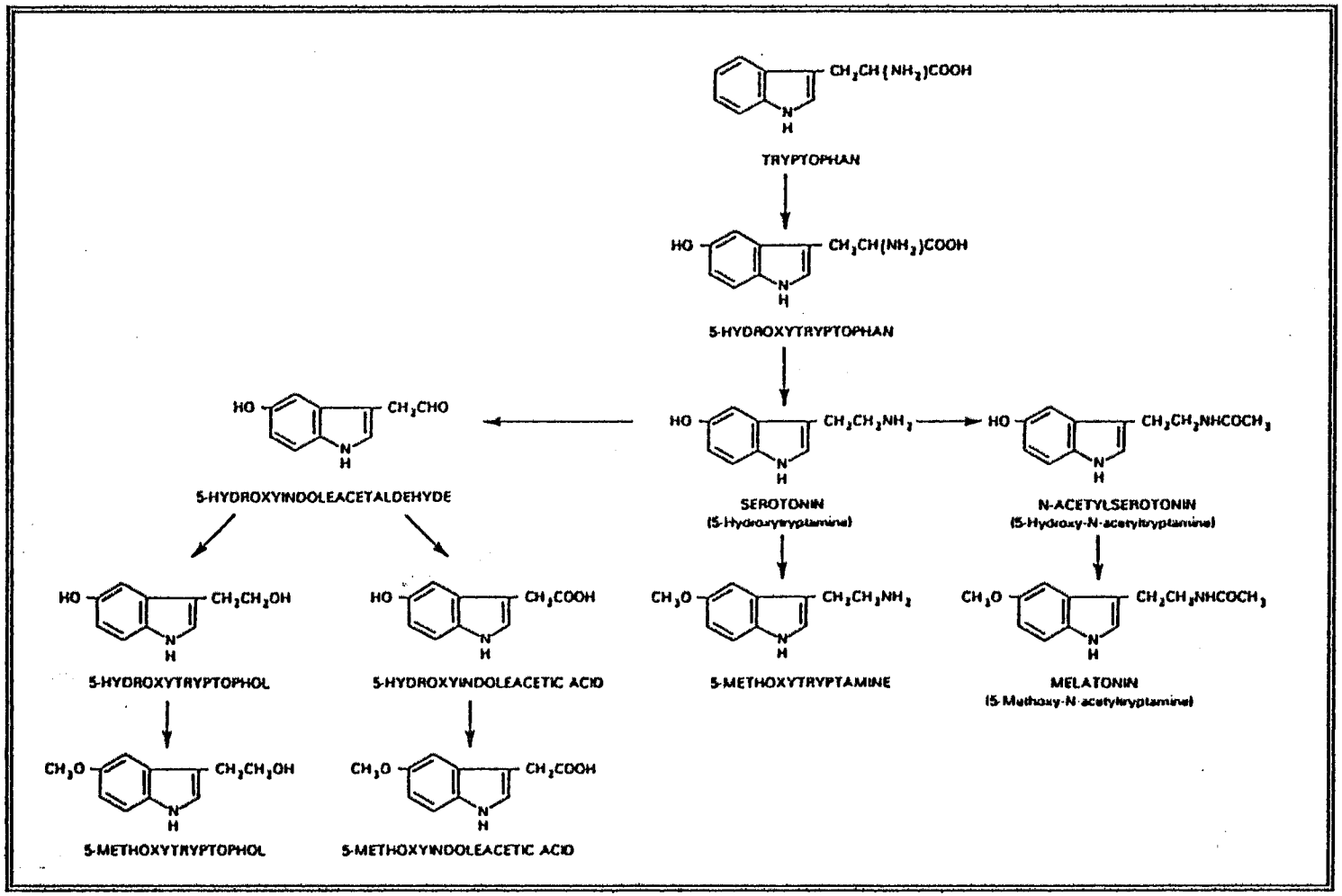

FIGURE 1.4 : Tryptophan Metabolism in the Pineal Gland

[Modified from Young and Silman, 1982]

group is donated by Acetyl CoA [Weissbach *et al*, 1960]. Finally, N-acetylserotonin is O-methylated to melatonin by HIOMT [Axelrod and Weissbach, 1960; Cardinali and Wurtman, 1972].

Melatonin is the pineal indoleamine of highest repute and the focus of most pineal research. It is considered by many to be the pineal hormone responsible for the principle endocrine effects associated with the pineal gland.

1.5.2 Pineal Melatonin Secretion and Catabolism

Melatonin has not been found to be stored in large pools in the pineal gland and events which alter melatonin synthesis are rapidly reflected in the blood [Arendt, 1988]. It is now generally accepted that the hormone is secreted into the bloodstream rather than into the cerebrospinal fluid. Although little is known about the mode of secretion, it appears to be secreted by simple diffusion as no active mechanism has been described. Melatonin is transported in the blood non-covalently bound to high-capacity, low-affinity binding sites on plasma albumin [Cardinali *et al*, 1972; Reiter, 1981]. It has a half life of about 20 minutes in the blood of rats [Gibbs and Vriend, 1981].

The liver is the primary site of melatonin catabolism, but the metabolic routes vary from species to species [Arendt, 1988]. In humans and rodents, melatonin is hydroxylated in the 6 position and then conjugated to sulphate and sometimes to glucuronide derivatives rendering 6-sulphatoxymelatonin as the major urinary metabolite. There is a very close correlation between plasma melatonin levels and both plasma and urinary 6-suphatoxymelatonin levels. This fact is sometimes used as a non-invasive technique of assessing melatonin levels in clinical situations.

1.5.3 Non-Pineal Sites of Melatonin Synthesis

Initially melatonin synthesis was thought to be confined to the pineal gland and when it was discovered to be synthesized in other organs, a hormonal role for pineal melatonin was discounted. However, organ specificity is not a requirement of a hormone and many hormones are synthesized in a variety of sites.

At present, melatonin is known to be synthesized in the retinas of the eyes, the Harderian gland, the intra-orbital lacrimal glands [Reiter, 1989] and the enterochromaffin cells of the gastro-intestinal tract [Raikhlin and Kvetnoy, 1976]. Many more tissues may also possess the enzymes required for melatonin synthesis. It is tentatively believed that pineal melatonin is solely, or at least predominantly responsible for the circulating melatonin levels in the blood. However, it is possible that the non-pineal sites may exhibit a compensatory increase in melatonin synthesis in pinealectomized animals.

1.6 CIRCADIAN RHYTHMS IN PINEAL MELATONIN SYNTHESIS

1.6.1 Description of the Circadian Rhythms in Melatonin Intermediates

Except where there is a genetically based enzyme deficiency, pineal indoleamines exhibit a distinctive and well described circadian rhythmicity.

1.6.1.1 Tryptophan: Primary Precursor in Melatonin Synthesis

A circadian rhythm in pineal tryptophan levels has not been well

defined. The levels of pineal tryptophan appear to be modulated by the concentration of tryptophan in circulation. Injections of tryptophan which increase serum tryptophan concentrations, can elevate pineal levels of the indole [Deguchi and Barchas, 1972]. As tryptophan is an essential amino acid, circulating concentrations depend largely on dietary intake. Being the least abundant amino acid in dietary protein, the mammalian body's supply is usually quite small [Fernstrom and Wurtman, 1974]. However, it is unique among amino acids in that 90% is transported bound to serum albumin, leaving only 10% freely diffusible [Bender, 1975]. The circulating serum levels of tryptophan therefore remain fairly constant. Furthermore, as the pineal gland is outside the blood brain barrier, tryptophan does not have to compete with the other large, neutral amino acids for an uptake system as it must to gain entry to the brain [Fernstrom and Wurtman, 1974].

1.6.1.2 Tryptophan Hydroxylase: Role in Melatonin Synthesis

Tryptophan hydroxylase has been located in the mitochondria of the pinealocytes [Lovenburg *et al*, 1967, 1968], and does not appear to be present in the nerve terminals. The enzyme has a low affinity for its substrate, tryptophan, and it has been proposed that it may act as a rate-limiting factor in the synthesis of serotonin [Reiter, 1989]. However, tryptophan hydroxylase activity in the pineal gland is relatively high compared with other tissues and its pteridine cofactor, tetrahydrobiopterin (BH_4), is present in higher concentrations than in any other tissues examined [Klein *et al*, 1981].

A circadian rhythm in pineal tryptophan hydroxylase activity has not been irrefutably established. Measurements of the biological half life of the enzyme suggest that it is synthesized continually [Sitaram and Lees, 1978]. Shibuya and co-workers [1978] reported

a ß-adrenergic receptor-dependent 1½-fold rise in enzyme activity in the middle of the dark period. Deguchi [1977] was unable to demonstrate a circadian rhythm in tryptophan hydroxylase activity and its existence remains controversial.

1.6.1.3 Aromatic L-Amino Acid Decarboxylase: Role in Melatonin Synthesis

This non-specific enzyme is confined mainly to the cytosol of pinealocytes where it is present in relatively high concentrations [Snyder and Axelrod, 1964a]. The activity of aromatic L-amino acid decarboxylase is only influenced by several days of constant exposure to environmental lighting [Snyder *et al*, 1965] and hence it does not appear to contribute to the circadian rhythms in the pineal gland.

1.6.1.4 Serotonin: Circadian Variations

Serotonin levels in the pineal gland are higher than in any other tissue in the body [Giarman and Day, 1959], being approximately 100-fold higher than those in the forebrain. This high concentration of serotonin serves to protect the pineal from any changes in circulating tryptophan and hence pineal serotonin levels are highly conserved [Daya *et al*, 1989]. However, in both nocturnal and diurnal animals, the levels of serotonin in the pineal exhibit a circadian variation (Figure 1.5). They are highest at midday and fall precipitously at night to a low level at about 11 pm [Quay, 1963]. Fiske [1964] reported day or light-time serotonin levels 13 times higher than those observed during the night or dark-time.

Pineal serotonin levels are conceivably regulated by three metabolic factors: (1) its rate of synthesis; (2) the conversion

of serotonin to oxidation products, and (3) the rate at which serotonin is converted to N-acetylserotonin by N-acetyltransferase. The latter has been proven to be the most important regulatory component [Klein and Weller, 1970]. Enhanced N-acetylation of serotonin to N-acetylserotonin is responsible for the dramatic decrease in pineal serotonin levels at night.

1.6.1.5 N-Acetyltransferase: Circadian Variations

Normally, after the onset of darkness, there is a 30 - 70 fold rise in the activity of N-acetyltransferase (NAT) [Axelrod, 1974; Arendt, 1988]. The rise in activity in the first hour of darkness is quite small. Thereafter, NAT activity increases rapidly to reach a maximum four hours into the dark period. This rise persists until the animal is exposed to light, usually the onset of the next light period. The regulation of NAT activity is fairly complex and will be discussed in greater detail in later sections.

1.6.1.6 N-Acetylserotonin: Circadian Variations

The circadian rhythm in pineal N-acetylserotonin is 180° out of phase with that of serotonin: levels are low during the day and elevated at night (Figure 1.5). This is due to the activity of N-acetyltransferase [Klein and Weller, 1970].

1.6.1.7 Hydroxyindole-O-methyltransferase: Role in Melatonin Synthesis

Pineal hydroxyindole-O-methyltransferase (HIOMT) is located almost entirely in the cytosol of the pinealocytes. The distribution of this methyltransferase is limited in mammals, but it is one of the most abundant single proteins in pinealocytes comprising 2 - 4% of the soluble protein fraction [Klein *et al*, 1981]. HIOMT has been

found to exhibit a small rise in activity at night or in the dark phase [Klein, 1979], (see Figure 1.5). During exposure to constant darkness there is a gradual increase in HIOMT activity and in constant light there is a gradual decrease in enzyme activity. The intracellular mechanisms involved in regulating HIOMT have not been clearly eludicated, and at this time it is not considered to be an important contributor to the melatonin circadian rhythm in the pineal gland. It may play a more important role in seasonally altering the amplitude of the melatonin cycles [Binkley and Mosher, 1989].

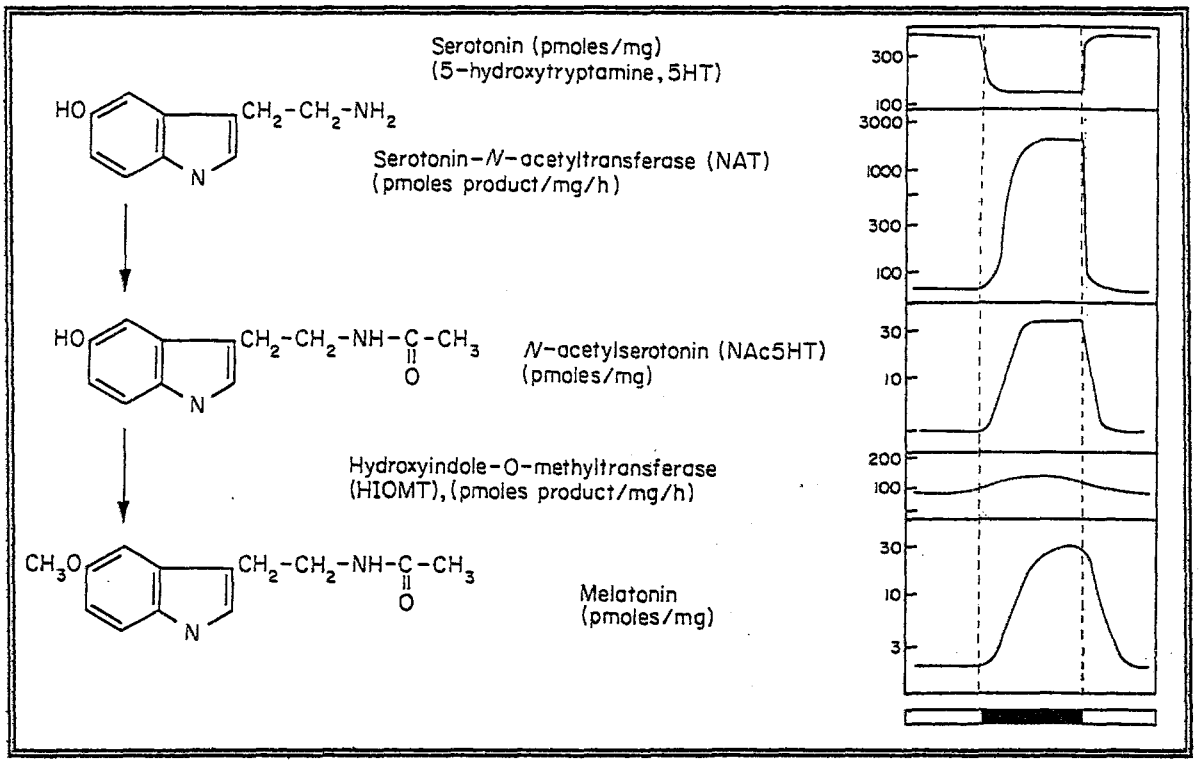

FIGURE 1.5 : <u>Diurnal Variation in the Components in the Pathway of Melatonin Synthesis from Serotonin</u>

[Arendt, 1988]

1.6.1.8 Melatonin: Circadian Variations

The pineal melatonin circadian rhythm is in phase with that of N-acetyltransferase and N-acetylserotonin (Figure 1.5). The magnitude of the nocturnal rise in pineal melatonin varies among species, with the trough to peak ratio usually ranging from 1:2 to 1:10 [Reiter, 1988].

When the circadian rhythms of melatonin in various mammalian species were compared, it was apparent that the nature of the rhythm varied in a species-specific manner [Reiter, 1988]. These 24 hour rhythms have thus been classified into 3 types: A, B and C, based on the nature of the nocturnal rise in melatonin [Reiter, 1983], (see Figure 1.6). The type A pattern of melatonin production is associated with a delayed rise in pineal melatonin following the onset of the dark period. Levels of the indole remain low for several hours into the dark period and then rise rapidly to reach a short-term peak in the latter half of the dark cycle. The peak is followed by a rapid reduction in melatonin to near basal levels by the onset of the light period. The type B pattern is associated with a gradual rise in melatonin concentrations at the beginning of, or in some cases just before, the onset of the dark period. The peak is established near the middle of the dark period after which there is a gradual decline in values to basal levels just prior to the onset of the next light phase. In the type C pattern, pineal melatonin levels increase rapidly after the onset of darkness, reaching a plateau which is maintained for the majority of the dark phase. Levels drop steeply to basal values just before the onset of the next light phase. Within certain limits, a change in the light : dark ratio alters the duration of the nocturnal rise in melatonin accordingly.

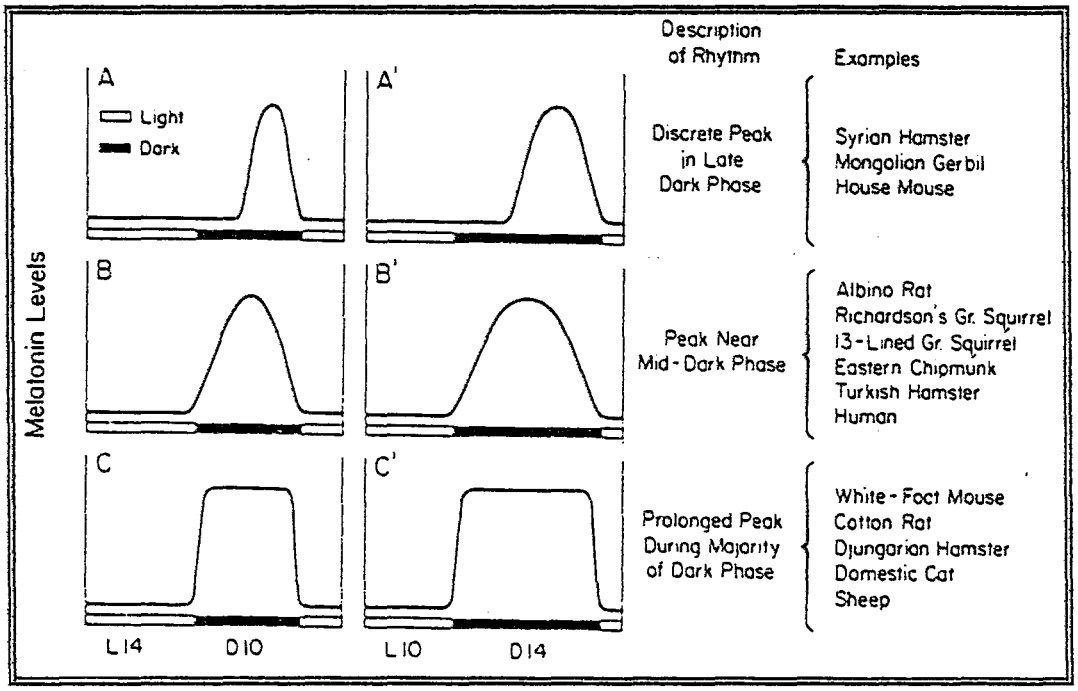

FIGURE 1.6 : <u>Patterns of Melatonin Production in Mammals</u>

[Reiter, 1989]

1.6.1.9 Norepinephrine: Daily Variations

A 24 hour rhythm has also been established for the turnover of norepinephrine in the sympathetic nerves innervating the mammalian pineal gland [Wurtman *et al*, 1967; Brownstein and Axelrod, 1974]. The highest turnover of norepinephrine apparently occurs at night.

Norepinephrine is synthesized from the amino acid tyrosine. The first and rate determining step in the pathway is the conversion of tyrosine to L-dopa catalysed by tyrosine hydroxylase. The latter is subsequently converted to dopamine by dopa decarboxylase and finally hydroxylated to norepinephrine by dopamine hydroxylase [Stryer, 1981]. The activity of tyrosine hydroxylase in the intrapineal nerve endings has been found to rise at night [Craft

et al, 1984]. The result is enhanced synthesis and release of norepinephrine at night.

1.6.2 Regulation of the Circadian Rhythm in Melatonin Synthesis

1.6.2.1 Light: The Primary Regulator

Fiske *et al* [1960] reported that continuous exposure to light resulted in a decrease in the mass of the pineal gland in rats. This was the first indication of an influence of light on the pineal gland. The normal daily variations in the components of the melatonin biosynthetic pathway mentioned above indicate that light is an important regulator of melatonin synthesis. The daily variations in serotonin, N-acetylserotonin, melatonin and norepinephrine persist unchanged in constant darkness and in blinded animals, but are completely abolished in animals exposed to continuous light [Snyder *et al*, 1965; Brownstein and Axelrod, 1974; Axelrod, 1974]. Reversal of the lighting scheme (lights kept on at night and off during the day-time hours) causes a complete inversion of the rhythms within six days [Snyder *et al*, 1967]. The rhythms have also been shown to adapt to changes in the lengths of the dark and light schedule.

The ability of light to modify the circadian rhythms in the pineal is thus evident. Animals born and raised in continuous darkness have been found to exhibit a circadian rhythm, but this may not be in phase with the external day : night rhythm. This suggests that the rhythms are endogenous and probably generated by the central nervous system. Light is not required to entrain the cyclic production of melatonin by the pineal gland, but modifies the rhythm to be in phase with the external environment.

Early investigations indicated that the principal target of the light-induced responses in the pineal gland was NAT. When stimulated, the enzyme depletes its substrate, serotonin, resulting in concomitant elevation of its main product, N-acetylserotonin, and subsequently melatonin. When rats were exposed to as little as one minute of light during the dark period, there was a precipitous fall in pineal NAT activity [Deguchi and Axelrod, 1972a; Klein and Weller, 1972]. However, exposing the animals to darkness during the normal light period when NAT activity was low did not necessarily induce an increase in the activity of the enzyme [Binkley *et al*, 1973]. There appeared to be a refractory period approximately coincident with the normal light period during which time darkness could not initiate a rise in NAT or melatonin levels. From this it was deduced that light could "switch off" NAT at any time once it has been stimulated and returning the animals to the dark would only initiate enzyme activity again during a sensitive period.

The brightness or irradiance of the light which effects the above responses in pineal indole metabolism, varies greatly among mammals [Reiter, 1986; 1988]. The pineal gland of the albino rat will respond to as little as 0.0005 $\mu Watts/cm^2$ of white light, while the pineal of the Richardson's ground squirrel requires an irradiance of at least 1850 $\mu Watts/cm^2$. All other species have light sensitivities which lie in between these two extremes. The human pineal responds to about 150 $\mu Watts/cm^2$. Environmental lighting which naturally controls pineal function ranges between full moonlight (0.03 - 0.05 $\mu Watts/cm^2$) to full sunlight which on a clear day can be as bright as 25000 to 50000 $\mu Watts/cm^2$. Normal room light is on average 50 - 100 $\mu Watts/cm^2$.

Besides brightness, the wavelength of the irradiance also determines its ability to effect pineal indole metabolism [Reiter,

1988]. Wavelengths of light in the blue range (500 - 510 Å) are most effective, while wavelengths of light in the red range are least effective.

1.6.2.2 Innervation: Facilitates Light Regulation

The principal pineal circadian rhythms are abolished by denervating the sympathetic nerves to the pineal or by interrupting the nerve impulses from the brain [Snyder *et al*, 1965; Klein *et al*, 1971], (see Figure 1.7].

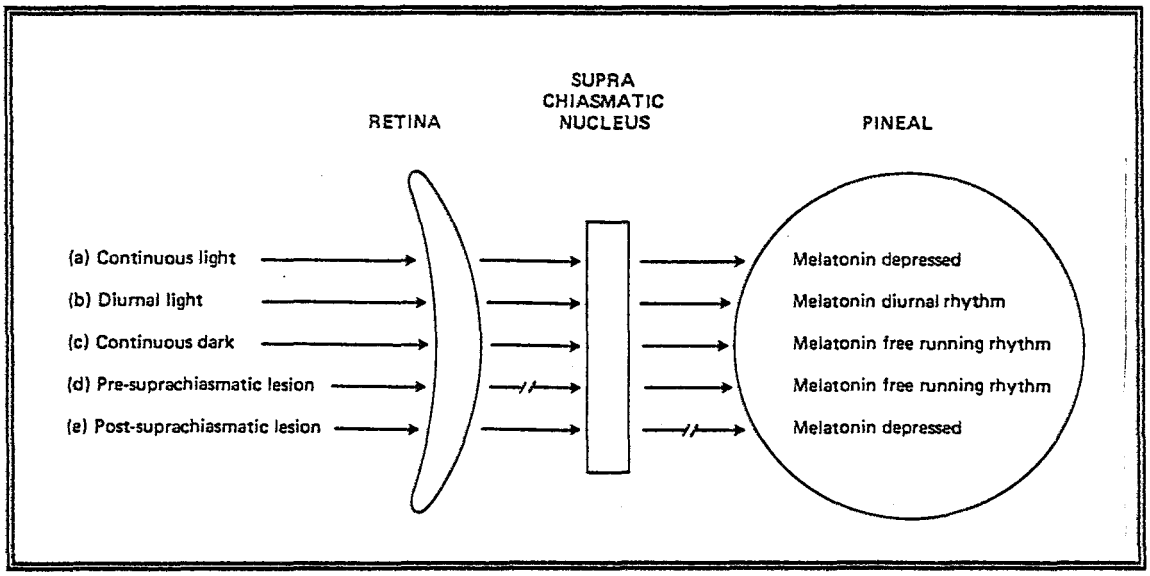

FIGURE 1.7 : Schematic Representation of Melatonin Output under conditions of alter Nervous Input

[Young and Silman, 1982]

The sympathetic innervation of the pineal gland has been previously described (section 1.3.3.2). Interruption of the sympathetic fibres at any point between the suprachiasmatic nucleus (SCN) and the pineal gland renders the gland non-functional [Reiter, 1988].

Lesions which completely destroy the SCN, abolish pineal rhythms while incomplete lesions which leave part of the SCN and its projections intact, do not affect the rhythms [Klein, 1978]. Severance of the optic nerves or optic tracts without affecting the retinohypothalamic projection will result in blindness, but will not disrupt any essential component of the retinal - pineal pathway [Klein 1978; Young and Silman, 1982]. If the SCN and the retina become uncoupled, the pineal rhythms follow the free-running rhythm of the SCN independent of environmental lighting stimuli [Klein and Weller, 1970].

The effects of light on pineal function are attributed to the inhibitory effects of light on the sympathetic nerves to the pineal gland [Klein, 1973].

1.6.2.3 Norepinephrine: Primary Messenger of Light Regulation

The importance of the sympathetic innervation for melatonin synthesis and the corresponding daily rhythm observed in pineal norepinephrine turnover implicated the neurotransmitter as a regulatory component of pineal indoleamine synthesis.

The two main neurotransmitters identified in the intrapineal nerve terminals are norepinephrine and serotonin. *In vitro* experiments showed that the addition of norepinephrine to pineal glands in culture initiated a significant increase in the synthesis of melatonin from a radioactive tryptophan precursor [Shein *et al*, 1967; Axelrod *et al*, 1969; Klein and Weller, 1970]. The same effect was achieved *in vitro* with other sympathomimetics: epinephrine, dopamine, octopamine and tyramine. The addition of serotonin to the culture media was completely ineffective. *In vivo* administration of reserpine, a drug that depletes catecholamines and serotonin from nerve terminals, has been found to prevent the

nocturnal rise in NAT activity. However, the administration of p-chlorophenylalanine, a compound which depletes nerve terminals of serotonin, has no effect on the nocturnal rise in NAT. This evidence has established norepinephrine as the neurotransmitter responsible for the sympathetic neural control of pineal indoleamine synthesis acting by a mechanism involving NAT. Light reduces the amount of norepinephrine released from sympathetic nerve endings in the gland [Brownstein and Axelrod, 1974] and this accounts for the reduction in NAT activity when the animals are exposed to light.

1.6.2.4 ß-Adrenergic Receptors: Transducers of Light Regulation

Having established that norepinephrine is responsible for the elevation in NAT activity in the pineal gland, it was necessary to determine the receptor population involved in the transduction of the neural signal. Norepinephrine is a multi-receptor agonist active at α_1-, α_2-, $ß_1$- and $ß_2$-adrenergic receptors.

In vitro studies revealed that the ß-adrenergic antagonist, propranolol, is able to prevent the norepinephrine-induced stimulation of NAT activity, but the α-adrenergic antagonist, phenoxybenzamine, has no effect [Wurtman *et al*, 1971]. *In vivo* administration of propranolol before the onset of the dark period inhibits the night-time rise in NAT activity. Similar treatment with α-blocking agents had no effect [Deguchi and Axelrod, 1972a]. Administration of isoprenaline, a ß-adrenergic agonist, during the day-time when NAT is at basal levels, results in a large increase in the activity of the enzyme [Deguchi and Axelrod, 1972b]. This effect is inhibited by ß-blocking agents. Furthermore, injection of isoprenaline at the end of the dark period prior to light exposure prevents the light induced decrease in NAT activity [Deguchi and Axelrod, 1972a]. Studies using selective $ß_1$- and

$ß_2$-adrenergic agonists and antagonists in pineal organ cultures demonstrate that the rat pineal responds to activity at the $ß_1$-adrenergic receptor subpopulation.

These findings clearly indicate that the effects of norepinephrine on NAT activity and therefore pineal metabolism are mediated primarily by ß-adrenergic receptors.

1.6.2.4.1 Supersensitivity and Subsensitivity of the ß-Adrenergic receptor

Deguchi and Axelrod [1973] found that the response of pineal ß-adrenergic receptors to nervous stimulation is dependent in previous exposure to norepinephrine. When the noradrenergic stimulation is abolished by denervation or severely diminished by reserpine administration, decentralization or continuous lighting, subsequent stimulation of the ß-adrenergic receptor results in greatly enhanced NAT activity. The maximum response is unchanged, but greatly reduced concentrations of norepinephrine are required to produce the same response in sensitized pineals relative to desensitized glands. Conversely, increased simulation of the ß-adrenergic receptor reduces the sensitivity of the receptor to subsequent stimulation. The phenomena of super- and sub-sensitivity are primarily attributed to an increase or decrease in the number of ß-receptors respectively [Kebabian *et al*, 1975; Romero *et al*; 1975b].

The diurnal, light-induced variation in norepinephrine release in the pineal gland induces a daily variation in the sensitivity of the ß-receptors [Romero and Axelrod, 1974]. The reduced release of norepinephrine during the day induces ß-receptor supersensitivity while increased stimulation of the ß-receptors at night leads to the phenomenon of subsensitivity. The rapid

increase in the number of ß-receptors towards the end of the dark period [Romero *et al*, 1975b] suggests that the ß-receptor density is regulated by an activation process possibly involving the internalization or uncoupling of the receptors, rather than the slower process of *de novo* receptor synthesis.

1.6.2.5 α-Adrenergic Receptors: Role in Regulation of Melatonin Synthesis

More recent data have shown that functional α_1-adrenergic receptors are also present in the pineal gland [Sugden and Klein, 1984]. These receptors do not exhibit a circadian variation or the phenomena of super- or sub-sensitivity. They are located postsynaptically and are involved in potentiating the ß-adrenergic response.

α_1-Adrenergic receptors have been implicated in enhanced turnover of phosphoinositol and elevation of cytosolic calcium by mediating the release of intracellular calcium and the entry of extracellular calcium into the pinealocytes.

α_2-Adrenergic receptors have also been demonstrated in the pineal gland. Pre-synaptic α_2-receptors regulate the release of norepinephrine via a classical negative feedback mechanism. These receptors have also been shown to mediate the inhibition of adenylate cyclase [Sabol and Niremberg, 1979].

1.6.2.6 Cyclic AMP: Second Messenger of Neural Regulation

Cyclic adenosine-3',5'-monophosphate (cyclic AMP) is a well recognized second messenger which initiates numerous intracellular events in response to hormonal or neural stimuli via receptor coupled adenylate cyclase. Adenylate cyclase is the enzyme which

catalyzes the conversion of adenosine triphosphate (ATP) to cyclic AMP. In other tissues, ß-adrenergic receptors had often been found to elicit numerous intracellular events coupled to adenylate cyclase and resulting from a classical rise in intracellular cyclic AMP concentrations. Early studies indicated that the same system was functional in the pineal gland.

Norepinephrine was found to stimulate adenylate cyclase in pineal homogenates [Weiss and Costa , 1967]. Furthermore, dibutyryl cyclic AMP, a cyclic AMP analogue, which unlike cyclic AMP is not catabolized by phosphodiesterase, was found to significantly stimulate melatonin formation from a radioactive tryptophan precursor *in vitro* [Shein and Wurtman, 1969]. Cyclic AMP itself only stimulated melatonin synthesis when theophylline, a phosphodiesterase inhibitor, was included in the culture medium [Strada *et al*, 1972]. Other studies showed that these effects were due to the ability to the cyclic nucleotide to enhance NAT activity.

Deguchi [1973] reported that injection of isoprenaline, a ß-adrenergic agonist, during the day-time resulted in a 15-fold increase in pineal cyclic AMP within 2 minutes. The elevated levels were maintained for 10 minutes before returning to basal levels 30 minutes after injection. A further 30 minutes after basal cyclic AMP had been attained, NAT activity started to rise and reached a maximum 3 hours after injection. Enzyme activity returned to normal day-time values after 5 hours. Injection of the ß-adrenergic antagonist, propranolol, before the isoprenaline, prevented the rise in both cyclic AMP and NAT activity. Propranolol even prevented the rise in NAT activity when it was injected 60 minutes after the isoprenaline and following the intracellular rise in cyclic AMP. Furthermore, propranolol administration during periods of maximal NAT activity resulted in

a rapid decline in the NAT activity. This indicates that elevation and maintenance of NAT activity is dependent not only on ß-receptor stimulated elevation of intracellular cyclic AMP levels, but also on continuous stimulation of the ß-receptor. This work provides evidence for a sensitive mechanism regulating NAT activity in which any event which interrupts the association of norepinephrine with the ß-receptor will decrease NAT activity.

The ß-adrenergic receptor stimulated rise in NAT activity at night or after the injection of isoprenaline is prevented by cyclohexamide, a protein synthesis inhibitor, and by actinomycin D, an antibiotic which prevents the transcription of RNA from a double helical DNA template. This suggests that the cyclic AMP in the pineal is involved in both transcriptional and translational processes leading to the activation of NAT. However, when NAT is re-induced by isoprenaline at night after its activity has been reduced by a brief exposure to light, the effect is blocked by cyclohexamide, but not by actinomycin D [Romero *et al*, 1975a]. Thus protein synthesis is always required for NAT activity, but RNA synthesis is only required for the initial induction of the enzyme. The lag period between the rise in cyclic AMP levels and the increase in NAT activity appears to reflect a period of RNA synthesis (probably mRNA) as actinomycin D is ineffective in preventing the rise of NAT when administered after this lag period. This lag period varies from species to species and depends on the length of the previous light exposure. The rapid return of cyclic AMP to basal levels within half an hour of stimulation suggests that RNA synthesis is the primary cyclic AMP-dependent event responsible for increasing NAT. It is unclear whether the RNA synthesis induces *de novo* synthesis of NAT itself or the synthesis of an NAT activator protein. The former is considered more likely [Reiter, 1991], although the two possibilities are not mutually exclusive.

A cyclic AMP - dependent protein kinase is present in the pineal gland [Fontana and Lovenberg, 1971; 1973]. The activity of this enzyme parallels the circadian variation in NAT. This phosphorylating enzyme is also involved in the induction of NAT.

More recently, the potentiation of β_1-adrenergic cyclic AMP response by α_1-adrenergic receptors has been demonstrated [Reiter, 1991]. Stimulation of β_1-adrenergic receptors results in a 10-fold increase in intracellular cyclic AMP levels while α_1-adrenergic receptor stimulation alone produces no changes in intracellular cyclic AMP levels. However, combined stimulation of α_1- and β_1-adrenergic receptors results in a 30 - 100 fold increase in intracellular cyclic AMP.

1.6.2.7 N-Acetyltransferase: Principal Target of Melatonin Regulation

N-acetyltransferase has been shown to play a key role in controlling pineal melatonin synthesis: it is the focus of all of the factors normally regulating the circadian variation in melatonin synthesis (see Figure 1.8). Neural pathways from the SCN relay episodic sympathetic nerve impulses to the pineal gland. The release of norepinephrine from the nerve varicosities in the pineal gland stimulates postsynaptic β_1-adrenergic receptors which are coupled to adenylate cyclase. The subsequent rise in intracellular cyclic AMP levels results in elevated NAT activity via a process involving protein transcription and translation. N-acetyltransferase then draws on its large supply of substrate to synthesize N-acetylserotonin, the direct precursor of melatonin. The availability of its substrate, serotonin, is not considered to be a rate-limiting factor as the latter is present in such high concentrations in the pineal gland. The activity of HIOMT which converts N-acetylserotonin to melatonin is also not considered to

be a rate-limiting step and hence control of melatonin synthesis is principally related to NAT activity.

The precise and reproducible nature of the daily melatonin rhythm is the result of the episodic release of norepinephrine in the pineal gland under the control of the SCN synchronized by environmental lighting and the changing responsiveness of the pinealocyte ß-receptors.

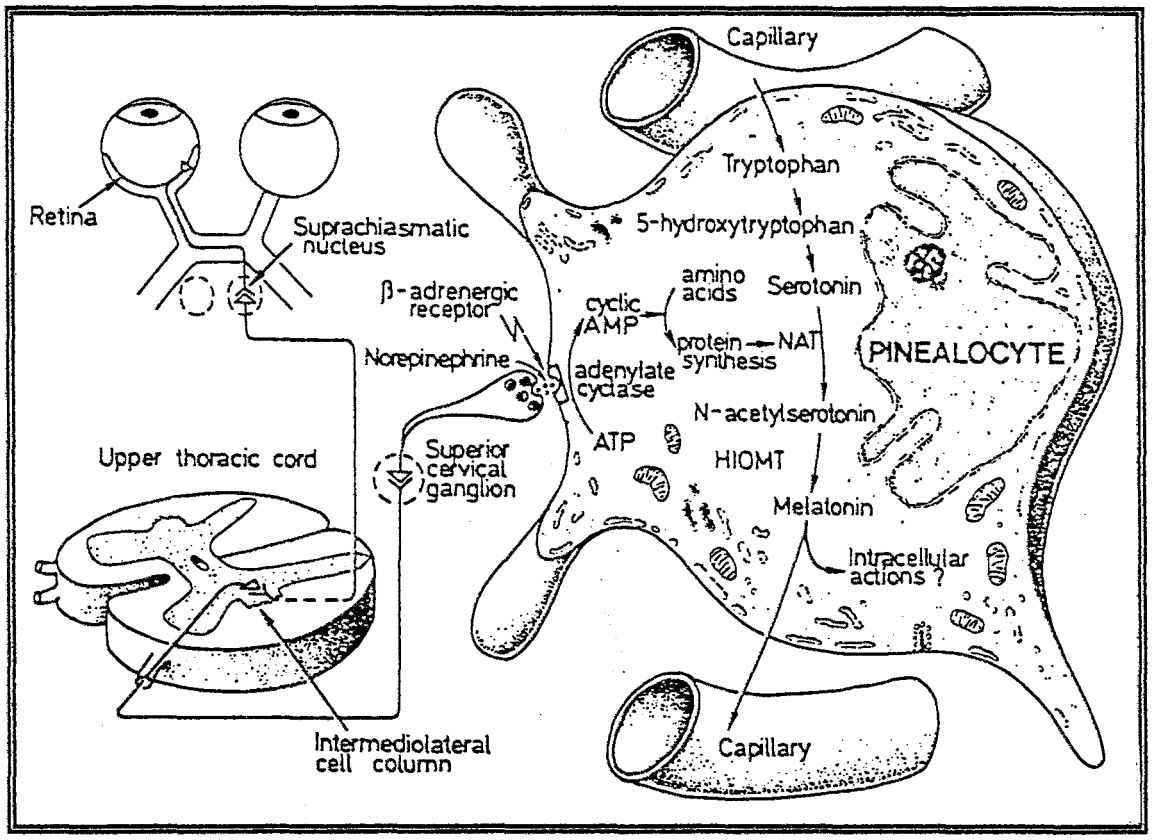

FIGURE 1.8 : <u>Diagrammatic Representation of Regulation of Pineal Melatonin Synthesis</u>

1.6.3 Extra-Retinal Routes of Pineal Indoleamine Regulation

There is a scarcity of information on the melatonin rhythms in lower vertebrates. Quantitatively indole metabolism appears to be important in these animals and it generates higher circulating melatonin levels than those found in more advanced animals. Non-pineal sites of melatonin synthesis are more prevalent with the retina of the eyes making an important contribution. This may also explain the failure of researchers to establish daily rhythms and obtain consistent effects of pinealectomy in all species. Pineal melatonin synthesis is probably influenced by light invading the skull and stimulating the pineal directly.

In chickens, lesion of the sympathetic nerves innervating the pineal gland does not affect the synchronization of NAT to the light : dark cycle, or the response of NAT to continuous light or brief exposure to light during the dark period. Furthermore, when chicken pineal glands were cultured in constant darkness, they exhibited a circadian NAT variation which was remarkably similar to that in the intact animal before sacrifice. It was hence proposed that the pineal glands of at least some birds have an innate timing ability independent of light and innervation [Binkley *et al*, 1977; 1978].

There is also evidence for a non-retinal pathway of light to the pineal gland in neonatal rats. As hooding abolished the effect of light in these animals, it appears that the pineal is directly photosensitive. This pathway probably controls physiological processes in the pineal gland before the eyes are fully developed [Binkley, 1981].

From the above discussion, it is evident that the evolutionary development in pineal photoreception in vertebrates reflects the

phylogenetic evolution of the anatomical structures in the pineal gland.

1.6.4 Significance of the Melatonin Rhythm

The ability of the pineal gland to convert incoming neural stimuli into outgoing hormonal messages led to the gland being classified as a neuroendocrine transducer, rather than being viewed as a true gland in the traditional sense. The latter classification would imply that it simply responds to substances in the bloodstream by secreting its own hormones into the blood. As a neuroendocrine transducer, the pineal gland transduces important photic information initially derived from the external environment into a hormonal message which can regulate numerous endogenous processes which cannot themselves perceive external, environmental conditions. The immediately obvious significance of this pineal function was related to the changing day-lengths associated with the annual progression of the seasons. By detecting the changing photoperiod, the pineal gland could control seasonal behavior by initiating physiological processes most appropriate to the external environment, e.g. reproduction and hibernation, and thereby ensure the survival of the individual and propagation of the species.

The mechanism whereby target organs interpret the daily oscillations in circulating melatonin levels is unclear. The oscillating nature of the melatonin signal may be essential for the endocrine activity of the hormone, as it has been shown in some mammals that continued availability of melatonin seems to down regulate the receptors on which it acts thereby rendering the indole physiologically impotent. A period of reduced melatonin availability is required for the receptors to become up-regulated and responsive to melatonin again [Reiter, 1989]. Three hypotheses have been proposed in an attempt to rationalize which component of

the melatonin signal is most important for the animal's physiological adjustments: the duration hypothesis, the coincidence hypothesis and the amplitude hypothesis [Reiter, 1987; 1988; 1989].

1.6.4.1 The Duration Hypothesis

The duration hypothesis proposes that it is the duration of the elevated melatonin levels that is responsible for end organ responses, particularly in relation to seasonal responses. The length of the night is directly proportional to the season of the year (short nights in summer and long nights in winter), therefore the length of the night-time rise in melatonin provides an important indication of the season (Figure 1.9). This hypothesis has been extended to include the direction of the change in elevated melatonin, i.e. whether the period is becoming longer (autumn) or shorter (spring). There is some evidence to suggest the duration of raised melatonin levels is important in controlling reproduction [Reiter, 1989].

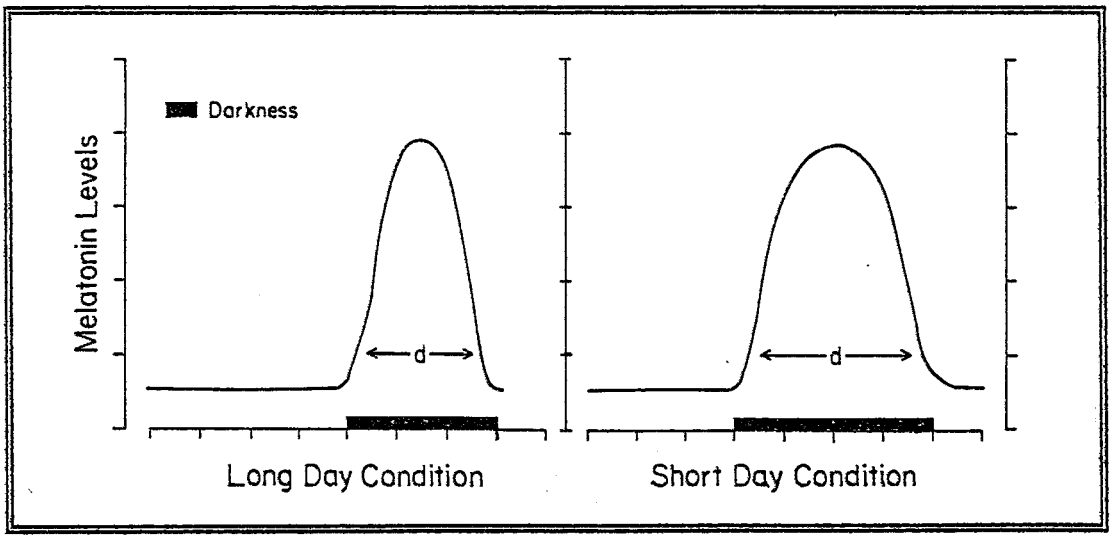

FIGURE 1.9 : Graphical Representation of the Duration Hypothesis [Reiter, 1989]

The duration hypothesis fails to explain why in many reported cases, target organs are only responsive to melatonin at certain times of the day [Redman *et al*, 1983].

1.6.4.2 The Coincidence Hypothesis

The coincidence hypothesis proposes that target organs exhibit a period or "window" of sensitivity to melatonin. According to this hypothesis, it is only when elevated melatonin levels coincide with the "window" of end-organ sensitivity, that the target organ is responsive to stimulation by the indole hormone (Figure 1.10). This theory allows target tissues to each have their own unique period of responsiveness to melatonin and explains why some

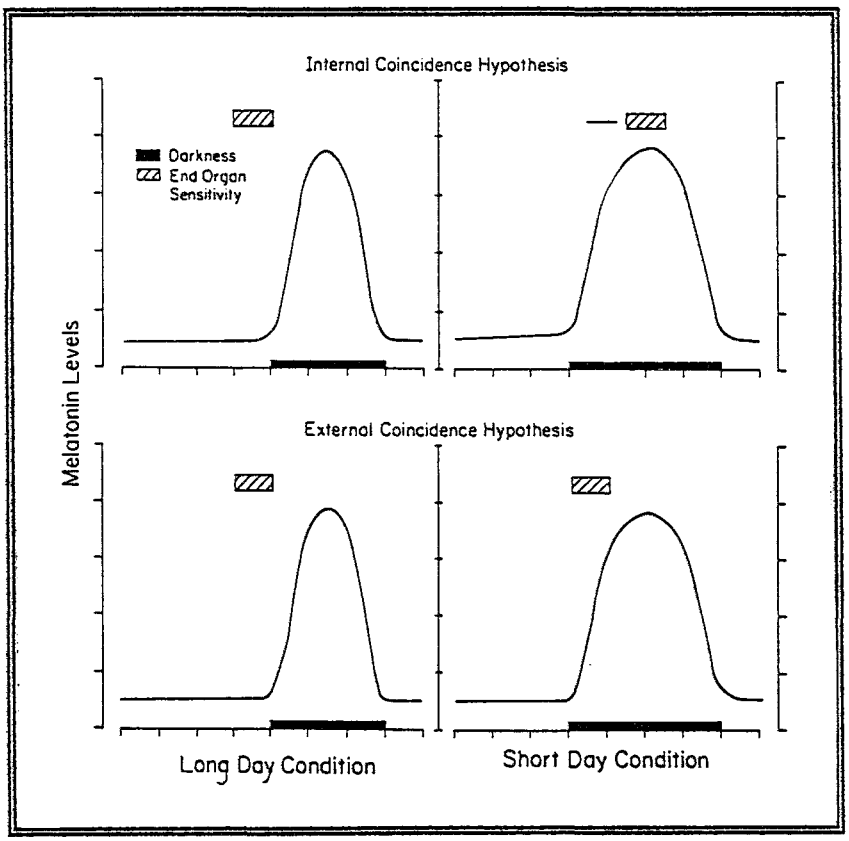

FIGURE 1.10 : <u>Graphical Representation of the Coincidence Hypothesis</u> [Reiter, 1989]

melatonin messages selectively modify the functioning of certain end organs but not others. This hypothesis requires that melatonin receptors be widespread throughout the body and only intermittently sensitive to melatonin. The receptors may be rendered insensitive by processes such as down regulation, internalization, degradation or uncoupling from effector components.

1.6.4.3 The Amplitude Hypothesis

The amplitude hypothesis suggests that it is the amplitude of the nocturnal rise in melatonin which cues different physiological processes. However, except in some human diseases, the amplitude of the rise in blood melatonin concentrations has not been shown to be highly variable.

1.7 ENDOCRINE EFFECTS OF THE PINEAL GLAND

The reported effects of the pineal indoles are numerous. However, the complex biological interactions *in vivo* have made the task of investigating the biochemical basis of the effects, a very difficult one. Moreover, pineal effects in different species are varied and generalizations are hence made with caution.

1.7.1 Tissue Distribution

An understanding of the tissue distribution of a compound is an important indication of its sites of action. In addition to its non-pineal sites of synthesis mentioned previously (section 1.5.3), melatonin has also been located in various sites in the brain, peripheral nerves and the ovaries. It is a highly lipophilic substance and hence its access to the central nervous system is not affected by the blood brain barrier. Intravenous injections of

[^{3}H]melatonin have revealed the ability of numerous organs to extract the indole from the blood. These include parts of the eye, peripheral nerves, the ovaries, the adrenal glands, and to a minor extent, other endocrine organs [cf. Burton, 1989; Reiter, 1989].

1.7.2 Binding Sites

The advent of radioligand binding assays have been an invaluable tool in identifying and quantifying melatonin receptors. Specific, saturable melatonin binding sites have been identified in synaptosomal preparations of the hypothalamus, SCN, striatum, medulla pons, hippocampus, cerebral cortex, choroid plexus, retina and the anterior pituitary [Zisapel *et al*, 1988; Vanecek, 1988]. High affinity binding sites have also been demonstrated in cytosolic fractions of the hypothalamus, hippocampus and striatum.

The presence of melatonin receptors in the hypothalamus is significant as a number of studies have implicated this region of the brain as the primary locus through which melatonin mediates many of its endocrine activities. Furthermore, a distinct diurnal rhythm in the density of melatonin binding sites has been reported in the hypothalamus [Zisapel *et al*, 1988]. This may be of physiological significance especially in respect of the coincidence theory of melatonin's influence on target tissues (section 1.6.4.2).

1.7.3 Effects of Pineal Compounds on Endocrine Axes

The effects of the pineal gland on three endocrine axes has attracted much research interest. They are the hypothalamic-pituitary-gonadal axis, the hypothalamic-pituitary-thyroid axis and the hypothalamic-pituitary-adrenal (HPA) axis. The central role of the hypothalamus in each of these axes as well as in

control of the retinal-hypothalamic-pineal axis, may provide evidence of a co-operative link between these endocrine systems.

1.7.3.1 Pineal Effects on Reproduction

The effect of the pineal gland on the reproductive system was historically the first to be recognized. In view of this, it is not surprising that the interaction between the pineal gland and the reproductive organs has enjoyed most of the research interest. This has resulted in a large accumulation of data which acknowledges the role of the pineal gland and pineal derivatives in modulation of the hypothalamic-pituitary-gonadal axis.

It has been conclusively demonstrated in both sexes that pinealectomy or any treatment which inhibits pineal function leads to premature physical maturation in juveniles, and to enlargement of the gonads and accessory organs in adults. The reproductive organs exhibit severe reduction in size and function following increased pineal activity. This antigonadotrophic effect has previously been attributed to melatonin, but recently, the involvement of other pineal indoles and peptides has been considered.

The pineal gland has been shown to depress various aspects of the hypothalamic-pituitary-gonadal axis [Blask, 1981]. The primary site of pineal-mediated antigonadal activity may be the hypothalamus. It has tentatively been proposed that melatonin acts through specific brain receptors to suppress the responsiveness of the pituitary to gonadotrophic releasing hormones and reduce the release of the gonadotrophic hormones from the pituitary.

Reiter and co-workers demonstrated that the pineal gland regulates the seasonal reproductive cycles in hamsters by utilizing the day-

length as a primary environmental cue. The shorter day-lengths which occur during the winter months enhances antigonadotrophic activity of the pineal gland. This is mirrored by the observation that arctic animals such as the seal, the walrus and the penguin have inordinately large pineal glands.

1.7.3.2 Pineal Effects on the Thyroid Gland

The thyroid gland is the end organ in the hypothalamic-pituitary-thyroid axis. The hypothalamus releases thyrotropin releasing hormone which stimulates the pituitary gland. The latter responds by releasing thyroid stimulating hormone which induces the synthesis and release of triiodothyronin and thyroxine from the thyroid gland. The thyroid hormones stimulate general metabolism in most body tissues and are essential for normal growth and development. Thyroxine stimulates enzymes in the Krebs Cycle and the Embden-Meyerhoff glycolytic pathway as well as increases lipid metabolism in rat pineal cultures [Milcu *et al*, 1968].

The general consensus is that the pineal gland exhibits an inhibitory action on the thyroid gland [Johnson, 1982]. Pinealectomy has been shown to result in enlargement of the thyroid gland in numerous mammalian species and significantly elevates plasma thyroxine levels relative to sham-operated controls [Narang *et al*, 1967; Vriend, 1983]. Intrinsic to thyroid function is the uptake of iodide from the blood and its incorporation into synthesized thyroid hormones. This factor has frequently been monitored to measure hormone production in the thyroid gland. Pinealectomy has been shown to increase iodide uptake by the thyroid gland.

The site of action of the pineal on the thyroid axis is uncertain. There is some evidence to suggest that the hypothalamus may be a

locus of pineal function [Johnson, 1982]. However, most evidence supports the hypothesis that the pineal interferes with thyroid function at the levels of the gland itself by modulating the synthesis and release of thyroid hormones.

Thyroxine and triiodothyronine added to the culture medium of incubated pineal glands have been found to increase the conversion of ^{14}C-tryptophan to ^{14}C-melatonin [Nir and Hirschmann, 1978]. The minimum effective doses of the thyroid hormones required to mediate the latter effects were considerably higher than the levels of these hormones normally present in serum. It has been suggested that the stimulation of melatonin synthesis by thyroid hormones may be a positive feedback regulatory system which would be operative under conditions of increased thyroid activity.

The HPA axis is intimately associated with the body's stress responses and will be discussed in detail in the subsequent sections.

1.8 NEUROENDOCRINOLOGICAL ASPECTS OF STRESS

The response of an animal to stress is multifaceted involving both adrenal cortical and medullary activities and generalized sympathetic discharge. Adrenocorticotropic hormone (ACTH) has been assigned a pivotal role in mediating physiological stress responses. In terms of this, a "stressor" is often defined as any noxious stimulus which results in an increase in the secretion of ACTH from the pituitary gland. The principle sites of ACTH action are the adrenal glands. In particular the adrenal cortex is known to play a vital role in maintaining physiological homeostasis and endows organisms with the capacity to resist many types of noxious stimuli and environmental changes which are essential to survival.

1.8.1 Stress and the Pineal Gland

In an attempt to define a role for the pineal gland which would explain its apparent multi-functionality, Romijn [1978] reviewed all the known and proposed endocrine activities of the pineal gland. He concluded that the pineal acts as a general tranquillizing gland to synchronize, stabilize and regulate homeostatic equilibrium in close association with changing environmental conditions. Many other researchers have proposed that the principle function of the pineal gland is to control, coordinate and regulate the physiological adaptation to stress [Milne, 1980; Armstrong *et al*, 1982], where stress is generally defined as the sum of the biological reactions to any adverse stimulus - physical, mental or emotional, internal or external - that tends to disturb an organism's homeostasis.

Theories implicating the pineal gland in the physiological response to stress are abundant, and while many are conceptually appealing, decisive evidence in their favour is still lacking. For example, a number of the lower vertebrates exhibit skin colour changes in response to some stressful stimuli to facilitate protective coloration or temperature regulation. It may be proposed that the possible involvement of melatonin in these responses reflects an antistressogenic role for the pineal gland in these animals [Johnson, 1982]. In higher vertebrates which have more sophisticated defense and adaptational systems, the target site of pineal function would no longer be the skin, but possibly a higher integrating centre such as the brain. The evolutionary changes in pineal gland structure and regulation described and discussed earlier would tend to support this theory.

Milne [1980] reported numerous physiological and cytological changes in the hypothalamus, pineal, adrenal and pituitary glands

of rats exposed to chronic auditory stress. They also examined these neuroendocrine organs from men who suffered from ulcer disease: gastric ulcer formation is considered an obvious peripheral consequence of exposure to prolonged stress. The findings were consistent with the idea that the pineal gland is antagonistic in function to the hypophysis exerting its anti-stressogenic effect by opposing the stress-induced hyperactivity of the hypothalamo-pituitary complex. Khan *et al* [1990] showed that pinealectomy exacerbates stress-induced gastric ulceration, an effect which could be counteracted by melatonin. It was further shown that rats exhibit a circadian variation in response to stress manifest by the significant reduction in gastric ulceration in rats stressed during the night relative to those stressed during the day. Lynch *et al* [1973] reported that factors which influence sympathetic activity, such as insulin-induced hypoglycaemia and immobilization stress, can rapidly increase pineal melatonin synthesis. As the rise in melatonin levels were inhibited by propranolol, it was concluded that the effects were mediated by catecholamines, either directly released onto the pineal gland or derived from sympathetic nerves terminating elsewhere in the body, or from the adrenal medulla. In view of the pivotal role of the adrenal glands in the stress response, if the pineal gland is a major regulator of physiological homeostasis and stress, an interaction between itself and the adrenal glands would be almost inevitable.

1.8.2 The Adrenal Glands

1.8.2.1 Structure of the Adrenal Glands

The adrenal glands are composed of two structurally and functionally distinct regions: a cortex responsible for the secretion of steroid hormones and a highly innervated medulla which

secretes catecholamines [Haynes and Larner, 1975; Ganong, 1979]. The cortex is further divided into three functional zones: the zona glomerulosa adjacent to the adrenal capsule and which is the site of mineralocorticoid production; the zona fasciculata which is the principal site for the synthesis and secretion of glucocorticoids; and finally, the innermost region, the zona reticularis, from which glucocorticoids and sex steroids are secreted.

1.8.2.2 Metabolism of Adrenocorticosteroids

The adrenocorticosteroids and adrenal androgens are synthesized from cholesterol via the pathway presented in Figure 1.11.

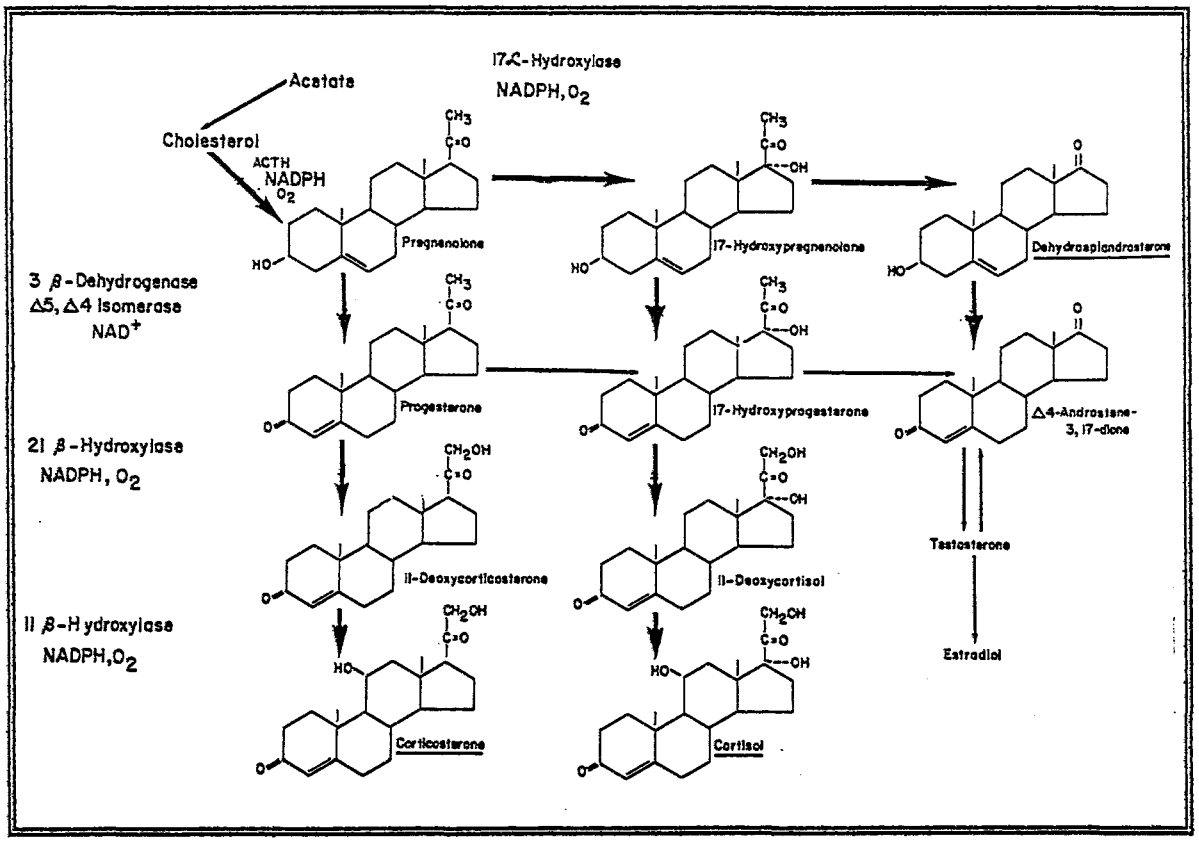

FIGURE 1.11 : Steroid Biosynthesis in the Adrenal Cortex

[Ganong, 1979]

The major adrenocorticosteroids secreted by all species of vertebrates are the mineralocorticoid, aldosterone, and the glucocorticoids, hydrocortisone or cortisol and corticosterone. The ratio of hydrocortisone to corticosterone varies from species to species. Birds, mice and rats mainly secrete corticosterone, while cats, sheep, monkeys and humans secrete predominantly hydrocortisone. Neither of the glucocorticoids are stored in the adrenal glands and for this reason the rate of their biosynthesis is tantamount to their rate of secretion and levels of physiological activity. Hydrocortisone, and to a lesser extent corticosterone, circulate in the blood system reversibly bound to an α-globulin called corticosteroid binding globulin (CGB) or transcortin. This glycoprotein has a high affinity but low total binding capacity. The corticosteroids also bind with low affinity to high capacity binding sites on serum albumin. The bound fraction appears to be inactive. It probably functions as a circulating reservoir of the hormones to lengthen their half life in the blood and maintain a readily available supply for the tissues. The half life of hydrocortisone in the blood is 60 - 90 minutes.

Glucocorticoids are principally catabolised in the liver. The ring structures are reduced to derivatives which are enzymatically conjugated to sulphate or glucuronic acid to form water-soluble sulphate esters or glucuronides which are predominantly secreted in the urine.

1.8.2.3 Actions of Adrenocorticosteroids

Glucocorticoids, like other steroid hormones, are thought to act by controlling the rate of synthesis of proteins. The glucocorticoids react with receptor proteins in the cytoplasm of sensitive cells to form a steroid-receptor complex. This complex

undergoes a conformational change and moves into the nucleus of the cells, where it binds to the chromatin material. In this way, glucocorticoids appear to stimulate the transcription and ultimately the synthesis of specific proteins. [Haynes and Larner, 1975; Ganong, 1979].

Glucocorticoids subserve widespread functions including the regulation of the mobilization of amino acids from tissue proteins, the metabolic degradation of amino acids in the liver, liver glycogen synthesis, the degradation of fats and the release of fatty acids into the bloodstream. These steroids perform a vital role in the stress response by causing tremendous shifts in carbohydrate metabolism throughout the body, which increase circulating energy substrates at the cost of stored energy. Glucocorticoids also increase cardiovascular tone, alter cognition and inhibit growth, the immune and inflammatory responses and reproduction. These changes are central to successful adaptation to acute and physical stress, as they increase readily available energy and supportive metabolism and defer energetically costly anabolism until less stressful times. The notorious fragility of organisms with adrenocortical insufficiency in adapting to stress testifies to the importance of glucocorticoids.

Mineralocorticoids play a major role in the regulation of body's sodium, potassium and water levels. The most important mineralocorticoid is aldosterone. This hormone acts directly on the kidney to increase both tubular reabsorption of sodium and urinary excretion of potassium. Sodium levels are in turn, a major determinant of water balance. Thus factors which affect mineralocorticoid levels have vital implications for the health and survival of the animal.

1.8.3 The Hypothalamic-Pituitary-Adrenal Axis

The adrenal glands are the end organs of the hypothalamic -pituitary-adrenal (HPA) axis [Haynes and Larner, 1975; Ganong, 1979]. The adrenal cortex secretes glucocorticoids as the final step in a neuroendocrine cascade initiated on perception of a stressor by the brain (see Figure 1.12). In response to a variety of stressors, the hypothalamus releases corticotropin-regulatory hormone (CRH) from nerve endings in the median eminence into the hypophyseal-portal blood vessels which transport it directly to the adenohypophysis or anterior glandular lobe of the pituitary gland. Here, CRH stimulates the release of ACTH which subsequently stimulates steroidogenesis and the secretion of corticosteroids from the cortices of the adrenal glands. ACTH acts via adenylate cyclase and a protein kinase to increase the amount of cholesterol that enters mitochondria and is converted to pregnenolone. This step is the rate determining step in the biosynthetic pathway of the adrenal steroid hormones.

Glucocorticoid levels in the blood as well as hypothalamic stores of CRH generally vary with a circadian rhythm [cf. Johnson, 1982]. In nocturnal species, levels are highest before the onset of darkness and reach their lowest levels in the early morning. In diurnal species, the glucocorticoid levels peak in the early morning and decline during the dark period [Touitou *et al*, 1982; 1983]. The daily fluctuations in the rates of secretion of hydrocortisone and corticosterone are determined by the fluctuations in the release of ACTH from the adenohypophysis. The latter is under the control of the hypothalamus and a diverse negative corticosteroid feedback mechanism involving both rapid rate-sensitive and delayed level-sensitive forms of regulation [Haynes and Larner, 1975; Sapolsky *et al*, 1986].

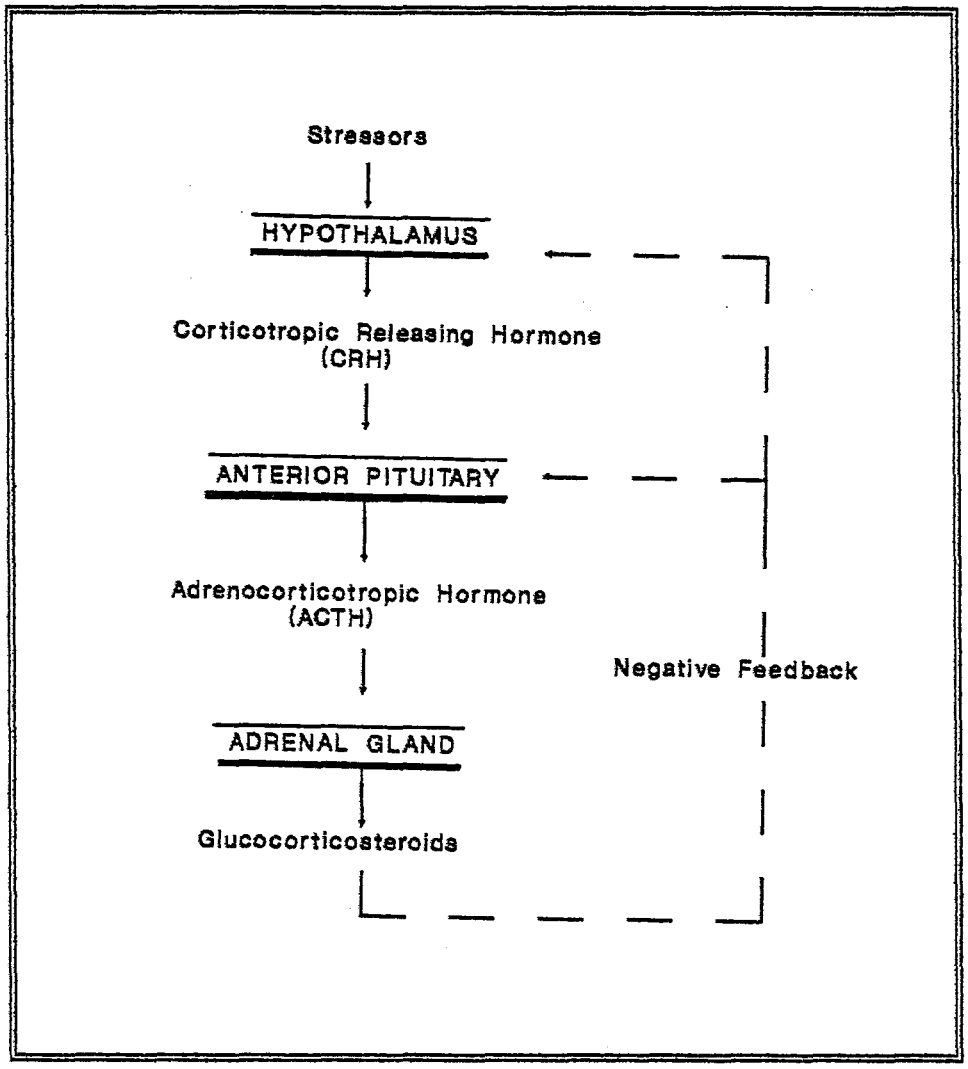

FIGURE 1.12 : The Hypothalamic-Pituitary-Adrenal Axis

[Haynes and Larner, 1975]

Glucocorticoids released from the adrenal cortex regulate the HPA axis by interacting with glucocorticoid receptors in the brain, pituitary and suprahypothalamic structures causing suppression of further CRH and ACTH release [Haynes and Larner, 1975, Keller-Wood and Dallman, 1984]. Of all the suprahypothalamic loci, the hippocampus has been most consistently implicated as an inhibitory influence upon the adrenocortical axis. It appears to regulate basal ACTH and glucocorticoid secretions as well as inhibiting the

stress-induced activation of the adrenal axis. Continual activation of the HPA axis eventually leads to a decrease in the sensitivity of this negative feedback system [Sapolsky *et al*, 1986]. This effect is due to the loss of glucocorticoid receptors in the hippocampus which subsequently results in an inability to terminate the adrenocorticoid response to stress-induced HPA activation. A major neuroendocrine consequence of long term stress is therefore the sustained elevation of circulating glucocorticoid levels (see Figure 1.13).

1.8.4 Neurotoxicity and Neuronal Degeneration

The loss of glucocorticoid receptors in the hippocampus following acute stress or elevated glucocorticoid levels, appears to be transient, presumably involving changes in receptor processing rates. However, the receptor losses following prolonged stress and in aging result from the permanent degeneration of neurons [Sapolsky *et al*, 1986].

1.8.4.1 Glucocorticoid Neurotoxicity

Sustained, elevated concentrations of circulating glucocorticoids have been shown to produce neuronal degeneration, particularly in the hippocampus. In an attempt to understand the cellular mechanisms underlying the vulnerability of neurons to glucocorticoids, Sapolsky *et al* [1986] found evidence to suggest that these steroids directly compromise neuronal viability by impairing the ability of neurons to survive damaging insults. The seemingly anomalous preference for the hippocampus may be explained by the fact that the structure has the highest concentration of corticosterone receptors in the brain [McEwen *et al*, 1968]. The actions of the glucocorticoids are at least partly mediated by glucocorticoid receptors. This is evidenced by the ability of

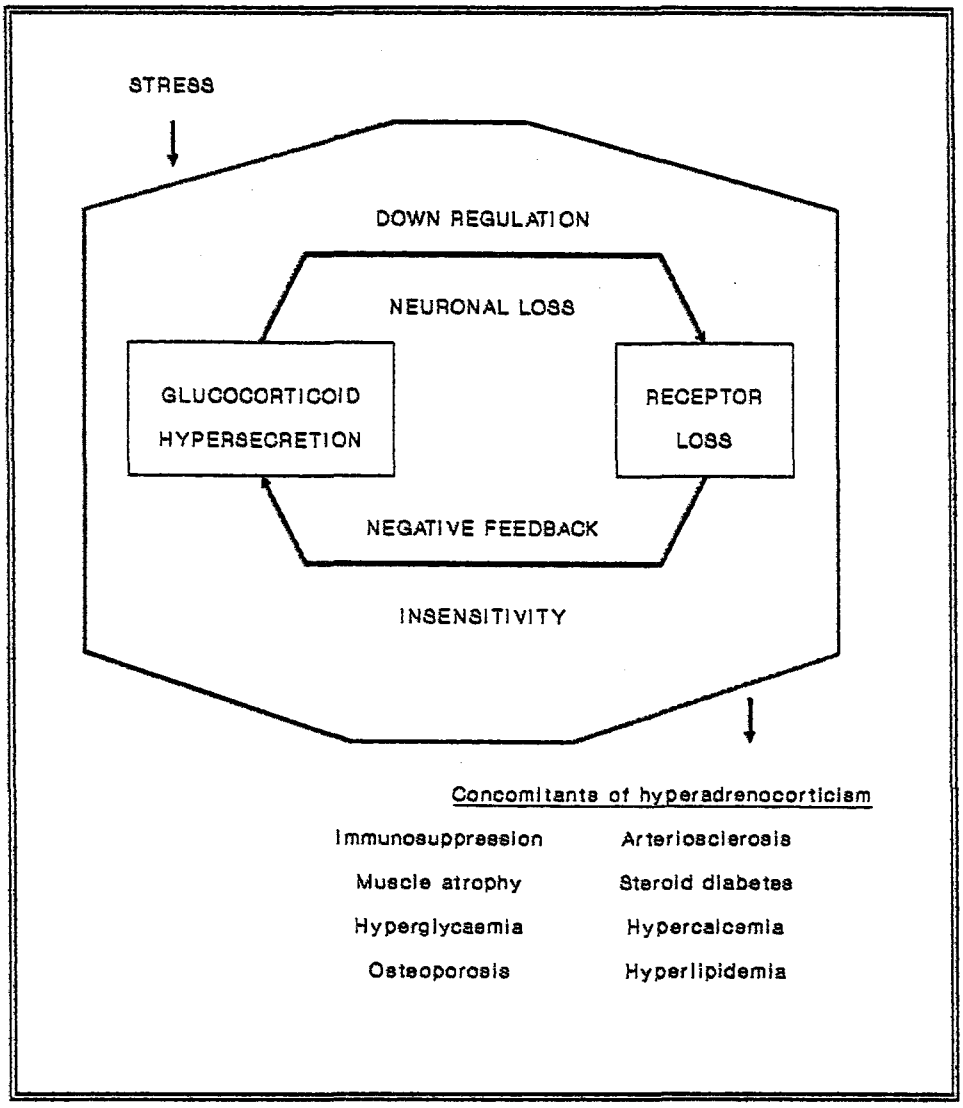

FIGURE 1.13 : Schematic Representation of the Effect of Stress on the Glucocorticoid Cascade Hypothesis

[Sapolsky *et al*, 1986]

glucocorticoid receptor antagonists to attenuate the enhanced insult-induced effects of glucocorticoids *in vitro*.

Some of the damaging actions of glucocorticoids arise from their disruption of hippocampal neuronal energy. Neurons consume energy at a high rate and are notoriously vulnerable to depletion of

energy supplies. They have limited abilities to store glycogen and are heavily reliant on blood glucose as an energy source. Glucocorticoids have been shown to significantly inhibit glucose uptake and utilization in the hippocampus, although measures of whole brain glucose content do not indicate a similar steroid action throughout the entire organ. In clinical studies, exogenous glucose has been shown to protect against hippocampal damage [cf. Sapolsky *et al*, 1986].

Glucocorticoids also contribute to neuronal degeneration by exacerbating the effects of neurotoxins [Sapolsky *et al*, 1986]. Two neurotoxins, kainic acid and 3-acetylpyridine, are more neurotoxic in rats with physiologically elevated corticosteroid levels [Sapolsky, 1985; Sapolsky and Pulsinelli, 1985]. Kainic acid is a glutamate receptor agonist. Glutamate, along with other acidic amino acids, is an excitatory neurotransmitter in the CNS [Johnson, 1972; Bradford, 1986] and glutamatergic neurons have been identified in the frontal cortex, septum, striatum and hippocampus [cf. Gilad *et al*, 1990].

1.8.4.2 Glutamate Neurotoxicity

Excitatory amino acid receptors have been divided into five subpopulations based on their affinity for a number of selective ligands [Watkins *et al*, 1990]. N-Methyl-D-aspartate (NMDA), D-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), kainate, L-2-amino-4-phosphonobutyrate (APB) receptors as well as a quisqualate metabotropic receptor subgroup have been defined. Pathological studies of neurodegenerative diseases have focused on the ion-channel-linked NMDA, AMPA and kainate receptors [Meldrum and Garthwaite, 1990]. The metabotropic receptor whose activation results in the generation of inositol triphosphates, may also be involved in neurodegeneration, but this has yet to be directly

demonstrated.

Glutamate, the most abundant excitatory amino acid in the brain, is an agonist at each of the receptor subgroups. Excitotoxicity mediated by glutamate has predominantly been ascribed to excessive activation of the NMDA receptor. Three types of neurotoxic processes have been distinguished [Schramm *et al*, 1990]. The first requires the action of high concentrations of glutamate which leads to an influx of sodium and chloride ions and water causing swelling of the neurons and lysis within 1 - 2 hours. The second is a delayed type of glutamate-induced neurotoxicity which extends over approximately 20 hours and results in extensive calcium mobilization and prolonged elevation of free cytosolic calcium levels. It only requires glutamate to initiate the process and then becomes inevitable, being unaffected by glutamate antagonists. In the third process, neuronal death occurs a few minutes after exposure to low concentrations of glutamate. The mechanism is unclear although swelling appears to be an important factor.

Glutamate released from pre-synaptic neurons is primarily inactivated by re-uptake into glial cells or astrocytes and presynaptic nerve terminals [Bradford, 1986; Gilad *et al*, 1990]. Autoradiographic and biochemical studies indicate that glutamate is preferentially taken up by glial cells. [^{3}H]Glutamate is selectively accumulated by glial cells while [^{3}H]glutamine is preferentially sequestered by neurons. [Bradford and Ward, 1976; Bradford 1986]. Both high affinity and low affinity transport systems have been identified. Glutamate is taken up into glia via the high affinity, sodium-dependent transport system and either oxidized through the Krebs cycle or converted to glutamine by glutamine synthetase. Once formed, glutamine is readily released into the extracellular space by simple diffusion from where it enters nerve terminals via a low affinity uptake system or by

diffusion. In the neurons it is converted back to glutamate by glutaminase and can be re-employed in neurotransmission. The cellular distribution of glutamine synthetase and glutaminase also favours the existence of the above mechanism of glutamate uptake and re-cycling, (see Figure 1.14).

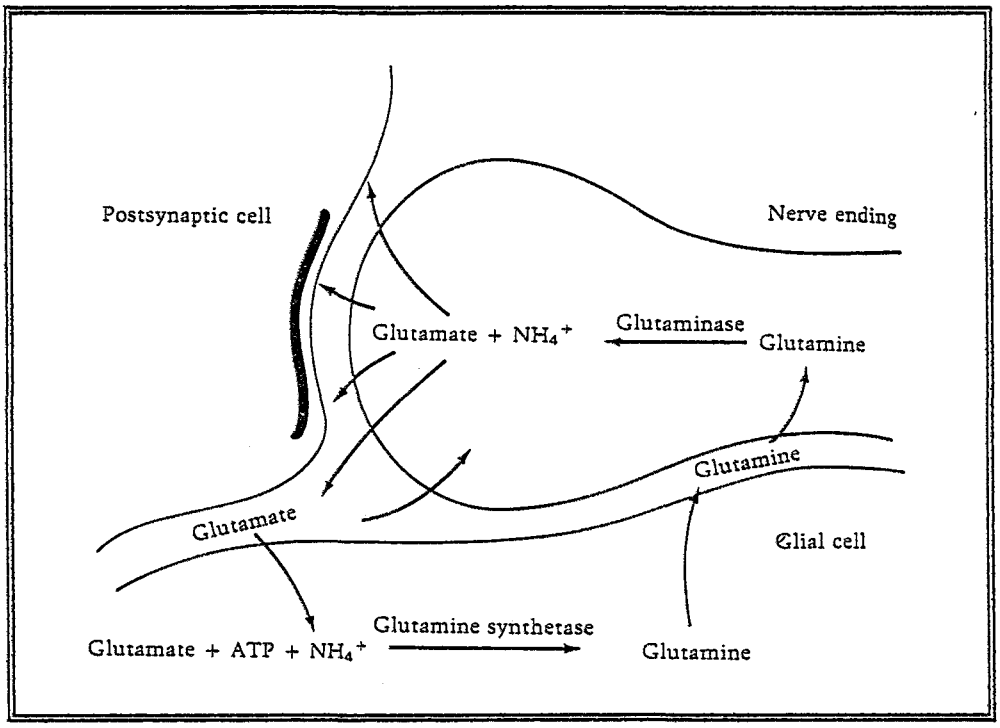

FIGURE 1.14 : <u>The Recycling of Glutamate between Neurons and Glial Cell in the Central Nervous System</u>

[Bradford, 1986]

Gilad *et al* [1990] examined changes in the glutamate release and high-affinity uptake systems in synaptosomes as an index of glutamatergic activity in various regions of the rat forebrain. The authors reported an increase in the release and high-affinity uptake of glutamate by synaptosomal preparations from several brain regions in rats which had been subjected to 1 and 2 hours of restraint stress. It was shown that restraint stress can lead to

increased glutamate uptake by neurons in the frontal cortex, septum and hippocampus, but not in the striatum. The authors concluded that forebrain glutamatergic neurons are activated by stressful stimuli in a regionally selective manner, and suggest that high-affinity uptake is important in clearing increased levels of released glutamate. However, it is known that glucocorticoids inhibit the uptake of excitatory amino acids by glial cells. Thus, in cases of chronic stress both cerebral glutamatergic activity and adrenocorticoid activity will increase. The high levels of circulating glucocorticoids may inhibit glutamate inactivation, leading to increased concentrations of the neurotoxin in the synapse and profound neuronal degeneration.

In a recent study, Marinova and co-workers [1991] investigated the effect of exogenous melatonin on glucocorticoid receptors in the hippocampus and hypothalamus of rats on long-term glucocorticoid treatment. They showed that melatonin significantly decreased the affinity of corticosterone receptors for their natural ligands. They proposed that by decreasing the sensitivity of glucocorticoid receptors, melatonin could possibly protect the neurons from the deleterious influence of elevated corticosterone concentrations as well as possibly prevent hippocampal receptor loss following chronic stress or long-term exposure to elevated circulating glucocorticoid levels. This finding strengthens the proposal that the pineal gland may play a role in regulating the effects of the adrenal glands.

1.9 PINEAL - ADRENAL GLAND INTERACTIONS

1.9.1 Effects of the Pineal Gland on Adrenal Function

The pineal gland is generally considered to exert an inhibitory or

regulatory influence on the adrenal glands. Unfortunately, much of the literature on the subject is contradictory and difficult to compare due to inadequacies in the experimental design. Conflicting evidence is likely to be due to the small magnitude of some of the reported significant differences, sex and species differences as well as variation in the length of the experiments. Most research on pineal - adrenal gland interactions has involved modifying pineal output and measuring the adrenal response.

1.9.1.1 Pineal Effects on Adrenal Hypertrophy

The first suggestion that the pineal gland might have an effect on the adrenal axis came when pinealectomized rats were reported to have enlarged adrenal glands [Wurtman *et al*, 1959]. Adrenal hypertrophy was demonstrated in mice following pinealectomy and found to be reversed by exogenous melatonin [Vaughan *et al*, 1972]. Similar effects have also been demonstrated in submammalian vertebrates. Light deprivation has been shown to decrease adrenal mass, an effect reversed by pinealectomy [Reiter and Hester, 1966]. However, these significant differences have not been universally found probably for the reasons mentioned above.

The effect of the pineal gland on compensatory adrenal hypertrophy has also been monitored. After a unilateral adrenalectomy, plasma glucocorticoid levels decrease. These lowered levels feed back on the hypothalamus and anterior pituitary gland to increase plasma ACTH levels. Hence the remaining adrenal gland becomes the target of increased ACTH levels and exhibits a compensatory increase in mass. Compensatory adrenal hypertrophy, and the thickness of the zona fasciculata in particular, are significantly reduced by treatments known to stimulate pineal function, such as blinding, and treatment with crude pineal extracts or exogenous melatonin. These effects are reversed by pinealectomy or ACTH treatment

[Dickson and Hasty, 1972; Vaughan *et al*, 1972; Johnson, 1982]. These data suggest that the site of pineal inhibitory action is at the level of the anterior pituitary or higher, since ACTH treatment reverses pineal inhibition. Furthermore, pineal extracts fail to inhibit adrenal hypertrophy resulting from exogenous administration of ACTH. It has therefore been hypothesized that pineal derivatives may regulate the adrenal axis by inhibiting the synthesis or release of this pituitary hormone.

1.9.1.2 Pineal Effects on Steroidogenesis and Plasma Corticosteroid Levels

Other researchers have proposed that the pineal functions directly by regulating steroidogenesis. Melatonin has been directly implicated in the metabolism and release of corticosteroids. Mehdi and Sandor [1977] demonstrated that melatonin could inhibit the transformation of carbon-labelled progesterone to hydrocortisone and aldosterone *in vitro*. Studies on the influence of the pineal on plasma glucocorticoid levels are difficult to interpret because of the limited number of time points assayed and the varying length of the photoperiods employed. Furthermore, it remains controversial whether manipulations of the photoperiod such as blinding, light deprivation or exposure to constant light, used to presumably alter pineal activity in many of the experiments, are able to directly induce phase shifts in the daily corticosteroid rhythm. This would lead to apparent differences in plasma corticosteroid levels which were independent of pineal activity. Although the results of these experiments must therefore be interpreted with caution, there is some evidence that the pineal gland can influence patterns of glucocorticoid secretion.

Lateral ventricular administration of melatonin and other pineal indoles was shown to reduce rat plasma corticosterone

concentrations [Motta *et al*, 1971]. Melatonin failed to affect the corticosterone response when brain catecholamines had been depleted by reserpine treatment. This finding suggested that ACTH is under the inhibitory control of a central adrenergic mechanism and that melatonin may act by stimulating this mechanism. The theory is supported by the finding that melatonin added to pituitary incubates *in vitro* does not alter ACTH secretion.

Kinson and co-workers [1967] indicated that the pineal gland may exert an inhibitory effect on the adrenal glands by demonstrating that the secretion rates of corticosterone and aldosterone were significantly elevated one month after pinealectomy. Niles and co-workers [cf. Armstrong *et al*, 1982] reported that blinding or exposure to short daily photoperiods flattened the normal diurnal plasma corticosteroid rhythm. This effect was partially reversed by pinealectomy, and completely reversed by immunization with melatonin and N-acetylserotonin antibodies. Immunization alone resulted in reduced corticosterone levels throughout the whole diurnal cycle. These findings show that the pineal gland does not simply restrict adrenal steroidogenesis, but that melatonin and N-acetylserotonin could somehow be involved in the regulation of the resting diurnal adrenocortical rhythm.

In the case of mineralocorticoids, the pineal gland has been reported to both stimulate and inhibit aldosterone secretion, while a number of researchers have failed to demonstrate any effect of the pineal gland on adrenal mineralocorticoid function. The predominant theme of research in this area has been the investigation of the pineal gland and hypertension [Johnson, 1982]. Pinealectomy results in small elevations in blood pressure from one to seven weeks after the operation, with normal levels possibly re-established after 3 months. Melatonin administered in the drinking water of pinealectomized rats from the time of surgery

prevented hypertension, and when administered after hypertension had been established, lowered the blood pressure [Zanoboni and Zanoboni-Muciaccia, 1967; Johnson, 1982]. Other studies have produced inconsistent and controversial findings which require further research.

1.9.1.3 Influence of Pineal Antigonadal effects on Adrenal Function

Another line of research proposes that the pineal gland may exert its inhibitory influences on the pituitary-adrenal axis, at least in part, by virtue of its antigonadal effects.

Ogle and Kitay [1976] investigated the influence of pinealectomy on adrenal function in intact and previously ovariectomized rats. Pinealectomy of previously ovariectomized rats appeared to increase the proportion of corticosterone secreted by altering intra-adrenal metabolism without altering the total steroid output, hypophyseal ACTH secretion or the corticosteroid feedback mechanism. However, in intact animals, pinealectomy enhanced both the resting plasma levels of corticosterone and the total steroid production, suggesting an increase in ACTH secretion. The enhancement of circulating estrogen levels which occur subsequent to pinealectomy, due to release of pineal inhibition on the ovaries, may be partly responsible for the increase in corticosterone levels. Estradiol is not only known to stimulate ACTH secretion, but is also a potent inhibitor of adrenal reductase activity. Thus the pineal gland could have affected ACTH secretion and adrenal steroid metabolism either directly or indirectly via estradiol.

1.9.1.4 Pineal influences on the stress-activated adrenal glands

There is a large body of evidence which implies that the pineal

gland functions to moderate stress-induced changes in adrenal cortical activity.

Motta *et al* [1971] showed that melatonin counteracts the increased secretion of ACTH effected by the injection of histamine, a potent stressful stimulus. They further showed that melatonin loses its ability to interfere with the stimulating effect of histamine on ACTH secretion in rats deprived of catecholamines. This, coupled with other findings, raised the suggestion that the effect of melatonin on the HPA axis might be mediated by the activation of a central adrenergic pathway normally inhibiting ACTH secretion.

In an attempt to assess the effect of the pineal on cold stress-induced changes in plasma corticosteroid levels, Rivest *et al* [1979] subjected male albino rabbits to pineal stalk lesion or sham-operation. Six days later, the rabbits were exposed to 7°C environmental temperatures. Although both groups displayed increased corticosteroid levels in response to the cold stress, the lesioned rabbits exhibited an earlier and a higher elevation. Previously Vaughan *et al* [1972] had reported that the adrenal glands of male, but not female, mice were significantly hypertrophied after exposure to 4 hour periods at 5°C for three successive days. This effect of cold stress could not be prevented by melatonin administration.

These data could suggest a role for the pineal gland in promoting homeostasis and stress adaptation, rather than a simple inhibitory effect of the pineal on the HPA axis.

1.9.1.5 Pineal Products Mediating Pineal-Adrenal Effects

Early attempts to elucidate the potential of pineal derivatives to influence the adrenal glands involved monitoring the effect of

crude pineal extracts on adrenal function. Both organic and aqueous extracts were shown to alter adrenocorticoid function. An aqueous extract was found to potently inhibit corticosterone release from incubated rat adrenal glands [Porter and Heiman, 1977]. The researchers concluded that the active substance could not be a lipophillic indole and substantiated their conclusion by showing that neither melatonin nor 5-methoxytryptophol could simulate the effects of the crude extract. The existence of a non-indole pineal factor responsible for influencing adrenocortical function has gained favour recently with the increased interest in pineal peptide hormones. The identity of many pineal peptide hormones remains to be elucidated. Arginine vasotocin is a physiologically active peptide which has been reported to inhibit compensatory adrenal hypertrophy in mice and block the action of CRH in cats, as well as inhibit ACTH release in vivo [cf. Johnson, 1982]. However, although arginine vasotocin is known to be present in submammalian vertebrates, its presence and hence functional significance in the mammalian pineal remains to be clarified.

There is extensive evidence implicating the principal pineal indole, melatonin, in modifying adrenal function. Melatonin has been demonstrated to reverse many of the effects of pinealectomy on adrenal hypertrophy and compensatory adrenal hypertrophy, although it could not significantly counteract adrenal enlargement following castration or cold stress. Melatonin treatment was reported to increase adrenal 5α-reductase activity in hypophysectomized and pinealectomized rats indicating that this indole acts at the level of the adrenal cortex to reduce glucocorticoid secretion from the gland [Ogle and Kitay, 1976; Johnson, 1982].

The injection and implantation of melatonin into various organs and sites in the central nervous system have led to reports of

inhibitory, indifferent and stimulatory effects of melatonin on adrenal function. Armstrong *et al* [1982] has cautioned that it may be necessary to distinguish between the effects of endogenous and exogenous pineal substances, and hence the effects of pineal deactivation by pinealectomy and administration of substances like melatonin. Furthermore it may also be necessary to distinguish between melatonin in the cerebral spinal fluid (CSF) and that circulating in the plasma, as it is possible that melatonin released into systemic circulation may have a different role and mode of action to that released in the CSF. When melatonin is introduced intraventricularly, evidence favours a mode of action dependent on central monoamine levels, while when melatonin is introduced peripherally, it appears to act independent of central structures.

1.9.2 Effects of the Adrenal Gland on Pineal Physiology

Relatively few studies have investigated any possible influence of adrenal derivatives on pineal function. It is conceivable that if the pineal gland were a regulator of the adrenal glands, the former should respond physiologically to altered adrenal output. Such "feedback" mechanisms are characteristic of most endocrine systems. Unfortunately, most of the scanty literature available on this aspect of pineal-adrenal interactions is contradictory, and many researchers have been unable to confirm reports of glucocorticoid effects on pineal physiology.

In 1982, Reiter and co-workers [cf. Johnson, 1982; Touitou, 1989] showed that bilateral adrenalectomy resulted in decreases in the night-time rise in pineal melatonin and N-acetyltransferase activity 8 hours into the dark phase. The inability of other researchers to confirm this finding may be the result of shorter experiments. As stress results in natural increases in the

secretions of ACTH and glucocorticoids, researchers have investigated the effect of various stresses on pineal physiology. A variety of studies have shown that exposure of rats to stressful stimuli increase N-acetyltransferase activity and melatonin production [Lynch *et al*, 1973; Vaughan *et al*, 1978]. Contrastingly, in human studies various stresses including insulin-induced hypoglycaemia, pneumonencephalography, electro-shock therapy, sleep deprivation, sprinting and psychosocial stress did not cause increases in melatonin secretion despite activation of the hypothalamic-pituitary adrenal axis [Touitou, 1989]. Studies of this nature are confounded by the number of variables involved in specific stress responses including the severity or duration of the stress, predictability of onset, control of onset or termination of the stress, preparedness in dealing with the stress, previous experience with similar or dissimilar stressors, species-specific defense mechanisms and species/strain differences. Results from these studies of pineal-adrenal interactions are thus very unlikely to produce conclusive evidence and a more direct approach is required.

Observations in disease states are equally inconclusive. An interaction between glucocorticoids and pineal derivatives has been suggested in depressive disorders. Wetterberg *et al*, [1982] reported an inverse correlation between high levels of serum hydrocortisone and low levels of serum melatonin in depressive patients. However, these correlations are not evident when patients are treated with antidepressant drugs [Touitou, 1989]. Adrenal insufficiency and pituitary dysfunction have been described in some patients with pineal tumour. However, in these cases, parameters such as age and sex are often variable, interfering factors. [Touitou, 1989].

From the preceding discussion, it is obvious that the detailed

nature and characteristics of pineal-adrenal interaction remain unclear at this time. Although much effort has been expended in its study, the complexity of the individual endocrine axes involved, the host of contradictory findings and the lack of a clear, unambiguous paradigm to dramatically and reproducibly demonstrate interactions between these two systems, have delayed the eludication of the relationship between the pineal and adrenal glands.

1.10 TRYPTOPHAN PYRROLASE AND THE PINEAL GLAND

Tryptophan pyrrolase is a major determinant of tryptophan levels in vivo. Recent evidence has indicated that it may be a peripheral mediator of pineal gland function.

1.10.1 Tryptophan Pyrrolase

Tryptophan pyrrolase (L-tryptophan oxygen 2,3-oxidoreductase, EC (1.13.11.11) is a haem-dependent liver cytosolic enzyme which catalyzes the oxidative cleavage of the pyrrole ring of L-tryptophan to form N'-formylkynurenine in the first and rate-determining step in the kynurenine-nicotinic acid pathway of tryptophan degradation [Badawy and Evans, 1975]. More than 90% of the body's total tryptophan is degraded in the liver via this pathway (see Figure 1.15). In the livers of certain, but not all animal species, tryptophan pyrrolase exists in at least two forms: the active reduced holoenzyme and the predominant, inactive apoenzyme which requires the addition of exogenous haematin for the demonstration of its activity [Badawy and Evans, 1974]. Both forms are present in the livers of rats, mice, chickens and possibly man, while the apoenzyme is absent from the livers of guinea pigs, hamsters, rabbits, sheep, frogs, cats and the gerbil. Activation

of the apoenzyme *in vitro* involves its conjugation with haem to form the oxidized (ferrihaem) holoenzyme which is then reduced to the active form in the presence of tryptophan.

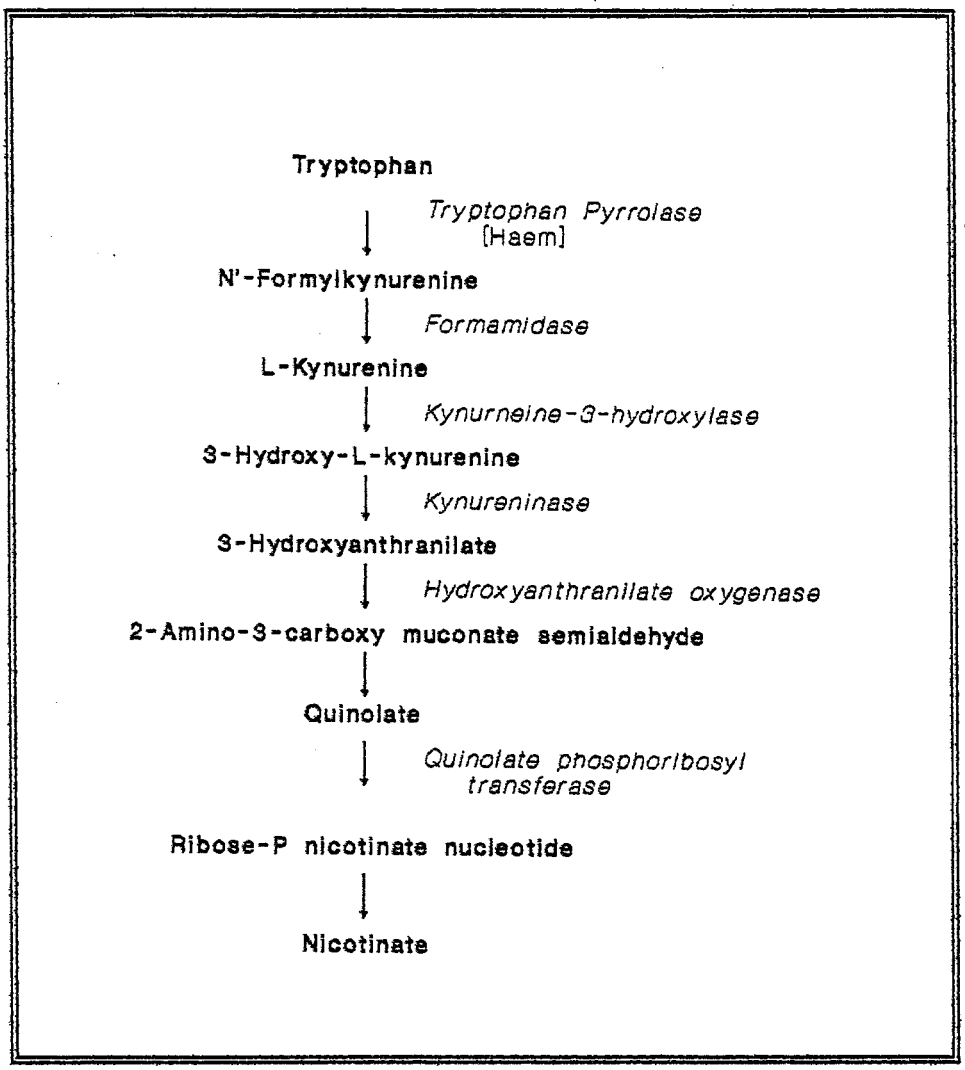

FIGURE 1.15 : The Kynurenine-Nicotinic Acid Pathway

[Bender, 1975]

1.10.2 Regulation of Tryptophan Pyrrolase

Rat liver tryptophan pyrrolase is regulated by four known mechanisms: hormonal induction by glucocorticoids, substrate

activation by tryptophan, cofactor activation by haem and feedback inhibition by NADPH [Badawy, 1979].

The glucocorticoids, hydrocortisone and corticosterone, cause a hormonal induction of tryptophan pyrrolase involving the synthesis of new apoenzyme [Knox and Auerbach, 1955; Young, 1981]. This hormonal induction mechanism is not found in species lacking the apoenzyme [Badawy and Evans, 1974]. Tryptophan pyrrolase exhibits a diurnal rhythm which correlates closely with plasma corticosteroid concentrations [Wurtman, 1974; Salter and Pogson, 1985]. However, it is clear that the basal capacity of the liver to metabolize tryptophan is unaffected by the absence of glucocorticoids although larger tryptophan loads are handled much less effectively by adrenalectomized rats. This is presumably due to the fact that they lack the ability to induce the steroid-mediated increases in tryptophan pyrrolase activity.

Glucocorticoids produce an RNA-dependent increase in total pyrrolase activity by stimulating the synthesis of new apoenzyme. Only about 50% of the new apoenzyme is saturated with haem so that the ratio of holoenzyme/apoenzyme is maintained at basal levels. Glucocorticoids do not increase haem synthesis or the saturation of apoenzyme with its cofactor.

The substrate activation of tryptophan pyrrolase involves decreased degradation of pre-existing apoenzyme in conjunction with its normal rate of synthesis. This is accompanied by an initial increase in haem saturation and stabilization of the enzyme [Badawy and Evans, 1975]. The tryptophan-mediated effects are inhibited by protein synthesis inhibitors although tryptophan pyrrolase synthesis itself appears to be unaffected. Some evidence suggests that tryptophan may increase the availability of haem for tryptophan pyrrolase by stimulating the synthesis of haem

biosynthetic enzymes which subsequently result in enhanced synthesis of the cofactor [Badawy *et al*, 1981].

Conjugation of tryptophan pyrrolase with its cofactor, haem, is the first step in the activation of the apoenzyme. The saturation of apo-tryptophan pyrrolase with haem is modified by treatments causing destruction, inhibition of synthesis, increased utilization and enhanced synthesis of liver haem. The administration of haematin or its precursor, 5-aminolaevulinate (5-ALA), enhances the activity of tryptophan pyrrolase [Badawy and Evans, 1975]. Utilization of endogenous haem, releases the negative feedback inhibition by this porphyrin on 5-aminolaevulinate synthase, the rate-limiting enzyme in its synthetic pathway. This leads to further synthesis of haem and enhanced activity of tryptophan pyrrolase. Drugs (e.g. barbiturates) which are detoxified in the liver by the action of microsomal haem-dependent cytochrome P450 increase hepatic turnover of haem and similarly enhance tryptophan pyrrolase activity.

1.10.3 Tryptophan Pyrrolase and Depression

Liver tryptophan pyrrolase activity is the major peripheral factor that influences circulating tryptophan levels. It has been established that the availability of tryptophan to the brain is in turn a major determinant of cerebral tryptophan and serotonin levels. This followed from the finding that tryptophan uptake by the brain is mediated by a saturable carrier system which is responsible for transporting all the large neutral amino acids across the blood brain barrier. Since one species of carrier molecule facilitates the transport of six amino acids, tryptophan must compete with tyrosine, phenylalanine, leucine, isoleucine and valine for attachment and uptake into the brain. Furthermore, tryptophan hydroxylase, the rate-limiting enzyme in the pathway of

serotonin synthesis, is not fully saturated with tryptophan under normal physiological conditions. Activation of liver tryptophan pyrrolase has been shown to decrease forebrain serotonin levels [Curzon, 1969; Young, 1981; Badawy *et al*, 1987; Daya *et al*, 1989; Van Wyk *et al*, 1991]

The cerebral serotoninergic system is intricately involved in various behavioural conditions of which its involvement in mental disorders has generated the most interest [Curzon, 1969; Goodwin and Post, 1974; Van Praag, 1982]. The highly substantiated indoleamine hypothesis of depressive disorders states that depression is associated with a deficiency of brain serotonin [Skene, 1979]. Numerous factors which stimulate liver tryptophan pyrrolase activity and subsequently reduce tryptophan availability to the brain, have been associated with depressive disorders. High plasma corticosteroid levels which stimulate tryptophan pyrrolase activity have been found to occur in depression [Hullin *et al*, 1967], and depressive episodes are frequently found in patients with primary disturbances of the hypothalamic-pituitary-adrenal axis. Immobilization stress which leads to an increase in circulating corticosteroid concentrations, has also been shown to cause tryptophan pyrrolase activity to rise and brain serotonin levels to fall [Curzon and Green, 1969]. Badawy and Evans, [1981] established that a large number of antidepressant drugs increase brain tryptophan concentrations by inhibiting liver tryptophan pyrrolase activity. This inhibition appears to be due to the prevention of the conjugation of the apoenzyme with haem.

1.10.4 Tryptophan Pyrrolase and the Pineal Gland

The diurnal rhythm of tryptophan pyrrolase activity is consistent with an inhibitory effect of the pineal gland on the enzyme. The highest activity is observable during the late hours of the light

period when melatonin levels are lowest. Enzyme activity is lowest towards the end of the dark period when melatonin levels are high [Curzon, 1979].

It has been shown that activation of liver tryptophan pyrrolase by the barbiturate, pentobarbitone [Van Wyk *et al*, 1991], and by 5-ALA [Daya *et al*, 1989], decreases forebrain serotonin levels without affecting pineal serotonin levels . Although derived from the same precursor, pineal serotonin and melatonin levels are highly conserved. Furthermore, Daya *et al* [1989] reported that forebrains from 5-ALA treated animals removed during the night-time, had significantly higher cerebral tryptophan and serotonin levels than those removed during the day-time. As the night-time rise in brain indoles coincided with the natural rise in pineal melatonin levels, it was proposed that melatonin could be responsible. However, the increases in brain indoles were not prevented by continuous exposure of the animals to light on the day of the experiment, and were reversed by injection of melatonin [Daya *et al*, 1990]. Although the results indicated that melatonin was not responsible for the nocturnal rise in brain indoles in the 5-ALA treated animals, the possibility of melatonin regulating tryptophan uptake by the brain could not be disregarded. Recently, Walsh and Daya [1991] demonstrated that melatonin displaces tryptophan from bovine serum albumin *in vitro* suggesting the possible potential of melatonin to effect the same displacement *in vivo*. This provides preliminary evidence for a mechanism whereby melatonin may increase tryptophan availability to the brain and elicit antidepressant effects.

Wurtman and Wurtman [1989] implicated melatonin in cyclic mood and appetite disorders like seasonal affective disorder (SAD) and carbohydrate craving obesity (CCO). They reported that the depression and carbohydrate craving associated with seasonal

affective disorder, a syndrome predominantly found during the winter months, could be effectively eliminated by extending the photoperiod and treating patients with supplemental light therapy. These reports are not necessarily in conflict with the antidepressant effects of melatonin suggested by the previous researchers. It is possible that prolonged elevation of melatonin results in large increases in plasma tryptophan levels and subsequent elevation of liver tryptophan pyrrolase activity.
A further possibility of melatonin acting directly on the central nervous system in depression, cannot be excluded.

1.11 EXPERIMENTAL OBJECTIVES

The objective of this study was to investigate the interaction between the pineal and adrenal glands. While much research has focused on the effect of the pineal gland on adrenal physiology, little work has been done on the effects of the adrenal steroids on pineal physiology and biochemistry. The first part of this study is based on the assumption that if the pineal gland were to regulate adrenal steroid output, it would probably be capable of detecting and responding to changes in plasma corticosteroid levels. The first aim was therefore to establish whether adrenal glucocorticoids can alter pineal physiology. The effect of chronic hydrocortisone treatment on rat pineal melatonin synthesis was studied.

The second objective was to determine whether the principal pineal indoleamine, melatonin, can protect against some of the deleterious effects of sustained, elevated plasma glucocorticoid levels. It has been proposed that such levels of corticosteroids may contribute to mental disorders by stimulating liver tryptophan pyrrolase to increase the catabolism of tryptophan, thereby

reducing the availability of this vital serotonin precursor to the brain. The effect of chronic hydrocortisone treatment and melatonin on rat liver tryptophan pyrrolase activity was investigated.

Another effect of chronically elevated plasma glucocorticoid levels, is to elicit neuronal degeneration by inhibiting the uptake of excitatory amino acid neurotransmitters from the synaptic clefts in the CNS. The resultant continuous stimulation of particularly postsynaptic glutamate receptors, causes an influx of ions and water into the neurons and ultimately, lysis of the cells. It was intended to ascertain the effect of chronic hydrocortisone treatment and melatonin on cerebral glutamate receptors in the male albino rat.

Finally, with the recent discovery of glutamate receptors in the pineal gland, an attempt was made to investigate the physiological significance of these receptors.

CHAPTER 2

EXPERIMENTAL PROCEDURES

2.1 ANIMALS

The animals employed throughout this study were male Wistar rats of the albino strain. Female rats were not used, in order to eliminate the variable influences of the female hormonal cycle. The animals were housed in opaque plastic cages with metal grid floors and covers. They had free access to Epol rat pellets and tap water. The animal room was well ventilated and artificially illuminated by cool white tubes which were automatically regulated to maintain a 12 hour light/ 12 hour dark cycle (lights on at 06h00). The intensity of the illumination during the light phase was approximately 300 $\mu Watts/cm^2$. The temperature of the animal room was maintained between 20 and 24°C. The rats were killed swiftly by cervical dislocation and rapidly decapitated. The top of the skull was removed by making an incision through the bone on either side of the head from the foramen magnum to near the orbit. The skull was lifted with a pair of forceps exposing the pineal gland and brain for removal with tweezers. Care was taken to remove all adhering tissue and visible traces of blood from the organs. To remove the livers, a mid-ventral incision was made through the abdominal musculature from the pelvic region to the posterior edge of the sternum. A transverse cut was made anteriorly to expose the liver which was carefully removed and dissected free of blood vessels and connective tissue.

When assays were not conducted immediately, all organs were frozen

in liquid nitrogen and stored at -70°C. Freezing did not alter the activity of the enzyme.

2.1.1 Administration of Hydrocortisone to rats

Hormonal treatment was provided for 7 days according to the scheme of Marinova *et al* [1991]. Hydrocortisone was suspended in absolute ethanol : glycerine (1 : 9) to give a homogeneous stock suspension containing 6 mg hydrocortisone/ml. Before use it was diluted in 0.5% saline to a final concentration of 60 µg/ml and provided to the animals as drinking fluid. Control animals received an equivalent volume of vehicle in 0.5% saline.

This technique is advantageous in that it avoids the stress effects of handling and injection. Hydrocortisone is effectively absorbed when given by mouth and may be administered orally in clinical treatments [Haynes and Larner, 1975]. The daily oral dose of on average 2.5 mg per rat exceeds that which has previously been shown to produce supraphysiological circulating levels of the glucocorticoid [Marinova *et al*, 1991].

2.2 THE MEASUREMENT OF RAT PINEAL GLAND SEROTONIN METABOLISM IN ORGAN CULTURE USING THIN LAYER CHROMATOGRAPHY

2.2.1 Introduction

Tissue and organ culture techniques are invaluable tools in biochemistry. They allow the researcher to finely control the experimental conditions *in vitro* and avoid the complications of complex *in vivo* interactions. The pineal gland is particularly suitable for organ culture studies by virtue of its small size and accessibility. Pineal organ culture systems have therefore been optimized and utilized by numerous researchers for various purposes [Klein and Rowe, 1970; Daya and Potgieter, 1982; Daya *et al*, 1989a]. The pineal gland in organ culture maintains its metabolic function and under the correct conditions may remain viable for more than six days. It is able to utilize an exogenous radioactive precursor such as [^{14}C]tryptophan or [^{14}C]serotonin, and synthesize various indole metabolites. Approximately 95% of the synthesized radioactive indoles are secreted into the culture medium during incubation. The radioactive indoles can then be isolated from the culture medium and quantified.

Previously, separation of pineal indoles was achieved using preparative organic extractions followed by chromatographic separations using combinations of thin layer adsorbants, paper and different solvents. Currently, the preferred method employs a bi-dimensional thin layer chromatography system [Klein and Notides, 1969]. It employs two organic solvents, the first of which separates MEL from NAS and the 5-hydroxyindoles from the 5-methoxyindoles. The inclusion of one part glacial acetic acid facilitates the complete separation of 5-MIAA from 5-HIAA. The second solvent improves the separation of MIAA and MTOH from MEL and the separation of HTOH and HIAA from NAS. The small amount of

acid which remains on the gel from the first solvent enhances the separation by the second solvent. Tryptophan, 5-HTP, 5-HT and 5-MT are unaffected by either solvent and remain at the origin. This chromatographic technique is quick and simple to use and effectively separates trace quantities of the indoles (1 pmole/10 µl).

In this study, pineal glands were cultured by the method previously described by Klein and Notides [1969] and modified by Daya and Potgieter [1982]. The radioactive indole metabolites were isolated using the bi-dimensional thin layer chromatography method developed by Klein and Notides [1969] and quantified using liquid scintillometry.

2.2.2 Materials

2.2.2.1 Animals

Male Wistar rats of the albino strain weighing 250 - 300 g were randomly assembled into test groups of five and maintained as described previously (Section 2.1). The animals were sacrificed between 10h00 and 11h00 for use in organ culture experiments.

2.2.2.2 Chemicals and Reagents

5-Hydroxy(side-chain-2-^{14}C)tryptamine creatine sulphate was purchased from Amersham International (England). The specific activity was 53 mCi/mmol and the radioactive concentration was 50 µCi/ml. BGJb culture medium (Fitton-Jackson modification) was purchased from Gibco (Europe) and aseptically fortified with the antibiotics streptomycin, penicillin and amphotericin B. The composition of this medium is presented in Table 2.1.

TABLE 2.1 : Composition of BGJb Culture Medium (Fitton-Jackson modification)

CONTENTS	CONCENTRATION (mg/ml)
Amino Acids	
L-Alanine	250.00
L-Arginine	175.00
L-Aspartic Acid	150.00
L-Cysteine HCl	90.00
L-Glutamine	200.00
Glycine	800.00
L-Histidine	150.00
L-Isoleucine	30.00
L-Leucine	50.00
L-Lysine HCl	240.00
L-Methionine	50.00
L-Phenylalanine	50.00
L-Proline	400.00
L-Serine	200.00
L-Threonine	75.00
L-Tryptophan	40.00
DL-Valine	65.00
Vitamins	
α-Tocopherol phosphate	1.00
Ascorbic Acid	50.00
Biotin	0.20
Calcium pantothenate	0.20
Choline chloride	50.00
Folic acid	0.20
Inositol	0.20
Folic acid	20.00
Para-aminobenzoic acid	2.00
Pyridoxal phosphate	0.20
Riboflavin	0.20
Thiamine HCl	4.00
Vitamin B_{12}	0.04
Inorganic Salts	
Dihydrogen sodium ortho-phosphate	90.00
Magnesium sulphate ($7H_2O$)	200.00
Potassium Chloride	400.00
Potassium dihydrogen phosphate	160.00
Sodium bicarbonate	3 500.00
Sodium chloride	5 300.00
Other Compounds	
Calcium lactate	555.00
Glucose	10 000.00
Phenol red	20.00
Sodium acetate	50.00

Aluminium 0.25 mm Kieselgel 60 G_{254} thin layer chromatography plates were purchased from Merck (Germany) and Packard® Scintillator 299™ liquid scintillation cocktail was purchased from Packard Instrument Co Inc. (Netherlands).

The Indole standard solution was prepared by dissolving approximately 1 mg of each of the serotonin metabolites: 5-HT, NAS, MEL, 5-HIAA, 5-HTOH, 5-MIAA and 5-MTOH in 2.5 ml absolute ethanol. This was mixed with 2.5 ml of 1% ascorbic acid in 0.1 N HCl as an antioxidant and stored at -20°C. The indoles were purchased from Sigma Chemical Co (St. Louis, USA).

Van Urk's Reagent was prepared by dissolving 2 g para-dimethylbenzaldehyde in 100 ml 25% HCl followed by the addition of 100 ml 95% ethanol.

Unless specified, all chemicals and reagents were purchased from local commercial sources.

2.2.3 Organ Culture of Rat Pineal Glands

A schematic representation of the organ culture procedure is presented in Table 2.2. After sacrifice, the pineal glands of the rats were swiftly removed as described previously (section 2.1). Each pineal gland was individually placed in a 10 mm x 12 mm Kimble tube containing 52 µl of BGJb culture medium and 0.4 µCi of [^{14}C]serotonin. In experiments involving the addition of a test compound, 10 µl of the test compound was added to the incubation medium to give the desired final concentration in the total volume of 70 µl. In control vials, the test compound was substituted with 10 µl of vehicle. A blank containing no tissue was also included in each series of incubations to determine background radioactivity. The incubation vials were then gassed with carbogen

(5% carbon dioxide : 95% oxygen), immediately sealed and incubated at 37°C in the dark for 24 hours. The incubation was terminated by removal of the pineal glands from the culture medium which was then analyzed using thin layer chromatography.

TABLE 2.2 : Scheme of Organ Culture Procedure

REAGENTS	BLANK	CONTROL GROUP	TEST GROUP
BGJb Medium	52 µl	52 µl	52 µl
	Introduce pineal gland into medium		
Test compound(s)	10 µl	---	10 µl
Vehicle(s)	---	10 µl	---
[^{3}H]Serotonin	8 µl	8 µl	8 µl
	Gas tubes with 5% CO_2 : 95 O_2 Incubate at 37°C for 24 hours Remove pineal from medium Store in freezer until assay		

2.2.4 Thin Layer Chromatography of Radioactive Indoles

A 10 µl aliquot of the culture medium was applied to a 10 x 10 cm chromatography plate to form a 4 - 5 mm spot. A 10 µl aliquot

of the solution containing the indole standards was subsequently spotted on top of the culture medium. The spotting process took place under a continual gentle stream of nitrogen to aid with drying the spots and prevent atmospheric oxidation of the indoles. The TLC plate was then placed in a saturated TLC tank containing chloroform : methanol : glacial acetic acid (93 : 7 : 1) and allowed to develop until the solvent front had moved approximately 9 cm. The plate was dried under nitrogen and re-developed in the same solvent a second time. Following this, the plate was again dried under nitrogen and developed once in ethyl acetate at right angles to the first direction. The total front movement in the second direction was approximately 5 cm.

After drying under nitrogen, the plate was sprayed lightly with Van Urk's reagent and dried in an oven at 60°C until the 6 spots coinciding with the 6 indole stands were clearly visible. The spots were cut out and placed into plastic scintillation vials. Thereafter, 3 ml of scintillation cocktail were added to each vial, which was then shaken for 30 minutes to elute the radioactive metabolites from the cuttings. The entire procedure was performed under subdued light. The radioactivity was quantified in a Beckman LS 2800 scintillation counter.

2.2.5 Results

A typical bi-dimensional thin layer chromatogram of the pineal indole metabolites is presented in Figure 2.1. The indoles are clearly separated from each other into characteristic green and blue spots. Approximately 90 - 95% of the radioactivity was recovered and blank values were negligible, representing less than 0.02% of the total radioactive counts.

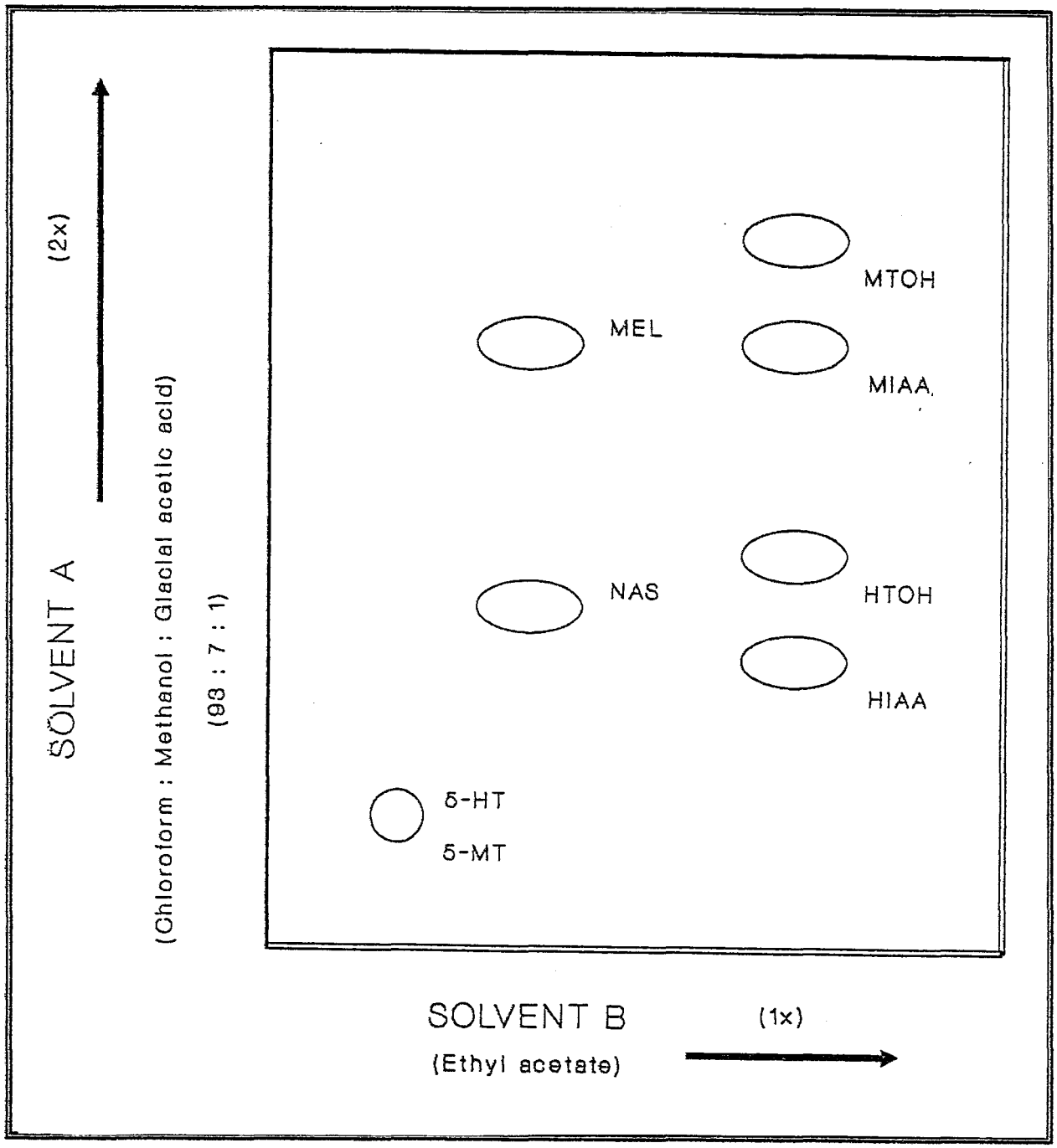

FIGURE 2.1 : A typical bi-dimensional thin layer chromatogram of the pineal indole metabolites

Abbreviations:

MEL	melatonin	**NAS**	N-acetylserotonin
5-HT	serotonin	**5-MT**	5-methoxytryptamine
HIAA	5-hydroxyindole acetic acid	**HTOH**	5-hydroxytryptophol
MIAA	5-methoxyindole acetic acid	**MTOH**	5-methoxytryptophol

Results from these studies are expressed as dpm/10 µl medium for each indole. The data were statistically analyzed by one-way analysis of variance and statistical differences between groups were determined using the Student's t-test. Results are expressed as the mean of the five separate incubations ± SEM.

2.2.6 Discussion

The pineal organ culture technique is a simple and effective method of monitoring pineal metabolism. Care should be taken to maintain aseptic conditions at all times and the culture medium should be analyzed as soon as possible after termination of the incubation to prevent sizable losses of radioactivity. These losses are probably predominantly due to oxidative degradation of the indole metabolites.

The bi-dimensional thin layer chromatography technique yields very good separations of the individual pineal indole metabolites when the original spots are kept as small as possible and the solvents are fresh and uncontaminated.

2.3 THE DETERMINATION OF PINEAL SEROTONIN N-ACETYLTRANSFERASE ACTIVITY

2.3.1 Introduction

Serotonin N-acetyltransferase (NAT) is an important, regulatory enzyme in the biosynthetic pathway of melatonin in the pineal gland. It is responsible for catalysing the conversion of serotonin to N-acetylserotonin with acetyl coenzyme A acting as the acetyl donor.

This enzyme has been assayed by a number of methods. The method used in this study is based on that developed by Deguchi and Axelrod [1972c]. NAT is quantified by the rate at which it catalyzes the N-acetylation of tryptamine HCl with [^{3}H]acetyl coenzyme A acting as the acetyl donor, to form radioactive N-acetyltryptamine. Tryptamine is used in place of the natural substrate, serotonin, because it is more readily utilized by the enzyme. The labelled N-acetyl-[^{3}H]tryptamine can then be extracted into an organic solvent and the radioactivity measured by liquid scintillometry.

2.3.2 Materials

2.3.2.1 Animals

Male Wistar rats of the albino strain weighing 250 - 300 g were randomly assembled into test groups of five and maintained as described previously (Section 2.1). The animals were sacrificed between 10h00 and 12h00.

2.3.2.2 Chemicals and Reagents

[^{3}H]Acetyl-coenzyme A was purchased from Amersham International (England). The specific activity was 3.9 Ci/mmol and the radioactive concentration was 50 µCi/200 µl. Tryptamine HCl was purchased from Sigma Chemical Co. (St. Louis, USA) and Packard^R Scintillator 299^{TM} liquid scintillation cocktail from Packard Instrument Co Inc. (Netherlands). All other chemicals were obtained from local commercial sources.

2.3.3 N-Acetyltransferase Assay Procedure

The pineal glands were swiftly removed from the rats after sacrifice as previously described (section 2.1). Each pineal gland was individually placed in a small glass homogenizer containing 100 µl of ice cold 50 mM sodium phosphate buffer, pH 6.8. The pineals were then homogenized on ice for approximately 30 seconds until a homogeneous solution was obtained. A 20 µl aliquot was transferred to an Eppendorf tube. 10 µl of sodium phosphate buffer containing 2.8 mM tryptamine HCl and 40 nCi [^{3}H]acetyl coenzyme A was placed on the side of the Eppendorf tube. The surface tension was sufficient to keep the drops of homogenate and working solution separate until the tubes were tapped and quickly vortexed to thoroughly mix the two liquids. This ensured a constant incubation time. Each assay was performed in triplicate and blanks were included in which the pineal homogenate was substituted with 20 µl of 50 mM sodium phosphate buffer, pH 6.8. The tubes were incubated at 37°C for 45 minutes in a shaking water bath. The reaction was terminated by the addition of 100 µl chilled 0.2 M borate buffer, pH 10. 1 ml of toluene : isoamyl alcohol (97 : 3) was then added to each tube which was subsequently sealed and shaken for 5 minutes. The resulting emulsion was centrifuged at 2000 x g for 30 seconds in a Hettich Universal II centrifuge. The supernatant

was washed by the addition of a further 100 µl of borate buffer, pH 10, re-shaken for 5 minutes and re-centrifuged at 2000 x g for 30 seconds. A 0.5 ml aliquot of the supernatant was transferred to a plastic scintillation vial containing 3 ml of scintillation cocktail. The vials were shaken for 10 minutes and the radioactivity quantified in a Beckman LS 2800 scintillation counter. The procedure is summarized in Table 2.3.

In experiments involving the evaluation of a test compound on N-acetyltransferase activity *in vitro*, pineal glands removed from the rats following sacrifice were incubated with the test compound for 6 hours in organ culture as described in section 2.2.3.

2.3.4 Results

The cpm obtained from the enzyme assay were converted to dpm by correcting for the counting efficiency of the scintillation counter and blank values were subtracted from the sample values. The dpm were converted to Curies (1 Curie = 2.2×10^{22} dpm) and subsequently to moles of N-acetyltryptamine using the specific activity of the [^{3}H]acetyl coenzyme A (1 mole = 3.9×10^2 Ci). Corrections were also made for using one fifth of the pineal homogenate and for incubating the enzyme for 45 minutes. The final results were expressed as pmoles N-acetyltryptamine formed per pineal gland per hour. Triplicate determinations were found to agree closely and were averaged before the data were statistically analyzed by one-way analysis of variance. Statistical differences between groups were determined using the Student's t-test. Results are expressed as the mean of the five incubations ± SEM. Preliminary time course studies indicated a linear increase in the amount of N-acetyl-[^{3}H]tryptamine formed with time. Blank values obtained in the absence of enzyme were low indicating that a negligible amount of [^{3}H]acetyl coenzyme A was extracted into the solvent.

TABLE 2.3 : **Schematic Representation of N-Acetyltransferase Assay**

REAGENTS	BLANK	SAMPLE
Homogenate	---	20 µl
Sodium phosphate buffer	10 µl	---
Working solution*	10 µl	10 µl
Vortex for 10 seconds Incubate at 37°C for 45 minutes		
Borate buffer	100 µl	100 µl
Toluene:Isoamyl alcohol	1 ml	1 ml
Shake for 5 minutes Centrifuge of 30 seconds at 2000 x g		
Borate buffer	100 µl	100 µl
Shake for 5 minutes Centrifuge for 30 seconds at 2000 x g Transfer 500 µl supernatant to a scintillation vial Add 3 ml scintillation cocktail Shake for 10 minutes and count radioactivity		

* The working solution comprised per sample: 2 µl 0.05M sodium phosphate buffer, pH 6.8, 4 µl [^{3}H]acetyl coenzyme A diluted to 0.01 µCi/µl with water and 4 µl tryptamine HCl (1.377 mg/ml in sodium phosphate buffer).

2.3.5 Discussion

An attempt to use chloroform as the organic solvent for the extraction of N-acetyl-[^{3}H]tryptamine was less successful than the toluene : isoamyl alcohol alternative. The radioactive counts obtained using the former were very much lower than those obtained with the latter solvent and there appeared to be inadequate extraction of the labelled product with chloroform. Furthermore, in the original method, cold acetyl coenzyme A was included in the incubation tubes to compete with the radioactive [^{3}H]acetyl coenzyme A for the enzyme. When cold acetyl coenzyme A was included, radioactive counts were not significantly different from the blank values. Omission of the cold coenzyme did not affect the blank readings, but increased the other radioactive counts while still resulting in a linear increase in the amount of product formed with time. It was therefore decided to omit the cold acetyl coenzyme A from the incubation mixture.

2.4 THE DETERMINATION OF PINEAL HYDROXYINDOLE-O-METHYLTRANSFERASE ACTIVITY

2.4.1 Introduction

Hydroxyindole-O-methyltransferase (HIOMT) is the final enzyme in the biosynthetic pathway of melatonin in the pineal gland. It is responsible for catalysing the conversion of N-acetylserotonin to melatonin with S-adenosyl methionine acting as the methyl donor.

The enzyme assay used in this study was a modification of the method of Axelrod and Weissbach [1961]. The assay is based on the transfer of the radioactive [^{14}C]-methyl group from S-adenosyl-L-[methyl-^{14}C]methionine to N-acetylserotonin by HIOMT to form N-acetyl-5-[^{14}C]methoxytryptamine or radioactive melatonin. The radiolabelled melatonin can then be extracted into an organic solvent and the radioactivity measured by liquid scintillometry. The amount of radioactive melatonin formed is an indication of HIOMT activity.

2.4.2 Materials

2.4.2.1 Animals

Male Wistar rats of the albino strain weighing 250 - 300 g were randomly assembled into test groups of five and maintained as described previously (Section 2.1). The animals were sacrificed between 10h00 and 12h00.

2.4.2.2 Chemicals and Reagents

S-Adenosyl-L-[methyl-^{14}C]methionine was purchased from Amersham International (England). The specific activity was 56.0 mCi/mmol

and the radioactive concentration was 25 µCi/ml. N-acetylserotonin was purchased from Sigma Chemical Co. (St. Louis, USA) and Packard® Scintillator 299™ liquid scintillation cocktail from Packard Instrument Co Inc. (Netherlands). All other chemicals were obtained from local commercial sources.

2.4.3 Hydroxyindole-O-methyltransferase Assay Procedure

The pineal glands were swiftly removed from the rats after sacrifice as previously described (section 2.1). Each pineal gland was individually placed in a small glass homogenizer containing 100 µl of ice cold 50 mM sodium phosphate buffer, pH 7.9. The pineals were then homogenized on ice for approximately 30 seconds until a homogeneous solution was obtained. A 20 µl aliquot was transferred to an Eppendorf tube. 10 µl of 0.05 mM sodium phosphate buffer containing 0.9 mM N-acetylserotonin and 2 nCi S-adenosyl-L-[methyl-[14C]methionine was placed on the side of the Eppendorf tube. The surface tension was sufficient to keep the drops of homogenate and working solution separate until the tubes were tapped and quickly vortexed to thoroughly mix the two liquids. This ensured a constant incubation time. Each assay was performed in triplicate and blanks were included in which the pineal homogenate was substituted with 20 µl 50 mM sodium phosphate buffer, pH 7.9. The tubes were incubated at 37°C for 45 minutes in a shaking water bath. The reaction was terminated by the addition of 100 ul chilled 0.2 M borate buffer, pH 10. 1 ml of toluene : isoamyl alcohol (97 : 3) was then added to each tube which was subsequently sealed and shaken for 5 minutes. The resulting emulsion was centrifuged at 2000 x g for 30 seconds in a Hettich Universal II centrifuge. The supernatant was washed by the addition of a further 100 µl of borate buffer, pH 10, re-shaken for 5 minutes and re-centrifuged at 2000 x g for 30 seconds. A 0.5 ml aliquot of the supernatant was transferred to a plastic scintillation vial

containing 3 ml of scintillation cocktail. The vials were shaken for 10 minutes and the radioactivity quantified in a Beckman LS 2800 scintillation counter. The procedure is the same as that summarized in Table 2.3 for NAT activity except that the working solution comprised per sample: 5 µl 0.05 mM sodium phosphate buffer, pH 7.9, 1 µl S-adenosyl-L-[methyl-^{14}C]methionine, 3 µl N-acetylserotonin (0.6549 mg/ml made up in phosphate buffer) and 1 µl deionized water.

In experiments involving the evaluation of a test compound on HIOMT activity *in vitro*, pineal glands removed from the rats following sacrifice were incubated with the test compound for 6 hours in organ culture as described in section 2.2.3.

2.4.4 Results

The actual cpm obtained from the enzyme assay were converted to dpm by correcting for the counting efficiency of the scintillation counter and the blank values were subtracted from the sample values. As in the case of the NAT assay, blank values obtained in the absence of enzyme were very low indicating that a negligible amount of the S-adenosyl-L-[methyl-^{14}C]methionine, was extracted into the solvent. The dpm were converted to Curies (1 Curie = 2.2 x 10^{22} dpm) and subsequently to moles of melatonin using the specific activity of the S-adenosyl-L-[methyl-^{14}C]methionine (1 mole = 56 Ci). Corrections were also made for using one fifth of the pineal homogenate and for incubating the enzyme for 45 minutes. The final results were expressed in terms of pmoles N-acetyl-5-[^{14}C]methoxytryptamine formed per pineal gland per hour. Triplicate determinations were found to agree closely and were averaged before the data were analyzed statistically by one-way analysis of variance. Significant differences between groups were determined using the Student's t-test. The results are expressed

as the mean of the five separate incubations ± SEM.

2.4.5 Discussion

As with the NAT assay, an attempt to use chloroform as the organic solvent for the extraction of N-acetyl-5-[^{14}C]methoxytryptamine in place of toluene : isoamyl alcohol was unsuccessful. Once again, cold S-adenosylmethionine was omitted for the same reasons the cold cofactor was omitted before (section 2.3.5). When the HIOMT and NAT assays are done on the same homogenate, the pineal gland is homogenized in 100 µl sodium phosphate buffer, pH 6.8. A 10 µl sample of this homogenate is removed for the HIOMT assay and its pH adjusted to 7.9 with approximately 10 µl of sodium phosphate buffer, pH 11.3. This provides a rapid, highly efficient method of quantifying both of these important enzymes in the melatonin biosynthetic pathway.

2.5 THE MEASUREMENT OF PINEAL CYCLIC ADENOSINE MONOPHOSPHATE (cAMP) LEVELS

2.5.1 Introduction

Although only discovered in the 1950's, cyclic AMP has made a major impact on many areas of biochemical research. It is recognized as a ubiquitous, yet vital mediator of numerous intracellular events playing a particularly important role as a second messenger in the transduction of hormonal responses. Methods to quantify cyclic AMP levels are therefore in great demand. Various researchers have employed a variety of techniques to assay for this cyclic nucleotide.

Cyclic AMP is synthesized from ATP by adenylate cyclase, a membrane bound enzyme, and degraded to AMP by a specific phosphodiesterase. The earliest cyclic AMP assays involved determining the activity of adenylate cyclase as an indirect index of cyclic AMP levels. The formation of radioactive cyclic AMP from its labelled precursor, [^{14}C]ATP, could be quantified radiochemically. Later, direct, competitive binding assays were developed which utilized purified specific cyclic AMP binding proteins. Brown *et al* [1971] used a binding protein, a protein kinase, with a very high specificity for cyclic AMP previously identified in adrenal glands, to develop a saturation assay for cyclic AMP. More recently, radioimmunoassays have been developed which use [^{125}I]antigen and antiserum to measure cyclic AMP levels. Although these radioimmunoassays are generally quick and simple to use, with a high degree of specificity and sensitivity, they are usually very expensive.

The assay used in this study was based on that developed by Brown *et al*[1971] with modifications by Daya [1985]. It is sensitive,

specific and relatively inexpensive.

2.5.2 Materials

2.5.2.1 Animals

Male Wistar rats of the albino strain weighing 250 - 300 g were randomly assembled into test groups of five and maintained as described previously (Section 2.1). The animals were sacrificed between 10h00 and 12h00.

2.5.2.2 Chemicals and Reagents

[8-^{3}H]Adenosine-3',5'-Cyclic Phosphate, ammonium salt was purchased from Amersham International (England). The specific activity was 28 Curies/mmol and the radioactive concentration was 1 mCi/ml. Adenosine -3',5'- Cyclic Phosphate was purchased from Boehringer Mannheim (Germany), theophylline, isoproterenol and bovine serum albumin (whole fraction) from Sigma Chemical Co (St. Louis, USA) and activated charcoal from Merck (Germany). All other chemicals were obtained from local commercial sources.

A [8-^{3}H]adenosine-3',5'-cyclic phosphate working solution was prepared by diluting the commercial stock to a 0.1 mCi/ml solution with ethanol : water (1:1). The final solution was obtained by adding 10 µl of this was to 6.24 ml of deionized water.

The homogenizing buffer, pH 7.4 was 50 mM Tris-HCl containing 0.25 M sucrose, 25 mM potassium chloride and 5 mM magnesium chloride.

The protein diluting buffer, pH 7.4 was 50 mM Tris-HCl containing 8 mM theophylline and 6 mM 2-mercaptoethanol.

10% w/v charcoal saturated with 2% BSA was prepared by dissolving 400 mg of whole crystalline bovine serum albumin in protein diluting buffer. To this, 2 g of activated charcoal was added and the mixture stirred. It was stored frozen.

2.5.3 Preparation of cAMP Binding Protein

Bovine adrenal glands were collected from the Grahamstown abattoir as soon as possible after slaughter and transported to the laboratory on ice. The adrenal cortices were dissected free and homogenized in a glass homogenizer in 1.5 volumes of ice cold homogenizing buffer. The homogenate was centrifuged at 2000 x g for 10 minutes at 4°C. The resulting supernatant was centrifuged at 5000 x g for 15 minutes at 4°C. The supernatant from this centrifugation was recovered and stored in 1 ml aliquots at -20°C for up to 3 months.

A protein dilution curve was constructed to determine the optimum cyclic AMP binding to give radioactive counts in the region of 1000 cpm. A series of dilutions of the binding protein was prepared in protein diluting buffer and assayed as described below. The protein dilution curve is presented in Figure 2.2 and shows that a 1:3 dilution of the protein provided optimum binding and radioactive counts. This dilution was used for all subsequent assays.

2.5.4 Preparation of Pineal Tissue and Incubation Procedure

The pineal glands were rapidly removed from the rats after sacrifice as described previously (section 2.1). Each pineal gland was carefully bisected into the two hemispheres. The one hemisphere was placed in 50 µl pre-warmed BGJb culture medium while the other hemisphere was transferred to culture medium containing

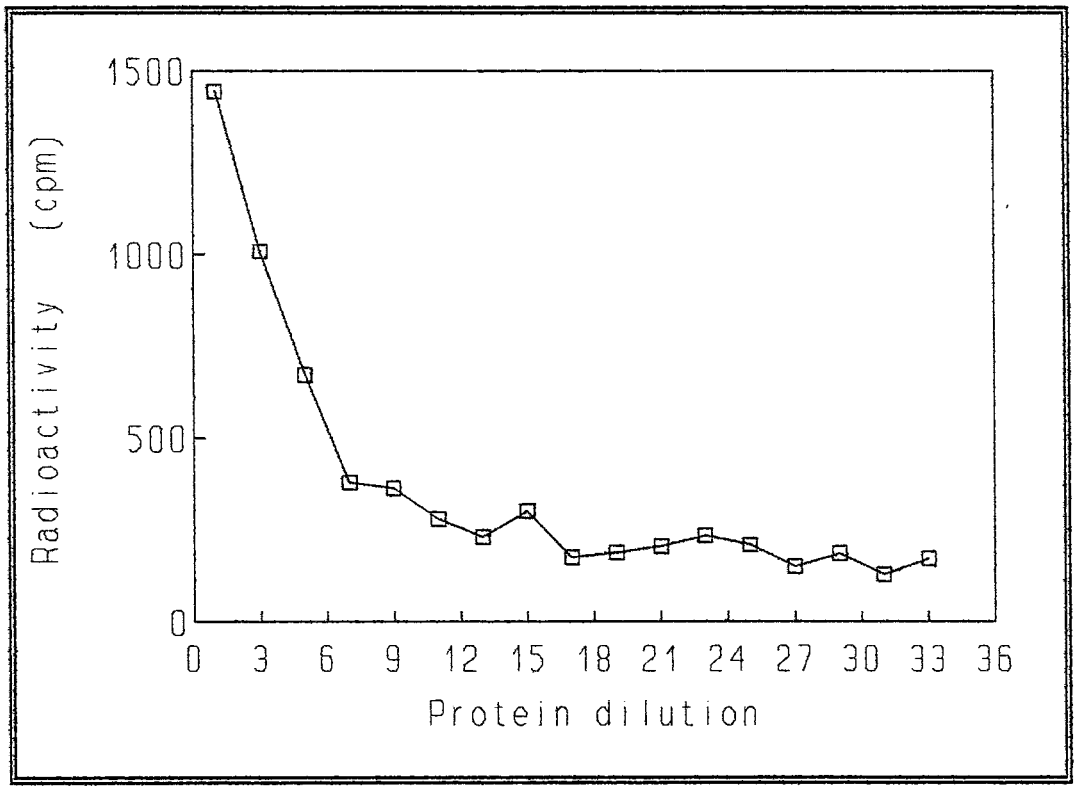

FIGURE 2.2 : A Typical Protein Dilution Curve

10 µM isoprenaline. Each hemisphere was then incubated at 37°C for 20 minutes. Following incubation, the pineal hemispheres were removed from the media and homogenized in 10 µl of ice cold buffer. A 50 µl aliquot of the homogenate was used for determination of cAMP levels.

2.5.5 Cyclic AMP Assay Procedure

The cyclic AMP assay procedure is summarized in Table 2.4. Reaction tubes were prepared containing 150 µl of the protein diluting buffer, 8 nCi [8-^{3}H]adenosine-3',5'-cyclic phosphate, 50 µl of cold cyclic standard in the range 0 - 8 pmoles or 50 µl of pineal homogenate and deionized water to give a final volume of 250 µl. The tubes were mixed gently. Where possible, assays were performed in duplicate. Blanks containing no cyclic AMP standard or pineal homogenate were included. 100 µl of diluted binding protein were added to each of tubes which were gently mixed and incubated at 4°C in a refrigerator or on ice for 100 minutes. Following incubation, 100 µl of the activated charcoal suspension were added to each tube and vortexed for 10 seconds. The tubes were centrifuged at 1200 x g for 15 minutes at 4°C and a 100 µl aliquot of the supernatant was added to scintillation vials containing 3 ml of scintillation cocktail. The vials were shaken for 5 minutes and the radioactivity quantified in a Beckman LS 2800 scintillation counter.

TABLE 2.4 : Scheme of the Cyclic AMP Assay

REAGENTS (µl)	BLANK	CYCLIC AMP STANDARDS (pmoles)							SAMPLE
		0	0.25	0.5	1.0	2.0	4.0	8.0	
Buffer	250	150	150	150	150	150	150	150	150
Standard: - 20 nM - 200 nM	--- ---	--- ---	12.5 ---	25 ---	50 ---	--- 10	--- 20	--- 40	--- ---
Sample	---	---	---	---	---	---	---	---	50
Water	50	50	37.5	25	---	40	30	10	---
[^{3}H]cAMP	50	50	50	50	50	50	50	50	50
	Mix Gently								
Protein	---	100	100	100	100	100	100	100	100
	Incubate at 4°C for 100 minutes								
Charcoal	100	100	100	100	100	100	100	100	100
	Vortex for 15 seconds Centrifuge at 1200 x g for 15 minutes at 4°C Add 100 µl of supernatant to 3 ml scintillation cocktail Shake for 10 minutes and count radioactivity								

2.5.6 Results

Blank values were subtracted from all readings. The Co/Cx ratio was calculated by dividing the radioactivity (cpm) of the zero standard (Co) by the radioactivity of the higher standards and test samples. A calibration curve was plotted using the Co/Cx ratio of the standards against the concentration of the cyclic AMP standards (pmoles). A typical standard curve is presented in Figure 2.3.

The interference of non-cyclic AMP material in the binding assay was estimated by incubating pineal homogenate with approximately 50 µg of phosphodiesterase for 1 hour at 37°C before conducting the assay to degrade all endogenous cyclic AMP. Cyclic AMP values obtained following incubation were not significantly different from the zero standard indicating negligible interference by non-cyclic AMP material.

The data from sample assays were statistically analyzed by one-way analysis of variance and the significant difference between the means determined using the Student's t-test.

2.5.7 Discussion

The results of this competition assay indicate a linear relationship between the binding of cold and radioactive cyclic AMP to a cyclic AMP binding protein. Furthermore, the assay was specific and sensitive down to 0.25 pmoles of cyclic AMP. It is preferred to the method of Brown *et al* [1971] because it does not require any additional preparatory treatment of the crude pineal homogenate.

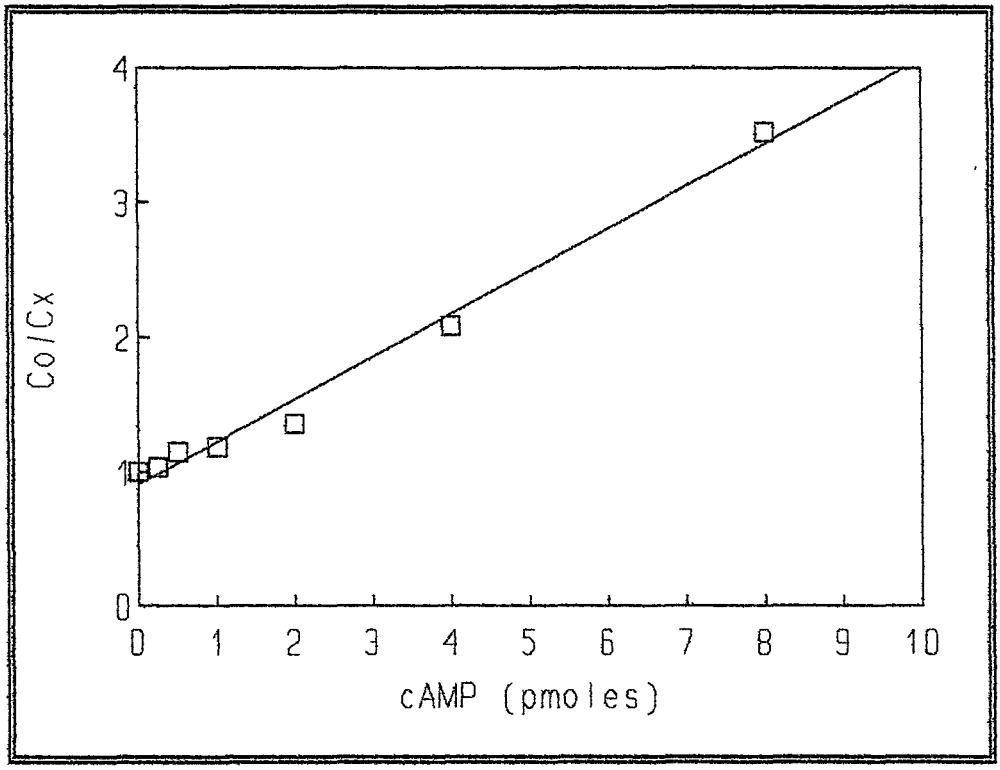

FIGURE 2.3 : A Typical Cyclic AMP Standard Curve

Each point is the mean of duplicate determinations.

2.6 THE MEASUREMENT OF PINEAL β-ADRENERGIC RECEPTOR BINDING USING [^{3}H]DIHYDROALPRENOLOL

2.6.1 Introduction

The observation that many cells respond in a highly selective way to minute concentrations of a particular chemical or drug, led different investigators to hypothesize about cell receptors or specific sites on cells which were the site of drug action [cf.Carman-Krzan, 1986]. Langley in 1905, studying the effect of nicotine and curare on the voluntary smooth muscle, postulated the existence of specific receptors or "receptive sites" on the cell which would receive the stimulus from the drug and transmit it to the cell, causing the effect. However, it was not until the 1950s that the receptor theory became a major area of research interest in pharmacology. The synthesis of structurally related analogues of drugs and endogenous substances expanded the field to work on the detailed structure-function relationship between drugs and their receptors. These studies established the structural requirements of agonists and antagonists and led to the distinguishing of competitive and non-competitive antagonists [Titeler, 1981; Carman-Krzan, 1986].

The beginning of radioligand receptor binding studies was in 1965 with the partly successful labelling of muscarinic cholinergic receptor sites in guinea-pig ileum with [^{3}H]atropine. Today, well-validated radioligand binding assays exist for labelling almost all known receptors and a wide variety of high affinity, highly specific radioactive ligands are available.

Previously available electrophysiological or biochemical techniques measured receptor activation indirectly by recording an event which may be several steps removed from the initial interaction.

Radioligand binding methodology facilitates the direct measurement of the ligand-receptor interaction in the absence of cellular influences and functionally coupled biological responses. Furthermore, the binding of ligand molecules to a receptor population is a second order reaction which can be quantified by applying kinetic analyses similar to those originally devised for the study of enzyme catalysed reactions. Thus the affinity of the ligand for the receptor and the total number of binding sites present can be readily assessed. The binding of the radioligand to receptor and non-receptor sites can be distinguished by examining the saturability and pharmacological specificity of the radioligand binding sites. If the binding sites are saturable, reversible and display the same stereospecificity and pharmacological characteristics as the receptor mediated biological response, it can be concluded that the radioligand has specifically labelled the recognition site of the receptor [Titeler, 1981; Foster and Fagg, 1984; Carman-Krzan, 1986].

Ligand binding techniques may be used to determine the affinity of various agents for the receptor site, the distribution of the receptors in different tissues and subcellular fractions as well as the plasticity of receptor parameters following drug treatments, experimental lesions and pathological states such as disease or injury. Additionally, binding studies can be used to monitor the isolation of the receptor molecule and determine its biochemical characteristics. An extension of these procedures is the recent development of autoradiographical techniques for the localisation of receptor-bound ligand in tissue sections. This has allowed detailed investigations of the regional distributions of a number of important receptor binding sites.

The study of ß-adrenergic receptors necessitates the use of a radioligand with a high affinity, specificity and specific activity

to identify the small number of ß-adrenergic receptors present in tissues. Many such competitive ß-adrenergic antagonists are available including [^{3}H]propranolol, [^{125}I]iodohydroxy-benzylpindolol, [^{3}H]CGP-12177 and [^{3}H]dihydroalprenolol (DHA).

The method used in this study was adapted for the pineal gland by Greenberg and Weiss [1978] and employs [^{3}H]DHA as the radioactive ligand.

2.6.2 Materials

2.6.2.1 Animals

Male Wistar rats of the albino strain weighing 250 - 300 g were randomly assembled into test groups of eight and maintained as described previously (Section 2.1). The animals were sacrificed between 10h00 and 12h00.

2.6.2.2 Chemicals and Reagents

1-[Propyl-2,3-^{3}H]dihydroalprenolol was purchased from Amersham International (England). The specific activity was 53.9 Ci/mmol and the radioactive concentration was 1.0 mCi/ml. dl-Propranolol hydrochloride was purchased from Sigma Chemical Co. (St. Louis, USA) and Packard^{R} Scintillator 299^{TM} liquid scintillation cocktail from Packard Instrument Co Inc. (Netherlands). All other chemicals were obtained from local commercial sources.

2.6.3 Preparation of Pineal Homogenate

The pineal glands were rapidly removed from the rats after sacrifice as described previously (section 2.1). Two similarly treated pineals were homogenized together in 5 ml of ice cold 50

mM Tris-HCl buffer, pH 8.0,containing 3 mM $MgCl_2.6H_2O$ in a glass homogenizer. The homogenate was centrifuged at 49000 x g for 15 minutes at 4°C in a Beckman L8-80M ultracentrifuge with a 70.1 Ti rotor. The pellet was washed once by resuspension in the original volume of buffer and re-centrifuged as before. The pellet was finally resuspended in 0.9 ml of Tris-HCl buffer. A 300 µl aliquot was frozen to be used for the protein determination while the remaining volume was used for the [^{3}H]DHA binding assay.

For the saturation curve, pineal glands from 8 rats were homogenized in 10 ml of ice cold Tris-HCl buffer in a glass homogenizer. The homogenate was centrifuged at 49000 x g for 15 minutes at 4°C. The pellet was washed once as described above and resuspended in 3.6 ml of buffer. A 300 µl aliquot was frozen to be used for the protein determination while the remaining fraction was used for the [^{3}H]DHA saturation curve.

2.6.4 [^{3}H]Dihydroalprenolol Binding Assay Procedure

The binding assay procedure is summarized in Table 2.5. A 100 µl aliquot of the prepared pineal homogenate was added to 10 mm x 12 mm Kimble tubes containing 50 µl of 8 nM [^{3}H]DHA and 50 mM Tris-HCl buffer, pH 8.0. For assessment of the total binding, the final volume was made up to 450 µl with buffer and for non-specific binding, 10 µl of buffer were substituted with 10 µl of 450 µM dl-propranolol. Each assay was performed in triplicate and a blank containing no pineal homogenate was included in each series of assays. The tubes were vortexed gently and incubated at 37°C for 10 minutes. The incubation was terminated by the addition of 5 ml of ice cold buffer. The samples were then rapidly filtered through pre-wetted Whatman GF/C glass microfilters in a 12 place Millipore sampling manifold. Each filter was washed five times with 4 ml of cold buffer. The filters were transferred to scintillation vials

TABLE 2.5 : Scheme of the [^{3}H]DHA Binding Assay

REAGENTS (µl)	TOTAL BINDING			NON-SPECIFIC BINDING		
	BLANK	STANDARD	SAMPLE	BLANK	STANDARD	SAMPLE
Buffer*	400	300	300	390	290	290
[^{3}H]DHA	50	50	50	50	50	50
Propranolol	---	---	---	10	10	10
Homogenate	---	100	100	---	100	100
	Incubate at 37°C for 45 minutes					
Buffer*	5 ml	5 ml	5 ml	5 ml	5 ml	5 ml
	Filter through Whatman GF/C glass filters Wash filters 5 x with 4 ml ice cold buffer*					
Scintillation cocktail	3 ml	3 ml	3 ml	3 ml	3 ml	3 ml
	Shake for 30 minutes and count radioactivity					

* 50 mM Tris-HCl Buffer, pH 8.0, containing 3 mM $MgCl_2.6H_2O$.

containing 3 ml scintillation cocktail and shaken for 30 minutes. Finally the radioactivity was quantified in a Beckman LS 2800 scintillation counter.

The saturation curve was prepared by incubating 100 µl of binding protein with 50 µl of varying concentrations of [^{3}H]DHA (0 - 20 nM)

made up to a final volume of 450 µl with 50 mM Tris-HCl buffer, pH 8.0. Non-specific binding was again assessed by replacing 10 µl of buffer with 10 µl of 450 µM dl-propranolol and blanks were included in which the binding protein was omitted. The samples were assayed as described above.

2.6.5 Protein Determination of Pineal Homogenate

The protein concentration of the pineal homogenates was determined by a modification of the method of Lowry *et al* [1951] for small samples. The scheme for the protein assay is summarized in Table 2.6. A 0.1 ml aliquot of protein sample was made up to 0.4 ml with 50 mM Tris-HCl buffer, pH 8.0. To this was added 2 ml of an alkaline solution prepared by mixing 2% Na_2CO_3 in 0.1 N NaOH with 0.5% $CuSO_4.5H_2O$ in 1% sodium tartrate in a ratio of 50 : 1. The tubes were mixed and left to stand at room temperature for 15 minutes. Folin-Ciocalteu reagent was diluted 1 : 1 with deionized water and 0.2 ml aliquots were added to the assay mixture. The tubes were vortexed gently and left to stand at room temperature for 30 minutes in the dark, after which the absorbance was measured at 750 nm. For the standard curve, protein standards containing 0 - 40 µg of bovine serum albumin were assayed as above. A typical standard curve thus obtained is presented in Figure 2.4. The assays were always performed in duplicate or triplicate.

2.6.6 Results

The actual cpm obtained from the binding study were converted to dpm by correcting for the counting efficiency of the scintillation counter. Blank values were subtracted from sample values. The saturable specific binding was calculated as the total binding minus the non-displaceable, non-specific binding determined in the presence of 10 µM propranolol. These dpm were converted to Curies

(1 Curie = 2.2 x 10^{22} dpm) and subsequently to moles of [^{3}H]DHA using the specific activity of the radioactive compound (1 mole = 53.9 x 10^3 Ci). The specific binding was expressed as fmoles [^{3}H]DHA bound/mg protein. The final result is the mean of the duplicate or triplicate determinations. The data were statistically analyzed by one-way analysis of variance and the significant difference between the means was determined using the Student's t-test.

TABLE 2.6 : Scheme of the Protein Assay

REAGENTS (ml)	BLANK	PROTEIN STANDARDS (µg)					SAMPLE
		2.5	5.0	10.0	20.0	40.0	
Protein Std: - 0.1 mg/ml	---	25	50	100	---	---	---
- 1.0 mg/ml	---	---	---	---	20	40	---
Unknown	---	---	---	---	---	---	100
Buffer#	400	375	350	300	380	360	300
Alkaline soln*	2000	2000	2000	2000	2000	2000	2000
	Stand at room temperature for 15 minutes						
FC Reagent**	200	200	200	200	200	200	200
	Stand at room temperature for 30 minutes Read absorbance at 750 nm						

\# 50 mM Tris-HCl buffer, pH 8.0
* 2% Na_2CO_3 in 0.1 N NaOH : 0.5% $CuSO_4.5H_2O$ in 1% sodium tartrate (50 : 1)
** Folin - Ciocalteu Reagent : Water (1 : 1)

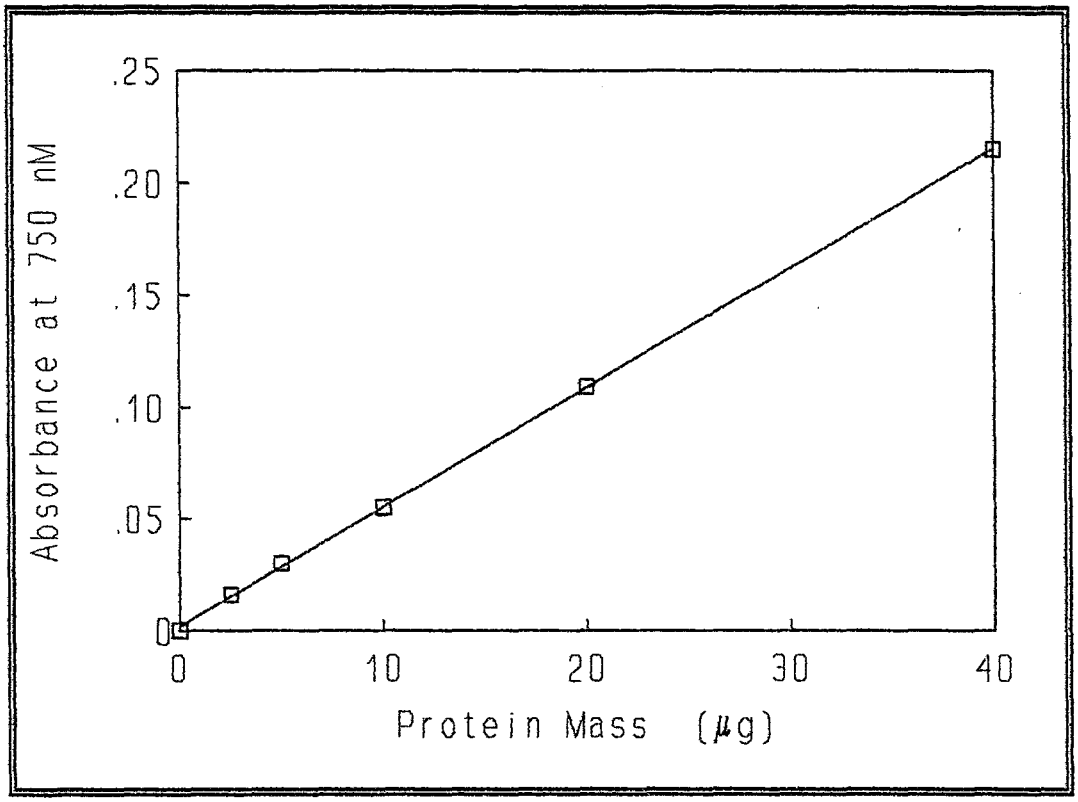

FIGURE 2.4 : <u>A typical protein standard curve</u>.

Each point is the mean of duplicate determinations.

2.6.7 Discussion

The specific binding was between 20 - 24% of the total binding. The saturation curve in Figure 2.5 shows the binding of [^{3}H]DHA to rat pineal membranes is saturable at 15 - 20 nM. The Scatchard plot in Figure 2.6 demonstrates the presence a single class of ß-adrenergic receptors with an equilibrium dissociation constant (K_D) of 8.3 nM and an apparent maximum number of binding sites (B_{max}) equivalent to 417.6 fmole/mg protein. These findings are in close agreement with previous reports [Greenberg and Weiss, 1978; Banoo, 1991].

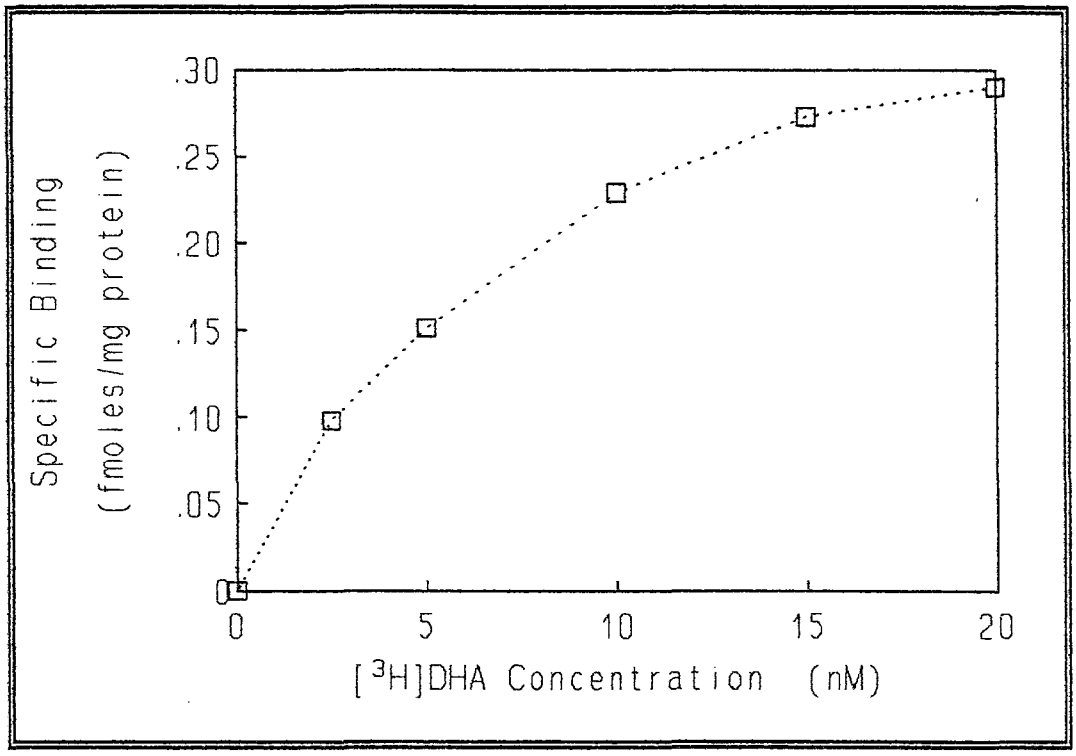

FIGURE 2.5 : **Saturation Curve of [^{3}H]DHA Binding to Rat Pineal Gland Membranes**

Each point represents the mean of triplicate determinations.

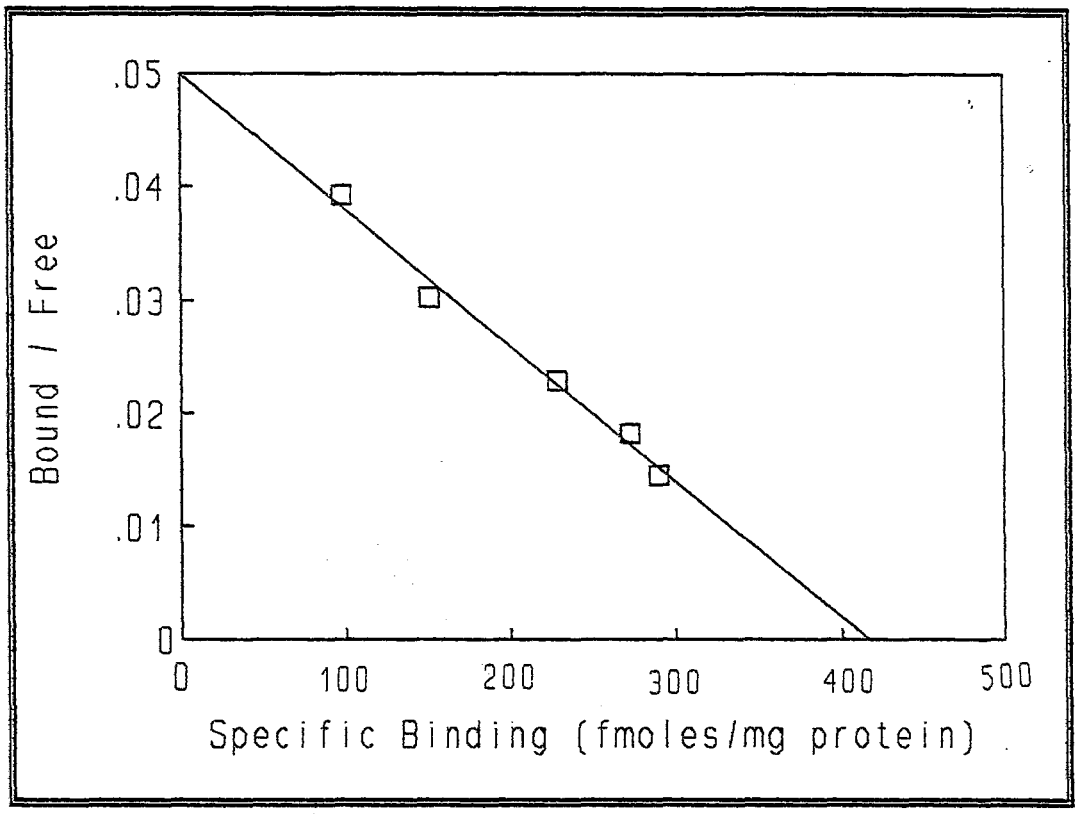

FIGURE 2.6 : **Scatchard Plot of [^{3}H]DHA Binding to Rat Pineal Membranes**

The K_D from the above slope is 8.3 nM and the B_{max} from the x-intercept is 417.6 fmol/mg protein.

The correlation coefficient is 0.993.

2.7 THE MEASUREMENT OF LIVER TRYPTOPHAN PYRROLASE ACTIVITY

2.7.1 Introduction

Tryptophan pyrrolase is a haem-dependent liver cytosolic enzyme which catalyzes the conversion of L-tryptophan to N'-formylkynurenine in the first and rate-determining step in the catabolic kynurenine-nicotinic acid pathway [Badawy and Evans, 1975]. It is the most important peripheral factor regulating plasma tryptophan levels *in vivo*. Tryptophan pyrrolase enzyme assays have provided important information about the various factors regulating both the activity of the enzyme and endogenous tryptophan levels in normal and diseased animals.

In the livers of some animal species, tryptophan pyrrolase exists in at least two forms: the active reduced holoenzyme which does not require the presence of a cofactor for the demonstration of its activity, and the inactive apoenzyme which requires the addition of exogenous haem for the demonstration of its activity [Badawy and Evans, 1974]. Activation of the apoenzyme *in vitro* involves its conjugation with haem to form the oxidized (ferrihaem) holoenzyme which is then reduced to the active form in the presence of tryptophan. When assessing the affect of a particular agent on tryptophan pyrrolase activity, it is important to measure both apoenzyme and holoenzyme activities in order to draw conclusions about the site of action of the agent. Both forms of the enzyme are present in the liver of rats, mice, chickens and possibly man, but the apoenzyme has not been found in guinea pigs, hamsters, rabbits, sheep, frogs, cats and the gerbil.

The tryptophan pyrrolase enzyme assay employed in this study was previously described by Badawy and Evans [1973; 1975]. A few modifications were made to increase the number of assay points

possible on each liver. The activity of the enzyme is determined by measuring the formation of kynurenine from L-tryptophan. The holoenzyme is measured in the absence of added haemin and total enzyme activity in the presence of exogenous haemin. The apoenzyme is calculated as the difference.

2.7.2 Materials

2.7.2.1 Animals

Male Wistar rats of the albino strain weighing 250 - 300 g were randomly assembled into test groups of five and maintained as described previously (Section 2.1). The animals were sacrificed between 10h00 and 12h00.

2.7.2.2 Chemicals and Solutions

L-Tryptophan, melatonin and haemin hydrochloride were purchased from Sigma Chemical Co. (St. Louis, USA). All other chemicals were obtained from local commercial sources.

The haemin solution was prepared by dissolving 7.8 mg haemin hydrochloride in a minimal amount of 4 M NaOH. The pH was carefully adjusted to 7.0 with 1 M HCl and the final volume made up to 10 ml. This solution was always prepared immediately prior to use.

2.7.3 Preparation of Rat Liver Homogenate

The procedure is summarized in Table 2.7. The liver was rapidly removed after sacrifice as described previously (section 2.1) and homogenized in 60 ml of 140 mM KCl - 2.5 mM NaOH homogenizing buffer, pH 7.0, with a Waring blender for 1 minute (1000

rev/minute). Care was taken not to allow excessive foaming associated with protein denaturation. The resulting suspension was further homogenized in a Dounce homogenizer with a loose-fitting glass pestle until a completely homogeneous solution was obtained. To this, 60 ml of 0.2 M sodium phosphate buffer, pH 7.0 was added and the mixture gently stirred. These procedures were performed on ice and as quickly as possible.

2.7.4 Determination of Tryptophan Pyrrolase Activity

To determine apoenzyme activity, a 15 ml aliquot of liver homogenate was added to a solution containing 12.5 ml of deionized water and 2.5 ml of 0.03 M L-tryptophan. To determine total enzyme activity, 0.1 ml of a 1.2 mM haemin solution was included to give a final overall concentration of 2 μM. To assess the conversion of endogenous tryptophan to kynurenine, controls were prepared in which the tryptophan was replaced with 2.5 ml 0.2 M sodium phosphate buffer. When a test compound was used, the water fraction or part thereof was replaced with a solution of the test compound. In these instances, a control was included which received the vehicle.

The rest of the assay was performed in triplicate. 3 ml aliquots of the assay mixture were transferred to boiling tubes and stoppered in an atmosphere of 95% O_2 : 5% CO_2. The tubes were incubated at 37°C for 0, 15, 30, 45, 60 and 75 minutes in a shaking water bath (120 oscillations/minute). The reaction was terminated by the addition of 2 ml of 0.9 M trichloracetic acid. The mixtures were shaken for a further 2 minutes and then filtered through Whatman No. 1 filter paper. 2.5 ml of the fitrates were transferred to tubes containing 1.5 ml of 0.6 M NaOH. The absorbance was measured at 365 nm in a Unicam SP 1001 spectrophotometer against a blank consisting of 2 ml of 0.9 M

trichloracetic acid and 1.5 ml of 0.6 M NaOH.

2.7.5 Results

Absorbance readings from the triplicate determinations were pooled and averaged. The concentration of kynurenine was calculated by applying Beer-Lamberts Law and measuring the increase in absorbance at 365 nm. The molar extinction coefficient of kynurenine is ε = 4540 litre/mole.cm. Results were expressed as mole kynurenine formed/mg protein per hour. Holoenzyme activity was that determined in the absence of haemin and total activity was that determined in the presence of added haemin. The difference was a measure of apoenzyme activity. The data were analyzed by one-way analysis of variance and statistical differences between the means of test groups were determined using the Student's t test.

The time course for the enzyme activity (Figure 2.7) indicated that the formation of product was linear for the first 60 minutes and 45 minutes was chosen to be the most suitable incubation time for all the studies.

Due to the long duration of the entire assay procedure, it was often not possible to perform all the required assays on one day. To determine the effect of freezing on tryptophan pyrrolase activity, the enzyme was assayed in fresh livers and in livers previously frozen in liquid nitrogen and stored at -70°C. The frozen livers exhibited a small, but consistent decrease in enzyme activity.

TABLE 2.7 : Scheme of the Tryptophan Pyrrolase Enzyme Assay

	TRYPTOPHAN	TRYPTOPHAN + HAEM
Homogenize liver in KCl-NaOH buffer	60 ml	
Add Na K monophosphate buffer	60 ml	
Add water to 100 ml reaction beakers	12.5ml	12.5ml
Transfer to 100 ml reaction beakers	15ml	15ml
Add haem to give 2µM solution	-	100 µl
Add 0.03 M tryptophan	2.5 ml	2.5 ml
CONTINUE IN TRIPLICATE		
Transfer to boiling tube	3 ml	3 ml
Incubate in atmosphere of carbogen for 1 hour at 37°C with shaking		
Stop reaction with 0.9M TCA	2 ml	2 ml
Shake for 2 minutes Filter through Whatman No. 1 filter paper in Buchner apparatus		
Remove filtrate to test tubes containing 1.5 ml NaOH	2.5 ml	2.5 ml
Read Absorbance at 365 nm		

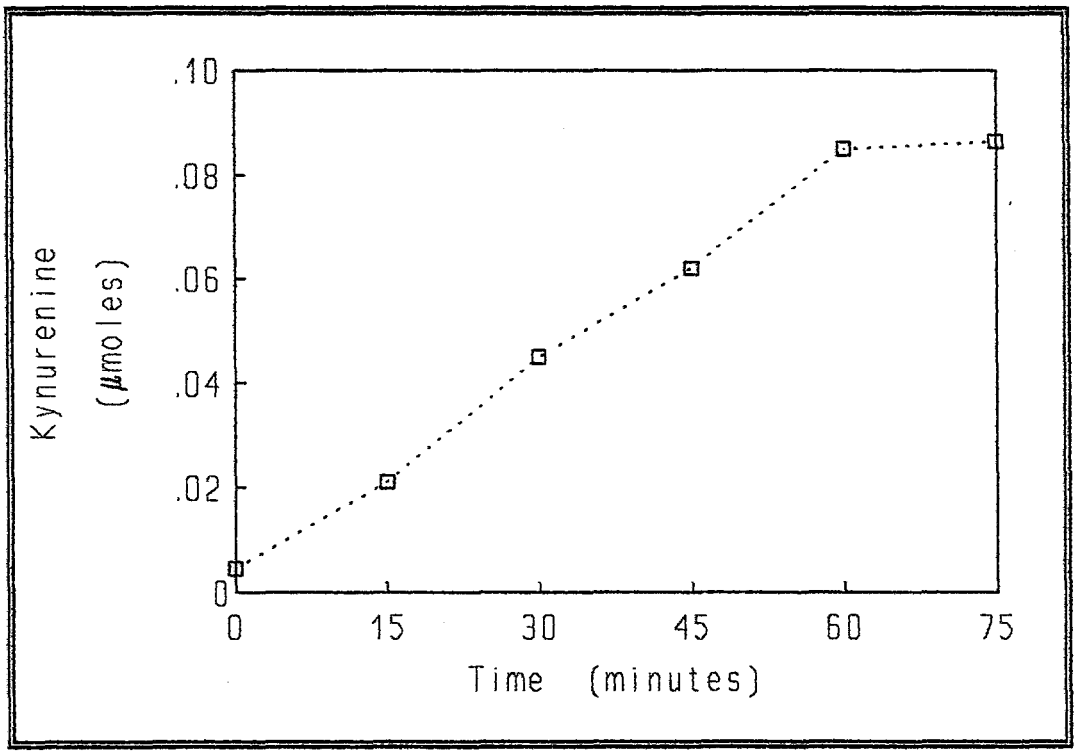

FIGURE 2.7 : <u>Effect of incubation time on tryptophan pyrrolase activity.</u>

Each point represents the mean of duplicate determinations.

2.7.6 Discussion

The tryptophan pyrrolase enzyme assay was shown to produce a linear increase in kynurenine formed with time. Due to the limited reduction in tryptophan pyrrolase activity following freezing, all livers were frozen in liquid nitrogen and stored at -70°C prior to the enzyme assay. This accommodated practical problems associated with the assay while still producing consistent, reproducible results.

2.8 THE MEASUREMENT OF GLUTAMATE BINDING TO RAT CEREBRAL MEMBRANES USING [^{14}C]GLUTAMATE

2.8.1 Introduction

A brief, general introduction to radioligand binding studies and their uses was presented in section 2.6.1.

Glutamate, aspartate and a number of related amino acids have the common ability to activate the central nervous system. The pharmacology of these excitatory amino acids has been extensively investigated using electrophysiological techniques, and it has become apparent that multiple receptors exist for these substances. The advantages to be gained using radioligand binding assays in the study of these receptors include the ability to directly quantify receptor occupancy *in vitro*, to monitor the isolation and purification of the receptor molecules and to kinetically characterize the binding sites.

The first investigations of L-glutamate binding to CNS membranes were conducted independently by Roberts [1974] and Michaelis *et al* [1974]. These studies indicated the binding of glutamate to a specific population of receptors was saturable, of reasonably high affinity and could be displaced by other neuroexcitatory amino acids. However, further progress was hampered by the low specific activity of L-[^{14}C]glutamate. When L-[^{3}H]glutamate with a higher specific activity became available, more detailed studies of glutamate binding to CNS membranes was possible. Comparison of the data obtained from these studies reveals a very wide range of values for the equilibrium dissociation constant (8 - 8300 nM) and maximum number of binding sites (0.13 - 381 pmole/mg protein) have been reported [cf. Foster and Fagg, 1984]. Some of the variation in these parameters can be explained by the different methods of

membrane preparation and the use of tissue from different areas of the brain from different species. However, these factors alone do not account for the wide variation in kinetic values. This suggests that within the procedures of tissue preparation and binding assay, are variables which determine the characteristics of glutamate binding.

An ideal preparation for use in binding assays is one which has a reasonably high density of binding sites and is free of endogenous contaminants which could interfere with the assay. Subcellular fractionation techniques are often designed to isolate synaptic membranes based on one or two morphological or biochemical criteria, but certainly do not isolate the total synaptic membrane fraction. This may result in the loss of potential binding sites during the fractionation procedures. However, the use of crude preparations leads to relatively high levels of non-specific binding which can make the accurate estimation of specific binding very difficult. The choice of a subcellular fractionation technique therefore should balance the need to prevent the loss of membraneous fractions with the need to reduce the levels of non-specific binding as far as possible.

Another important aspect of the membrane preparation is the removal of endogenous compounds which interfere with the binding assay. Soluble endogenous substances, trapped in membrane vesicles or tightly bound to membrane components, may be carried through several stages of the subcellular fractionation procedure. Thus a crucial step in membrane preparation is the lysis of synaptosomes and membrane vesicles by osmotic or mechanical shock, and subsequent washing by repeated centrifugations to rid the membranes of such contaminants.

The methodology used in this study to prepare cerebral synaptosomal

membranes and obtain optimal conditions for the glutamate binding assay was experimentally developed using the principles employed by Cotman and Taylor [1972], Jones and Matus [1974], Foster *et al* [1981], Monaghan and Cotman [1986] and Brose *et al* [1989; 1990].

2.8.2 Materials

2.8.2.1 Animals

Male Wistar rats of the albino strain weighing 250 - 300 g were randomly assembled into test groups of ten and maintained as described previously (Section 2.1). The animals were sacrificed between 10h00 and 12h00.

2.8.2.2 Chemicals

L-[G-^{3}H]Glutamate was purchased from Amersham International (England). L-Glutamic acid was purchased from Sigma Chemical Co. (St. Louis, USA). All other chemicals were purchased from local commercial stores.

2.8.3 Preparation of Cerebral Synaptosomal Membranes

The brains were rapidly removed from the rats after sacrifice as described previously (section 2.1) and the forebrains dissected free. The ten forebrains were homogenized together in ice cold 10% (w/v) 0.32 M sucrose in a glass homogenizer with a Teflon pestle. All procedures were strictly performed on ice and all centrifugations performed at 4°C. The homogenate was centrifuged at 1500 x g for 5 minutes in a Beckman centrifuge with a JA 21 rotor. The supernatant was set aside on ice while the pellet was washed by resuspending it in a similar volume of ice cold sucrose and re-centrifuging as before. The supernatants from the first

two steps were combined and centrifuged at 17000 x g for 10 minutes in a Beckman L8 - 80M ultracentrifuge with a 70 Ti rotor. The supernatant was discarded while the pellet was resuspended in 20 ml of 200 µM tris acetate buffer, pH 7.2, and incubated for 15 minutes at 4°C. After incubation, the suspension was centrifuged at 50000 x g for 25 minutes in a Beckman L8 - 80M ultracentrifuge with a 70 Ti.1 rotor. The pellet was recovered and washed four times by resuspending it in 200 µM tris acetate buffer, pH 7.2, and re-centrifuging as before. The final pellet was resuspended in 10% sucrose and layered onto a discontinuous sucrose density gradient consisting of 10 ml of each of 0.85M, 1.0 M and 1.3 M. As the centrifuge tubes were made non-wettable polyallomer, the gradient was layered by adding the lightest sucrose solution first. A syringe with a long needle was used to apply successive sucrose concentrations of increasing density to the apex of the tube and float the lighter concentrations up from the bottom. Loading non-wettable tubes from the top by allowing solutions to run down the side of the tube causes mixing. The gradient was centrifuged in a Beckman L8 - 80M ultracentrifuge with a SW 28 rotor. The synaptic plasma membrane fraction at the 1.0 - 1.3 M sucrose interface was collected, diluted with 200 µM tris acetate buffer, pH 7.2, and centrifuged at 50000 x g for 20 minutes in a Beckman L8 - 80M ultracentrifuge with a 70 Ti.1 rotor. The resulting pellet was resuspended in 50 mM tris acetate buffer, pH 7.2 to give a protein concentration of 1 mg/ ml.

2.8.4 Protein Determination of Pineal Homogenate

The protein concentration of the cerebral homogenates were determined by the method of Lowry *et al* [1951]. A 1.2 ml aliquot of protein sample was placed in a test tube. To this was added 6 ml of an alkaline solution prepared by mixing 1 ml of a 1% $CuSO_4.5H_2O$ solution, 1 ml of a 2% sodium tartrate solution and 98

ml of a 2% Na_2CO_3 in 0.1 N NaOH in order. The tubes were mixed and left to stand at room temperature for 10 minutes. 0.3 ml of Folin-Ciocalteu reagent was added to each of the tubes which were vortexed gently and left to stand at room temperature for 30 minutes in the dark. After the incubation, the absorbance was measured at 750 nm. For the standard curve, protein standards containing 0 - 0.5 mg of bovine serum albumin were assayed as above. The assays were always performed in duplicate or triplicate. A typical standard curve thus obtained is presented in Figure 2.2.

2.8.5 Saturation Curve for Cerebral Glutamate Receptors

The binding assay procedure is summarized in Table 2.8. The saturation curves were prepared by adding 200 µl aliquots of the prepared cerebral homogenate to 10 x 12 mm Kimble tubes containing 40 µl of varying concentrations of [^{3}H]glutamate (0 - 500 nM) made up to a final volume of 250 µl with 50 mM tris acetate buffer, pH 7.2. Non-specific binding was assessed by replacing 10 µl of the tris acetate buffer with 10 µl of 2.5 mM cold glutamate solution. Blanks were included which omitted the binding protein. The tubes were incubated at 30°C for 20 minutes. The incubation was terminated by the addition of 4 ml of ice cold buffer. The samples were then rapidly filtered through pre-wetted Whatman GF/C glass microfilters in a 12 place Millipore sampling manifold. Each filter was washed 3 times with 4 ml of cold buffer. The filters were transferred to scintillation vials containing 3 ml of scintillation cocktail and shaken for 30 minutes. Finally the radioactivity was quantified in a Beckman LS 2800 scintillation counter.

TABLE 2.8 : Scheme of the [^{3}H]Glutamate Binding Assay

REAGENTS (µl)	TOTAL BINDING			NON-SPECIFIC BINDING		
	BLANK	STANDARD	SAMPLE	BLANK	STANDARD	SAMPLE
Buffer*	210	10	10	200	---	---
[^{3}H]Glutamate	40	40	40	40	40	40
Glutamate	---	---	---	10	10	10
Homogenate	---	200	200	---	200	200
	Incubate at 30°C for 20 minutes					
Buffer*	4 ml	4 ml	4 ml	4 ml	4 ml	4 ml
	Filter through Whatman GF/C glass filters Wash filters 3 x with 4 ml ice cold buffer*					
Scintillation cocktail	3 ml	3 ml	3 ml	3 ml	3 ml	3 ml
	Shake for 30 minutes and count radioactivity					

* 50 mM Tris-acetate buffer, pH 7.2.

2.8.6 Results

The actual cpm obtained from the binding study were converted to dpm by correcting for the counting efficiency of the scintillation

counter. Blank values were subtracted from samples values. The saturable specific binding was calculated as the total binding minus the non-displacable, non-specific binding determined in the presence of 0.1 mM cold glutamate. These dpm were converted to Curies (1 Curie = 2.2 x 10^{22} dpm)and subsequently to moles of [^{3}H]glutamate using the specific activity of the radioactive compound (1 mole = 45 x 10^3 Ci). The specific binding was finally expressed in terms of fmoles [^{3}H]glutamate bound/mg protein. The final result is the mean of duplicate or triplicate determinations for n = 10.

2.8.7 Discussion

The receptor purification process took approximately 12 hours. As it was not always possible to complete the whole purification procedure on the day of sacrifice, it was often necessary to store the forebrains or a subcellular fraction overnight. As freezing has been reported to not affect NMDA binding sites, intermediate steps were stored frozen at -20°C when necessary. This did not effect a marked change in total or specific glutamate binding, although it did appear to increase the protein concentration of the synaptic plasma membrane fraction. Further purification of synaptic structures from the synaptic plasma membrane fraction by detergent treatment, which results in the removal of non-synaptic membranes, has been reported to lead to large enrichments in glutamate binding sites [Monaghan and Cotman, 1986]. Detergent treatment was not attempted in this study due to the limited amount of protein obtained from the sucrose gradient.

CHAPTER 3

THE EFFECT OF CHRONIC HYDROCORTISONE TREATMENT ON PINEAL PHYSIOLOGY AND BIOCHEMISTRY

3.1 INTRODUCTION

Careful and complete elucidation of pineal - adrenal gland interactions awaits further research. Much of the literature proposes that there is an interaction between these two endocrine glands and supports an inhibitory or regulatory role for the pineal gland on adrenal function [Romijn, 1978; Armstrong *et al*, 1982]. While much attention has been focused on the possible influence of the pineal on the adrenal glands, few researchers have addressed the reverse influence of the adrenals on the pineal gland. The latter issue is worthy of deeper understanding because if the pineal gland were to regulate adrenal function, it would be necessary for the pineal to detect and respond to adrenal output.

The adrenal glands secrete catecholamines and glucocorticoids as the final step in a neuroendocrine cascade initiated on perception of a stressor by the brain [Haynes and Larner, 1975; Ganong, 1979]. In view of the anti-stressogenic role proposed for the pineal gland, it was of interest to determine whether either of these secretions could induce any physiological response from the pineal. Lynch *et al* [1973] demonstrated that factors which increase sympathetic activity could rapidly increase pineal melatonin levels. As the rise in melatonin levels in rats could be prevented by propranolol, a ß-adrenergic antagonist, it was

concluded that this effect was mediated by catecholamines. As an end organ of the sympathetic nervous system with a rich blood supply, the pineal gland would be an obvious target for the action of catecholamines derived from nervous or peripheral sources. Thus, an increase in pineal activity following stress could indicate a non-specific response to increased circulating catecholamine levels resulting from enhanced stress-induced sympathetic activity, or an important regulatory mechanism initiated to modulate the neuroendocrine consequences of stress. Other researchers have proposed that a high affinity uptake system in the pineal gland protects the organ against the action of generalized sympathetic activity during the day by ensuring the rapid sequestration of catecholamines by presynaptic nerve terminals [Armstrong *et al* [1982].

The effect of glucocorticoids on the pineal gland has not been directly assessed, although sustained elevation of circulating glucocorticoid levels are the major neuroendocrine consequence of long term stress. The aim of this study was to directly assess the effect of glucocorticoids on pineal indoleamine metabolism, and the secretion of the putative pineal hormone, melatonin.

3.2 THE EFFECT OF CHRONIC HYDROCORTISONE TREATMENT ON RAT PINEAL AND ADRENAL GLAND WEIGHT AND PINEAL INDOLEAMINE METABOLISM IN ORGAN CULTURE

3.2.1 Introduction

The change in mass of an organ is often associated with altered physiological processes within the organ. Generally, hyperplasia or an increase in mass indicates increased activity and metabolism within the organ, while hypoplasia or a decrease in mass is

associated with a decrease in the metabolic activities within the organ. This parameter has been used in many experiments as a crude indication of a possible effect of treatment on a particular organ.

As discussed previously, pineal organ culture provides an ideal tool with which to examine pineal indoleamine metabolism. When incubated in the presence of a radioactive [^{14}C]serotonin precursor, the pineal gland utilizes this substrate to synthesize radioactively labelled metabolites. These are secreted into the culture medium from where they can be isolated and finally measured using liquid scintillometry.

In this experiment, an attempt was made to ascertain whether chronic hydrocortisone treatment would induce any marked weight changes in the pineal or adrenal glands indicative of enhanced or reduced activity. The pineal glands were then cultured *in vitro* to determine the effect of the hydrocortisone treatment on indoleamine metabolism.

3.2.2 Materials and Methods

Two groups of five male Wistar rats of the albino strain were randomly assembled. The test group was treated for seven days with hydrocortisone according to the schedule described in section 2.1.1. The control group received the appropriate vehicle. The animals were sacrificed on the eighth morning and their pineal and adrenal glands rapidly removed and weighed. The pineal glands were then cultured *in vitro* for 24 hours in the presence of radioactive serotonin as described in section 2.2.3. After 24 hours, the incubation was terminated and the culture medium was analyzed for radioactive metabolites of pineal metabolism using thin layer chromatography and liquid scintillometry as outlined in section 2.2.4.

3.2.3 Results

The mass of the pineal and adrenal glands from the normal and hydrocortisone treated animals is tabulated in Tables 3.1 and 3.2. respectively. The difference between the means of the control and treated groups was statistically analyzed using the Student's t test. There was no change in pineal weight after seven days of hydrocortisone treatment, but the weight of the adrenal glands from the treated animals was significantly reduced ($p < 0.05$).

TABLE 3.1 : Weights of Pineal Glands from Hydrocortisone Treated Rats

TREATMENT	WEIGHT ± SEM (mg)
Control	1.8 ± 0.25
Hydrocortisone	2.1 ± 0.30

TABLE 3.2 : Weights of Adrenal Glands from Hydrocortisone Treated Rats

TREATMENT	WEIGHT ± SEM (mg)
Control	67.7 ± 5.28
Hydrocortisone	49.3 ± 3.74 *

* $p < 0.05$ compared with the control.

The radioactivity corresponding to each of the indole metabolites isolated from the culture medium following the incubation of the pineal glands with [^{14}C]serotonin are tabulated in Table 3.3 and graphically presented in Figure 3.1.

TABLE 3.3 : Pineal [^{14}C]Indole Metabolism by Pineal Glands in Organ Culture following Chronic Hydrocortisone Treatment

INDOLE	dpm/10 µl medium ± SEM		P VALUE
	CONTROL	HYDROCORTISONE	
Melatonin	2701 ± 464	4660 ± 690	< 0.05
N-acetylserotonin	1232 ± 306	2513 ± 383	< 0.05
Hydroxyindole acetic acid	56231 ± 2566	40516 ± 4590	< 0.05
Hydroxytryptophol	14539 ± 809	21893 ± 1264	
Methoxyindole acetic acid	345 ± 48	403 ± 78	
Methoxytryptophol	453 ± 38	688 ± 75	< 0.05

The difference between the means of the control and treated groups for each particular indole was statistically analyzed using the Student's t test. The pineal glands from the hydrocortisone

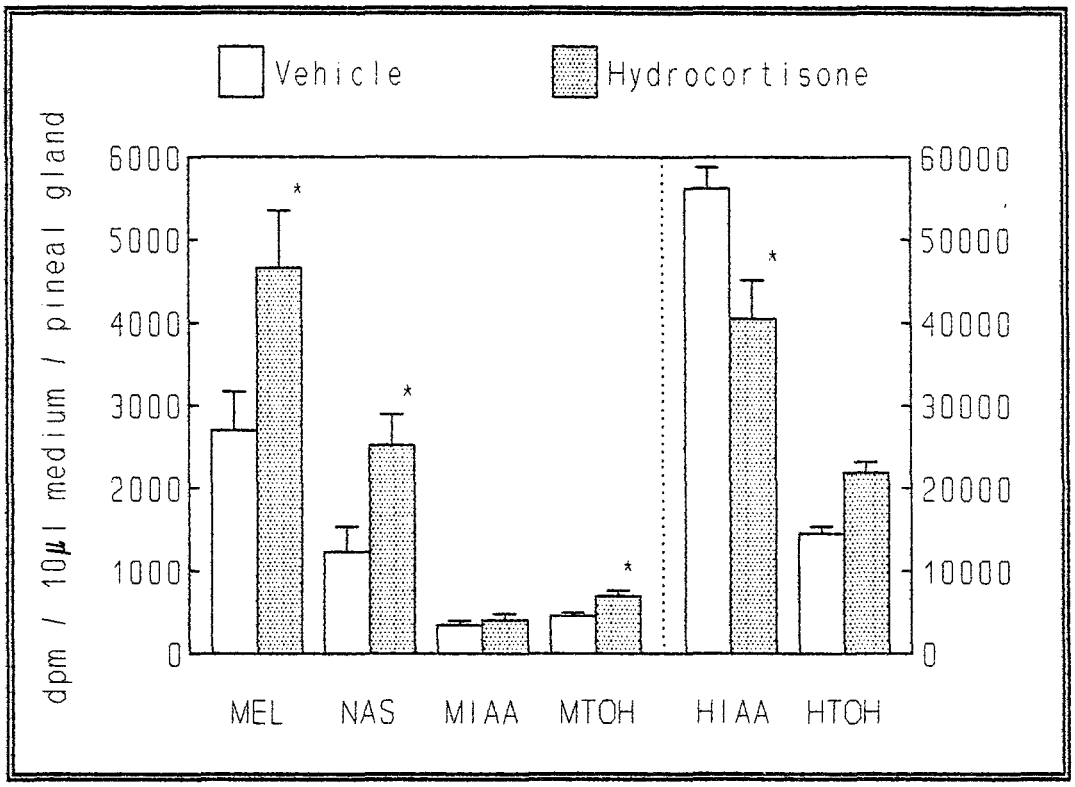

FIGURE 3.1 : **<u>Effect of Chronic Hydrocortisone Treatment on [^{14}C]Serotonin Metabolism by Rat Pineal Glands in Organ Culture</u>.**

Values represent the mean + SEM for n = 5. Comparison of hydrocortisone treatment with relavent control:

* $p < 0.05$ for MEL, NAS, MTOH and HIAA.

treated animals synthesized considerably more N-acetylserotonin, melatonin and 5-methoxytryptophol than the control pineal glands. The levels of hydroxyindole acetic acid were lower in the pineals from the normal rats than those from the treated animals, while metabolism of hydroxytryptophol and methoxyindole acetic acid was unaffected by the steroid treatment.

3.2.4 Discussion

As hydrocortisone is absorbed through the gastrointestinal tract, it may be assumed that the oral hydrocortisone treatment resulted in a significant increase in plasma hydrocortisone levels. Marinova *et al* [1991] reported that the treatment schedule used in this study can be expected to produce supraphysiological levels of circulating glucocorticoid. The decrease in adrenal mass is consistent with decreased stimulation of the adrenal glands by ACTH released from the pituitary gland. Elevated circulating hydrocortisone levels would be expected to interact with glucocorticoid receptors in the hypothalamus, reducing the release of CRH, and subsequently the release of ACTH from the pituitary gland. The reduced stimulation of ACTH on the adrenal glands would characteristically result in inactivity and hypoplasia of these organs. The observed hypertrophy therefore confirms that circulating glucocorticoid levels were significantly elevated by the hydrocortisone treatment.

Although there was no significant increase in pineal mass, the oral hydrocortisone treatment increased the ability of the pineal gland to synthesize N-acetylserotonin, melatonin and 5-methoxytryptophol. This observation suggests that a positive feedback mechanism is operative between the adrenal glands and melatonin metabolism. It suggests that glucocorticoids derived from the adrenal glands during periods of increased adrenal activity, may exert a

stimulatory effect on pineal metabolism to increase melatonin synthesis. An increase in melatonin synthesis in response to increased circulating glucocorticoid levels such as those associated with exposure to stress, supports the involvement of melatonin in the adaptational response to stress.

3.3 THE EFFECT OF CHRONIC HYDROCORTISONE TREATMENT ON RAT PINEAL N-ACETYLTRANSFERASE ACTIVITY

3.3.1 Introduction

Having repeatedly established that chronic hydrocortisone treatment significantly enhances the ability of the pineal gland to synthesize N-acetylserotonin and melatonin *in vitro*, a possible mechanism of action was explored. NAT is responsible for catalysing the conversion of serotonin to N-acetylserotonin and has been shown to play a vital role in regulating pineal melatonin synthesis. NAT *in vivo* is primarily regulated by rises in intracellular cyclic AMP levels elicited by ß-adrenergic stimulation. The episodic release of norepinephrine from nerve variscosities terminating on the pineal gland, stimulates postsynaptic $ß_1$-adrenergic receptors which are coupled to adenylate cyclase. The subsequent rise in intracellular cyclic AMP levels elevates NAT activity via a process involving protein transcription and translation.

The purpose of this experiment was to determine whether chronic hydrocortisone treatment could affect pineal NAT activity. In addition to monitoring basal NAT activity during the daytime when it is generally low, a ß-adrenergic agonist, isoprenaline, was used to mimic the nighttime rise in enzyme activity. This made it possible to determine the effect of the hydrocortisone treatment

on ß-adrenergic receptor stimulated NAT activity.

3.3.2 Materials and Methods

Four groups of five male Wistar rats of the albino strain were randomly assembled. Two groups were treated for seven days with hydrocortisone according to the scheme described in section 2.1.1 and two groups received the vehicle. The animals were sacrificed at 12h00 on the eighth morning. Three hours prior to sacrifice, a set of the hydrocortisone-treated and a set of the control rats were injected intraperitoneally with isoprenaline (5mg/kg), while the remaining two groups were injected with the vehicle. Following sacrifice, the pineal glands were swiftly removed and assayed for NAT activity according to the scheme described in section 2.3.

3.3.3 Results

The enzyme activity, expressed in terms of pmoles N-acetyltryptamine formed per pineal gland per hour, was calculated as described in section 2.3.4. The data were analyzed by one-way analysis of variance and statistical differences between groups were determined using the Bonferroni multiple range test. Values, which represent the mean ± SEM (n = 5), are tabulated in Table 3.4 and graphically presented in Figure 3.2.

Hydrocortisone treatment alone did not significantly affect NAT activity during the day. Isoprenaline effected a 7 - 8 fold increase in NAT activity in both control and hydrocortisone treated rats ($p < 0.0001$). Furthermore, hydrocortisone significantly increased isoprenaline stimulated NAT activity ($p < 0.0001$).

TABLE 3.4 : N-Acetyltransferase Activity in Normal and Hydrocortisone treated rats

TREATMENT	pMoles N-ACETYLTRYPTAMINE formed per hour ± SEM
Control	0.25 ± 0.017
Isoprenaline	1.72 ± 0.171
Hydrocortisone	0.34 ± 0.032
Hydrocortisone + Isoprenaline	2.67 ± 0.208

Con. vs Iso. : $p < 0.0001$ HC vs HC + Iso. : $p < 0.0001$

HC vs Iso. : $p < 0.0001$ Iso. vs HC + Iso. : $p < 0.0001$

3.3.4 Discussion

Although the hydrocortisone treatment did not affect basal NAT activity during the day, it significantly enhanced ß-adrenergic receptor stimulated enzyme activity. From this it appears that glucocorticoids sensitize NAT to ß-adrenergic receptor stimulation. This could indicate a mechanism whereby pineal indole metabolism may be enhanced under stressful conditions. Glucocorticoids released from the adrenal cortex could sensitize pineal NAT activity to stimulation by catecholamines derived from sympathetic nervous activity or the adrenal medullae during the normal physiological response to stress.

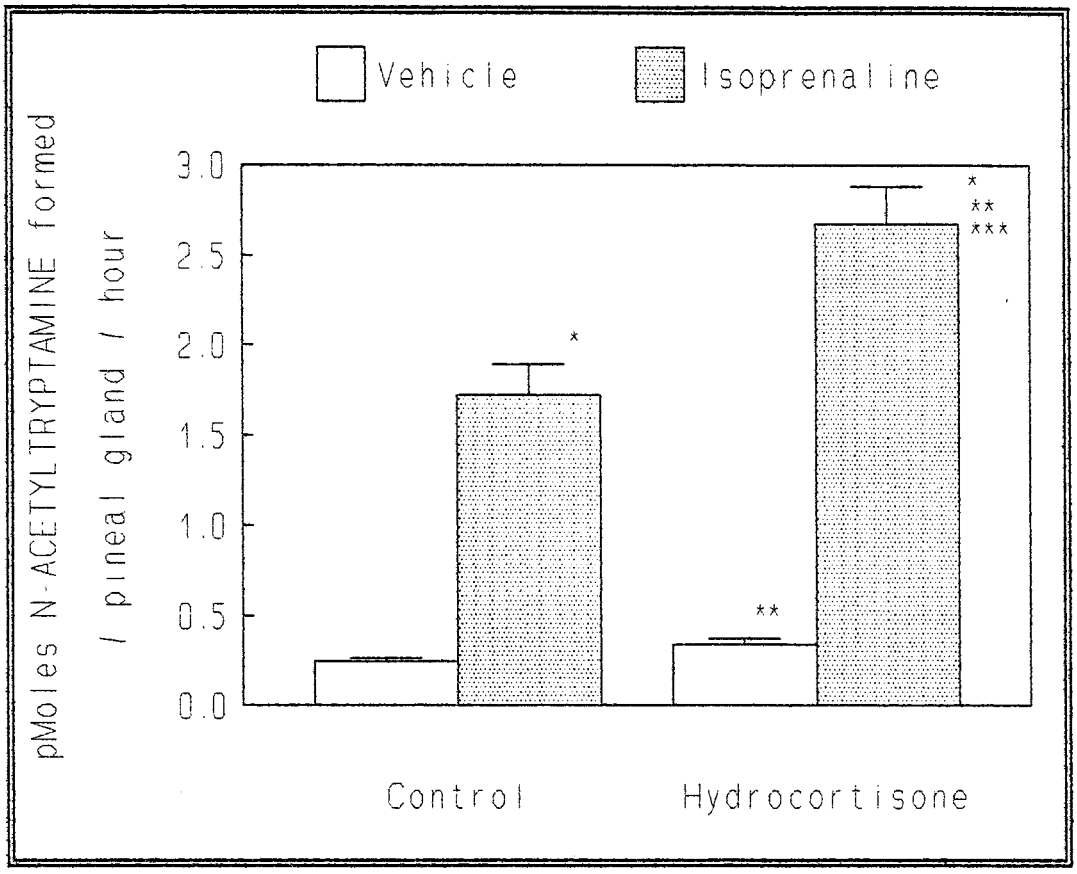

FIGURE 3.2 : **Effect of Chronic Hydrocortisone Treatment on Isoprenaline-stimulated N-Acetyltransferase Activity in Rat Pineal Gland.**

Values represent the mean + SEM of triplicate determinations for n = 5.

* $p < 0.001$ compared with the control

** $p < 0.001$ compared with the isoprenaline-stimulated pineals

*** $p < 0.001$ compared with the hydrocortisone-treated pineals

Water was used as the vehicle.

3.4 THE EFFECT OF CHRONIC HYDROCORTISONE TREATMENT ON RAT PINEAL CYCLIC AMP LEVELS

3.4.1 Introduction

Having established that the increase in melatonin metabolism in pineal organ culture is accompanied by elevation in NAT activity, the mechanism responsible for the increase in NAT activity was investigated. The hydrocortisone could be acting by binding to cytosolic steroid receptors, inducing a classical rise in protein synthesis and general enzyme proteins within the pinealocytes. This could explain the rise in N-acetylserotonin and melatonin as well as the rise in 5-methoxytryptophol. Another possibility is that the hydrocortisone could be stimulating NAT activity by increasing the cyclic AMP responsiveness of ß-adrenergic receptors. The two mechanisms are not mutually exclusive.

Cyclic AMP is synthesized from ATP by adenylate cyclase. NAT is controlled *in vivo* by changes in cyclic AMP levels elicited by ß-adrenergic receptor-linked adenylate cyclase. Chronic stimulation of the ß-adrenergic receptors results in decreased sensitivity of the receptors to further stimulation. Daya and Joubert [1984] showed that ß-adrenergic receptor subsensitivity resulting from chronic stimulation by isoprenaline was reversed by a single dose of hydrocortisone 16 hours before sacrifice. The authors concluded that their findings implied that glucocorticoids might have a feedback relationship on the pineal gland by altering the cyclic AMP responsiveness of pineal ß-adrenergic receptors.

The ability of chronic hydrocortisone treatment to alter the cyclic AMP responsiveness of ß-adrenergic receptors was investigated by examining the basal and ß-adrenergic agonist stimulated cyclic AMP levels in pineal homogenates from rats chronically treated with

hydrocortisone.

3.4.2 Materials and Methods

Two groups of five male Wistar rats of the albino strain were randomly assembled. The test group was treated for seven days with hydrocortisone according to the scheme described in section 2.1.1 and the control group received the vehicle. The animals were sacrificed at 10h00 on the eighth morning and their pineal glands swiftly removed. The pineals were carefully bisected and cultured *in vitro* as described in section 2.2.3. A set of the hydrocortisone treated pineal hemispheres and a set of the control pineal hemispheres were incubated in the presence of 100 µM isoprenaline while the remaining two groups of hemispheres were incubated in the presence of the vehicle. The incubation was terminated after 6 hours by freezing the pineal tissue on dry ice. The hemispheres were then homogenized and assayed for cyclic AMP according to the schedule described in section 2.5.

3.4.3 Results

The pmoles of cyclic AMP in each pineal gland were calculated as described in section 2.5.6. The data were analyzed by one-way analysis of variance and statistical differences between groups were determined using the Bonferroni multiple range test. Results are presented as the mean ± SEM (n = 5), and are tabulated in Table 3.5 and graphically presented in Figure 3.3.

Hydrocortisone treatment significantly increased daytime basal cyclic AMP levels ($p < 0.05$). Isoprenaline increased basal cyclic AMP levels in both normal ($p < 0.01$) and hydrocortisone ($p < 0.001$) treated rats.

TABLE 3.5 : Cyclic AMP levels in Normal and Hydrocortisone Treated Rats

TREATMENT	pMoles CYCLIC AMP formed per hour ± SEM
Control	2.155 ± 1.424
Isoprenaline	35.055 ± 5.337
Hydrocortisone	20.093 ± 5.743
Hydrocortisone + Isoprenaline	64.775 ± 10.443

Con. vs HC : $p < 0.05$ Iso vs HC + Iso. : $p < 0.01$
Con. vs Iso. : $p < 0.01$ HC vs HC + Iso. : $p < 0.001$

3.4.4 Discussion

Cyclic AMP is an important intracellular messenger which, in the pineal gland, is intimately associated with regulating NAT activity. Hydrocortisone caused an increase in both basal and isoprenaline-stimulated cyclic AMP levels in rat pineal glands. The increase in basal cyclic AMP evoked by hydrocortisone was significantly less than the nighttime rise found in rats not treated with the steroid. Viewed together with the inability of hydrocortisone to increase basal NAT activity found previously, it can be deduced that the rise in basal cyclic AMP levels was not sufficient to invoke a significant rise in the activity of this enzyme. However, it is likely that limited stimulation of the ß-adrenergic receptors will be required to increase cellular cyclic

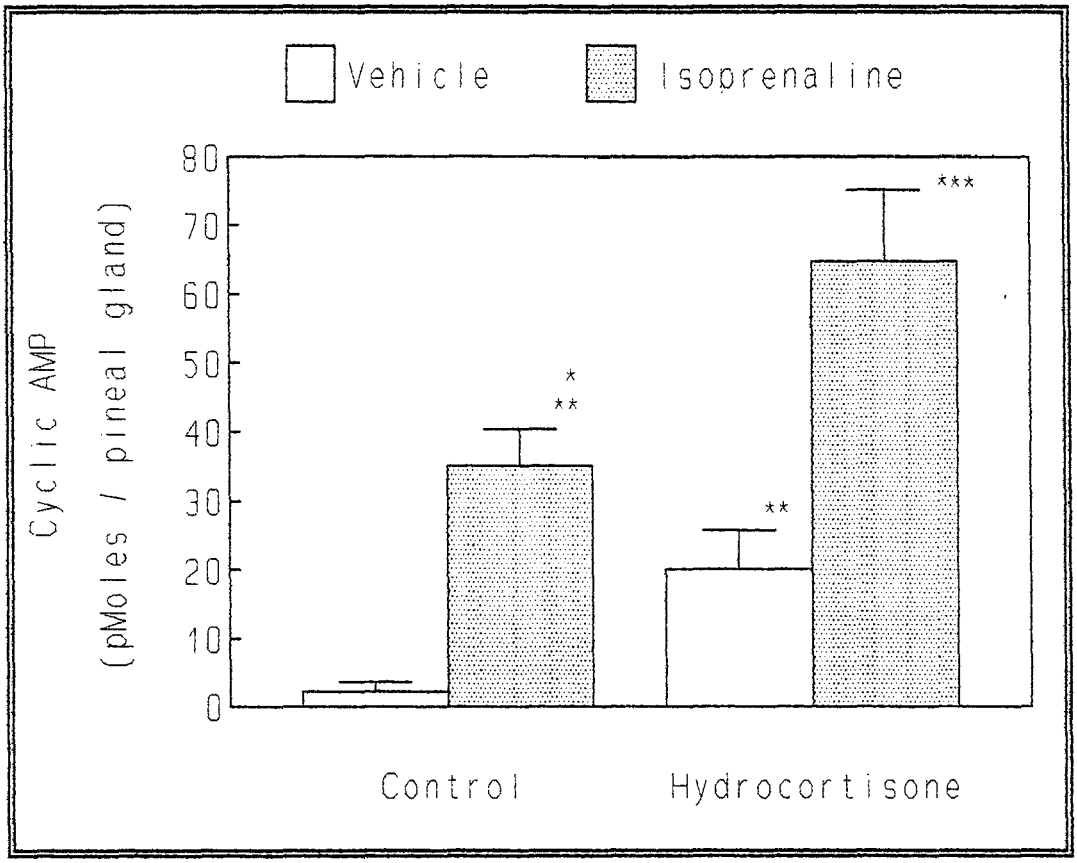

FIGURE 3.3 : **Effect of Chronic Hydrocortisone Treatment on Daytime Basal and Isoprenaline-stimulated cyclic AMP levels in Rat Pineal Glands**

Values represent the mean + SEM for n = 5.

* $p < 0.05$ compared with the control
** $p < 0.05$ compared with the hydrocortisone and isoprenaline-stimulated pineals
*** $p < 0.001$ compared with the control

Water was used as the vehicle.

AMP levels above the "threshold" necessary for NAT induction. This is a further indication of a mechanism whereby hydrocortisone does not directly increase NAT activity and the synthesis of melatonin, but rather sensitizes the pineal gland to future stimulation by ß-adrenergic agonists.

3.5 THE EFFECT OF CHRONIC HYDROCORTISONE TREATMENT ON RAT PINEAL ß-ADRENERGIC RECEPTOR BINDING SITES

3.5.1 Introduction

The pineal gland is richly supplied with ß-adrenergic receptors. They have been found to exhibit a circadian rhythm and display the phenomena of super- and sub-sensitivity. As such they play an important role in regulating dark-induced stimulation of pineal indoleamine metabolism. In the dark or at nighttime, norepinephrine released from postganglionic sympathetic nerves terminating on the pineal gland, interacts with ß-adrenergic receptors on pinealocytes to initiate a classical rise in intracellular cyclic AMP. The cyclic AMP in turn stimulates the synthesis of NAT and subsequently leads to an increase in the conversion of serotonin to N-acetylserotonin and melatonin.

Having established that the hydrocortisone-induced rise in melatonin synthesis was accompanied by both a rise in NAT activity and cyclic AMP levels, it was necessary to investigate whether the effect was proximal or distal to the ß-adrenergic receptors, i.e. whether hydrocortisone enhances cyclic AMP levels by influencing the interaction of ß-adrenergic agonists with adenylate cyclase-linked ß-adrenergic receptors, or by a mechanism independent of the ß-receptors.

In this experiment, the specific binding of [^{3}H]DHA to pineal ß-adrenergic receptors was determined in both normal and hydrocortisone treated rats.

3.5.2 Materials and Methods

Two groups of eight male Wistar rats of the albino strain were randomly assembled. The test group was treated for seven days with hydrocortisone according to the scheme described in section 2.1.1. The control group received the appropriate vehicle. The animals were sacrificed on the eighth morning and their pineal glands rapidly removed. Two similarly treated pineal glands were homogenized together and the specific binding of [^{3}H]DHA to ß-adrenergic receptors was measured according to the method described in section 2.4 using 8 nM [^{3}H]DHA.

3.5.3 Results

The specific binding of [^{3}H]DHA to the pineal ß-adrenergic receptors, expressed as fmole/mg protein, was calculated as described in section 2.6.6. The statistical difference between the means from the two groups was determined using the Student's t-test. Values represent the mean of quadruplicate determinations ± SEM (n = 4). The effect of chronic hydrocortisone treatment on ß-adrenergic receptor binding is tabulated in Table 3.6 and graphically presented in Figure 3.4.

The hydrocortisone treatment resulted in a small, but significant, increase in the specific binding of [^{3}H]DHA to pineal ß-adrenergic receptors ($p < 0.05$).

TABLE 3.6 : **Binding of [^{3}H]DHA to ß-Adrenergic receptors in the Pineal Glands of Normal and Hydrocortisone treated rats**

TREATMENT	SPECIFIC BINDING (fmoles/mg protein)
Control	0.104 ± 0.0064
Hydrocortisone	0.134 ± 0.0085

Control vs HC : $p < 0.05$

3.5.4 Discussion

The present results indicate that hydrocortisone increases pineal melatonin synthesis by a mechanism involving the ß-adrenergic receptors. Hydrocortisone increases the specific binding of ß-adrenergic agonists to pineal ß-adrenergic receptors. Whether this is due to an increase in affinity or number of binding sites, remains to be established. The number of ß-adrenergic receptors may be increased by processes involving the externalization of the receptors on the cell membrane or an increase in the synthesis of receptors.

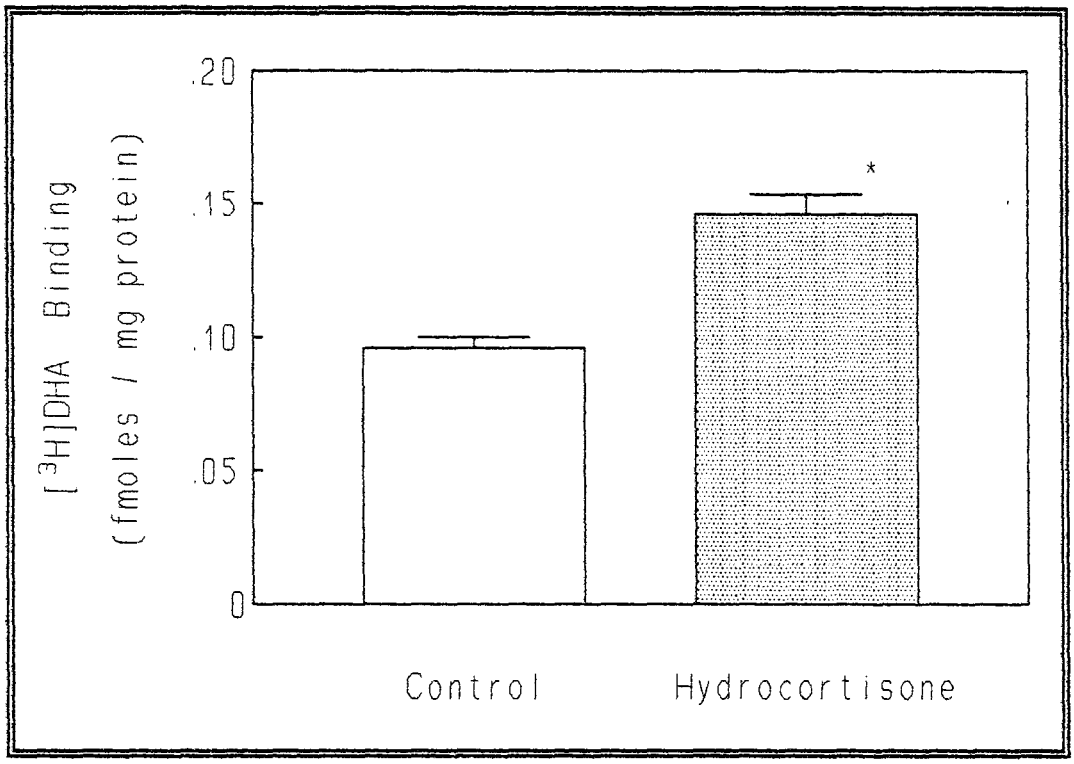

Figure 3.4 : **Effect of Chronic Hydrocortisone Treatment on Specific Binding of [^{3}H]DHA to Rat Pineal Membranes**

Values represent the mean + SEM of triplicate determinations for n = 5.

* $p < 0.05$ compared with the control

3.6 DISCUSSION

It is apparent from the preceding series of experiments that the glucocorticoid, hydrocortisone, is able to endow pinealocytes with the ability to increase melatonin and 5-methoxytryptophol synthesis. The rise in indole synthesis could be the result of at least two possible mechanisms: hydrocortisone could bind to steroid receptors in the pinealocyte cytosol [Haynes and Larner, 1975; Ganong, 1979] and increase the synthesis of enzymes involved in the biosynthesis of melatonin, or hydrocortisone could act by altering the responsiveness of ß-adrenergic receptors to their natural ligands and thereby enhance the ß-receptor stimulated rise in intracellular cyclic AMP, NAT and finally melatonin synthesis [Daya and Joubert, 1984]. Although it does not explain the rise in 5-methoxytryptophol, it appears as if at least the latter mechanism is true. Hydrocortisone increased the specific binding of a radiolabelled ß-adrenergic agonist to pineal membranes *in vitro*. Increases in cyclic AMP levels and NAT activity were also observed following chronic hydrocortisone treatment.

The hydrocortisone treatment resulted in insignificant or small increases in the basal NAT activity and cyclic AMP levels, but dramatically increased these parameters under ß-adrenergic receptor stimulation. This suggests that rather than directly increasing melatonin synthesis, glucocorticoids, like hydrocortisone, increase the sensitivity of the pineal gland to stimulation by ß-adrenergic agonists. This would explain the ability of ß-adrenergic antagonists to block the increase in pineal melatonin following stress responses. Such observations have lead previous researchers to attribute the increase in melatonin synthesis following stress, to catecholamines [Lynch *et al*, 1973].

In this study, circulating glucocorticoid levels were increased by

the oral administration of hydrocortisone. No particular stress was employed to elevate serum corticosteroid levels as has been the pattern in previous research [Johnson, 1982; Touitou *et al*, 1983]. This excluded the variable parameters associated with individual responses to different stresses.

Although it has been clearly shown that the glucocorticoid, hydrocortisone, can endow the biochemical machinery of pinealocytes with the ability to increase melatonin production, it remains to be established whether this is purely a pharmacological effect or whether physiological levels of the glucocorticoid can elicit the same effect. This would clarify the possibility of stress-elevated glucocorticoid levels inducing an increase in pineal melatonin synthesis. Furthermore, it remains to be established that it is the high concentration of systemic glucocorticoids that is producing the effect and not the ratio of corticosterone to hydrocortisone as has been proposed for other steroid effects. Corticosterone is the predominant glucocorticoid in rats, while hydrocortisone is the predominant glucocorticoid in humans. Therefore, it is possible that either the hydrocortisone alone, or the increased hydrocortisone : corticosterone ratio presumably induced by the steroid treatment which increased the ability of the pineal gland to synthesize melatonin and other indoles. In view of this, it is essential that in future research, serum levels of the glucocorticoids should be measured.

An attempt was also made to ascertain whether the increase in melatonin production observed *in vitro* also occurred *in vivo* during the hydrocortisone treatment. As melatonin is not stored in the pineal gland, increases in melatonin synthesis are rapidly reflected in the blood. A radioimmunoassay was employed to measure plasma melatonin levels. Unfortunately, the method of Fraser *et al* [1983] which was used, had a number of drawbacks. Primarily

used for measuring melatonin levels in humans, it required a relatively large amount of plasma to be extracted from each test individual. This was often difficult to achieve when working with rats. The second major problem was that of haemolysis. Haem released on lysis of the red blood cells inconsistently interfered with the assay by causing quenching problems during radioactive counting. It was, therefore, not possible within the duration of this study to overcome all the problems and get the procedure to produce consistent and valid results. However, preliminary, individual, trial experiments appear to indicate that basal plasma melatonin levels in hydrocortisone treated animals are unaffected during the daytime, but may rise above normal nighttime levels at night. If this effect could be conclusively demonstrated, it would further support the theory that glucocorticoids sensitize the pineal gland to ß-adrenergic stimulation, rather than leading directly to increased indoleamine metabolism. The particular radioimmunoassay used was appealing because of its reputed sensitivity and simplicity. It only required isolation of the blood plasma fraction from the test animals without any additional extraction or purification procedures. However, the possibility of using urinary 6-sulphatoxymelatonin as an index of plasma melatonin should be investigated. This method would have the advantage of eliminating the sacrifice of the animals and hence it would be possible to assay more time points over a longer experimental duration. The use of such a method is proposed in view of the fact that a more dynamic picture of endogenous events could be obtained without the costly use of large numbers of animals. One of the major criticisms of research in this field is the use of time-point studies which provide limited information with intervals of undefined activity. Furthermore, comparisons are difficult when different researchers choose different time intervals or time points within the photoperiod.

CHAPTER 4

THE INFLUENCE OF MELATONIN ON PERIPHERAL EFFECTS OF CHRONIC HYDROCORTISONE TREATMENT

4.1 INTRODUCTION

Elevated circulating levels of glucocorticoids have been associated with numerous undesirable conditions, the most serious of which is damage to the brain and disruption of brain function. Elevated circulatory glucocorticoid levels occur endogenously in times of stress [Haynes and Larner, 1975; Ganong, 1979]. In response to a variety of stressors, the hypothalamus releases CRH, which stimulates the release of ACTH from the anterior pituitary. The latter subsequently stimulates the adrenal glands to release glucocorticosteroids. In a healthy individual, the glucocorticoids interact with receptors in the hypothalamus and pituitary gland to suppress further CRH and ACTH release and thereby regulate further stimulation of the adrenal cortex [Haynes and Larner, 1975]. However, following chronic, or continual long-term activation of this feedback system such as that experienced by chronically stressed or by aged animals, the sensitivity of the negative feedback mechanism is reduced and eventually results in the inability to terminate the adrenocorticoid response to subsequent stimulation [Sapolsky *et al*, 1986]. The continuously elevated glucocorticoid levels have a number of physiological side effects which include myopathy, steroid diabetes, hypertension, immunosuppression, infertility, inhibition of growth, depression and other mental disorders.

Although an interaction between the pineal gland and the adrenal glands has not been conclusively established, a large body of evidence suggests that the pineal gland exerts an inhibitory influence on the adrenal glands. Enhanced pineal activity, pineal extracts and treatments with exogenous melatonin may inhibit adrenocorticosteroid synthesis and release. The initial investigations in the present study suggest that the pineal gland responds to increased circulating glucocorticoid levels by increasing its potential to synthesize melatonin and 5-methoxytryptophol. This indicates a possible physiological role for melatonin in conditions of elevated circulating glucocorticoid levels.

The aim of the following series of experiments was to investigate the possible physiological significance of the glucocorticoid-mediated increase in pineal melatonin synthesis, by determining whether melatonin could protect against two of the deleterious consequences of sustained elevated hydrocortisone concentrations.

4.2 EFFECT OF CHRONIC HYDROCORTISONE TREATMENT AND MELATONIN ON RAT LIVER TRYPTOPHAN PYRROLASE ACTIVITY

4.2.1 Introduction

Liver tryptophan pyrrolase is responsible for catalysing the rate-determining step in the catabolic pathway of tryptophan degradation [Badawy and Evans, 1975]. It is thus the major peripheral determinant of free serum and brain tryptophan. Factors which enhance the catabolism of this essential amino acid, reduce the circulating levels of tryptophan, and concomitantly, its availability to the brain. In the brain, tryptophan is utilized

in the synthesis of serotonin, an important neurotransmitter involved in various behavioural activities. Reduced availability of tryptophan to the brain, leads to behavioural and mental disorders associated with deficiencies of the cerebral serotoninergic system [Van Praag, 1982].

Glucocorticoids are one of several factors which increase tryptophan pyrrolase activity. These steroids cause a hormonal induction of the enzyme by mediating the synthesis of new apoenzyme [Knox and Auerbach, 1955; Young, 1981]. Young [1981] demonstrated that high tryptophan pyrrolase activity induced by hydrocortisone diminishes brain tryptophan and serotonin. Patients with depressive disorders have frequently been reported to exhibit abnormally high plasma corticosteroid concentrations. Badawy and Evans [1981] reported that a large number of antidepressant drugs act by inhibiting tryptophan pyrrolase activity. Inhibition of this catabolic pathway, increases brain tryptophan and serotonin levels, alleviating the depression.

The pineal gland has been implicated in both depressive mental disorders [Wurtman and Wurtman, 1989] and in the physiological response to stress [Romijn, 1978; Johnson, 1982; Armstrong *et al*, 1982]. An inverse relationship exists between tryptophan pyrrolase activity and pineal melatonin synthesis [Curzon, 1979]. Furthermore, it has been shown that activation of tryptophan pyrrolase by the haem precursor, 5-ALA [Daya *et al*, 1989], and by the barbiturate, pentobarbitone [Van Wyk *et al*, 1991], decreases rat forebrain serotonin without affecting pineal serotonin levels. Although derived from the same precursor, pineal serotonin is highly conserved. The resultant stability of melatonin levels in adverse conditions is supportive of the homeostatic, regulatory role proposed for the pineal gland.

The aim of this experiment was to determine the effect of melatonin and chronic hydrocortisone treatment on rat liver tryptophan pyrrolase activity.

4.2.2 Materials and Methods

Two groups of five male albino Wistar rats were randomly assembled. The test group was treated for seven days with hydrocortisone according to the schedule described in section 2.1.1. The control group received the appropriate vehicle. The animals were sacrificed on the eighth morning and their livers rapidly removed as described in section 2.1 and frozen at - 70°C in liquid nitrogen. The livers were assayed for tryptophan pyrrolase activity using the schedule described in section 2.7. The effect of melatonin (100 nM - 1 mM) on tryptophan pyrrolase activity in normal and hydrocortisone treated animals was also assessed.

4.2.3 Results

Enzyme activity, expressed as µmoles kynurenine formed/mg protein per hour, was calculated as described in section 2.7.5. Holoenzyme activity was determined in the absence of haemin and total activity in the presence of added haemin. The difference was a measure of apoenzyme activity. The data were analyzed by one-way analysis of variance. Statistical differences between the means were determined using the Students t-test. Values represent the mean ± SEM (n = 5). The results of the enzyme assay are tabulated in Table 4.1 and graphically presented in Figure 4.1. The effect of melatonin on tryptophan pyrrolase activity in normal and hydrocortisone treated rats is tabulated in Table 4.2 and graphically presented in Figures 4.2 and 4.3.

TABLE 4.1 : **Effect of Chronic Hydrocortisone Treatment on Rat Liver Tryptophan Pyrrolase Activity**

ENZYME ACTIVITY	µMoles KYNURENINE formed per mg protein per hour ± SEM		P VALUE
	CONTROL	HYDROCORTISONE	
Total enzyme	5.656 ± 1.0	10.392 ± 1.2	< 0.001
Holoenzyme	4.500 ± 1.2	7.080 ± 0.8	
Apoenzyme	1.156 ± 0.4	3.478 ± 0.8	< 0.05

Tryptophan pyrrolase activity was enhanced in the presence of haemin. There was nearly a 50% increase in total tryptophan pyrrolase activity in the livers from the hydrocortisone treated rats relative to the untreated controls ($p < 0.001$). This was mainly due to a significant increase in apoenzyme activity in the treated animals ($p < 0.05$) as the activity of the holoenzyme fractions were not significantly different.

Haemin increased the activity of tryptophan pyrrolase in both normal and hydrocortisone treated animals. Melatonin caused a dose-dependent inhibition of haemin-induced tryptophan pyrrolase activity in both groups of rats to levels approximating those observed in the absence of haemin. Tryptophan pyrrolase was significantly inhibited by melatonin concentrations above 200 µM in both normal ($p < 0.05$) and hydrocortisone treated rats ($p < 0.001$). The percentage inhibition in both groups of rats was approximately the same.

TABLE 4.2 : Effect of Melatonin on Tryptophan Pyrrolase Activity in Normal and Hydrocortisone Treated Rats

TREATMENT	HOLOENZYME	TOTAL ENZYME	MELATONIN CONCENTRATION				
			1 mM	200 µM	50 µM	1 µM	100 nM
	µMoles KYNURENINE formed per mg protein per hour						
Control	4.9 ± 0.8	6.2 ± 1.0	3.2 ± 0.6	4.7 ± 1.0	5.4 ± 1.1	5.8 ± 1.0	5.8 ± 0.9
Hydrocortisone	7.6 ± 0.7	11.1 ± 0.6	5.6 ± 0.6	8.5 ± 1.0	9.4 ± 0.8	10.5 ± 1.1	11.1 ± 1.1
Percentage of Total Activity							
Control	79.5	100.0	51.1	73.2	83.5	92.0	94.0
Hydrocortisone	68.1	100.0	50.5	75.9	84.8	94.6	99.9

Control: Total enzyme vs 1 mM : $p < 0.01$
Total enzyme vs 200 µM : $p < 0.05$
Hydrocortisone : Total enzyme vs 1 mM : $p < 0.001$
Total enzyme vs 200 µM : $p < 0.001$

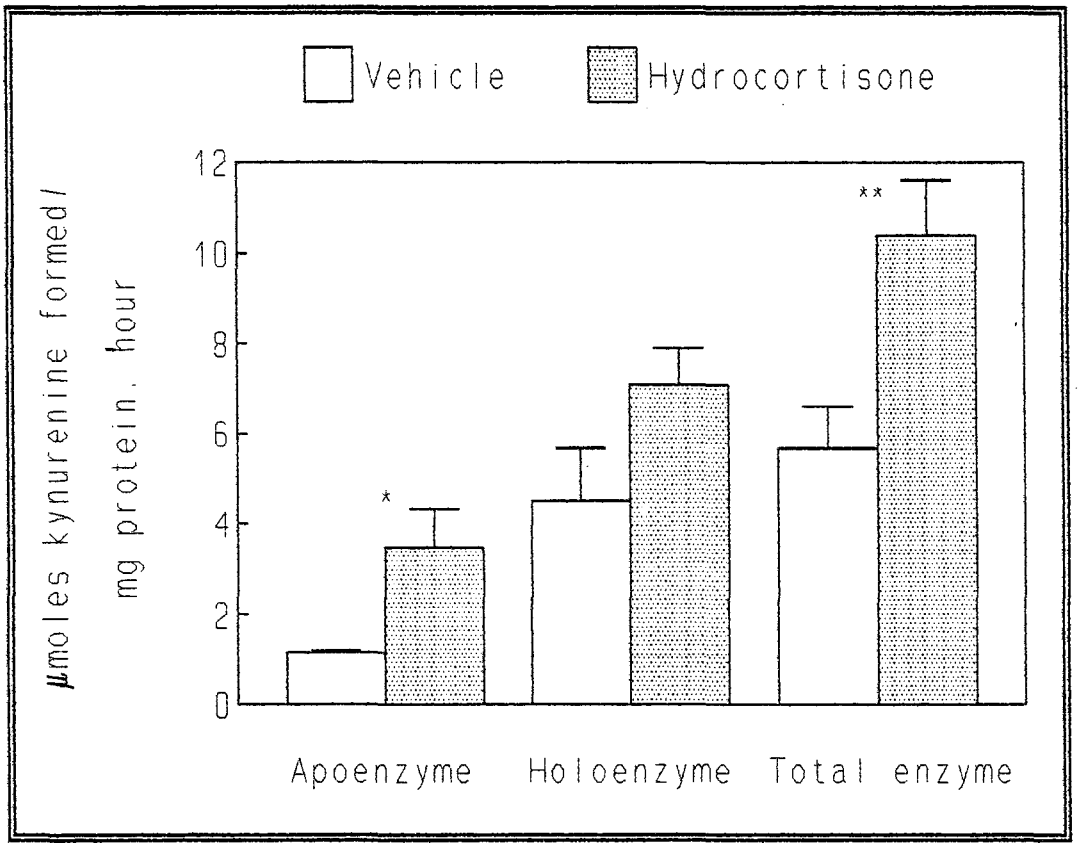

FIGURE 4.1 : **Effect of Chronic Hydrocortisone Treatment on Rat Liver Tryptophan Pyrrolase Activity**

Values represent the mean + SEM of triplicate determinations for n = 5.

Comparison of hydrocortisone treatment with the relevant control:

* $p < 0.05$
** $p < 0.001$

The hydrocortisone was suspended in absolute ethanol : glycerine (1:9) and diluted in 0.5% saline.

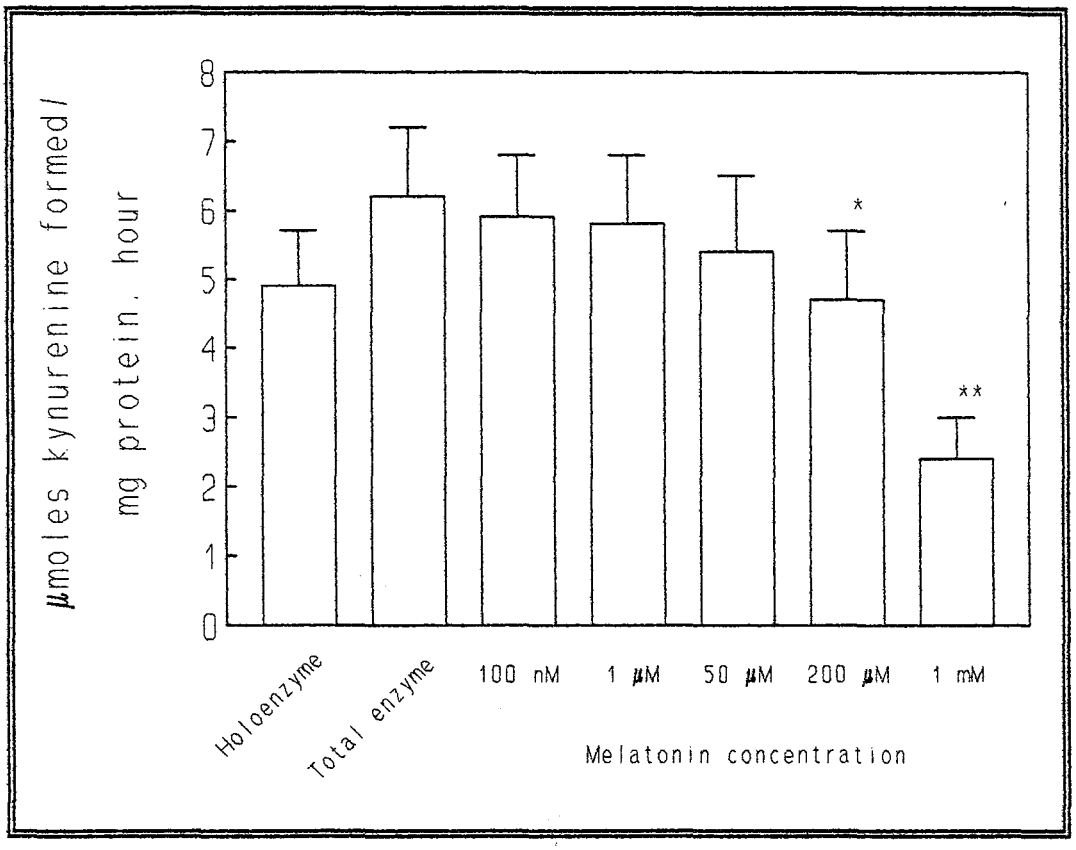

FIGURE 4.2 : Effect of Melatonin on Rat Liver Tryptophan Pyrrolase Activity in Untreated Rats

Values represent the mean + SEM for n = 5.

* $p < 0.05$ compared with total activity

** $p < 0.01$ compared with total activity

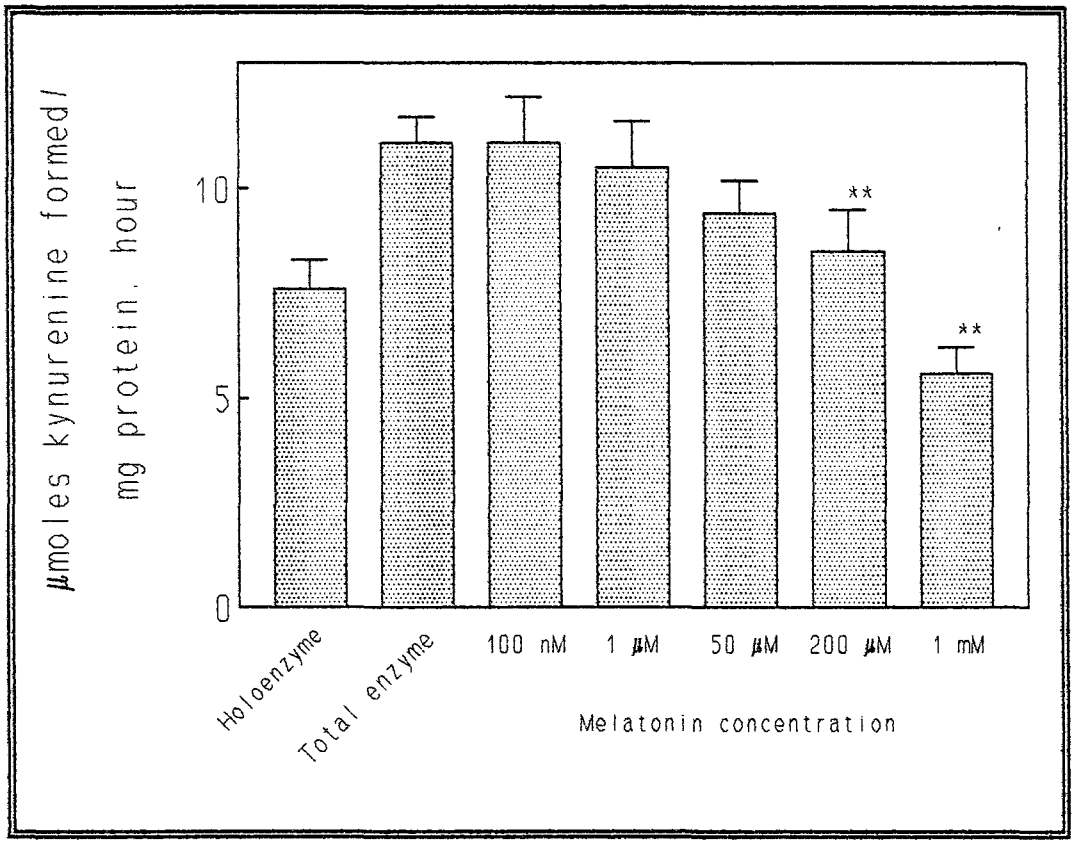

FIGURE 4.3 : **Effect of Melatonin on Rat Liver Tryptophan Pyrrolase Activity in Hydrocortisone Treated Rats**

Values represent the mean + SEM for n = 5.

** $p < 0.001$ compared with total activity

4.2.4 Discussion

Tryptophan pyrrolase is a haem-dependent liver cytosolic enzyme and the addition of the haemin cofactor produced the expected rise in tryptophan pyrrolase activity. Typically, hydrocortisone increases tryptophan pyrrolase apoenzyme activity [Young, 1981]. This was confirmed in the present study. Apoenzyme and total activity were significantly increased while holoenzyme activity was unaffected by the hydrocortisone treatment.

Melatonin, the principal pineal hormone, caused a dose-dependent inhibition of haemin-activated tryptophan pyrrolase activity, with indole concentrations in excess of 200 μM completely reversing the haemin-induced activation of the apoenzyme. This observation is confirmed by the findings of Walsh *et al* [1991]. According to these authors, melatonin significantly inhibits tryptophan pyrrolase activity at indole concentrations above 50 μM. The ability of melatonin to inhibit activation of the apoenzyme suggests that the indole is interfering with the availability of haem to the enzyme. The mechanism of this inhibition requires further investigation.

The fact that hydrocortisone increases apoenzyme activity, and that melatonin inhibits activation of the apoenzyme, suggests that melatonin may play a protective role in preventing excessive tryptophan catabolism resulting from the glucocorticoid-induced increase in tryptophan pyrrolase activity. Young [1981] demonstrated that although glucocorticoids also have an action on protein metabolism that is primarily catabolic, and causes the release of tryptophan from protein to augment the free tryptophan stores, the glucocorticoid-mediated increase in tryptophan pyrrolase activity causes a major decline of total free tryptophan stores in the whole animal. Thus melatonin may play an important

role in preventing excessive loss of this essential amino acid from the body.

4.3 EFFECT OF CHRONIC HYDROCORTISONE TREATMENT AND MELATONIN ON GLUTAMATE BINDING IN RAT BRAIN

4.3.1 Introduction

Another deleterious effect of glucocorticoids, is inhibition of the re-uptake of excitatory amino acid neurotransmitters from the synaptic clefts in the CNS. The resultant accumulation of especially glutamate in the synapse, causes continuous stimulation of postsynaptic glutamate receptors. This effects excessive mobilization of calcium and the influx of ions and water into the neurons which ultimately leads to lysis of the cells. The excitotoxic effects of glutamate are predominantly attributed to stimulation of the NMDA receptor. NMDA- mediated neuronal degeneration is associated with the pathology of cerebral ischaemia and trauma, hypoglycaemia, anoxia, epilepsy, Huntington's disease, senile dementia of the Alzheimer type, Parkinsonism and amyotrophic lateral sclerosis [Schramm *et al*, 1990; Meldrum and Garthwaite, 1990].

The aim of this experiment was to determine the effect of chronic hydrocortisone treatment on cerebral NMDA receptors.

4.3.2 Materials and Methods

Two groups of ten male albino Wistar rats were randomly assembled. The test group was treated for seven days with hydrocortisone according to the schedule described in section 2.1.1. The control group received the appropriate vehicle. The animals were

sacrificed on the eighth morning and their brains rapidly removed. The forebrains were dissected free and measured for glutamate binding sites according to the method described in section 2.8. Saturation curves were prepared by measuring the specific binding of 0 - 500 nM [^{3}H]glutamate to cerebral membranes derived from both normal and treated rats.

4.3.3 Results

The specific binding, expressed as fmoles [^{3}H]glutamate bound/mg protein, was calculated as described in section 2.8.6. Saturation curves and Scatchard plots were derived for the data obtained from both normal and treated rats. The data is tabulated in Table 4.3 and graphically presented in Figures 4.4 - 4.7.

As is shown in Figure 4.4, the binding of [^{3}H]glutamate to cerebral membranes from untreated rats was not completely saturating in the range tested. Scatchard analysis of the data (Figure 4.5) demonstrated the presence of glutamate receptors with an apparent equilibrium dissociation constant (K_D) of 327.3 nM and a maximal number of binding sites (B_{max}) equivalent to 1.127 pmoles/mg protein. The hydrocortisone treatment caused a 30-fold increase in the specific binding of glutamate to rat cerebral membranes. The binding of [^{3}H]glutamate was not saturating in the range tested (Figure 4.6). From the Scatchard analysis (Figure 4.7), the apparent equilibrium dissociation constant was approximately 825.3 nM and the maximum number of binding sites was equivalent to 35.834 pmoles/mg protein.

TABLE 4.3 : Specific Binding of [^{3}H]Glutamate to Cerebral Membranes from Normal and Treated Rats

FREE GLUTAMATE (nM)	SPECIFIC BINDING (fmoles/mg protein)	
	CONTROL	HYDROCORTISONE
25	80.8	650.4
50	146.3	1266.0
100	263.1	4111.9
200	463.2	6177.1
300	499.6	10496.0
400	636.7	11715.0
500	671.4	12903.0

4.3.4 Discussion

The equilibrium dissociation constant (0.327 μM) and maximum number of binding sites (1.127 pmoles/mg protein) obtained for the binding of [^{3}H]glutamate to cerebral membranes from untreated rats are in agreement with previous reports of glutamate binding to NMDA-sensitive binding sites [Monaghan and Cotman, 1986]. The receptor purification procedure employed was theoretically fairly specific for NMDA receptors. Firstly, ions such as calcium, sodium

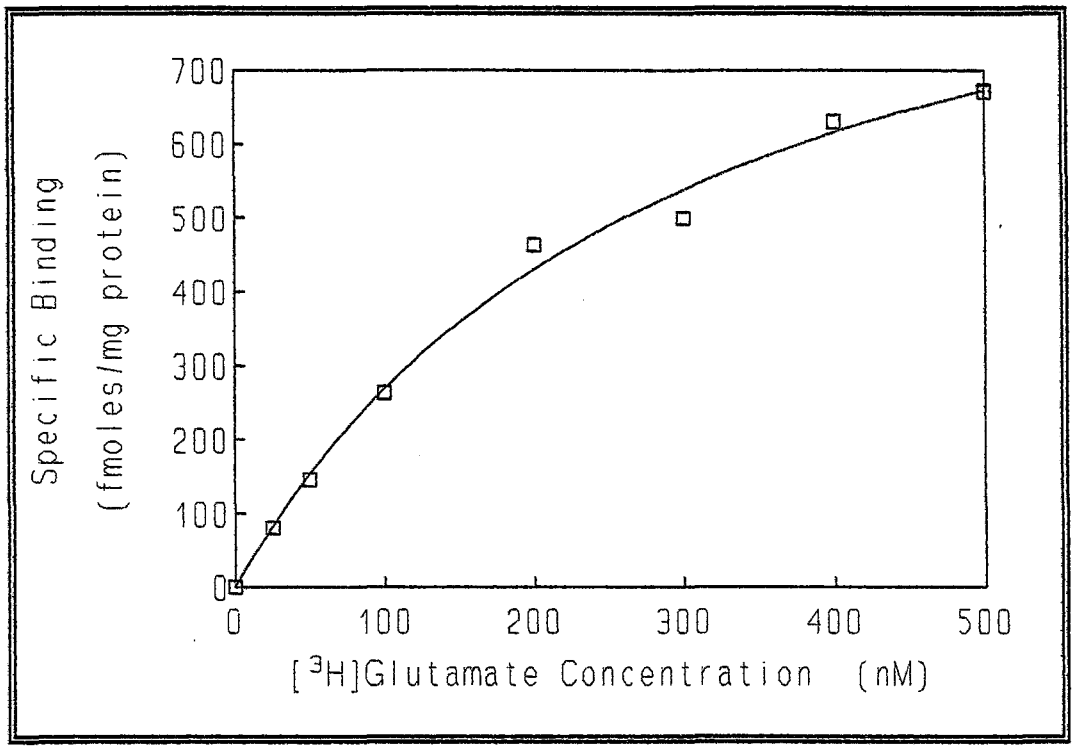

FIGURE 4.4 : **Saturation Curve of [^{3}H]Glutamate Binding to Cerebral Membranes from Normal, Untreated Rats**

Each point represents the mean of duplicate determinations.

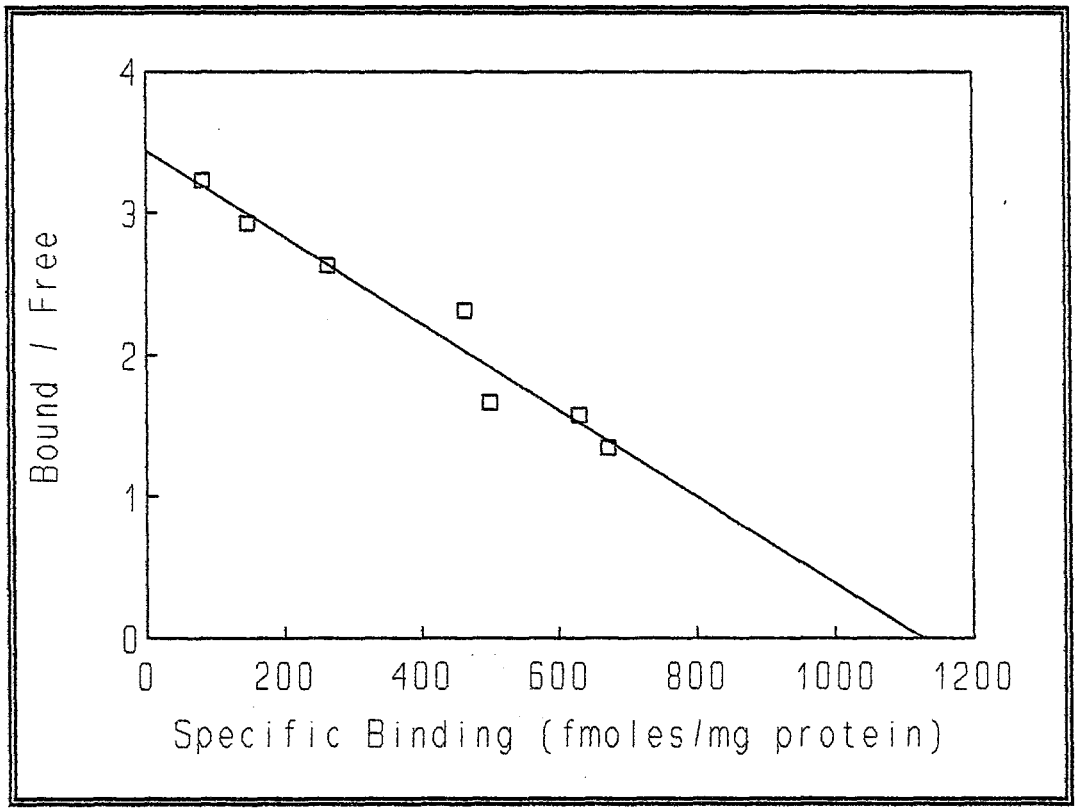

FIGURE 4.5 : **Scatchard Plot of [^{3}H]Glutamate to Rat Cerebral Membranes from Normal, Untreated Rats**

The K_D from the above slope is 327.3 nM and the B_{max} from the x-intercept is 1127.1 fmoles/mg protein.

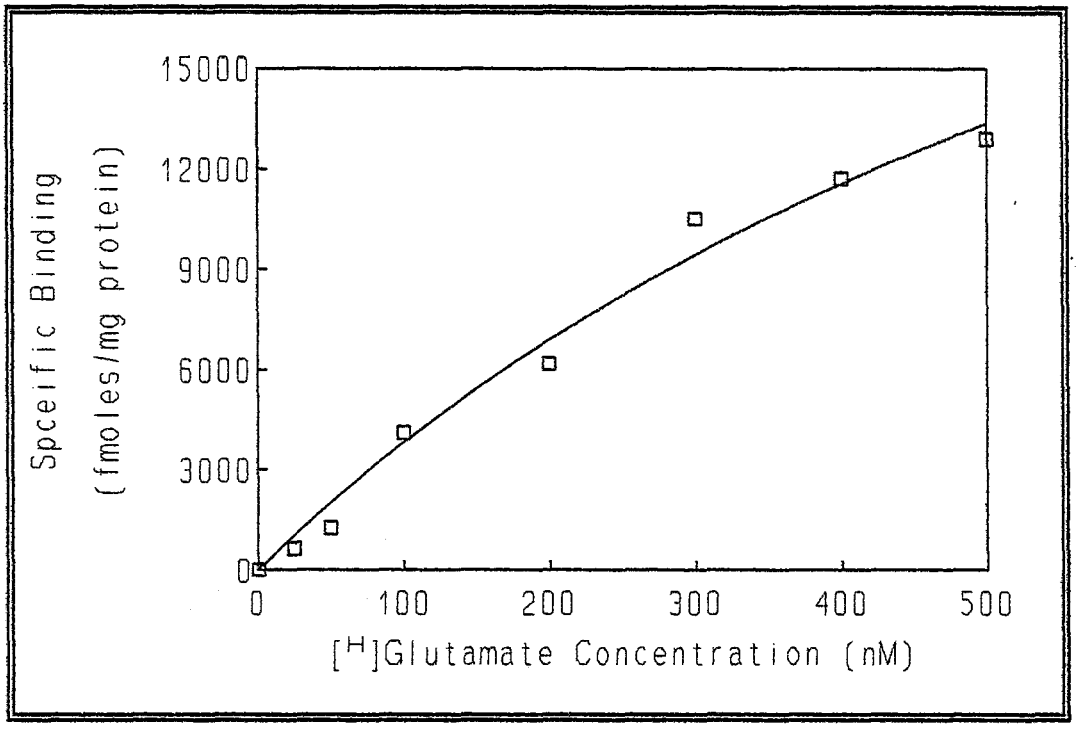

FIGURE 4.6 : **Saturation Curve of [^{3}H]Glutamate Binding to Cerebral Membranes from Hydrocortisone Treated Rats**

Each point represents the mean of duplicate determinations.

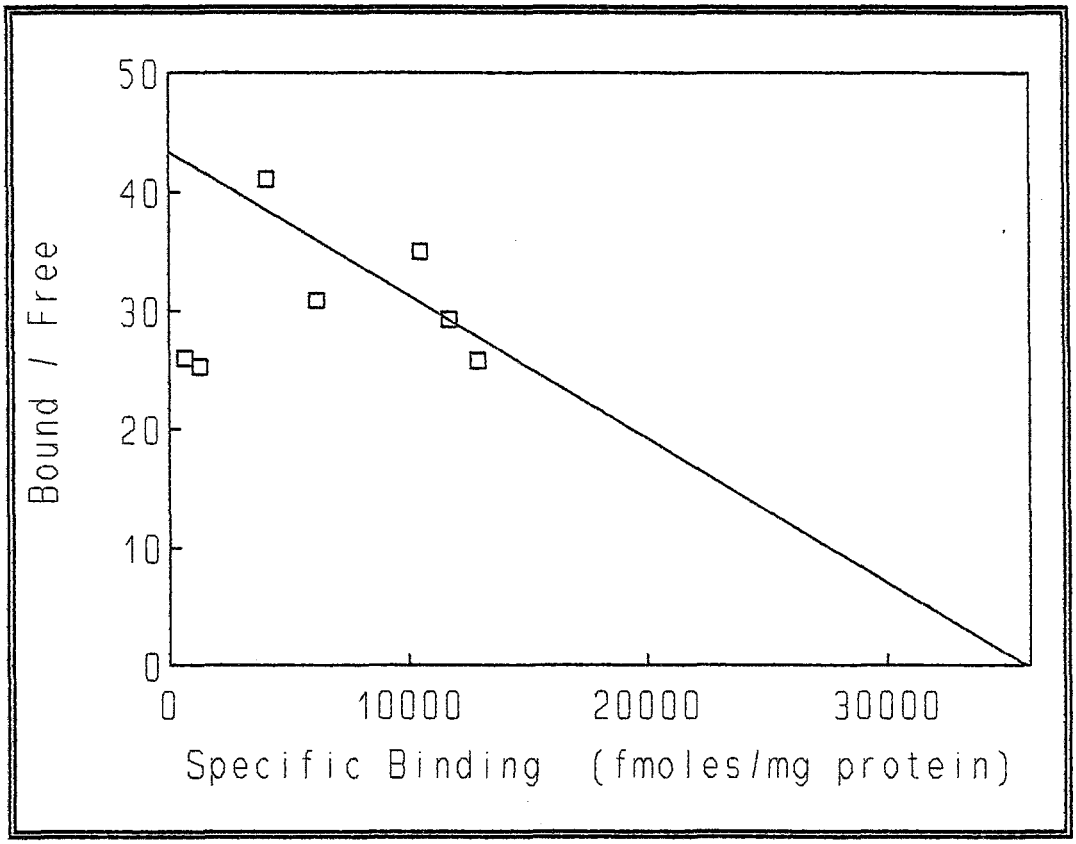

FIGURE 4.7 : **Scatchard Plot of [^{3}H]Glutamate Binding to Cerebral Membranes from Hydrocortisone Treated Rats**

The K_D from the above slope is 825.3 nM and the B_{max} from the x-intercept is 35834.7 fmoles/mg protein.

and chloride, known to enhance the binding of glutamate to non-NMDA binding sites, were omitted from all buffers and solutions [Foster and Fagg, 1984; Monaghan and Cotman, 1986]. Secondly, the brains and receptor homogenates were frozen at various stages. Freezing is also known to diminish non-NMDA binding sites without affecting NMDA-sensitive binding [Foster and Fagg, 1984]. Numerous attempts to characterize the glutamate receptors by performing competition studies with 10^{-11} - 10^{-3} M NMDA were unsuccessful, as were attempts to determine the effect of 10^{-11} - 10^{-3} M melatonin on [^{3}H]glutamate binding to rat cerebral membranes. Both of these failures may be due to the filtration method used to separate the bound from the free radioactive ligand. Due to the relatively low affinity of glutamate for the glutamate receptors, centrifugation should be the preferred and recommended method for the separation of bound and free radioactive ligand.

The hydrocortisone treatment decreased the affinity of glutamate binding sites for its ligand and caused a 30-fold increase in the maximum number of glutamate receptors in rat cerebral membranes. As saturation of the receptors with [^{3}H]glutamate was not fully achieved, no conclusions can be drawn about the number of classes of glutamate receptor isolated, although it is likely that they are NMDA receptors because of the purification procedures employed.

Glucocorticoids, like other steroid hormones are known to control the rate of synthesis of proteins and receptors. They react with proteins in the cytoplasm of sensitive cells to form a steroid-receptor complex. This complex undergoes a conformational change and moves into the nucleus where it binds to the chromatin material and stimulates the transcription and translation of specific proteins [Haynes and Larner, 1975]. It is thus likely that hydrocortisone increased the number of glutamate binding sites by inducing an increase in the rate of synthesis of the receptors.

From this study, it can be seen that although hydrocortisone apparently causes a dramatic decrease in affinity of glutamate receptors for its ligand in rat forebrain, it causes a massive increase in the number of glutamate binding sites. Glucocorticoids are also known to cause increases in synaptic glutamate, by inhibiting the uptake of the amino acid by glial cells. Whether the rise in synaptic glutamate concentration would be sufficient to cause excessive stimulation of the glutamate receptors, in view of the decrease in affinity of the receptors for glutamate, remains to be established. Excessive stimulation of especially NMDA receptors in the brain results in neuronal degeneration. The potential of this mechanism to produce excessive neural damage warrants further attention.

4.4 DISCUSSION

The findings of these experiments, confirm that elevated circulating glucocorticoid levels can adversely affect normal physiology to the detriment of cerebral function. Hydrocortisone depletes free tryptophan stores by enhancing the catabolism of this essential amino acid by liver tryptophan pyrrolase. This has been shown previously to decrease brain serotonin which causes behavioural and mental disorders associated with deficiencies of the cerebral serotoninergic system [Young, 1981]. The ability of melatonin to inhibit tryptophan pyrrolase indicates a mechanism whereby the pineal gland may protect against excessive loss of free tryptophan and the reduction in brain serotonin.

It was also shown that chronic hydrocortisone treatment causes a large increase in the number of glutamate receptors in the brain. Taken together with the finding that glucocorticoids inhibit the uptake of glutamate from synaptic clefts and thereby increase synaptic glutamate concentrations, it is possible that increased circulating glucocorticoid levels may result in increased

stimulation of cerebral glutamate receptors. Glutamate receptors in the central nervous system have been implicated in numerous neurodegenerative diseases, and protection against excessive stimulation of these receptors would be vital in preventing, or at least delaying, the degenerative loss of brain cells. Unfortunately, within the duration of this study, it was, for technical reasons, not possible to show that melatonin either interferes with the binding of glutamate to cerebral glutamate receptors, or affects the hydrocortisone-mediated increase in glutamate receptor numbers. However, in view of the cumulative evidence favouring an anti-stressogenic role for the pineal gland, and the ability of melatonin to alter the affinity of other cerebral receptors for their natural ligands (eg GABA [Niles, 1989] and corticosterone [Marinova *et al*, 1991]), the ability of melatonin and other pineal indoles to affect the binding of glutamate to cerebral glutamate receptors, warrants further research.

CHAPTER 5

EFFECT OF GLUTAMATE AND GABA ON PINEAL GLAND BIOCHEMISTRY

5.1 INTRODUCTION

Glutamate is present in significant concentrations in the pineal gland [Collin and Oksche, 1981], and the proportion of this neurotoxic excitatory amino acid increases in the pineal gland with pineal maturation and aging. It has tentatively been suggested that glutamate exhibits a mild circadian rhythm in rats with nighttime levels being marginally lower than daytime levels. [Quay, 1981].

Previously, the presence of glutamate in the pineal gland was attributed to the fact that it is the precursor of gamma-amino butyric acid (GABA). GABA, known to be present in the pineal, is synthesized from glutamate by a pyridoxal phosphate-dependent glutamate decarboxylase. High and low affinity GABA receptors have been identified in the pineal gland. Chan and Ebadi [1980] showed that GABA exerts a chloride-dependent inhibition of the norepinephrine-induced stimulation of NAT activity in a dose-dependent manner. Foldes *et al* [1984] also reported that preincubation with GABA inhibits the norepinephrine-induced increase in NAT activity in pineal slices. However, the precise functional significance of GABA in the pineal gland, is unknown. Recently, glutamate receptors have also been described in rat [Kus *et al*, 1990] and bovine [Ebadi *et al*, 1986] pineal glands. The functional significance of these receptors remains to be established. Investigation into the role and function of glutamate

receptors in the pineal gland is warranted, not only to gain greater insight into the functioning and regulation of the pineal itself, but especially in view of the damaging toxic effects attributed to glutamate in the central nervous system.

The aim of this series of experiments was to investigate the effect of glutamate and GABA on pineal biochemistry.

5.2 THE EFFECT OF GLUTAMATE ON RAT PINEAL INDOLEAMINE METABOLISM IN ORGAN CULTURE

5.2.1 Introduction

Kus *et al* [1990], monitoring melatonin synthesis by pineal glands in an *in vitro* perfusion system, recently reported that the rise in melatonin production stimulated by the addition of the α- and ß-adrenergic agonists, isoprenaline and phenylephrine respectively, was prevented when the pineal glands were preincubated with glutamate. The authors showed that the effect was not prevented by glutamate agonists to any of the known glutamate receptor populations. It was further shown, that the inhibition of melatonin production was not manifest when glutamate was administered together with isoprenaline and phenylephrine or when the pineals were stimulated with isoprenaline alone. The authors concluded that the inhibition of melatonin production was possibly mediated via a glutamate metabolite, such as GABA.

In the first experiment, the effect of glutamate on isoprenaline-stimulated pineal glands was investigated in organ culture.

5.2.2 Materials and Methods

Four groups of 5 male Wistar rats of the albino strain were randomly assembled. The animals were sacrificed between 10h00 and 12h00 and their pineal glands were cultured *in vitro* for 24 hours in the presence of radioactive serotonin as described in section 2.2.3. The first group of pineals was incubated in the presence of 100 µM isoprenaline, and the second group, in the presence of 100 µM isoprenaline and 100 µM L-glutamate. The third group was incubated with 100 µM glutamate for 4 hours before 100 µM isoprenaline was added. The control pineals were incubated in the presence of the vehicle. After 24 hours, the incubation was terminated and the culture medium was analyzed for radioactive metabolites of pineal metabolism using thin layer chromatography and liquid scintillometry as outlined in section 2.2.4.

5.2.3 Results

The radioactivity corresponding to each of the indole metabolites isolated from the culture medium following the incubation of the pineal glands with [^{14}C]serotonin are tabulated in Table 5.1. The data were analyzed by one-way analysis of variance and statistical difference between groups were determined using the Bonferroni multiple range test. Values represent the mean ± SEM for n = 5. The indole metabolites, where significant differences were recorded, are graphically presented in Figures 5.1 - 5.6.

Isoprenaline induced a significant increase in pineal N-acetylserotonin and melatonin synthesis from [^{14}C]serotonin in pineal organ cultures. Preincubation of pineal glands with

TABLE 5.1 : Effect of Glutamate on Pineal Indoleamine Metabolism in Organ Culture

INDOLE	dpm / 10 µl medium / pineal gland ± SEM			
	CONTROL	ISOPRENALINE	GLUTAMATE + ISOPRENALINE	GLUTAMATE + 4 HOUR ISOPRENALINE
N-acetylserotonin	2439 ± 504	4611 ± 574	1231 ± 393	1391 ± 197
Melatonin	2047 ± 250	5187 ± 305	1805 ± 406	2444 ± 227
Hydroxyindole acetic acid	37112 ± 5188	17739 ± 2039	21566 ± 2900	20365 ± 4270
Hydroxytryptophol	15984 ± 4727	4179 ± 620	5363 ± 478	9701 ± 2359
Methoxyindole acetic acid	293 ± 41	75 ± 14	102 ± 12	107 ± 7
Methoxytryptophol	290 ± 48	195 ± 37	161 ± 34	269 ± 56

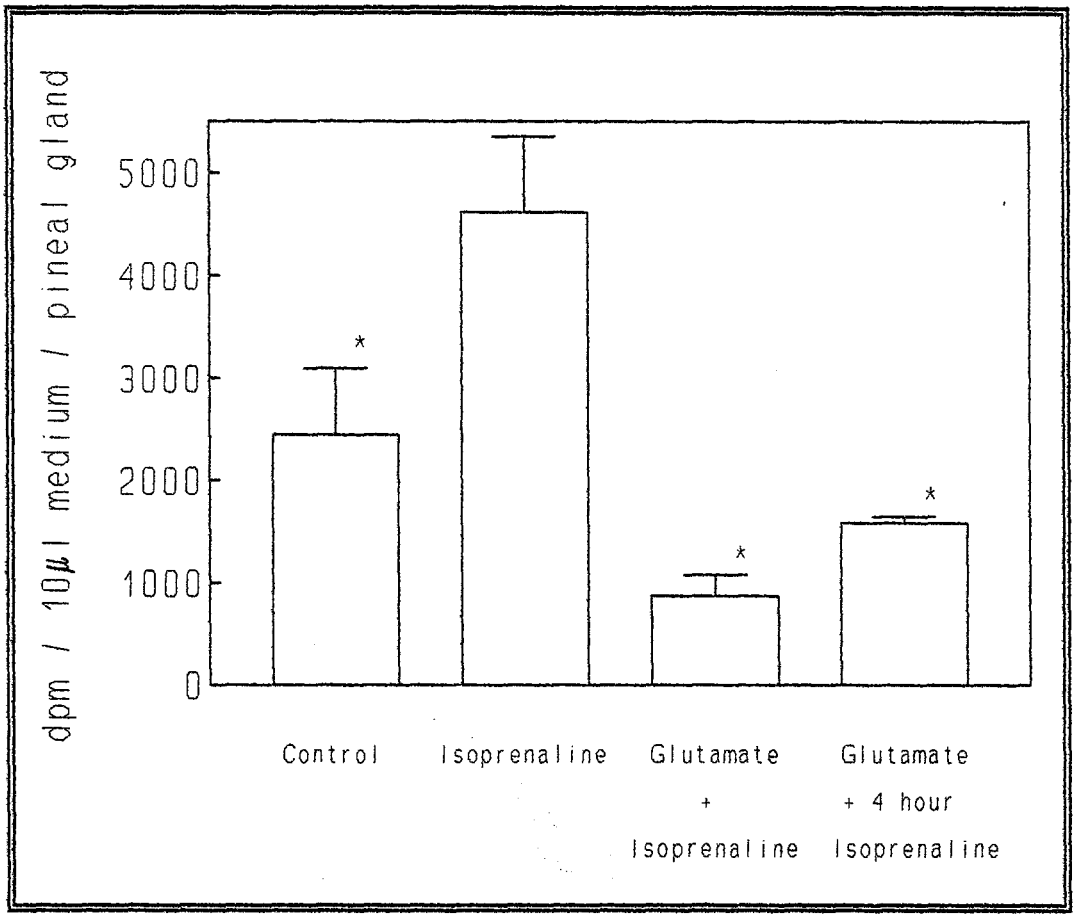

FIGURE 5.1 : **Effect of Glutamate on Isoprenaline-induced N-Acetylserotonin Synthesis in Rat Pineal Organ Cultures**

* $p < 0.001$ compared with Isoprenaline-stimulated.

Values represent the mean + SEM for n = 5.

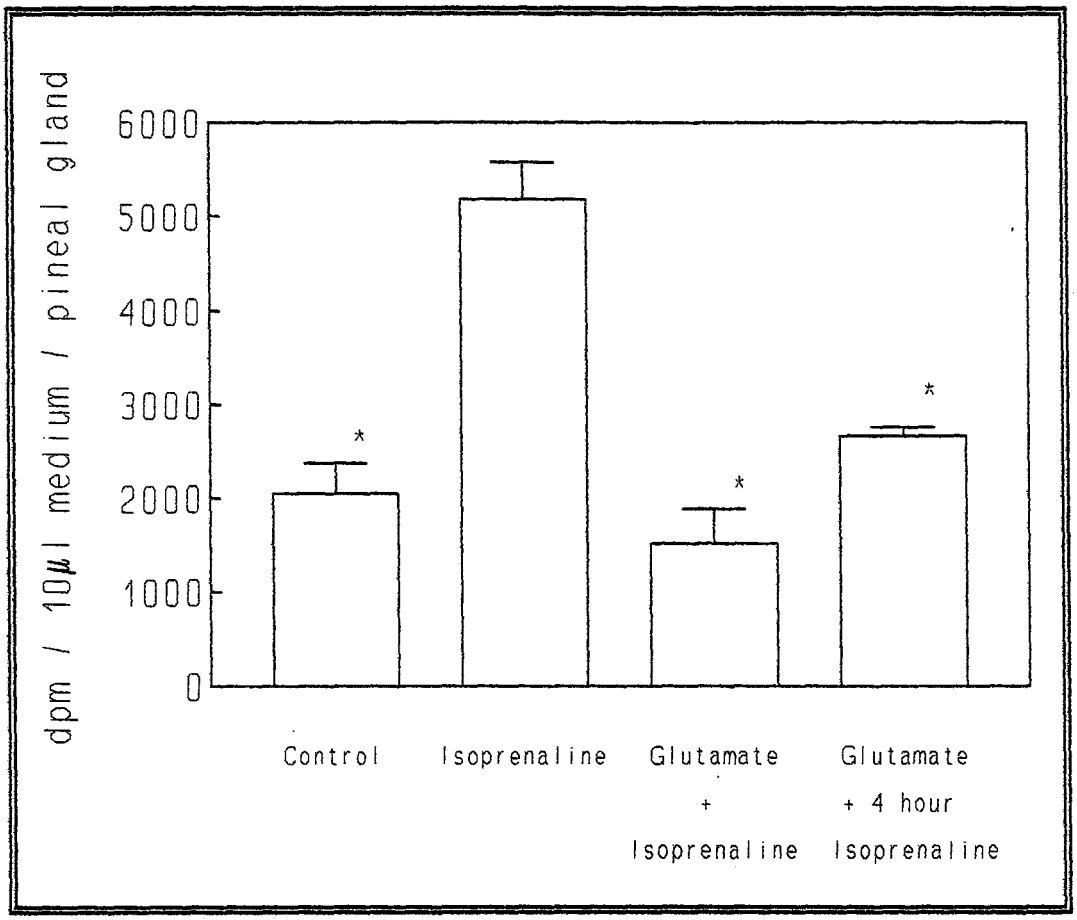

FIGURE 5.2 : **Effect of Glutamate on Isoprenaline-induced Melatonin synthesis in Rat Pineal Organ Cultures**

* $p < 0.001$ compared with Isoprenaline-stimulated.

Values represent the mean + SEM for n = 5.

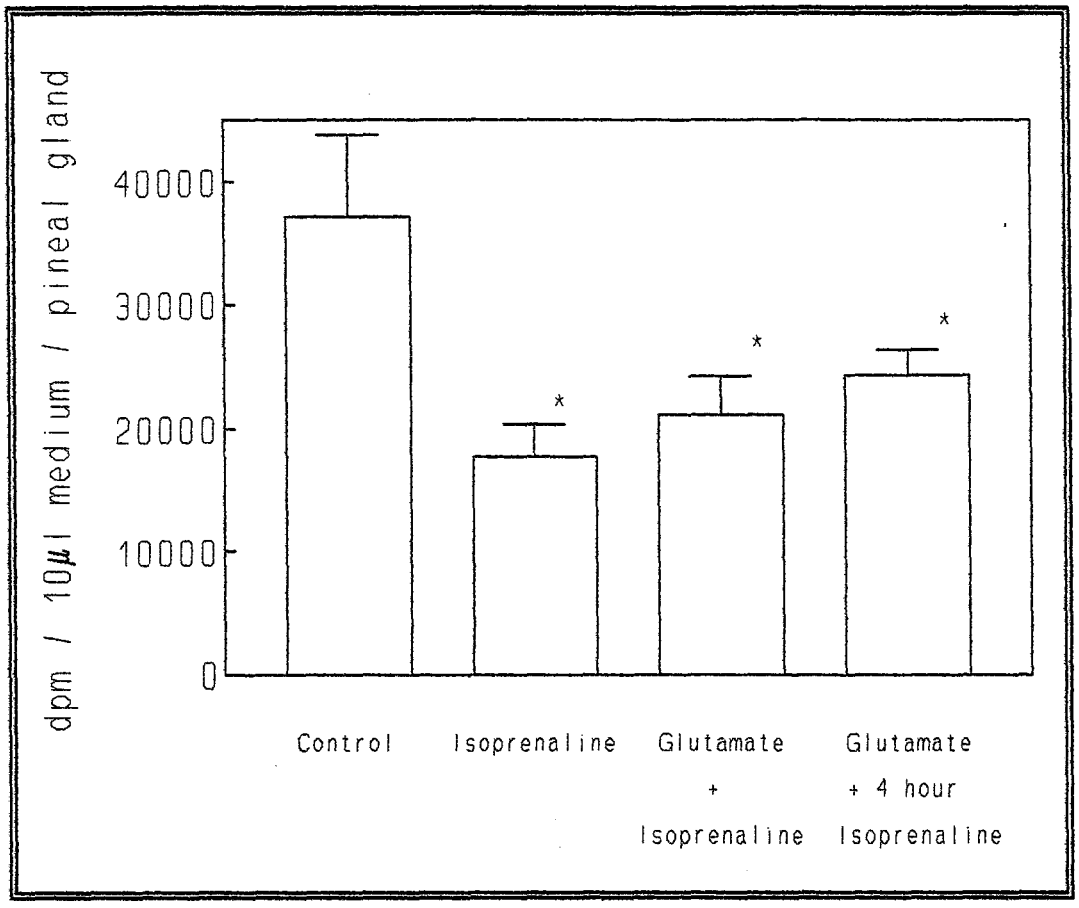

FIGURE 5.3 : **Effect of Glutamate on Isoprenaline-induced 5-Hydroxyindole Acetic Acid synthesis in Rat Pineal Organ Cultures**

* $p < 0.05$ compared with the control.

Values represent the mean + SEM for n = 5.

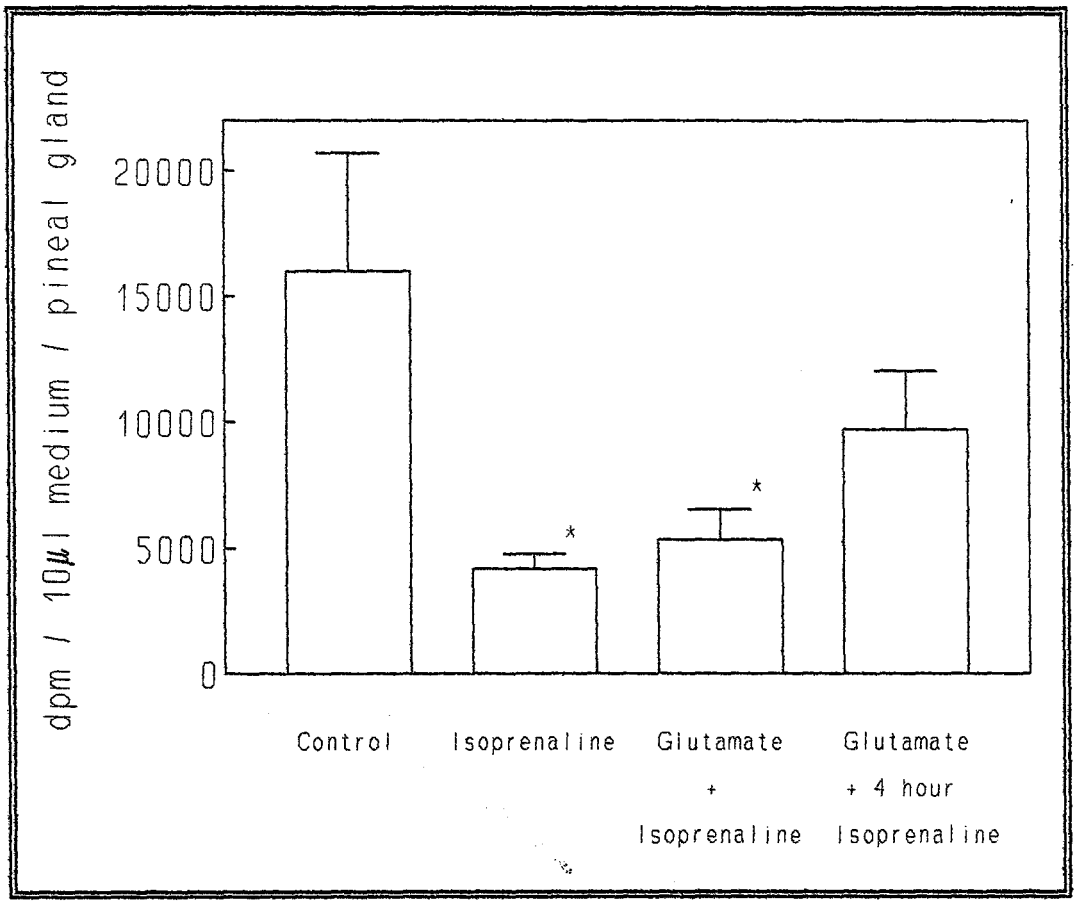

FIGURE 5.4 : **Effect of Glutamate on Isoprenaline-induced 5-Hydroxytryptophol Synthesis in Rat Pineal Organ Cultures**

* $p < 0.05$ compared with the control.

Values represent the mean + SEM for n = 5.

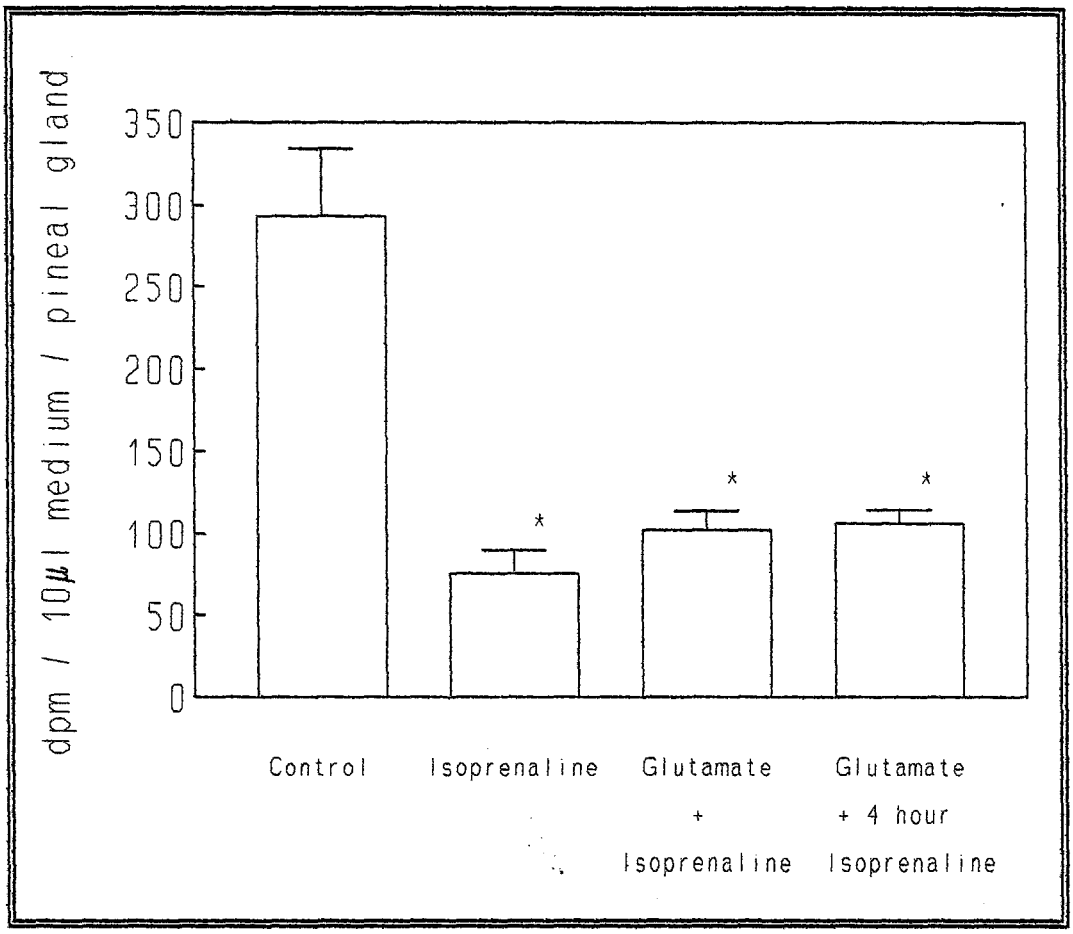

FIGURE 5.5 : **Effect of Glutamate on Isoprenaline-induced 5-Methoxyindole Acetic Acid Synthesis in Rat Pineal Organ Cultures**

* $p < 0.05$ compared with the control.

Values represent the mean + SEM for n = 5.

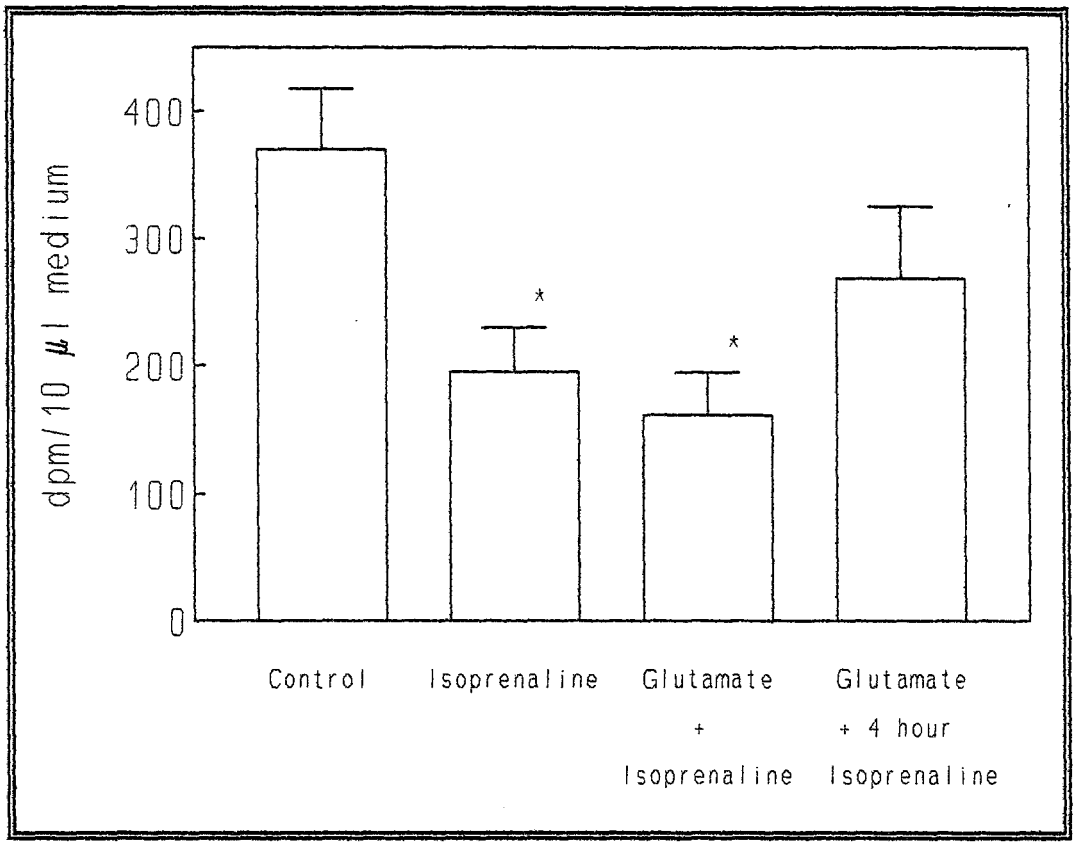

FIGURE 5.6 : **Effect of Glutamate on Isoprenaline-induced 5-Methoxytryptophol synthesis in Rat Pineal Organ Cultures**

* $p < 0.01$ compared with the control.

Values represent the mean + SEM for n = 5.

glutamate for four hours, as well as simultaneous administration of glutamate and isoprenaline, significantly inhibited the isoprenaline-induced increase in N-acetylserotonin and melatonin production ($p < 0.001$). Neither of the glutamate treatments affected the isoprenaline-induced decrease in 5-hydroxyindole acetic acid ($p < 0.05$), 5-hydroxytryptophol ($p < 0.05$) or 5-methoxyindole acetic acid production ($p < 0.01$). 5-Methoxytryptophol synthesis was unaffected by any of isoprenaline or glutamate treatments.

5.2.4 Discussion

The results from this study indicate that glutamate inhibits the isoprenaline-stimulated rise in pineal melatonin production. This is in agreement with the findings of Kus *et al* [1990]. Additionally, the inhibition of melatonin synthesis was demonstrated in the absence of α-adrenergic receptor stimulation, and also when glutamate and isoprenaline were administered simultaneously. The latter observation suggests that glutamate itself could, at least partly, be responsible for the inhibition of melatonin production. However, in contrast with the perfusion system employed by Kus *et al* [1990] which enabled the continual measurement of pineal melatonin synthesis over short time periods, the organ culture method employed in this study, measures the quantity of melatonin which accumulates over the 6 hour incubation period. Therefore, it is possible that, even when glutamate and isoprenaline were administered simultaneously, the glutamate was converted to a metabolite with inhibitory effects on melatonin synthesis.

5.3 THE EFFECT OF GABA ON RAT PINEAL INDOLEAMINE METABOLISM IN ORGAN CULTURE

5.3.1 Introduction

It had previously been suggested that the principal effects of glutamate in the pineal gland were mediated by a glutamate metabolite, since preincubation of the pineal gland with glutamate was essential for the demonstration of its inhibitory influence on melatonin synthesis [Kus *et al*, 1990]. The incubation time was deemed necessary for the conversion of glutamate to its physiologically active metabolite. With the knowledge that GABA receptors have been demonstrated in the pineal gland [Chan and Ebadi, 1980], GABA was considered the most likely candidate.

In the initial experiment in this study, it was demonstrated that simultaneous administration of glutamate and isoprenaline could inhibit the isoprenaline-induced rise in N-acetylserotonin and melatonin production in organ culture. Due to the fact that in an organ culture system, there would be time for glutamate to be converted to a physiologically active metabolite, which could, after the required lag period, decrease the accumulation of melatonin, it is possible that glutamate itself could be exerting the inhibitory influences on pineal melatonin synthesis.

The aim of this experiment was to determine whether GABA, an important metabolite of glutamate, could mimic the effects of glutamate observed in the previous experiment.

5.3.2 Materials and Methods

Three groups of 6 male Wistar rats of the albino strain were randomly assembled. The animals were sacrificed between 10h00 and

12h00 and their pineal glands were cultured *in vitro* for 24 hours in the presence of radioactive serotonin as described in section 2.2.3. The first group of pineals was incubated in the presence of 100 µM isoprenaline, and the second group, in the presence of 100 µM isoprenaline and 100 µM GABA. The control pineals were incubated in the presence of the vehicle. After 24 hours, the incubation was terminated and the culture medium was analyzed for radioactive metabolites of pineal metabolism using thin layer chromatography and liquid scintillometry as outlined in section 2.2.4.

5.3.3 Results

The radioactivity corresponding to each of the indole metabolites isolated from the culture medium following the incubation of the pineal glands with [^{14}C]serotonin are tabulated in Table 5.2. The data were analyzed by one-way analysis of variance and statistical difference between groups were determined using the Bonferroni multiple range test. Values represent the mean ± SEM for n = 5. The indole metabolites, where significant differences were recorded, are graphically presented in Figures 5.7 - 5.9.

Isoprenaline induced a significant increase in pineal N-acetylserotonin and melatonin synthesis from [^{14}C]serotonin in pineal organ cultures. GABA significantly inhibited the isoprenaline-induced increase in N-acetylserotonin synthesis ($p < 0.05$), but effected only a marginal, insignificant decrease in the isoprenaline-stimulated rise in melatonin production, so that it was no longer significantly different from control values. Furthermore, GABA enhanced the isoprenaline-induced decrease in 5-hydroxytryptophol production by pineal organ cultures ($p < 0.05$). 5-Hydroxy- and 5-methoxy-indole acetic acid and 5-methoxytryptophol were unaffected by either isoprenaline or GABA.

TABLE 5.2 : Effect of GABA on Pineal Indoleamine Metabolism in Organ Culture

INDOLE	dpm / 10 µl medium / pineal gland ± SEM		
	CONTROL	ISOPRENALINE	GABA + ISOPRENALINE
N-acetylserotonin	2299 ± 542	4558 ± 1031	2787 ± 712
Melatonin	2607 ± 347	4639 ± 751	4417 ± 582
Hydroxyindole acetic acid	21985 ± 2270	27483 ± 4109	30864 ± 4562
Hydroxytryptophol	16638 ± 5077	11684 ± 2499	4226 ± 582
Methoxyindole acetic acid	224 ± 47	131 ± 35	169 ± 46
Methoxytryptophol	196 ± 14	201 ± 43	148 ± 17

5.3.4 Discussion

Although GABA could mimic the inhibitory effect of glutamate on the isoprenaline-stimulated accumulation in N-acetylserotonin, it did not significantly inhibit melatonin production. A possible explanation is that GABA, rather than exert inhibitory effects, actually enhances the conversion of N-acetylserotonin to melatonin. This would also explain why there was less serotonin precursor available for conversion to 5-hydroxytryptophol. Balemans and co-workers [cf. Ebadi *et al*, 1986] demonstrated that the addition of GABA together with an acetyl donor, resulted in a significant

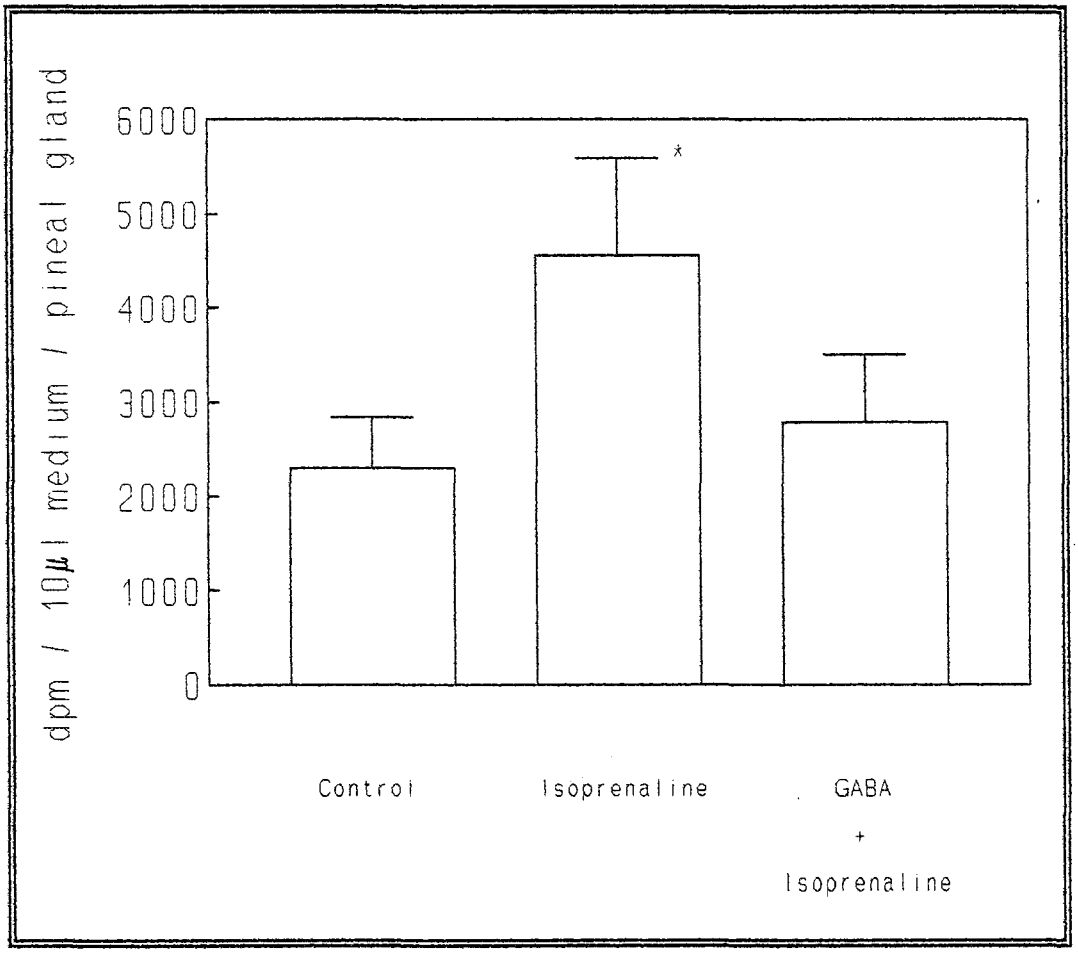

FIGURE 5.7 : **Effect of GABA on Isoprenaline-induced N-Acetylserotonin synthesis in Rat Pineal Organ Cultures**

* $p < 0.05$ compared with the control.

Values represent the mean + SEM for n = 5.

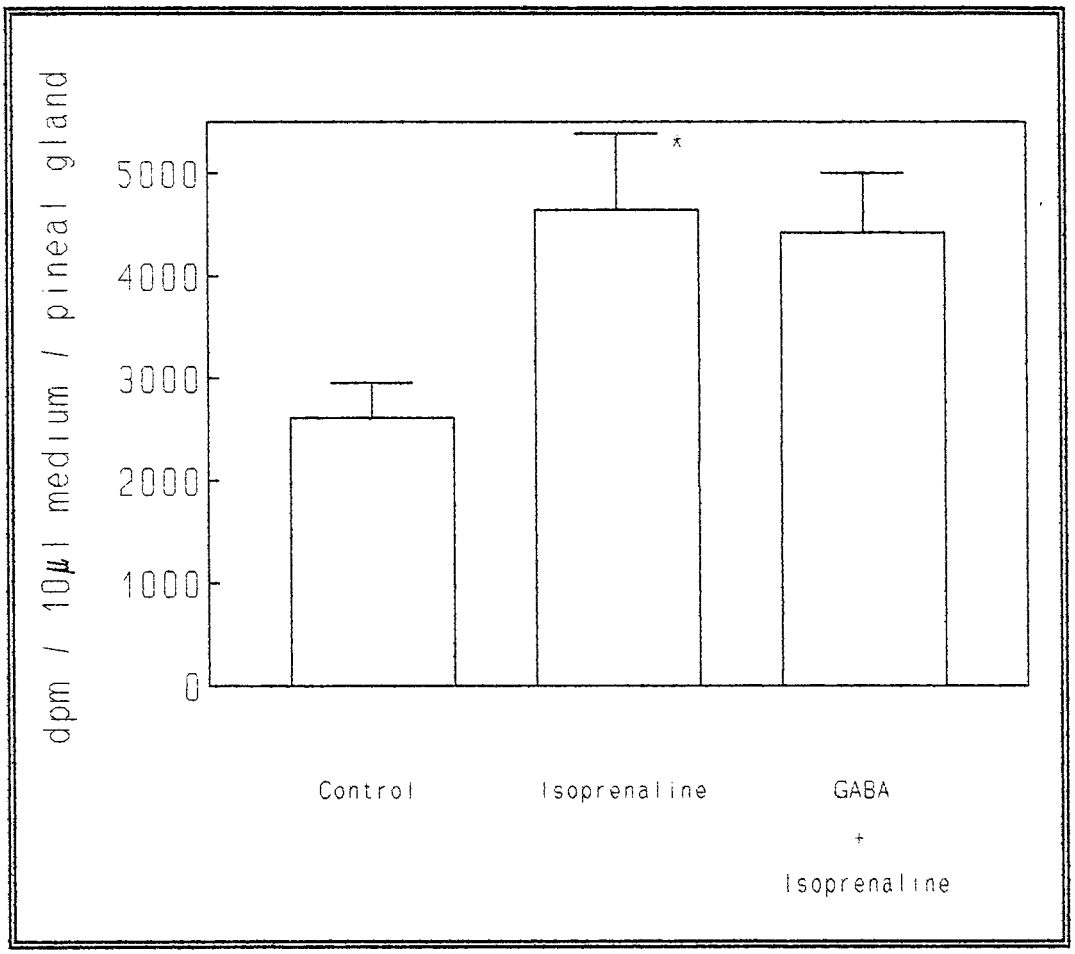

FIGURE 5.8 : **Effect of GABA on Isoprenaline-induced Melatonin Synthesis in Rat Pineal Organ Cultures**

* $p < 0.05$ compared with the control.

Values represent the mean + SEM for n = 5.

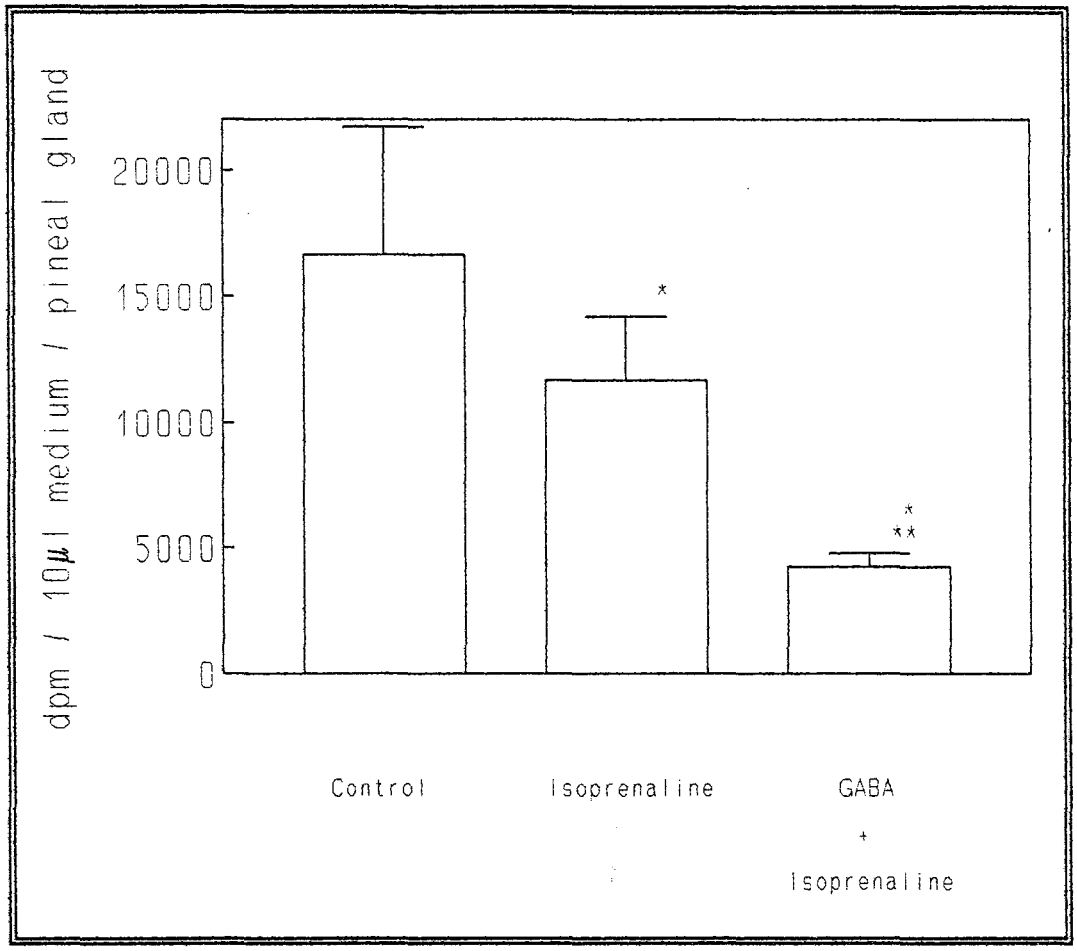

FIGURE 5.9 : **Effect of GABA on Isoprenaline-induced 5-Hydroxytryptophol synthesis in Rat Pineal Organ Cultures**

* $p < 0.05$ compared with the control.

** $p < 0.05$ compared with the isoprenaline-stimulated pineals.

Values represent the mean + SEM for n = 5.

increase in the synthesis of the N-acetylated products. Hence there are indications that GABA may stimulate rather than inhibit pineal indoleamine synthesis. Typically the amounts of the methoxyindoles are very small and the amount of hydroxyindole acetic acid, very large. Hence, the failure of the treatments to induce significant differences in these indoles is not unusual.

5.4 THE EFFECT OF GLUTAMATE ON RAT PINEAL N-ACETYLTRANSFERASE ACTIVITY

5.4.1 Introduction

It has thus far been demonstrated that the influence of glutamate on pineal N-acetylserotonin and melatonin synthesis in pineal organ cultures is primarily inhibitory. GABA was able to mimic the inhibitory effect of glutamate on N-acetylserotonin, but not on melatonin accumulation. A possible explanation is that GABA enhances the conversion of N-acetylserotonin to melatonin, while glutamate inhibits the melatonin biosynthetic pathway at a point prior to the synthesis of N-acetylserotonin. To investigate this possibility, the effect of glutamate on NAT, the principal regulatory enzyme in the melatonin biosynthetic pathway, was examined. NAT is responsible for converting serotonin to N-acetylserotonin. In addition to monitoring basal NAT activity during the day when it is generally low, a ß-adrenergic agonist, isoprenaline, was used to mimic the nighttime rise in enzyme activity. This made it possible to determine the effect of glutamate on ß-adrenergic stimulated NAT activity.

5.4.2 Materials and Methods

Four groups of five male Wistar rats of the albino strain were randomly assembled. The animals were sacrificed between 10h00 and 12h00 and their pineal glands were cultured *in vitro* for 6 hours. The first group of pineals was incubated in the presence of 100 µM isoprenaline; the second group, in the presence of 100 µM glutamate and the third group, in the presence of 100 µM isoprenaline and 100 µM L-glutamate. The control pineals were incubated in the presence of the vehicle. After 6 hours, the incubation was terminated by freezing the pineal glands on dry ice. The pineals were then assayed for NAT activity according to the scheme described in section 2.3. This freezing procedure is known not to alter NAT activity.

5.4.3 Results

The enzyme activity, expressed as pmoles N-acetyltryptamine formed per pineal gland per hour, was calculated as described in section 2.3.4. The data were analyzed by one-way analysis of variance and statistical differences between groups were determined using the Bonferroni multiple range test. Values which represent the mean ± SEM (n = 5), are tabulated in Table 5.3 and graphically presented in Figure 5.10.

Glutamate significantly increased basal NAT activity ($p < 0.05$). Isoprenaline also effected a significant increase in basal NAT activity of similar magnitude in both control and glutamate treated pineal glands ($p < 0.01$).

TABLE 5.3 : Effect of Glutamate on N-Acetyltransferase Activity in Rat Pineal Glands

TREATMENT	pMoles N-ACETYLTRYPTAMINE formed / pineal gland / hour
Control	0.172 ± 0.0063
Isoprenaline	0.268 ± 0.0148
Glutamate	0.251 ± 0.0143
Glutamate + Isoprenaline	0.345 ± 0.0333

5.4.4 Discussion

The ability of glutamate to increase basal pineal NAT activity, is in conflict with the finding that the amino acid inhibits the accumulation of N-acetylserotonin and melatonin in pineal organ cultures. No explanation is immediately obvious.

It is possible to propose that glutamate may be converted to GABA during the organ culture incubation and the stimulation of NAT activity observed is actually due to GABA. This theory would be in keeping with the findings of Balemans and co-workers [cf. Ebadi *et al*, 1986] that GABA enhances the ß-adrenergic stimulated synthesis of N-acetylated products in the pineal gland. However, this would not explain the ability of glutamate to inhibit melatonin synthesis in organ culture. It would probably have been advisable to include a glutamate decarboxylase inhibitor in the

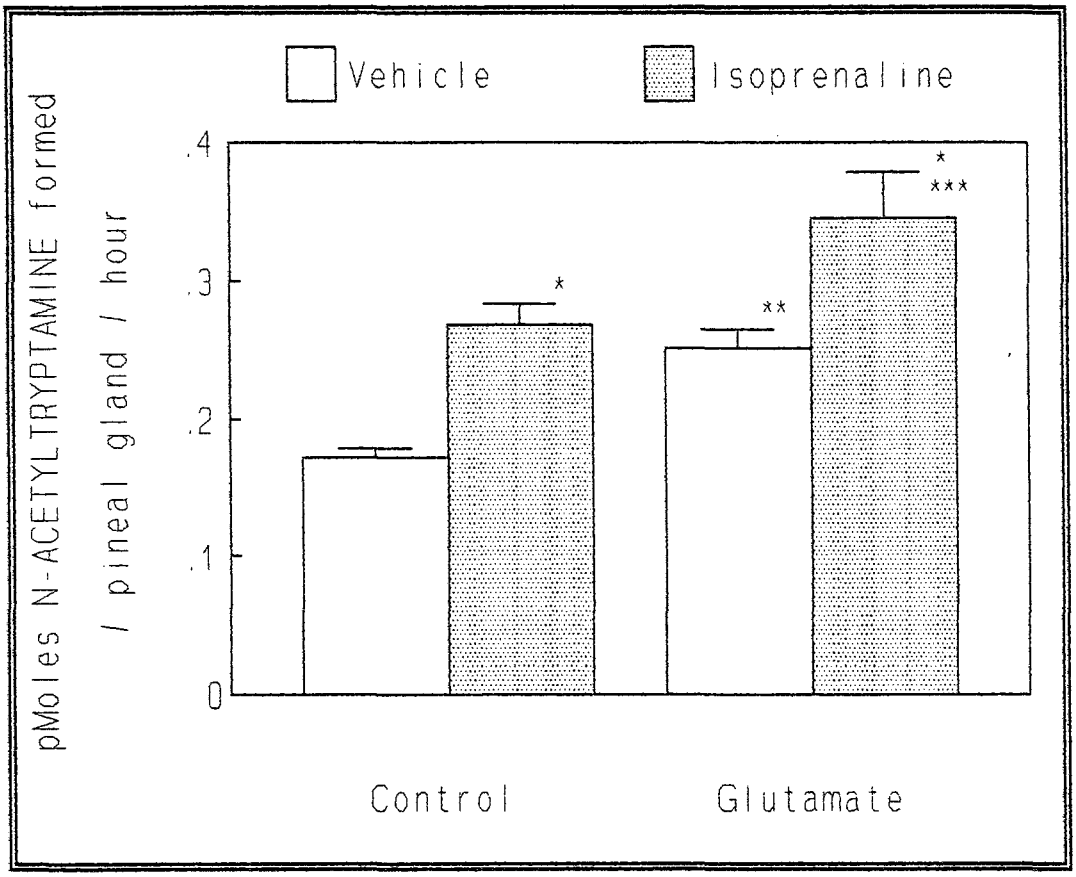

FIGURE 5.10 : **Effect of Glutamate on Isoprenaline-stimulated N-Acetyltransferase Activity in the Rat Pineal Gland.**

Values represent the mean + SEM of triplicate determinations for n = 5.

* $p < 0.02$ compared with the control

** $p < 0.05$ compared with the control

*** $p < 0.01$ compared with the glutamate treated pineals

Water was used as the vehicle.

culture medium to prevent any possible conversion of glutamate to GABA.

Another possibility is that glutamate only inhibits melatonin synthesis in intact tissue and that the effect is lost when the cell membranes are disrupted. Rather than acting directly on the biosynthesis of melatonin, glutamate may be acting via receptors to initiate an intracellular series of events which leads to the inhibition of melatonin synthesis. For example, glutamate is an agonist at NMDA receptors. NMDA receptors are ion-channel linked and have been associated with extensive intracellular mobilization of calcium [Meldrum and Garthwaite, 1990]. Recently, Olivieri and Daya [1992] proposed that intracellular calcium concentrations are important for the regulation of pineal NAT activity. These authors implied that high levels of intracellular calcium exert inhibitory effects on NAT activity. This would subsequently lead to decreased melatonin synthesis. Development of this idea awaits greater understanding of the pineal glutamate receptor population involved.

5.5 THE EFFECT OF GLUTAMATE ON RAT PINEAL HYDROXYINDOLE-O-METHYLTRANSFERASE

5.5.1 Introduction

Having demonstrated that glutamate stimulates NAT activity although it causes a decrease in N-acetylserotonin and melatonin synthesis from serotonin by pineal glands in organ culture, the effect of glutamate on HIOMT activity was investigated. HIOMT is responsible for the conversion of N-acetylserotonin to melatonin, and it is possible that the glutamate-induced decrease in melatonin synthesis could result from a possible inhibition of HIOMT by glutamate.

5.5.2 Materials and Methods

Two groups of five male Wistar rats of the albino strain were randomly assembled. The rats were sacrificed at 10h00 and their pineal glands were rapidly removed. The pineals were assayed for HIOMT activity according to the scheme described in section 2.4.

5.5.3 Results

The enzyme activity, expressed as pmoles [^{14}C]melatonin formed per pineal gland per hour, was calculated as described in section 2.4.4. The data were analyzed by one-way analysis of variance and statistical difference between groups were determined using the Students t-test. Values which represent the mean ± SEM (n = 5), are tabulated in Table 5.4 and graphically presented in Figure 5.11.

TABLE 5.4 : Effect of Glutamate on Hydroxyindole-O-Methyltransferase Activity in Rat Pineal Glands

TREATMENT	pMoles MELATONIN formed / pineal gland / hour
Control	13.055 ± 12.088
Glutamate	13.363 ± 11.041

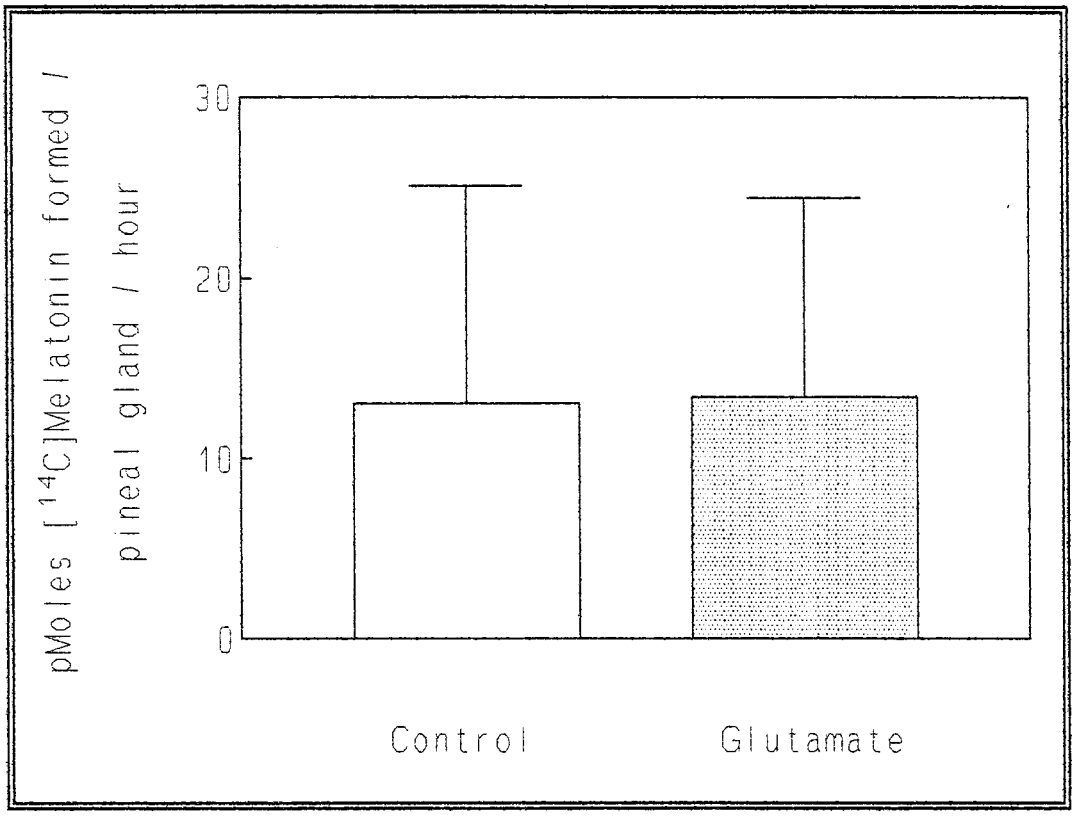

FIGURE 5.11 : Effect of Glutamate on Hydroxyindole-O-Methyltransferase Activity

Values represent the mean + SEM of triplicate determinations for n = 5.

Glutamate did not significantly affect HIOMT activity *in vitro*.

5.5.4 Discussion

The finding that glutamate did not influence pineal HIOMT activity is not unexpected. The enzyme appears to be very stable and does not exhibit rapid or extreme changes in activity. It is not believed to be involved in the pineal melatonin circadian rhythm and does not respond rapidly to changes in the photoperiod as NAT is known to do. Most evidence suggests that HIOMT activity is only affected by long term treatments.

5.6 DISCUSSION

The presence of large concentrations of glutamate in the pineal gland, has been appreciated for many years. Its function in the pineal was primarily considered to be that of precursor to GABA, an amino acid with high and low affinity binding sites previously described in the pineal gland [Chan and Ebadi, 1980]. However, recently glutamate receptors have been described in the pineal gland and although their functional significance remains to be defined, a more direct influence of glutamate on the pineal is implied. In view of the important regulatory role ascribed to the pineal gland, and the toxic effects of glutamate in the central nervous system, the identity, function and operation of pineal glutamate receptors requires attention.

Previously, preincubation of pineal glands with glutamate was found to inhibit the increase in melatonin synthesis following the administration of isoprenaline and phenylephrine [Kus *et al*, 1990]. In this study, glutamate was found to inhibit the increase in melatonin synthesis in organ culture when the pineal glands were stimulated with isoprenaline alone, and when the pineals were not

preincubated with glutamate. It is, however, difficult to distinguish between the effects of glutamate and the possible action of its metabolite, GABA, in organ culture. Presumably, glutamate is converted to GABA by glutamate decarboxylase in the pineal glands during the incubation period. In an attempt to ascertain whether the effects of glutamate on melatonin synthesis in pineal organ culture could be simulated by GABA, it was found that the inhibition of isoprenaline-stimulated melatonin accumulation by GABA was insignificant. Although the isoprenaline stimulated N-acetylserotonin levels were significantly lower in the presence of GABA, this could possibly indicate enhanced conversion of N-acetylserotonin to melatonin. It thus appeared that the inhibition of melatonin synthesis was directly due to the action of glutamate rather than GABA.

An attempt was then made to determine the point at which glutamate inhibits the conversion of serotonin to N-acetylserotonin and melatonin. In conflict with the findings from the organ culture experiment, glutamate increased both basal and isoprenaline stimulated NAT activity, although it did not affect HIOMT activity. It can only be proposed that the ability of glutamate to inhibit melatonin biosynthesis is lost when the intact organ is disrupted by homogenization.

CHAPTER 6

SUMMARY

A general review of pineal gland anatomy, biochemistry and function reveals that this once disregarded, diminutive structure on top of the brain has evolved into a sophisticated, neuroendocrine gland capable of eliciting a multitude of effects. These multiple influences have been collectively interpreted as reflecting a general regulatory role for the pineal gland maintaining homeostasis in close association with changing environmental conditions. The long-standing role played by the adrenal glands in modulating homeostatic equilibrium *in vivo* is undisputed and an interaction between the pineal gland and the adrenal glands has been inferred, but not conclusively established. Manipulation of pineal output has produced results consistent with an inhibitory influence of the pineal gland on adrenal function. However, there is a scarcity of information available on the influence of modified adrenal output on pineal function and much of this literature is inconclusive and controversial. This matter is worthy of deeper understanding because if the pineal gland were to regulate adrenal function, it would be necessary for the pineal to detect and respond to altered adrenal output.

The secretion of glucocorticoids is a major neuroendocrine consequence of adrenal gland stimulation, especially in response to stressful stimuli which challenge homeostatic equilibrium. The primary objective of this study was therefore to examine the possible influence of increased circulating glucocorticoid levels on pineal indole metabolism. The animals were treated orally with the glucocorticoid, hydrocortisone, to overcome the variable influences arising from subjecting the animals to any of a variety of stressors, as has been the pattern in previous research. The

results show that seven days of oral hydrocortisone treatment endows the pineal gland with the ability to increase the synthesis of [^{14}C]melatonin from [^{14}C]serotonin in pineal organ cultures. This increase in melatonin synthesis was accompanied by a rise in NAT activity, cyclic AMP levels and enhanced binding of ß-adrenergic agonists to the pineal ß-adrenergic receptors. The hydrocortisone treatment resulted in small increases in basal NAT and cyclic AMP levels, but dramatically increased these parameters under ß-adrenergic stimulation. In view of this, it was proposed that hydrocortisone, rather than directly increasing melatonin production via a mechanism involving cytosolic steroid receptors, acted by sensitizing the pineal gland to ß-adrenergic agonists rendering the pineal more responsive to ß-adrenergic stimulation.

Under conditions of chronic or prolonged stress, the ability of the HPA axis to terminate the release of glucocorticoids from the adrenal glands, is severely impaired. The result is elevated circulating glucocorticoid levels which produce numerous deleterious physiological side effects, the most serious of which is damage to the brain and disruption of cerebral function. The second objective of this study was to investigate whether the principal pineal indole, melatonin, could protect against the deleterious effects of elevated circulating hydrocortisone levels.

The results show that chronic oral hydrocortisone treatment significantly increases liver tryptophan pyrrolase apoenzyme activity. The catabolism of tryptophan by tryptophan pyrrolase is an important peripheral determinant of tryptophan availability to the brain, and subsequently of brain serotonin levels. The results further show that melatonin inhibits both basal and hydrocortisone-stimulated liver tryptophan pyrrolase activity in a dose-dependent manner. This inhibition suggests that melatonin may protect against excessive loss of tryptophan from circulation and against

deficiencies in the cerebral serotoninergic system which have been associated with mood and behavioural disorders.

Another deleterious effect of sustained elevated glucocorticoid levels is inhibition of the re-uptake of excitatory amino acid neurotransmitters from the synaptic clefts in the CNS. Glutamate is a neurotoxic, excitatory amino acid which accumulates in the synaptic clefts and excessive stimulation by this neurotransmitter at especially NMDA receptors, has been associated with potent neural damage. The results show that chronic hydrocortisone treatment decreased the affinity of glutamate binding sites in the rat brain for glutamate, but caused a 30-fold increase in the number of glutamate receptors in rat cerebral membranes. The ability of melatonin to protect against either the binding of glutamate to cerebral glutamate receptors, or to interfere with the hydrocortisone-mediated increase in glutamate receptor numbers could not be demonstrated due to practical problems with the radioligand binding assay. However, the possible influence of the pineal gland in protecting against glutamate-induced neural degeneration should not be overlooked by future research.

In view of the neurotoxic effects of glutamate on the CNS, the functional significance of recently described glutamate receptors in the pineal gland was investigated. The results show that 10^{-4}M glutamate significantly inhibits isoprenaline-stimulated N-acetylserotonin and melatonin synthesis in organ cultures. This effect was demonstrated when the pineal glands were pre-incubated with glutamate prior to the addition of isoprenaline, and when glutamate was administered together with isoprenaline *in vitro*. GABA, a glutamate metabolite could not mimic the glutamate-induced decrease in isoprenaline-stimulated melatonin synthesis by pineal organ cultures, and it is likely that the observed effect was directly due to glutamate. Incubation of the pineal glands with

10^{-4}M glutamate did not affect HIOMT activity in pineal homogenates, but significantly elevated both basal and isoprenaline-stimulated NAT activity in pineal homogenates *in vitro*. It was proposed that glutamate only inhibits melatonin synthesis in intact tissue, possibly by a receptor-mediated series of events and that the effect is lost when the pinealocytes are disrupted by homogenization.

The present study has provided further support for an interaction between the pineal and the adrenal glands, by showing that pineal biochemistry is altered by enhanced adrenocorticoid activity. It has also shown that the principal pineal indole, melatonin, may protect against at least one of the adverse effects arising from elevated circulating hydrocortisone levels, such as those associated with chronic or long-term stress. There is an ever increasing likelihood that melatonin is an anti-stressogenic hormone and that the pineal gland may have a protective role to play in the pathology of stress-related diseases. In view of the severity of such diseases in modern day societies, the anti-stressogenic effects of the pineal gland are deserving of further investigation.

REFERENCES

Arendt, J (1988); *Clin. Endocrinol.*; **29**; 205 - 229.

Ariëns Kappers, J (1971); IN The Pineal Gland; Edited by Wolstenholme, G E W and Knight, J; Churchill Livingston - London; 3 - 33.

Armstrong, S, Ng, K T and Coleman, G J (1982); IN The Pineal Gland: Extra-Reproductive Effects : Volume III; Edited by Reiter, R J; CRC Press Inc. - Florida; 81 - 106.

Axelrod, J (1974); *Science*; **184**; 1341 - 1348.

Axelrod, J and Weissbach, H (1960); *Science*; **131**; 1312.

Axelrod, J and Weissbach, H (1961); *J. Biochem. Chem.*; **236**; 211 - 213.

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

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

Badawy, A A B and Evans, M (1973); *Biochem. J.*; **133**; 585 - 591.

Badawy, A A B and Evans, M (1974); *Biochem. J.*; **138**; 445 - 451.

Badawy, A A B and Evans, M (1975); *Biochem. J.*; **150**; 511 - 520.

Badawy, A A B and Evans, M (1981); *Biochem. Pharmacol.*; **30**; 1217 - 1222.

Badawy, A A B, Welch, A N and Morgan, C J (1981);*Biochem. J.*; **198**; 309 - 314.

Badawy, A A B, Morgan, C J and Davis, N R (1987); *Biochem. J.*; **248**; 293 - 295.

Banoo, S (1991); PhD Thesis; Rhodes University.

Bender, D A (1975); Amino Acid Metabolism; John Wiley and Sons Ltd - London; 155 - 191.

Binkley, S A (1981); IN The Pineal Gland : Anatomy and Biochemistry : Volume 1; Edited by Reiter, RJ; CRC Press Inc. - Florida; 155 - 172.

Binkley, S and Mosher, K (1989); IN Advances in Pineal Research : Volume 3; Edited by Reiter, R J and Pang, S F; John Libbey and Co Ltd - London; 107 - 112.

Binkley, S, Klein, D C and Weller, J (1973); *Experientia*; **29** 1339 - 1340.

Binkley, S, Rievman, J and Reilly, K (1977); *Science*; **197**; 1181 - 1183.

Binkley, S, Rievman, J and Reilly, K (1978); *Science*; **202**; 1198 - 1200.

Blask, D (1981); IN The Pineal Gland: Reproductive Effects: Volume II; Edited by Reiter, RJ; CRC Press Inc. - Florida; 189 - 216.

Bradford, H F (1986); Chemical Neurobiology: An Introduction to Neurochemistry; W H Freeman and Company - New York.

Bradford, H F and Ward, H K (1976); *Brain Res.*; **110**; 115 - 125.

Brose, N, Halpain, S, Suchanek, C and Jahn, R (1989); *J. Biol. Chem.*; **264**; 9619 - 9625.

Brose, N, Thomas, A, Weber, M G and Jahn, R (1990); *J. Biol. Chem.*; **265**; 10604 - 10610

Brown, B L, Albano, J D M, Ekins, R P and Sgherzi, A M (1971); *Biochem. J.*; **121**; 561 - 562.

Brownstein, M and Axelrod, J (1974); *Science*; **184**; 163 - 165.

Burton, S F (1989); MSc Thesis; Rhodes University.

Cardinali, D and Wurtman, R J (1972); *Endocrinology*; **91**; 877 - 886.

Cardinali, D P, Lynch, H J and Wurtman, R J (1972); *Endocrinology*; **91**; 1215 - 1218.

Carman-Krzan, M (1986); IN Progress in Medicinal Chemistry: Vol 23; Edited by Ellis, G P and West, G B; Elsevier Science Publishers - Amsterdam; 41 - 89.

Chan, A and Ebadi, M (1980); *Neuroendocrinology*; **31**; 244 - 251.

Collin, J P (1971); IN The Pineal Gland; Edited by Wolstenholm, GEW and Knight, J; Churchill Livingstone - Edinburgh; 79.

Collin, J P and Oksche, A (1981); IN The Pineal Gland: Anatomy and Biochemistry : Volume 1; Edited by Reiter, RJ; CRC Press Inc. - Florida; 27 - 67.

Cotman, C W and Taylor, D (1972); *J. Cell Biol.*; **55**; 696 - 711.

Craft, C M, Morgan, W W and Reiter, R J (1984); *Neuroendocrinology*; **38**; 193 - 198.

Curzon, G (1969); *Brit. J. Psychiat.*; **115**; 1367 -1374.

Curzon, G (1979); *J. Neural Transm. Suppl.*; **15**; 81 - 92.

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

Dafny, N (1977); *Exp. Neurol.*; **55**; 446 - 457.

Daya, S (1982); MSc Thesis ; Rhodes University.

Daya, S (1985); PhD Thesis; Medunsa.

Daya, S and Joubert, P H (1984); *IRCS Med. Sci.*; **12**; 1057.

Daya, S and Potgieter, B (1982); *S. Afr. J. Sci.*; **78**; 174.

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

Daya, S, Banoo, S, Brown, C and Potgieter, B (1989a); IN Advances in Pineal Research : Vol 3; Edited by Reiter, R J and Pang, S F; John Libbey and Co Ltd; 103 - 106.

Daya, S, Nonaka, K O and Reiter, R J (1990); *Neurosci. Lett.*; **114**; 113 - 116.

Deguchi, T (1973); *Mol. Pharmacol.*; **9**; 184 - 190.

Deguchi, T (1977); *J. Neurochem.*; **28**; 667.

Deguchi, T and Axelrod, J (1972a); *Proc. Natl. Acad. Sci.*; **69**; 2547 - 2550.

Deguchi, T and Axelrod, J (1972b); *Proc. Natl. Acad. Sci.*; **69**; 2208 - 2211.

Deguchi, T and Axelrod, J (1972c); *Anal. Biochem.*; **50**; 174 - 179.

Deguchi, T and Axelrod, J (1973); *Proc. Natl. Acad. Sci.*; **70**; 2411 - 2414.

Deguchi, T and Barchas, J (1972); *Mol. Pharmacol.*; **8**; 770 - 779.

Dickson, K L and Hasty, D L (1972); *Acta Endocrinol.*; **70**; 438 - 444.

Ebadi, M, Gavitrapong, P and Murrin, L C (1986); IN Advances in Pineal Research : Vol 1; Edited by Reiter, R J and Karasek, M; John Libbey and Co Ltd.; 99 - 109.

Fernstrom, J D and Wurtman, R J (1974); *Sci. Amer.*; **230**; 84 - 91.

Fiske, V M (1964); *Science*; **146**; 253 - 254.

Fiske, V M, Bryant, K and Putman, J (1960); *Endocrinology*; **66**; 489 - 891.

Foldes, A, Maxwell, C A, Rintoul, A J and Edols, R W (1984); *Neuroendocrinology*; **38**; 206 - 211.

Fontana, J A and Lovenberg, W (1971); *Proc. Natl. Acad. Sci.*; **68**; 2787 - 2789.

Fontana, J A and Lovenberg, W (1973); *Proc. Natl. Acad. Sci.*; **70**; 755 - 758.

Foster, A C and Fagg, G E (1984); *Brain. Res. Rev.*; **7**; 103 - 164.

Foster, A C, Mena, E E, Fagg, G E and Cotman, C W (1981); *J. Neurosci.*; **1**; 620 - 625.

Fraser, S, Cowen, P, Franklin, M, Franey, C and Arendt, J (1983); *Clin. Chem.*; **29**; 396 - 397.

Ganong, W F (1979); Review of Medical Physiology; Lange Medical Publications - California; 277 - 300.

Giarman, N J and Day, M (1959); *Biochem. Pharmacol.*; **1**; 235.

Gibbs, F and Vriend, J (1981); *Endocrinology*; **109**; 1796 - 1798.

Gilad, G M, Gilad, V H, Wyatt, R J and Tizabi, Y (1990); *Brain Res.*; **525**; 335 - 338.

Goodwin, R K and Post, R M (1974); IN Advances in Biochemical Psychopharmacology : Vol I; Edited by Costa, E, Gessa, G and Sandler, M; Raven Press - New York; 341 - 355.

Goldman, H and Wurtman, RJ (1964); *Nature*; **203**; 87 - 88.

Greenberg, L and Weiss, B (1978); *Science*; **201**; 61 - 63.

Haynes, R C and Larner, J (1975); IN The Pharmacological Basis of Therapeutics : 5th Ed.; Edited by Goodman, L S and Gilman, A; MacMillan Publishing Co. - New York; 1472 - 1503.

Hendrickson, A E, Wagoner, N and Cowan, W M (1972); *Z. Zellforsch. Mikrosk. Anat.*; **135**; 1.

Hodde, K C (1979); *Prog. Brain Res.*; **52**; 39 - 44.

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

Jequier, E, Robinson, D S, Lovenberg, W and Sjverdsma, A (1969); *Biochem. Pharmacol.*; **18**; 1071 - 1081.

Johnson, J L (1972); *Brain Res.*; **37**; 1 - 19.

Johnson, L Y (1982); IN The Pineal Gland: Extra-Reproductive Effects : Volume III; Edited by Reiter, RJ; CRC Press Inc. - Florida; 107 - 152.

Jones, D H and Matus, A I (1974); *Biochim. Biophys. Acta*; **356**; 276 - 287.

Karasek, M and Marek, K (1978); *Cell Tissue Res.*; **188**; 133 - 141.

Karasek, M and Vollrath, L (1989); IN Advances in Pineal Research : Volume 3; Edited by Reiter, R J and Pang, S F; John Libbey and Co Ltd - London; 21 - 26.

Kebabian, J W, Zata, M, Romero, J A and Axelrol, J (1975); *Proc.Natl. Acad. Sci.*; **72**; 3735 - 3739.

Keller-Wood, M and Dallman, M (1984); *Endocr. Rev.*; **5**; 1.

Khan, R, Daya, S and Potgieter, B (1990); *Experentia*; **46**; 860 - 862.

Kinson, G, Wahid, A K and Singer, B (1967); *Gen. Comp. Endocrinol.*; **8**; 445 - 454.

Klein, D C (1973); IN The Neurosciences; Edited by Schmitt, F O and Worden, F C; MIT Press - Cambridge; 509 - 516.

Klein, D C (1978); IN The Hypothalamus; Edited by Riechlin, S, Baldessarini, RJ and Martin, JB; Raven Press - New York; pp 303 - 317.

Klein, D C (1979); IN Endocrine Rhythms; Edited by Krieger, D T; Raven Press - New York; 203 - 223.

Klein, D C and Notides, A (1969); *Anal. Biochem.*; **31**; 480 - 483.

Klein, D C and Rowe, J (1970); *Mol. Pharmacol.*; **6**; 164 - 171.

Klein, D C and Weller, J L (1970); *Science*; **169**; 1093 - 1095.

Klein, D C and Weller, J L (1972); *Science*; **177**; 532 - 533.

Klein, D C, Berg, G R and Weller, J (1970); *Science*; **168**; 979 - 980.

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

Klein, D C, Auerbach, D A, Namboodiri, M A A and Wheler, G H T (1981); IN The Pineal Gland: Anatomy and Biochemistry : Volume 1; Edited by Reiter, RJ; CRC Press Inc. - Florida; 199 - 227.

Knox, W E and Auerbach, V H (1955); *J. Biol. Chem.*; **214**; 307 - 313.

Krstic, R (1973); *Acta Anat.*; **86**; 320.

Kus, L, Handa, R I and McNulty, L A (1990); Abstract from the 20th Annual Meeting of the American Society for Neuroscience; St. Louis - Missouri (USA); 1194.

Lerner, A B and Case, JD (1960); *Fed. Proc.*; **19**; 590 - 593.

Lovenberg, W, Jequier, E and Sjoerdsma, A (1967); *Science*; **155**; 217 - 219.

Lovenburg, W, Jequier, E and Sjoerdsma, A (1968); *Adv. Pharmacol.*; **6**; 21 - 27.

Lovenberg, W, Weissbach, H and Undenfriend, A (1962); *J. Biol. Chem.*; **237**; 89 - 93.

Lowry, O H, Rosebrough, NJ, Farr, A L and Randall, R J (1951); *J. Biol. Chem.*; **193**; 265 - 275.

Lynch, H J, Eng, J P and Wurtman, R J (1973); *Proc. Natl. Acad. Sci.*; **70**; 1704 - 1707.

Marinova, C, Persengiev, S, Konakchieva, R, Ilieva, A and Patchev, V (1991); *Int. J. Biochem.*; **23**; 479 - 481.

Matsushima, S and Morisawa, Y (1978); *Cell Tissue Res*; **195**; 461.

McEwen, B, Weiss, J and Schwartz, L (1968); *Nature*; **220**; 911 -912.

McIsaac, W M and Page, I (1959); *J. Biol. Chem.*; **234**; 858 - 864.

Mehdi, A Z and Sandor, R (1977); *J. Steroid Biochem.*; **8**; 821 - 823.

Meldrum, B and Garthwaite, J (1990); *TiPS*; **11**; 379 - 387.

Michaelis, E K, Michaelis, M L and Boyarski, L L (1974); *Biochem. Biophys. Acta.*; **367**; 338 - 348.

Milcu, S M, Petrescu, R and Tasca, C (1968); *Histochemie*; **15**; 312 - 317.

Milne, R (1980); *J. Neural Transmission*; **47**; 191 - 220.

Milne, R, Krstic, R and Devecerski, V (1968); *Acta Anat.*; **71**; 352.

Monaghan, D T and Cotman, C W (1986); *Proc. Natl. Acad. Sci.*; **83**; 7532 - 7536.

Motta, M, Schiaffini, O, Piva, F and Martini, L (1971); IN The Pineal Gland; Churchill Livingston Press - London; 279 - 301.

Narang, G C, Singh, D V and Turner, C (1967); *Proc. Soc. Exp. Biol. Med.*; **125**; 184 - 188.

Niles, L P (1989); IN Advances in Pineal Research : Volume 3; Edited by Reiter, R J and Pang, S F; John Libbey and Co Ltd; 201 - 206.

Nir, I and Hirschmann, N (1978); *J. Neural. Transm.*; **42**; 117 - 126.

Ogle, T F and Kitay, J E (1976); *Endocrinology*; **98**; 20 - 24.

Oksche, A, Korf, H W and Rodrîguez, E M (1987); IN Advances in Pineal Research : Vol 2; Edited by Reiter, R J and Fraschini, F; John Libbey and Co.; 1 - 18.

Olivieri, G and Daya, S (1992); *Med. Sci. Res.*; **20**; 303 - 304.

Pévet, P (1981); IN The Pineal Gland: Anatomy and Biochemistry : Volume 1; Edited by Reiter, RJ; CRC Press Inc. - Florida; 121 - 154.

Porter, J R and Heiman, M (1977); *Life Sci.*; **20**; 1363.

Quay, W B (1963); *Gen. Comp. Endocr.*; **3**; 473 - 479.

Quay, W B (1981); IN The Pineal Gland: Anatomy and Biochemistry : Volume 1; Edited by Reiter, RJ; CRC Press Inc. - Florida; 173 - 198.

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

Redman, J, Armstrong, S and Ng, K T (1983); *Science*; **219**; 1089 - 1090.

Reiter, R J (1981); *Am. J. Anat.*; **162**; 287 - 313.

Reiter, R J (1983); IN The Pineal Gland and its Endocrine Role; Edited by Axelrod, J, Fraschini, F and Velo, G P; Plenum - New York; 227 - 241.

Reiter, R J (1986); IN Advances in Pineal Research : Vol 1; Edited by Reiter, R J and Karasek, M; John Libbey and Co Ltd.; 77 - 87.

Reiter, R J (1987); *Life Sci.*; **60**; 2119 - 2131.

Reiter, R J (1988); IN ISI Atlas of Science: Plants and Animals: Volume 1; pp 111 - 116.

Reiter, R J (1989); IN Advances in Pineal Research : Volume 3; Edited by Reiter, R J and Pang, S F; John Libbey and Co. Ltd.; 165 - 173.

Reiter, R J (1991); *Mol. and Cell. Endocrinol.*; **79**; C153 - 158.

Reiter, R J and Hester, R J (1966); *Endocrinology*; **79**; 1168.

Rivest, R W, Roberts, K D and Leporé, F (1979); *Psychol. Rep.*; **44**; 883.

Roberts, P J (1974); *Nature*; **252**; 399 - 401.

Romero, J A and Axelrod, J (1974); *Science*; **184**; 1091 - 1092.

Romero, J A, Zatz, M and Axelrod, J (1975a); *Proc. Natl. Acad. Sci.*; **72**; 2107 - 2111.

Romero, J A, Zatz, M and Kebabian, J W (1975b); *Nature*; **258**; 435 - 436.

Romijn, H J (1978); *Life Sci.*; **23**; 2257 - 2274.

Sabol, S L and Niremberg, M (1979); *J. Biol. Chem.*; **254**; 1913 - 1920.

Salter, M and Pogson, C I (1985); *Biochem. J.*; **229**; 499 - 504.

Sapolsky, R M (1985); *Brain Res.*; **359**; 300.

Sapolsky, R M and Pulsinelli, W (1985); *Science*; **229**; 1397 - 1400.

Sapolsky, R M, Krey, L C and McEwen, B S (1986); *Endocrine Reviews*; **7**; 284 - 301.

Schramm, M, Eimerl, S and Costa, E (1990); *Proc. Natl. Acad. Sci.*; **87**; 1193 - 1197.

Shein, H M and Wurtman, R J (1969); *Science*; **166**; 519 - 520.

Shein, H M, Wurtman, R J and Axelrod, J (1967); *Nature*; **213**; 730 - 731.

Shibuya, H, Taru, M and Watanabe, S (1978); *Brain Res.*; **138**; 364 - 368.

Sitaram, B R and Lees, G J (1978); *J. Neurochem.*; **31**; 1021.

Skene, D J (1979); MSc Thesis; Rhodes University.

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

Snyder, S H and Axelrod, J (1964b); *Fed. Proc.*; **23**; 206.

Snyder, S H and Axelrod, J (1965); *Science*; **149**; 542 - 544.

Snyder, S H, Axelrod, J, Wurtman, R J and Fischer, J E (1965); *J. Pharmacol. Exp. Ther.*; **147**; 371.

Snyder, S H , Axelrod, J, and Zweig, M (1967); *J. Pharmacol. Exp. Ther.*; **158**; 206 - 213.

Snyder, S H, Zweig, M, Axelrod, J and Fischer, J E (1965); *Proc. Natl. Acad. Sci.*; **53**; 301 - 305.

Strada, S, Klein, D C, Weller, J and Weiss, B (1972); *Endocrinololgy*; **90**; 1470 - 1475.

Stryer, L (1981); Biochemistry - 2nd Ed.; W H Freeman and Company - New York; 895.

Sugden, D and Klein, D C (1984); *Endocrinology*; **114**; 435 - 440.

Titeler, M (1981); In Research Methods in Neurobiochemistry; Edited by Marks, N and Rodnight, R; Plenum Press - New York; 39 - 73.

Touitou, Y (1989); In Advances in Pineal Research : Volume 3; Edited by Reiter, R J and Pang, S F; John Libbey and Co. Ltd.; 241 - 246.

Touitou, Y, Sulon, J, Bogdan, A, Touitou, C, Reinberg, A, Beck, H, Sodoyez, J C, Demey-Ponsart, E and Cauwenberge, H (1982); *J. Endocr.*; **93**; 201 - 210.

Touitou, Y, Sulon, J, Bogdan, A, Reinberg, A, Sodoyez, T C and Demey-Ponsart, E (1983); *J. Endocr.*; **96**; 53 - 63.

Ueck, M (1979); *Prog. Brain. Res.*; **52**; 45 - 88.

Vanêcêk, J (1988); *J. Neurochem.*; **51**; 1436 - 1440.

Van Praag, H M (1982); *The Lancet*; (December); 1259 - 1264.

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

Vaughan, M K, Vaughan G M, Reiter, R J and Benson, B (1972); *Neuroendocrinology*; **10**; 139 - 154.

Vaughan, G M, Allen, J P, Tullis, W, Sackman, J W and Vaughan, M K (1978); *Neurosci. Lett.*; **91**; 83 - 87.

Vriend, J (1983); *Neuroendocrinology*; **36**; 68 - 78.

Wake, K, Ueck, M and Oksche, A (1974); *Cell Tiss. Res.*; **154**; 423 - 442.

Walsh, H and Daya, S (1991); *Med. Sci. Res.*; **19**; 745 - 746.

Walsh, H, Daya, S and Whiteley (1991); *Med. Sci. Res.*; **19**; 849 - 850.

Watkins, J C, Krogsgaard-Larsen, P and Honoré, T (1990); *TiPS*; **11**; 25 - 33.

Weiss, B and Costa, E (1967); *Science*; **156**; 1750 - 1752.

Weissbach, H, Redfield, B G and Axelrod, J (1960); *Biochim. et Biophys. Acta*; **43**; 343 - 353.

Wetterberg, L, Aperia, B, Beck-Friis, J, Kjellman, B F, Ljunggren, J G, Nilsonne, A, Petterson, U, Tham, A and Umden, F (1982); *The Lancet*; **ii**; 100.

Wolfe, D E (1965); *Prog. Brain Res.*; **10**; 332 - 376.

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

Wurtman, R J and Axelrod, (1967); *Adv. Pharmacol.*; **6**; 141 - 156.

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

Wurtman, R J and Wurtman, J J (1989); *Sci. Amer.*; **264**; 50 - 57.

Wurtman, R J, Altschule, M D and Holmgren, U (1959); *Am. J. Physiol.*; **197**; 108 - 110.

Wurtman, R J, Axelrod, J, Sedvall, G and Moore, R Y (1967); *J. Pharmac. Exp. Ther.*; **157**; 487 - 492.

Wurtman, R J, Shein, H M and Larin, F (1971); *J. Neurochem.*; **18**; 1683 - 1687.

Young, S N (1981); *Br. J. Pharmac.*; **74**; 695 - 700.

Young, I M and Silman, R E (1982); IN The Pineal Gland: Extra-Reproductive Effects : Volume 3; Edited by Reiter, RJ; CRC Press Inc. - Florida; 189 - 218.

Zanoboni, A and Zanoboni-Muciaccia, W (1967); *Life Sci.*; **6**; 2327.

Zisapel, N (1988); *J. Neural Transm.*; **73**; 1 - 5.