REGULATION OF THE INDOLEAMINES BY SEX STEROIDS

THESIS

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ABSTRACT

Alteration of serum tryptophan leads to parallel alterations in brain tryptophan levels. Such changes in brain tryptophan levels has been shown to lead to mood disturbances. The primary enzyme responsible for altering serum tryptophan levels is the liver cytosolic enzyme, tryptophan pyrrolase. Activation of this enzyme is responsible for the enhanced catabolism of circulating tryptophan.

The purpose of the present study was firstly to establish whether there is a link between sex steroids and tryptophan pyrrolase activity especially since sex steroids are also known to cause mood disturbances and secondly to determine the effects of sex steroids on brain indolamine metabolism.

The results show that all three sex steroids induce the activity of tryptophan pyrrolase implying that they decrease serum tryptophan levels by the activation of tryptophan pyrrolase, thus making less tryptophan available for uptake by the brain. It was also shown that the sex steroids enhance the uptake of ^{14}C-tryptophan by brain synatopsomes. In addition, the sex steroids influenced the pattern of metabolism of serotonin by organ cultures of rat pineal glands.

It is possible that the sex steroids regulate the availability and uptake of indoleamines in the brain.

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DEDICATION

I dedicate this thesis to my son, **George Awah.**

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ABBREVIATIONS USED

5-ALA	5-aminolevulinic acid
AIA	allylisopropylacetamide
AMP	adenosine monophosphate
cAMP	cyclic adenosine monophosphate
Ci	Curie
CNS	central nervous system
CoA	Coenzyme A
CSF	cerebrospinal fluid
CVS	cardiovascular system
DHT	5α-dihydroxytestosterone
DNA	deoxyribonucleic acid
d.p.m.	disintegrations per minute
FSH	follicle-stimulating hormone
FSH-RF	follicle-stimulating hormone releasing factor
GABA	gamma aminobutyric acid
GH	growth hormone
GMP	guanosine monophosphate
HIOMT	hydroxyindole-O-methyltransferase
HIAA	5-hydroxyindoleacetic acid
HPA	hypothalamic-pituitary-adrenal
HTOH	5-hydroxytryptophol
5-HT	serotonin
5-HTP	5-hydroxytryptamine
i.p.	intraperitoneal
i.v.	intravenous
L-DOPA	dihydroxyphenylalanine

LH	luteinizing hormone
LH-RH	luteinizing hormone releasing factor
MAO	monoamine oxidase
MEL	melatonin
MIAA	5-methyoxyindole acetic acid
MIF	melanocyte-stimulating hormone release-inhibiting factor
MSH	melanocyte-stimulating hormone
MTOH	5-methoxytryptophol
MT	5-methoxytryptamine
NA	Noradrenaline
NAS	N-acetylserotonin
NE	Norepinephrine
N.S.	Not significant
pCPA	p-chlorophenylalanine
PKC	protein kinase C
RNA	ribonucleic acid
SCN	suprachiasmatic nuclei
SNAT	serotonin-N-acetyltransferase
S-N-K	student-Newman-Keul's test
TH	thyroid hormone
TLC	thin-layer chromatography
TP	testosterone propionate
TRH	thyrotropin releasing hormone
TSH	thyroid stimulating hormone

APPENDIX LEGENDS

CHAPTER 1

LITERATURE REVIEW

1.1 HISTORY OF PINEAL RESEARCH

The history of the pineal gland has been fraught with events which were as exciting as they were intriguing, almost bordering on the fringes of mystery.

Galen, writing in the second century A.D., quoted studies of earlier Greek anatomists, particularly Heropilos (325-280 B.C.), who were impressed with the fact that the pineal was perched atop the aqueduct of the cerebrum and was a single structure rather than a paired one; he concluded that it served as a valve to regulate the flow of thought out of its 'storage bin' in the lateral ventricles of the brain. René Descartes (1596-1650) embellished this notion; he believed that the pineal housed the seat of the rational soul. In his formulation, the eyes perceived the events of the real world and transmitted these to the pineal by way of "strings" in the brain. The pineal responded by allowing humours to pass down hollow tubes to the muscles, where they produced the appropriate responses. This mechanistic theory of perception by Descartes may be primitive, but one cannot fail to admire this prophetic formulation of the pineal as a neuroendocrine transducer with the hindsight of 300 years of pineal research.

In the late 19th and early 20th centuries, the pineal fell from

its exalted metaphysical state. Otto Heubner (1898), a German physician, published a case study of a young boy who had precocious puberty and was also found to have a pineal tumour. Prior to this, De Graaf (1886), Horschelt and Spencer (1905) have reported on the phylogenetic regression of the pineal gland. Studmika (1905) similarly reported that the pineal develops from a photoreceptor into a complicated gland having an enigmatic function. Berblinger (1920) and Engel (1936) further observed the antigonadotropic effects of the pineal gland. All the foregoing references were cited from The Pineal Gland, Wurtman and Axelrod (1965).

1.1.1 ENDOCRINE FUNCTIONS OF THE PINEAL GLAND

Beyond a few years back, the state of knowledge about the gland was

(i) it appeared to be a photoreceptor in the frog (Nils Holmgren, 1918)

(ii) it had something to do with sexual function in rats and in humans

(iii) it contained a factor that blanched pigment cells in the tadpole (McCord and Allen, 1927).

With the discovery of the endocrine glands by Claude Bernard (1813-1878) and Brown-Sequard (1817-1894) and the subsequent intensive research over the years, it became evident that it would be incorrect to define the pineal gland from the concept of a pure gland without taking into account its endocrine

functions. The term neuroendocrine transducer means an organ that secretes its own hormone in response to a locally released neutrotransmitter. In view of the various classical functions of the pineal, this definitive term seemed more appropriate in describing the pineal gland. The role of the pineal gland appears to convert a neural input, nonadrenaline released from sympathetic neurons into a hormonal output, melatonin.

1.2 PINEAL ANATOMY

1.2.1 LOCATION AND STRUCTURE

Ariëns Kappers (1960) published a meticulous study that demonstrated that although the pineal gland originates from the brain in the development of the embryo, it loses all nerve connections with the brain soon after birth. The pineal gland of the rat is superficially situated in the brain just rostral to the cerebellum and between the occipital poles of the cerebral hermispheres. Dorsally, it is covered by the confluence of the superior sagittal and transverse sinuses. The anlage of the pineal diverticulum lies in the dorsal median area of the neural tube at the level of the neuroaxis, which will become the diencephalon. The diverticulum characteristically originates in the area between the posterior and habenular commissures i.e. an intercommissural position (Bergman, 1965). Although definitively, the pineal is an azygous midline structure, in the strictest sense there are actually two pineal anlagen, one on either neural fold of the developing neural tube

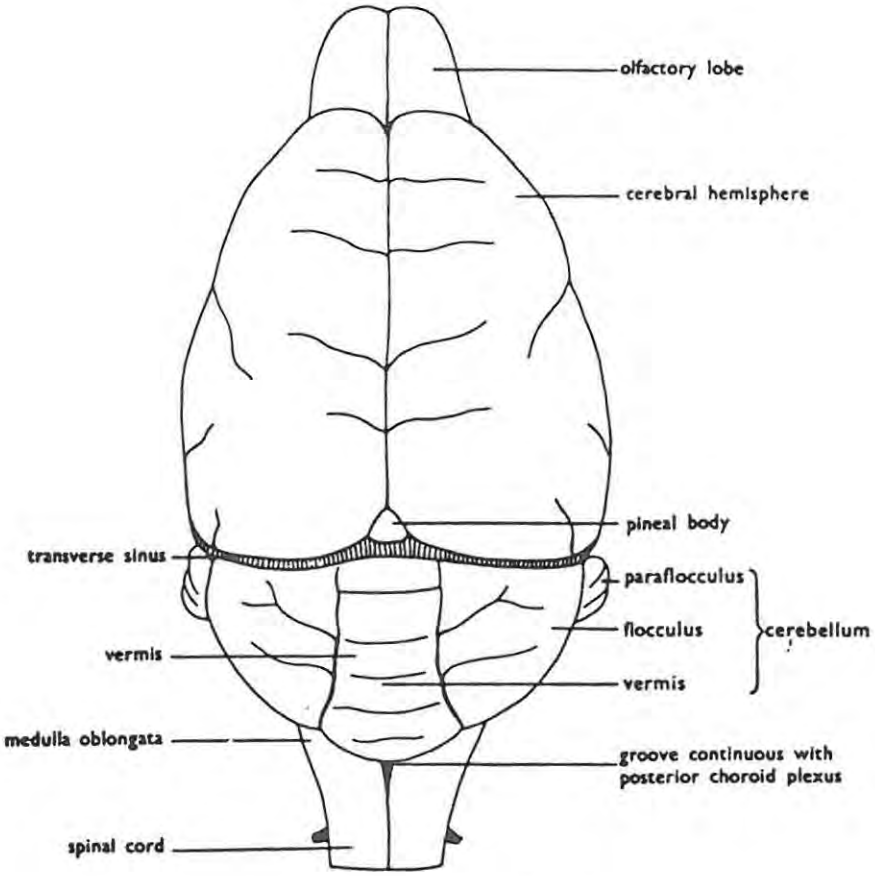


FIG. 1. Dorsal view of the rat brain.

(Rowett, 1968)

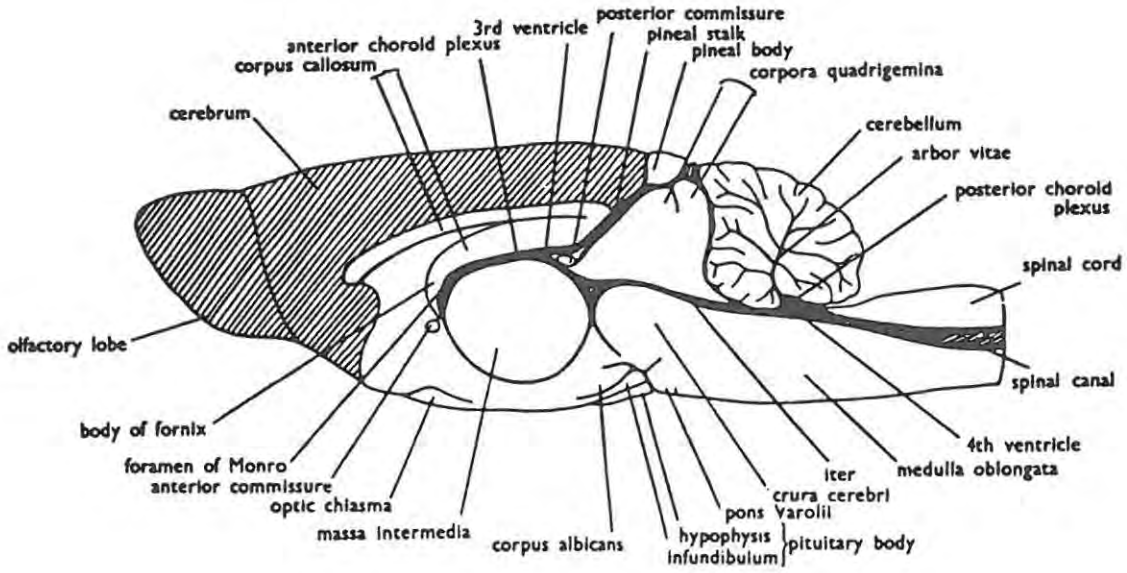


FIG. 2. Median sagittal view of the rat brain.

(Rowett, 1968)

(Reiter, 1981). The pinealocytes, the endocrine units, are not randomly arranged in the pineal gland (Vollrath, 1981 and 1984). Connective tissue septae, which are continuous with the capsule of the gland, separate the parenchymal cells into cords or lobules, the lobules may appear as follicles.

1.2.2 HISTOLOGICAL VARIATIONS IN HUMANS

There is great individual variation in the histological appearance of the human pineal gland and this variability becomes even more evident with age (Reiter, 1989). In advanced age the connective tissue septae typically become more prominent and in many individuals calcified structures - concretions, corpora arenacea, calcium deposits - appear (Welsh, 1985). The functional significance of these structures remains unknown, although in experimental animals, both the development and the persistence of the corpora arenacea require that the sympathetic innervation of the pineal gland remain intact (Champney *et al.*, 1985).

1.2.3 THE PINEAL CELL

There are two categories of pinealocytes and although these can be easily identified, there is a considerable confusion in the nomenclature owing to ignorance of exact pineal function. The pineal gland is chiefly composed of the first category of cells: light parenchymal pinealocytes which are characterized by the presence of granular vesicles. Phylogenetically, Pévet and

Collin (1976), have shown that the cells originated from the neurosensory photoreceptor cells present in the pineal organ of submammalian vertebrates (Collin, 1971a and 1971b), and that they belong to the sensory cell line as defined by Collin (1971). In consequence, this category of cells can be termed true pinealocytes or pinealocytes *sensu stricto* (Wolfe, 1965). The second category of pineal parenchyma cells, called interstitial or glial cells are characterized by their location to the perivascular space and absence of granular vesicles (Pevét, 1977). The granular vesicles in the pinealocyte proper are believed to store the antigonadrotropic hormones (Upson *et al.*, 1976).

1.2.4 ULTRASTRUCTURE OF THE PINEAL CELL

1.2.4.1 Cytoplasm

Four to twelve clusters of ribosomes are dispersed throughout the cytoplasm and form no link with the membranes. A few ribosomes are associated with paired cytoplasmic membranes and with the outer layer of the nuclear envelope to form the rough endoplasmic reticulum. Milofsky (1957) observed 'giant' mitochondria which are fairly abundant in epiphyseal cells. The cytoplasm contains vernate bodies in which variable numbers of glycogen particles, lipid droplets, mitochrondria, vesicles and grumose granules are present.

Synaptic ribbons occur in the perikarya and polar processes of

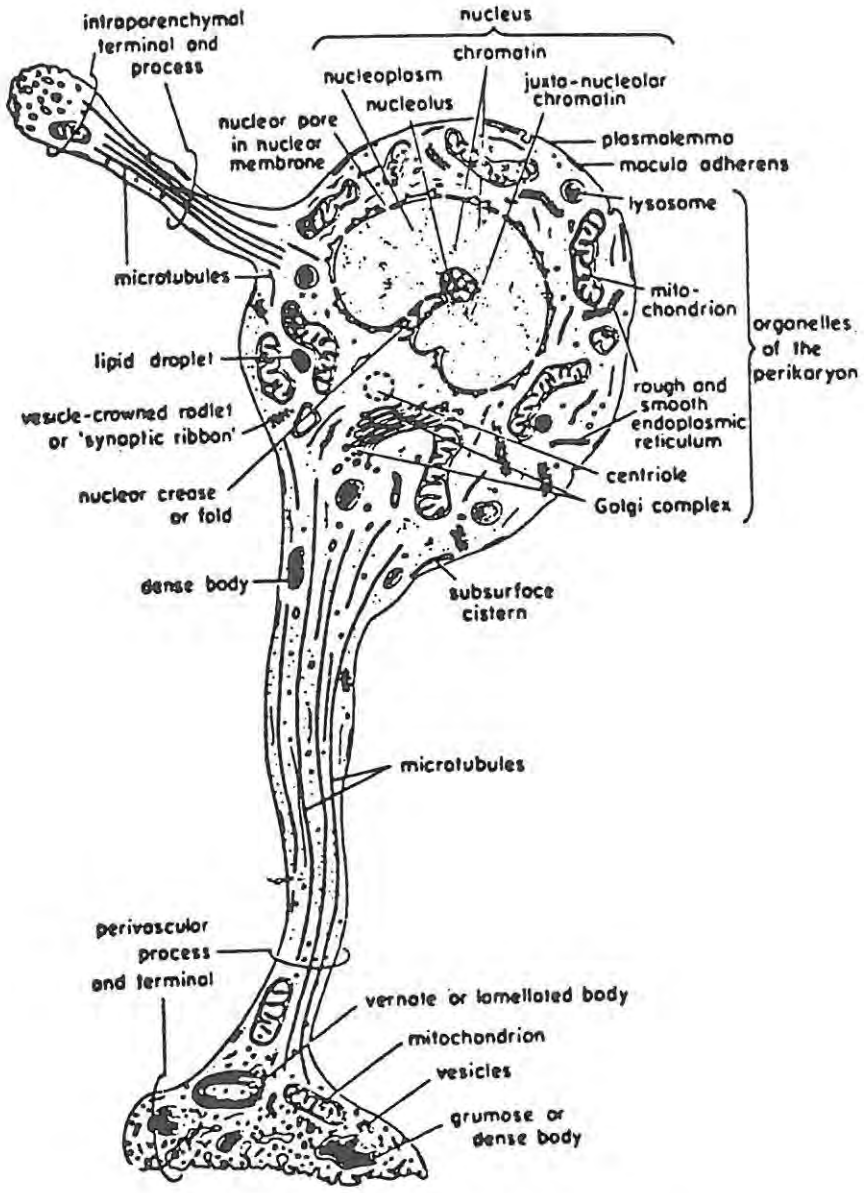


Fig. 3. Ultrastructure of generalised mammalian pinealocyte. Not all organs are drawn to scale.

(Quay, 1974b)

these cells, usually situated beneath the plasmalemma. Membranes of the Golgi apparatus occur randomly in the perikaryon of epiphyseal cells. The acanthosome or spiny body, is the only cytoplasmic organelle constantly associated with the Golgi apparatus, resulting in invaginations resembling micropincytosis. Grumose globules, containing fine membranous and granular matter distributed evenly in the dense amorphous matrix are present but usually sparsely distributed.

1.2.4.2 Nucleus

The nuclear enveloppe consists of the usual perinuclear cisterna bridged by nuclear pores. Chromatin is localized in an uneven marginal zone sporadically confluent with areas deeper in the nucleus. Chromatin areas are filled with material arranged in meshworks, granular masses and helical strands. The nucleoli are composed of amorphous areas surrounded by a dense nucleolonema and ribonucleoprotein granules are present (Milofsky, 1958).

1.3 NERVOUS INNERVATION

The neural connections of the pineal gland have been of special interest because both the synthetic (Wurtman *et al.*, 1965) and the endocrine (Reiter and Hester, 1966) capabilities of the gland rely on an intact sympathetic innervation. Early physiological (Thiéblot *et al.*, 1947) and anatomical data (Kappers, 1960) provided proof that the cell bodies of the

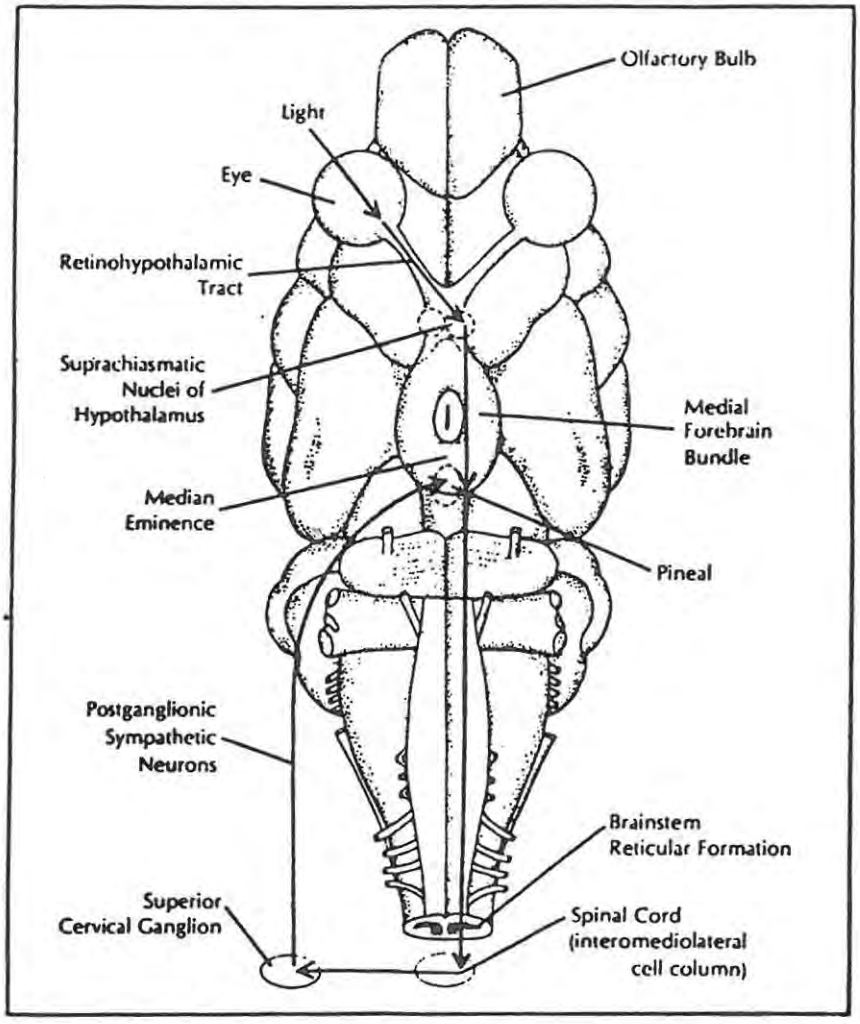


FIG. 4. Neural pathways to the pineal gland.

(Wurtman, 1980)

postganglionic sympathetic neurons were in the superior cervical ganglia and that processes of these cells terminated within the pineal gland. The course of the postganglionic fibres from the superior cervical ganglia to the pineal is via blood vessels (the internal carotid plexus). As the fibres near the gland, they enter the tentorium cerebelli and eventually form one or two relatively discrete nerves, the nervi cornarii, before entering the gland (Kappers, 1960). They usually penetrate the capsule near the distal end or apex of the gland. The nerve fibres ramify among the pinealocytes and terminate especially in pericapillary spaces and occasionally between parenchymal cells (Romijn, 1973b; Matushima and Reiter, 1977). Contrary to an earlier view (Wolfe, 1965), in most species, the nerve terminals do not form morphological synapses with pinealocytes, rather they end primarily in the vicinity of pinealocyte processes, often around capillaries.

1.4 TRANSLATION OF PHOTOPERIODIC INFORMATION INTO A CHEMICAL MESSENGER

Unlike a number of other endocrine organs, the pineal gland relies heavily on its innervation for its endocrine activity (Reiter, 1989). In particular, sensory information perceived by the eyes is essential in determining the production of pineal melatonin. The pineal gland is the site at which light:dark information is translated, or transduced, into a chemical messenger (Reiter, 1991b). In general, light suppresses melatonin production, whereas darkness is associated with the

biosynthesis of several indoleamines (Reiter, 1984). Information about ambient lighting conditions is transferred from the retinas to the pineal gland over a complex series of neurons that are located in both the central and peripheral nervous system (Kappers, 1965). This neuronal route includes the retinohypothalamic tract, the suprachiasmatic nuclei (SCN), and the pre- and postganglionic fibres of the peripheral sympathetic nervous system (Reiter, 1981).

After transduction into a neural signal in the photoreceptors of the retinas, the message is sent to the hypothalmus via ganglion cell axons, which form the retinohypothalamic tract (Moore, 1978). At the level of the optic chiasma these fibres diverge from the classic optic system and terminate in the suprachiasmatic nuclei of the anterior hypothalamus (Sonofriew and Weindl, 1982). After synapsing in this location, fibres project possibly to the paraventricular nuclei of the hypothalamus (Bittman, 1984). Long descending actions then supposedly carry the neural message to the intermediolateral cell column of the upper thoracic spinal cord (Swanson and Sawchenki, 1983). The axons of these preganglionic perikarya leave the spinal cord and pass up the sympathetic trunk to synapse on postganglionic cell bodies in the superior cervical ganglia; the postganglionic fibres enter the skull and among other organs, terminate within the pineal gland (Kappers, 1965).

The melatonin synthetic and secretory activity of the pineal gland is determined primarily by the release of noradrenaline

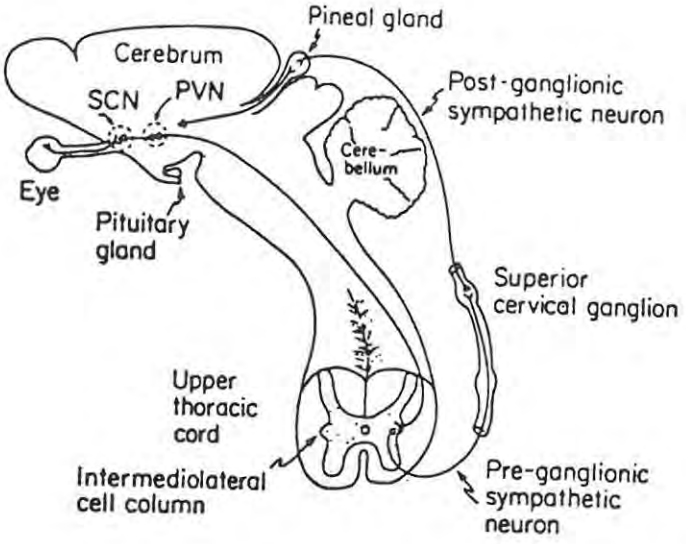


Fig. 5. Proposed neural connections between the eyes and the pineal gland in mammals. The postganglionic sympathetic innervation is essential to many of the metabolic rhythms observed in the pineal gland as well as the endocrine capabilities of the organ.

(Reiter, 1989)

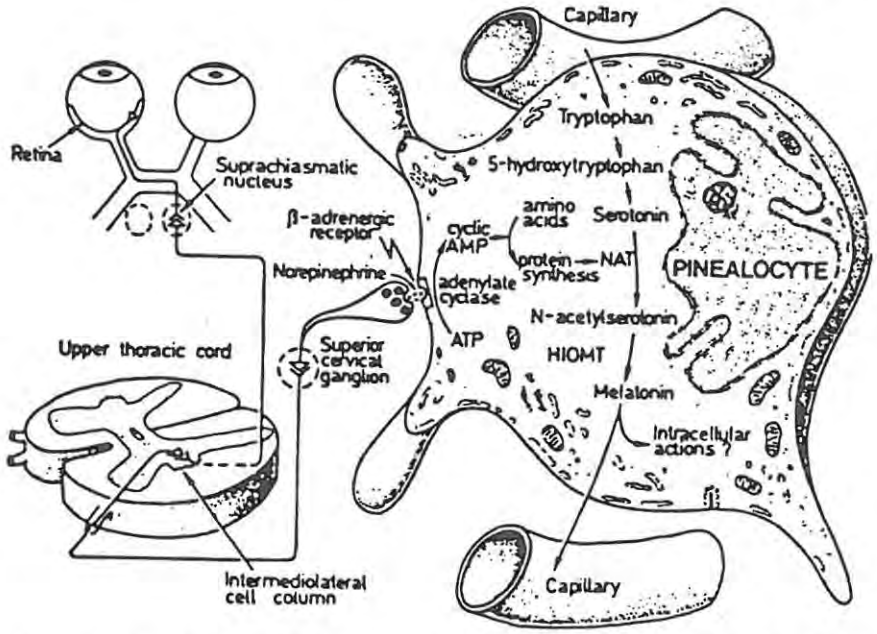


Fig. 6. Sympathetic neural interaction with the pinealocyte, the endocrine elements of the pineal gland. On the left are summarised the connections between the eyes and the pineal. In the pinealocyte is a summary of the uptake of tryptophan and its conversion to and secretion of melatonin.

(Dr. Radivoj Kirstic)

(Axelrod, 1974) from the postganglionic sympathetic nerve endings.

The sympathetic neural connections between the SCN and the pineal gland must remain intact in order for the pineal to function in its normal capacity. Interruption of the neural connection caudal to the SCN in either the central or peripheral nervous systems interferes with the cyclic production of melatonin as well as with the endocrine capability of the pineal gland (Kreisley and Moskowitz, 1978).

1.5 THE BIOCHEMISTRY OF THE PINEAL GLAND

1.5.1 CIRCADIAN RHYTHMS AND THE PINEAL GLAND

Pineal function and pineal biochemistry are linked by one general theme; the function and biochemistry involve circadian rhythms. Circadian rhythms are found in the metabolic pathways of the pineal gland and circadian rhythms have been implicated in three areas of pineal function: control of locomotor activity, circadian time measurements in photoperiodism, and colour changes.

Gaston and Menaher (1968) suggested that the pineal gland was a possible "biological clock" or "circadian pacemaker" after observing that the pineal gland was necessary for the normal persistence of locomotor activity in house sparrows. This result was verified in white-crowned sparrows (McMillan, 1972),

lizards (Underwood, 1977), and starlings (Gwinner 1978; Rutledge, 1978). Although, pinealectomy did not abolish circadian rhythms of locomotor activity in chickens (MacBride, 1973) or in rats (Quay, 1968), it did produce faster phase shifting in rats (Quay, 1968; Kincl *et al.*, 1970).

Reiter (1977) proposed that the pineal gland of hamsters may be the source of antigonadal hormone which plays a role in the photoperiod response; hamster testes regress in constant dark or on short days, an effect which is reversed by pinealectomy. Reiter (1977) further postulated that melatonin may be one of the antigonadal hormones responsible for this effect. Photoperiodism in the hamster reproductive system is dependent upon circadian rhythms for time measurement (Elliot, 1972 and 1976).

1.5.2 MELATONIN

McCord and Allen in 1917 discovered that extracts from bovine pineals contained a substance which lightened the skin colour of amphibians. Lerner and coworkers (1958) isolated this substance which they characterized as N-acetyl-5-methoxytryptamine (Lerner *et al.*, 1959; Lerner *et al.*, 1960) and was termed melatonin because of its blanching effect on melanophores. Its synthesis undergoes a diurnal variation in relation to the photoperiod, the level being highest during the dark phase of the photoperiod (Wurtman *et al.*, 1968).

1.5.2.1 Tissue distribution

Most of the melatonin in mammals is localized in the pineal gland. Since it is very lipophilic, its access to the CNS is not impeded by a blood brain barrier.

In addition to the pineal, other endocrine tissues, peripheral nerves and the sympathetic nervous chain also selectively take up labelled melatonin, but to a lesser extent. Adipose tissue contains the lowest concentration of ^{3}H-melatonin of any tissue, which indicates that the ability of such organs as the ovary, pineal and adrenal to concentrate the indole is unrelated to their relatively high lipid contents (Wurtman *et al.*, 1964b).

Other tissue/organs of the mammal in which the presence of melatonin has been established include peripheral nerves (Barchas and Lerner, 1964), brain (Wurtman, 1969; Koop *et al.*, 1980), lactating mammary tissue (Reppert and Klein, 1978), retina and Harderian gland (Bubenik *et al.*, 1978; Pang *et al.*, 1980). Cardinali *et al.*, (1972) have also established that 60-80% of melatonin in the plasma is bound to serum albumin. There exists a state of dynamic equilibrium between "free" tryptophan and tryptophan bound to serum albumin.

1.5.3 BIOSYNTHESIS OF PINEAL INDOLES

1.5.3.1 Melatonin

The ability of the pineal gland to produce large quantities of methoxyindoles is related at least partially to the high levels of tryptophan hydroxylase within the pineal gland (Lovenberg *et al.*, 1967; Deguchi, 1977). After the uptake of the amino acid in the pinealocyte the hydroxylating enzyme converts it to 5-hydroxytryptophan. Following this step 5-hydroxytryptophan is decarboxylated by the enzyme L-aromatic amino acid decarboxylase, which is rather widely distributed in the organism (Lovenberg *et al.*, 1962). This reaction results in the formation of serotonin, the content of this amine is higher in pineal gland than in any other organ in the body (Quay, 1963; Saavedra *et al.*, 1973). Serotonin is the common precursor of a number of indoles formed within the pineal gland. In a 2-step process, serotonin is N-acetylated by the enzyme SNAT (Klein *et al.*, 1971; Buda and Klein, 1975). The product of this reaction is N-acetylserotonin. This step requires an acetyl group which is provided by acetyl CoA. The formation of melatonin from N-acetylserotonin is catalyzed by the enzyme HIOMT (Axelrod and Weissbach, 1960; Axelrod *et al.*, 1965). During this conversion, the methyl group is provided by 5-adenosylmethionine (Axelrod and Weissbach, 1960).

The conversion of serotonin to melatonin is under control of light:dark cycle to which the animals are exposed via its action

on the peripheral sympathetic nervous system (Reiter, 1981). Characteristically, periods of light are associated with high pineal serotonin levels (Quay, 1963, 1966) and low SNAT activity (Klein and Weller, 1970; Rudeen *et al.*, 1975). Conversely, during the daily period of darkness the amount of serotonin within the gland is diminished, presumably because it is being converted to N-acetylserotonin by the increased activity of the acetylating enzyme (Reiter, 1981). The magnitude of the day:night change in NAT activity varies among species: uniformly however, darkness is associated with the rise in the activity of this enzyme (Klein *et al.*, 1971a; Deguchi and Axelrod, 1972; Rudeen *et al.*, 1975). As a consequence of the increased SNAT activity at night pineal levels of N-acetylserotonin are also augmented (Klein and Weller, 1973).

Considering the large amplitude rhythm in the activity of the acetylating enzyme throughout each 24-hour period, it has been proposed as the rate-limiting enzyme in melatonin production (Klein and Weller, 1973); but other workers (Wurtman and Ozaki, 1978) dispute this. HIOMT, the enzyme that catalyzes the conversion of N-actylserotonin to melatonin, was initially thought to exhibit a circadian rhythm as well (Wurtman *et al.*, 1963). However, it is now conceded that if it does vary throughout the 24-hour period, the rhythm is of very low magnitude (Klein *et al.*, 1981).

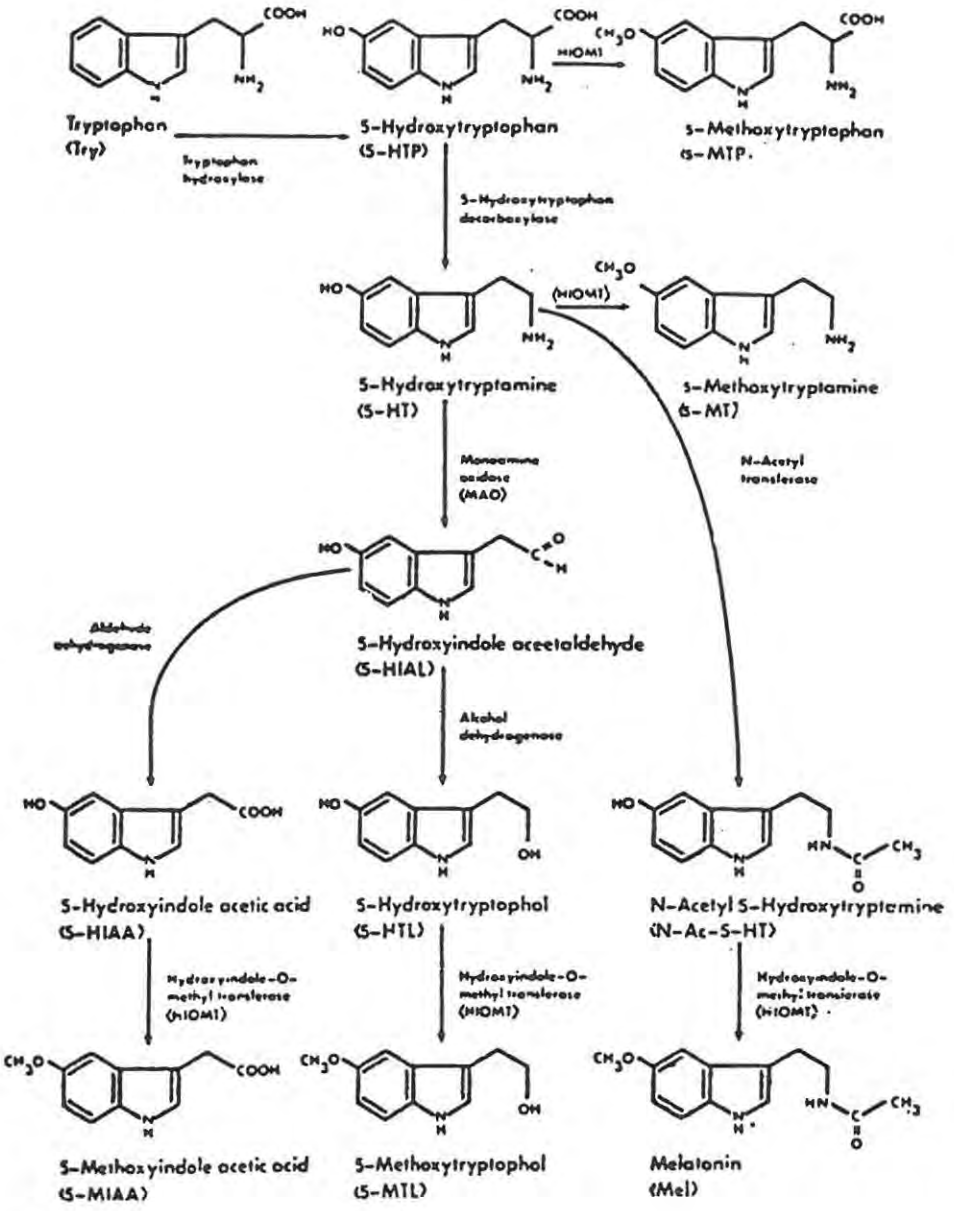


Fig. 7. The indole metabolism in the pineal gland.

1.5.3.2 Synthesis of other pineal indoles

Serotonin is converted to 5-hydroxyindole acetaldehyde by monoamine oxidase (Axelrod *et al.*, 1969). Some of this is then converted to 5-hydroxyindoleacetic acid by aldehyde dehydrogenase (Wurtman and Larin, 1968; Lerner and Case, 1960). This is then O-methylated in the 5 position by HIOMT to form 5-methoxyindole acetic acid (Wurtman and Axelrod, 1967). Some of the 5-hydroxyindole acetaldehyde is also converted to 5-hydroxytryptophol by alcohol dehydrogenase (McIsaac and Page, 1959) and is then methoxylated by HIOMT to form 5-methoxytryptophol (Wurtman and Axelrod, 1967). Serotonin can also be methoxylated by HIOMT to form 5-methoxytryptamine.

1.5.3.3 The role of ß- and α-adrenergic mechanisms in melatonin production

The α- and ß-receptors exhibit 24h rhythms in their density (Pangerl *et al.*, 1990); such flunctuations are generally believed to be determined by NE itself which, following its release causes the desensitization or internalization of the receptors (Romero *et al.*, 1975; Gonzalez-Brito *et al.*, 1988; Pangerl *et al.*, 1989). The involvement of two receptors in the regulation of intracellular cAMP, is a feature common to many cells (Reiter, 1991). cAMP is the essential intracellular second messenger required for the rise in nocturnal melatonin production (Axelrod, 1974; Klein *et al.*, 1981; Santana *et al.*, 1988).

ß-adrenergic stimulation of the pinealocyte causes a rise in intracellular accumulation of cAMP due to its ability to increase the activity of adenylate cyclase (O'Dea and Zatz, 1976). Activation of ß-adrenergic receptors alone induces up to 10-fold increase in cAMP in the pineal gland, conversely stimulation of α-adrenergic receptors alone is without any effect on cyclic nucleotide accumulation (Vanecek *et al.*, 1985). However, when combined stimulation of both ß- and α-adrenergic receptors by NE occurs, the rat pineal response in terms of cAMP is greatly potentiated with nucleotide content increasing up to 100-fold (Klein, 1985). These findings are consistent with the view that, in the pinealocyte, ß-receptors activation is a requirement for cAMP accumulation with α-receptor stimulation amplifying the response. The dual receptor regulation of cAMP in the pineal gland also translates into a similar stimulation (by ß-adrenergic receptors) and augmentation (by α-adrenergic receptors) of the activity of the rate-limiting enzyme in melatonin production, N-acetyltransferase (Klein *et al.*, 1981), as well as of melatonin itself (Santana *et al.*, 1988).

By observing the fact that ribosylation of the G-protein by cholera toxin can substitute for ß-adrenergic receptor activation (Sungden, 1989), it was believed that a G-protein is involved in the ß-receptor-mediated stimulation of adenylate cyclase.

Stimulation of α_1-receptors increases both the diacylglycerol and intracellular calcium concentrations; these two processes,

in a synergistic manner, stimulate the translocation of protein kinase C(PKC) (Sugden, 1989). The resultant activation of PKC increases the efficiency of activation of adenylate cyclase most probably through phosphorylation of the cyclase (Reiter, 1991).

α-adrenergic receptor stimulation only slightly augments the actual amount of melatonin formed, which is essentially a result of ß-adrenergic receptor stimulation (Reiter, 1991). Thus marked interactive effect of ß- and α-adrenergic stimulation of the second messenger cAMP does not carry over it a similar large augmentation of the amount of melatonin formed (Reiter, 1991).

1.5.3.4 Factors affecting melatonin synthesis

1.5.3.4.1 Drug effects

1.5.3.4.1.1 Catecholaminergic drugs

Axelrod *et al.*, (1969) have shown that the rate at which cultured rat pineals synthesize ^{14}C-melatonin from ^{14}C-tryptophan is increased by catecholaminergic drugs, such as d-noradrenaline, l-epinephrine, dopamine, tyramine, octopamine and tryptamine. Catecholaminergic drugs influence the release of noradrenaline from the sympathetic nerves innervating the pineal gland and hence these drugs exert a positive influence on melatonin synthesis. In addition, pheniprazine (Axelrod *et al.*, 1969) and harmine (Klein and Rowe, 1970), all MAO inhibitors have been observed to influence melatonin synthesis.

1.5.3.4.1.2 Psychotomimetic drugs

These drugs which include dimethyltryptamine, bufotenine, mescaline, lysergic acid diethylamide (LSD) and dimethoxyphenylethylamine (Hartley and Smith, 1973) as well as amphetamine (Bäckström and Wetterberg, 1973) and cocaine (Holtz *et al.*, 1974) are all known to increase melatonin synthesis. However, neuroleptics have been found to decrease this synthesis (Hartley *et al.*, 1972).

1.5.3.4.1.3 The adrenergic blockers

Wurtman *et al.*, (1971) had shown that ß-adrenergic blockers, such as propranolol can block the noradrenaline-induced increase in melatonin synthesis in cultured rat pineals. Phenoxybenzamine showed no effect. Wurtman *et al.*, (1974) further observed that neither propranolol nor phenoxybenzamine could affect the increase in melatonin synthesis caused by dibutyryl cAMP. However, cycloheximide, a protein synthesis inhibitor and actinomycin block this effect (Berg and Klein, 1971).

1.5.3.4.1.4 Sympathomimetics and related agents

The effects of sympathomimetic drugs on the rat pineal gland have been exhaustively investigated. Fiske (1964) and Klein *et al.*, (1971) have established that, denervation, by bilateral superior cervical ganglionectomy abolishes the nocturnal rise in

indoleamine metabolism. Axelrod *et al.*, (1969) and Klein *et al.*, (1970) also established the converse observation that, addition of norepinephrine to cultured glands increases the synthesis and secretion of melatonin. The actions of norepinephrine are mediated by the ß-adrenergic receptor (Wurtman *et al.*, 1971; Deguchi and Axelrod, 1972a). In contrast, other neurotransmitters, though present in the gland, are ineffective in stimulating the gland. These include serotonin (Klein and Weller, 1973; Deguchi and Axelrod, 1972a), GABA (Mata *et al.*, 1976) and histamine (Klein and Weller, 1973). Other agents which usually are not thought of as sympathomimetics, can still stimulate the pineal through weak interaction with the ß-adrenergic receptors. Examples include taurine (Wheler *et al.*, 1979), octopamine (Klein and Weller, 1973) and possibly DOPA (Deguchi and Axelrod, 1972a; Lynch *et al.*, 1973).

Agents which by-pass the ß-adrenergic receptor have also been useful in elucidating regulatory mechanisms. Induction of SNAT activity by cAMP analogues (Klein *et al.*, 1970; Deguchi and Axelrod, 1972b) and by phosphodiesterase inhibitors (Klein and Weller, 1973; Deguchi and Axelrod, 1972) strengthens the conclusion that cAMP mediates the effects of ß-adrenergic stimulation. Agents which block the ß-adrenergic receptors, or result in reduced concentration of norepinephrine at the receptors, have been shown to block the stimulation of indoleamine metabolism. Such agents include reserpine (Deguchi and Axelrod, 1972b) which depletes the sympathetic nerve endings

of catecholamines, and propranolol (Deguchi and Axelrod, 1972b; Parfitt *et al.*, 1976), which specifically blocks the ß-adrenergic receptors.

The ß-adrenergic receptors are similarly blocked by dichloroisoprenaline and they are inhibited to some degree by trifluoperazine and chloropromazine but they are not affected by the alpha lytics:phentolamine and phenoxybenzamine (Weiss and Costa, 1968; Uzunov and Weiss, 1971). Auerbach *et al.*, (1981) have shown that the relative potencies of norepinephrine and isoprenaline on pineal cyclic AMP levels appear to be dose-dependent. The norepinephrine-induced elevation of cAMP activity is potentiated by introducing theophylline, a phosphodiesterase inhibitor to the culture medium (Strada *et al.*, 1972).

Probenecid blocks the cultures efflux of both cAMP and cGMP whilst desmethylimipramine blocks the stimulated accumulation and efflux of cGMP (O'Dea *et al.*, 1976). Chloragen increases adenyl cyclase and cAMP levels and decreases cGMP levels in pineal organ cultures (Minneman, 1977).

1.5.4 PINEAL ENZYMES

Pineal enzymes represent five major categories on the basis of the nature of their catalytic effects:

(i) oxidoreductases

(ii) transferases

(iii) hydrolases

(iv) lyases

(v) isomerases

Quay (1974) has compiled a detailed survey of pineal enzymes within each of these categories.

1.5.4.1 Tryptophan-5-hydroxylase

This enzyme hydroxylates tryptophan to 5-hydroxytryptophan. Hori *et al.*, (1976) demonstrated that the enzyme is localized in the mitochondrial fractions. Romijn *et al.*, (1977) observed *in vitro*, a spherical enlargement of the mitochondria in the light pinealocytes of rabbit after addition of p-chlorophenylalanine, an inhibitor of tryptophan-5-hydroxylase (Besinger *et al.*, 1974; Lovenberg *et al.*, 1967; Deguchi and Barchas, 1972).

1.5.4.2 Serotonin N-acetyltransferase (SNAT)

SNAT is metabolically the most important of the transferases in the pineal gland. SNAT (EC2.3.1.5., acety CoA: argylamine-N-acetyltransferase), catalyzes the rate-limiting step in the pathway from 5-HT to its 5-methoxy derivatives. The pineal gland is a rich source of the enzyme system known as cAMP-dependent protein kinase which includes a regulatory protein (cAMP-binding) and a catalytic protein (protein kinase). When activated by cAMP, this system phosphorylates histones. It has important roles in the induction of some pineal enzymes, including SNAT (Fontana and Lovenberg, 1971; Fontana and

Lovenberg, 1971; Winters *et al.*, 1977).

SNAT activity in rat pineal cultures has been shown to be increased by noradrenaline (Klein *et al.*, 1971; Deguchi and Axelrod, 1973; Klein and Weller, 1973), DOPA (Deguchi and Axelrod, 1972) and isoprenaline (Deguchi, 1973), octopamine (Klein and Weller, 1970), theophylline and the MAO inhibitors pargyline and latron (Deguchi and Axelrod, 1972). Propranolol however abolished the rise in activity of SNAT by blocking the ß-adrenergic receptors of the pineal cells.

It appears that the retinohypothalamic projection is responsible for the synchronization of the circadian rhythm in pineal SNAT. Destruction of the suprachiasmatic nucleus abolished the rhythm in SNAT activity in the pineal gland. Evidently, the suprachiasmatic nucleus represents a central rhythm generator (Moore and Klein, 1974), an endogenous oscillator or biological clock (Klein, 1978).

The stimulatory effect of the catecholamine, norepinephrine on SNAT (Klein *et al.*, 1970a, b) occurs only during the night (Shibuya *et al.*, 1975; Klein and Weller, 1970). In contrast to the maximal amount of norepinephrine present at the end of the night, the activity of SNAT decreases as the end of the night approaches. This may probably be explained in the following way: the sensitivity of the ß-receptor increases during daytime (Romero and Axelrod, 1974, 1975). Since norepinephrine rapidly decreases during the daytime, cAMP is not produced in sufficient

quantity. After the onset of darkness, however, the increasing amount of norepinephrine produces a rapid increase in quantity of cAMP, although the maximal activity of SNAT during the night results in a high synthesis of N-acetylserotonin (Klein and Weller, 1970). Lynch *et al.*, (1973a) and Sampson (1975), have shown that rats immobilzed for several hours or made hypoglycaemic with insulin have high levels of melatonin as well as SNAT. These increases are due to the release of catecholamines from the sympathetic nerve terminals.

1.5.4.3 Hydroxyindole-O-methyltransferase (HIOMT)

This enzyme is responsible for the formation of: MIAA from HIAA; MTOH from HTOH and melatonin from N-acetylserotonin. Noradrenaline (Klein, 1969), when added to pineal cultures and dimethyltryptamine (Hartley and Smith, 1973) when added to pineal homogenates, increase HIOMT activity. Administration of DOPA similarly increases HIOMT activity (Lynch *et al.*, 1973).

The inhibitors of HIOMT include substituted N-benzoyltryptamines and N-phenylacetyltryptamines (Ho *et al.*, 1971), haloperidol (Hartley *et al.*, 1972; Ho *et al.*, 1973), fluperazine, GABA (Hartley *et al.*, 1972) and oxypertine (Ho *et al.*, 1973).

1.5.4.3.1 Effect of pteridines on HIOMT activity

Cremer-Bartels (1962a, b), and Cremer-Bartels and Balazs (1973) have established the presence of pteridine-like pigments in the

mammalian eye tissues. Ebels *et al.*, (1979) have also shown the presence of pteridines and pteridine-like pigments in the mammalian pineal gland. Cremer-Bartels *et al.*, (1975), further observed that commercial triamterene (2,4,7-triamino-6-phenylpteridine) affects retinal HIOMT in the process of light adaptation and therefore suggested that pteridines may play a role in the regulation of melatonin biosynthesis. The drug triamterene is applied in man and assumed to affect corticosteroid levels (Klein *et al.*, 1978). Melatonin itself, applied in rats, was shown experimentally to decrease the serum corticosteroid level (Minneman and Wurtman, 1976). The combination of these two phenomena suggests that triamterene affects pineal HIOMT and in this way may be involved in endocrine regulation (Cremer-Bartels, 1979).

1.5.5 ENDOCRINE EFFECTS OF THE PINEAL GLAND

1.5.5.1 Effect on the pituitary gland

1.5.5.1.1 The pineal gland and the regulation of gonadotropin secretion

Kitay (1954), Kitay and Altschule (1954), and Wurtman *et al.*, (1959) have demonstrated the induction of ovarian hypertrophy following pinealectomy. The induction was inhibited by the injection of crude, or protein-free, pineal extracts. Data from numerous literature reports (Reiter, 1973; Altschule, 1975; Minneman and Wurtman, 1975; Relkin, 1976; Mess *et al.*, 1978)

have established the inhibitory role of the pineal gland on both FSH and LH.

Fraschini *et al.*, (1968a, b) demonstrated a significant increase in pituitary LH content following pinealectomy. The extent of this increase was about the same as that observed after castration. The implantation of pineal tissue or of melatonin into either the medium eminence or the reticular substance of the mesencephalon equally depressed the castration-induced rise of pituitary LH. The fact that the implantation of melatonin or of some melatonin derivative into various locations within the brain stem inhibits LH secretion but not implantation of the same substances into the adenohypophysis, indicates that pineal hormone(s) act as sites within the central nervous system (Trentini *et al.*, 1979).

Narang *et al.*, (1967) and Vaughan *et al.*, (1971) reported a decrease in pituitary mass after melatonin administration. This was however not observed by De Prospo and Hurley (1971a).

Melatonin administration has been shown to block the pre-ovulatory surge in serum LH (Reiter and Sorrentino, 1971; Ying and Fiske, 1972) as well as decrease serum FSH (Sorrentino, 1968; Kamberi *et al.*, 1971).

Moszkowska *et al.*, (1973) put foward a mechanism to explain the above effects. They observed that microinjections of melatonin into the rat lateral ventricle resulted in a significant

decrease in hypothalamic releasing factor (RF) levels, notably, FSH-RF content. They therefore suggested that melatonin may act by decreasing the synthesis or release of the gonadotropin RFs in the hypothalamus. This finding was consistent with the studies of Kamberi *et al.*, (1970) who showed that melatonin injected directly into the pituitary gland had no effect on serum LH levels. However, there were contradictory reports by Martin *et al.*, (1977) and Martin and Klein (1976), which show that melatonin can act directly on the neonatal pituitary gland and suppress the response to LH releasing hormone (LH-RH) which, in turn suppresses pituitary LH release. The inhibitory role of the pineal gland exerted on the TSH-thyroid system, ACTH-adrenocortical mechanisms and GH-secretion is in many respects equivocal. The antigonadotrophic activity of the pineal gland and its hormones seem to be more unequivocal.

1.5.5.2 Adrenal glands

1.5.5.2.1 Glucocorticoids

The precise effect of melatonin administration on the production of ACTH and corticosterone are contradictory. Kinson and Singer (1967a, b) observed that pinealectomized rats secrete an increased amount of corticosterone and concluded that melatonin exerts an inhibitory effect on adrenal function. By contrast, Gromora *et al.*, (1967a, b) were able to show that the acute administration of melatonin significantly elevated corticosterone levels. This effect, however, disappeared

following hypophysectomy which suggested that melatonin has a stimulatory effect on ACTH. Fraschini *et al.*, (1968) established that there was a decrease in adrenal mass following intrahypothalamic implantation of melatonin. Barchas *et al.*, (1969) however observed no correlation between chronic or acute parenteral melatonin administration and the plasma levels of corticosterone, ACTH or pituitary stores of ACTH. De Prospo and Hurley (1971a) injected melatonin (100μg/day) into the lateral cerebral ventricles of female rats for 10 days. The idea here was to increase the amount of melatonin gaining access to the neuroendocrine axis. The melatonin, however, failed to depress the pituitary-adrenal axis.

Contrary to these reports Motta *et al.*, (1971) showed that an acute intraventricular injection of melatonin brought about a significant reduction in the levels of plasma corticosterone in normal male rats. Further work has established that melatonin similarly suppresses stress-induced corticosterone production. Following reserpine treatment, the corticosterone response was however not affected by melatonin treatment. These results suggest that ACTH is under inhibitory control by a central adrenergic mechanism (Giuliani *et al.*, 1966) and that melatonin might possibly exert its actions via this inhibitory mechanism. A central action of melatonin is supported by the *in vitro* evidence, that when the hormone is added to pituitary incubates, it does not change ACTH secretion (Jouan and Samperez, 1965). Melatonin has also been shown to alter the synthesis of corticosteroids in incubated adrenal slices (Giordano *et al.*,

1967, 1970).

Daily intraperitonal injections of melatonin (2 x 50 μg/day) stimulated adrenal 5-α-reductase activity in ovariectomized and hypophysectomized rats (Ogle and Kitay, 1977) suggesting that melatonin may play a role in the physiological regulation of adrenal steroidogenesis.

In many diurnal species, maximum levels of cortisol, corticosterone and ACTH occur just before daylight and around the onset of darkness in nocturnally active rodents (Krieger, 1979; Touitou *et al.*, 1982; 1983). Different types of physical or psychological stress are able to cause ACTH release whereas cortisol and synthetic glucocorticoids inhibit ACTH secretion through a negative feedback mechanism. Neither pneumoencephalography, electroshock therapy, sleep deprivation, psychological stress nor sprinting, elevated melatonin secretion despite the activation of the HPA axis (Vaughan *et al.*, 1979; Touitou *et al.*, 1983).

In a study of the effect of cortisol synthesis blockade by metyrapone, a drug which inhibits 11ß-hydroxylase activity which results in a fall in the production of cortisol, inducing a compensatory increase in ACTH secretion, Brisma *et al.*, (1985) established that in two patients with asymptomatic moderate hyperparathyroidism and prolactin-secreting microadenoma respectively, urinary melatonin levels remained unchanged whereas these levels increased in two healthy volunteers. Medhi

and Sandor (1969), using bovine adrenal cortex *in vitro* demonstrated that melatonin inhibits the conversion of radiolabelled progesterone to cortisone and aldosterone. Komissarenko *et al.*, (1979) also reported the inhibition of corticosteroidogenesis by an epiphyseal factor isolated from bovine pineal glands.

All these reports suggest that the pineal gland may play a modulatory role in corticosteroidogenesis.

1.5.5.2.2 Aldosterone

Gromora *et al* (1967a and b) have demonstrated that the acute administration of melatonin significantly reduced aldosterone production, while hypophysectomy abolished this effect. Farrel (1959 and 1960) isolated two pineal extracts. The first which he named, adrenoglomerulotropin enhances aldosterone release. The second extract which bears close structural identity with melatonin (Farrel and McIsaac, 1960), inhibits aldosterone secretion. Haulica *et al.*, (1980), have postulated the existence of positive and negative feedback relationships between the brain isorenin-angiotensin system (angiotensin II enhances the increase in serotonin levels in the pineal, the hypothalamus and the brain stem in dogs) and serotonin metabolism in the pineal gland. Earlier, Kinson *et al.*, (1967) reported significant increases in aldosterone levels one month after pinealectomy.

In view of the contentious nature and contradictions in these foregoing reports, it would not be possible to draw a precise and justifiable conclusion on the influence of the pineal gland and its putative hormone, melatonin on the secretions of aldosterone.

1.5.5.3 Thyroid Gland

Vriend (1978) has recently formulated a relationship between pineal function and thyroid activity. This was as a result of his observation that, if pinealectomized hamsters are subjected to darkness, short photoperiods or blindness, their gonads do not involute and plasma thyroxine levels remain normal, in contrast to sham-operated controls in which depression of thyroxine levels and gonadal involution occur.

Prior to this Milan *et al.*, (1968) and Csaba and Bernard (1972) have respectively demonstrated that low concentrations of thyroxine in the incubation medium increased the activity of the enzymes in the glycolytic pathway and lipid metabolism of cultured pineal gland; and that thyroxine enhanced serotonin metabolism in the pineal organ cultures. Milne (1963) also suggested that the feedback information from the thyroid gland influences not only the hypothalamic-hypophyseal axis but also the habenular-pineal complex. This hypothesis has recently received support from observations on melatonin synthesis in incubated pineal glands. Nir and Hirschman (1978) reported that thyroxine or triidothyronine added to the incubation medium

increased ^{14}C-tryptophan metabolism to melatonin and in the presence of 10mM norepinephrine, labelled precursor metabolism to N-acetylserotonin. At higher concentrations of thyroid hormone, the effects were not observed, implying biphasic effects.

All these reports suggest a direct influence of the thyroid gland on pineal function. On the other hand, the pineal gland appears, from previous reports to influence the physiological manifestations of the thyroid gland. Scépovic, (1963) demonstrated that the administration of pineal extract brings about a reduction in thyroid mass as well as ^{131}I accumulation by the thyroid gland and further that pinealectomy results in increased ^{131}I uptake. In a similar finding, Baschieri *et al.*, (1963) showed that there was a significant reduction in ^{131}I uptake by the thyroid gland after daily parenteral administration of melatonin. Singh and Turner, (1962) observed a reduction in thyroxine secretion in melatonin-treated rats. Another finding by Baschieri *et al.*, (1963) which was confirmed by Reiter *et al.*, (1965) showed that melatonin blocked the thyroid hyperplasia caused by methylthiouracil. Panda and Turner, (1968) reported that chronic melatonin administration brings about a reduction in pituitary levels and increase plasma levels of thyrotropic hormone. De Prospo *et al.*, (1968, 1969) similarly reported reduced ^{131}I uptake following melatonin administration which they found unrelated to the inhibitory effect of melatonin on gonadal secretions.

However, there have been a few reports which contradict the foregoing reports on melatonin effects on the thyroid. Naber *et al.*, (1969) demonstrated that melatonin did not affect ^{131}I uptake into the thyroid gland. Thiéblot *et al.*, (1966) showed that melatonin administered to prepuberal rats produces marked hypertrophy of the thyroid gland. Narang *et al.*, (1967) found that the TH secretion rate in rats was inhibited by chronic melatonin administration. Gordon *et al.*, (1980) have demonstrated that chronic melatonin increases the thyroid gland size and thyroxine content of the thyroid.

The view of the various workers on the effect of pineal function on the thyroid is that the pineal plays a modulatory role on the thyroid with respect to the release of TH.

1.5.5.4 Prolactin

Melatonin (500μg, twice daily for six days) is required from an intact pineal gland to augment serum prolactin level inasmuch as the effect of the indoleamine was negated by pinealectomy (Cardinali *et al.*, 1979). Increased prolactin release into circulation was observed (Kamberi *et al.*, 1971) following melatonin administration into the rat cerebral ventricles. This effect was not observed when melatonin was injected directly into the anterior pituitary and therefore the workers suggested that melatonin might block the release of prolactin inhibiting factor (dopamine) from the hypothalamus which in turn would explain the observed elevation in plasma levels of prolactin (Lu

and Meites, 1973). The intravenous administration of 5-HTP or 5-HT raises serum prolactin levels and the diurnal changes in prolactin secretion run parallel to pineal activity. Prolactin levels are high during the dark period (Willonghby, 1980).

Pinealectomy does not affect the diurnal rhythm but it delays the fall in the mean prolactin concentration occuring during the light period (Niles *et al.*, 1977). The reduced levels of prolactin caused by 5-MTOH and N-acetylserotonin in intact rats were abolished by pinealectomy. Of the various compounds studied for their effects on prolactin secretion, melatonin was found to influence the secretion of prolactin. Arginine vasotocin, a polypeptide with antigonadotropic properties also induced prolactin secretion in a biphasic manner. Serotonin and 5-HTP showed no effect on prolactin secretion (Hanen *et al.*, 1980).

From these reports, it can be suggested that the pineal gland can influence prolactin secretion.

1.5.5.5 Growth Hormone (GH)

Malm *et al.*, (1959) observed an increase in body weight of pinealectomized rats. Prior to this it has been known that total darkness or blinding inhibits the growth of rats (Luce-Clausen and Brown, 1939). Giammanco *et al.*, (1968) later showed that anosmia provokes the same growth-retarding effect. Relkin (1972b) demonstrated decreased pituitary and plasma GH levels

and retarded body growth in animals exposed to constant darkness. This effect was abolished by pinealectomy. Ronnelkeiv and McCann (1978) found that pinealectomy reduces the level of GH during the day but not in the night. Smythe and Lazarus, (1973) concluded from a series of studies that the ability of melatonin to increase serum GH in man is due to interaction with serotonin receptor sites in the hypothalamus which, in turn, induces the release of GH from the pituitary. They postulated that melatonin can act as a blocker or competitive antagonist at serotonin receptor sites. This might explain why melatonin and serotonin have opposite effects on GH secretion.

1.5.5.6 Melanocyte-stimulating Hormone (MSH) and Melanocyte-stimulating Hormone Release-Inhibiting factor (MIF)

Kastin and Schally (1967) found that melatonin decreased pituitary MSH. They suggested that the release of MSH from rat pituitaries is stimulated by an increased concentration of circulating melatonin and inhibited by raised levels of circulating MSH.

The exact mechanism is not clear but Kastin *et al.*, (1972b) proposed a possible link between the pineal gland, hypothalamus and pituitary involving melatonin, MSH and MIF.

Tilders and Smelik (1975) however found no relationship between melatonin administration and pituitary MSH. From an earlier

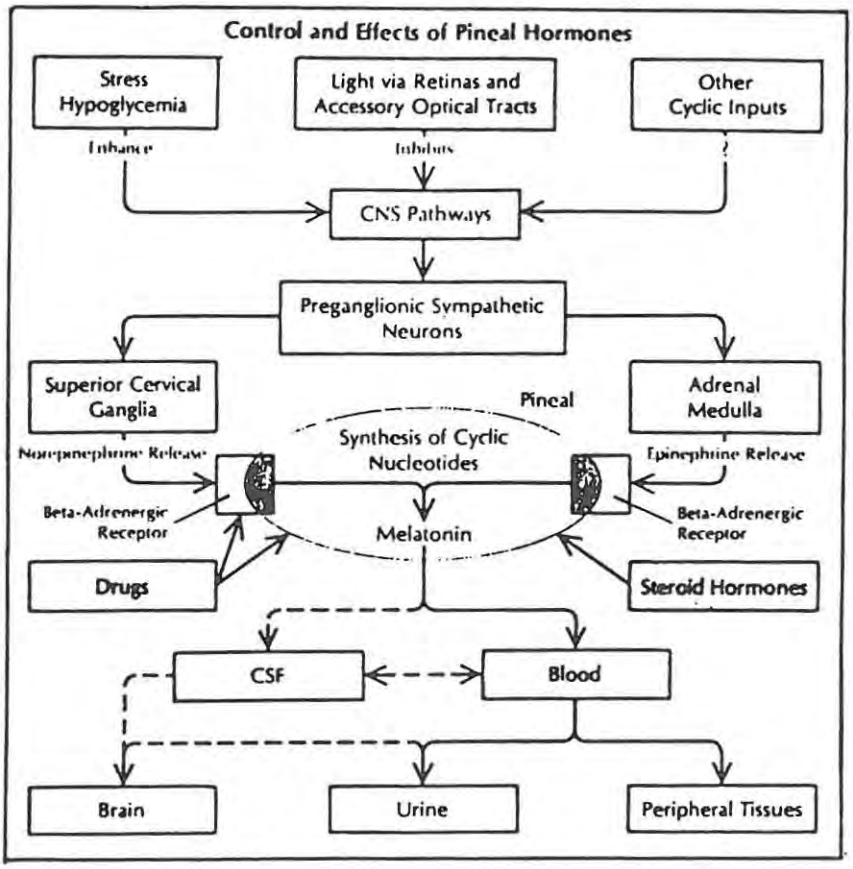


FIG. 8. Control and effects of pineal hormones. Diagram showing the areas of action of hormones on the pineal and of pineal hormones on other areas.

(Wurtman, 1980)

observation by Tilders (1973) that melatonin has no effect on diurnal variation of pituitary MSH levels, Tilders and Smelik (1975) concluded that the rhythm as well as resting MSH levels were not controlled by melatonin.

1.6 GONADAL SEX STEROIDS AND THE PINEAL GLAND

1.6.1 INTRODUCTION TO STEROIDS

1.6.1.1 Definition of Steroids

Steroids are a class of organic compounds biologically derived from six isopentyl pyrophosphate units and containing the perhydrocyclopentanephenanthrene nucleus i.e. three six-membered rings and one five-membered ring.

1.6.2 THE STEROID CYCLE

At present, steroid hormone production is believed to be initiated by the releasing factors of the hypothalamus of the brain which travel to the anterior lobe of the hypophysis (pituitary gland) in the portal blood stream of the pituitary stalk, where they cause the secretions of trophic hormone into the blood stream. The trophic hormone travels to the sight of steroid synthesis e.g. adrenal gland, ovary, testes, and stimulates the production of steroids which are released into the blood stream to travel to the target organ e.g. kidney and skin. It is believed that steroids are transformed in the

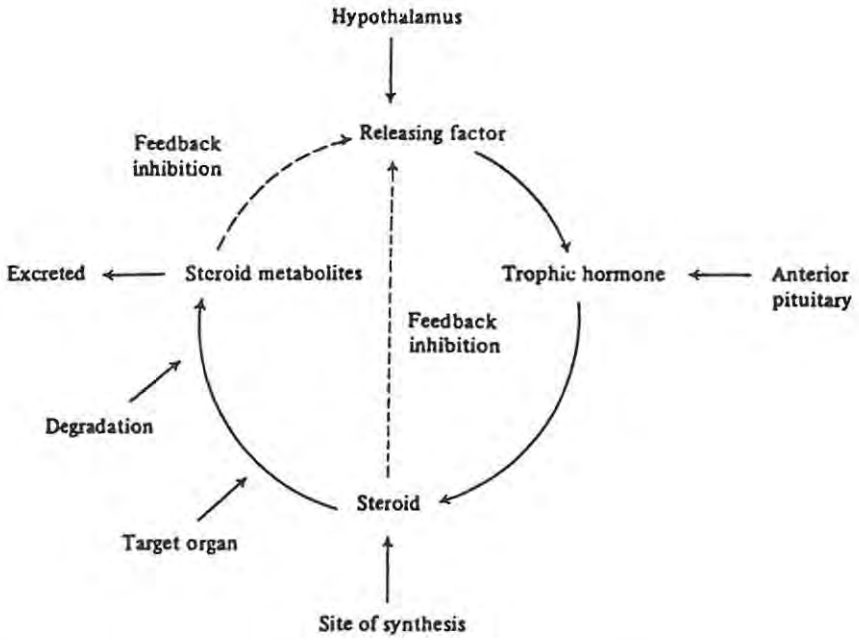


Fig. 9 . The steroid cycle

(Briggs and Brotherton, 1970)

target cells and metabolites released into the blood stream. Steroids are further degraded and rendered inactive at special degrading sites e.g. liver and are then excreted.

There is a feedback inhibition system whereby no releasing factor is produced when the blood steroid levels are too high. Each type of steroid hormone is believed to have its own steroid cycle, the nomenclature of which is based on the organ of the site of synthesis e.g. the corticosteroid cycle is initiated by the corticotrophin releasing factor which causes the release of the (adreno-)corticotrophic hormone (corticotrophin or ACTH), which in turn stimulates the cortex of the adrenal gland to produce corticosteroids. Similarly, luteotrophin releasing factor causes the release of the luteotrophic hormone, and this stimulates the corpus luteum of the ovary to produce its steroids. The evidence for feeback inhibitions by androgens and estrogens is still not conclusive, although oral contraceptives are belived to act by suppressing the hypothalamic releasing factors (Briggs and Brotherton, 1970).

1.6.3 EFFECT OF GONADAL SEX STEROIDS ON PINEAL MORPHOLOGY AND ADENYL CYCLASE

Histochemical and ultrastructural studies of chicks injected with the sex steroids suggested that the sex steroids influence precocious maturity of the pineal gland (Boya *et al.*, 1980).

The pineal adenyl cyclase system of ovariectomized rats was

found to be more susceptible to stimulation by noradrenaline. Prior to this Weiss and Crayton (1970) have shown that noradrenaline exerts a higher level of stimulation of male rats than female rats. The adenyl cyclase system is inhibited by chronic administration of estradiol. Testosterone and progesterone has no such effect on the system.

1.6.4 ESTRADIOL [1.3.5(10)-Estratrien-3, 17α-diol]

Zweens, (1965) reported the reversal of the pineal lipid content that followed ovariectomy in rats. Since then estradiol treatment in rats has been reported to influence a number of pineal constituents, including size and matrix of pinealocyte mitochondria (Clementi *et al.*, 1965), nucleic acid and protein content (Nir *et al.*, 1970; Nir *et al.*, 1972), protein synthesis (Cardinali *et al.*, 1974), 5-HT and NE turn over rates (Vacas and Cardinali, 1980; Cardinali *et al.*, 1975), NE-induced increase of adenylate cyclase activity (Weiss and Crayton, 1970) and of cAMP content (Davis, 1978), NE-induced changes of pinealocyte electrical activity (Marks *et al.*, 1972), HIOMT levels (Wurtman *et al.*, 1965; Cardinali *et al.*, 1974; Wallen and Yochim, 1974) and melatonin release *in vivo*, (Ozaki and Wurtman, 1978) and *in vitro* (Wilkinson and Arendt, 1978).

Two groups of workers (Marks *et al.*, 1972; Nagle *et al.*, 1972), independently reported indications about the existence of receptors for estradiol in the rat pinealocyte. The first group reported that ^{3}H-estradiol was bound to soluble macromolecules

by pineal homogenates *in vitro* whilst the second group reported that pineal glands of oophorectomized rats, given a pulse injection of ^{3}H-estradiol took up and retained the labelled steroid to the same extent as the uterus.

David *et al.*, (1975) have demonstrated the ^{3}H-estradiol uptake *in vivo* and *in vitro* and its binding to receptor proteins in rhesus moneky pineal glands resembling those of rats. This finding was confirmed a year later by Murthy *et al.*, (1976) who reported the ^{3}H-estradiol binding to cytosol proteins. Autographic analysis of rat pineal glands after *in vivo* administration of ^{3}H-estradiol revealed nuclear concentrations of labelled grains in still undefined population of pineal cells. Even though this finding is compatible with the biochemical data on cytoplasmic and nuclear estraphillic receptors, it is not clear whether these cells are pinealocytes or glial cells (Vernadakis *et al.*, 1978). These reports confirm the presence of specific estradiol receptors on the pinealocyte.

1.6.4.1 Effect of estradiol on pineal enzymes

1.6.4.1.1 HIOMT

Wurtman *et al.*, (1965a) found that there was a significant decrease of pineal HIOMT activity 24 hours after a single dose of 10μg of estradiol benzoate administration to ovariectomized rats. Alexander *et al.*, (1970) injected 50μg of estradiol for 3 days and found that this inhibited the postcastration rise of

pineal HIOMT activity in rats oophorectomized at the twenty-eighth day of life and sacrificed three weeks later. Cardinali, (1977) determined that 2μg of estradiol should be taken as the highest dose to be injected to examine changes in pineal activity within the physiological range, since the injection of 2μg of estradiol into ovariectomized rats produced translocation of pineal hormone-receptor complexes from cytoplasm to nuclei indistinguishable from normal rats in proestrus.

It is rather important to distinguish between pharmacological and physiological concentrations of estradiol in studies relating to pineal functions.

Cardinali *et al.*, (1974) reported that administration of 0,05-1,0μg/day of estradiol for three days increased HIOMT activity whereas greater pharmacological doses (5μg/day) decreased it (Nagle *et al.*, 1972). Administration of the weak estrogen, estrone, in a dose of 10μg restores the mean rate of pineal HIOMT activity depressed following ovariectomy in female cycling rats (Wallen and Yochim, 1974); therefore these doses of estrone seem to mimic low rather than high doses of estradiol. The conclusion from these reports is, estradiol appears to exert a biphasic, dose-dependent effect on pineal HIOMT activity in rats; low doses being stimulatory and high doses being inhibitory.

1.6.4.1.2 SNAT

Against the backdrop of the fact that estradiol has definite effects on the NE activation of adenylate cyclase (Weiss and Crayton, 1970), it is surprising that SNAT does not respond to estradiol treatment as has been suggested by numerous reports. Illnerová, (1975) found that estradiol administration did not alter light-dark cycles of SNAT nor its induction by isoprenaline *in vivo*. Wilkinson and Arendt, (1978) also reported that the estrogen-progesterone priming of ovariectomized adult rats resulted in marginal although significant increase of SNAT sensitivity to isoprenaline *in vitro*. Again estradiol treatment in *Corturnix* quail, did not affect pineal SNAT activity (Preslock, 1977). These reports suggest that estradiol has no effect on pineal SNAT activity.

1.6.4.1.3 MAO

Vacas and Cardinali, (1980) reported that there was a decrease in intraneuronal MAO activity following estradiol treatment. However, an opposite effect to the above finding was reported by Urry *et al.*, (1976) after a prolonged treatment of rats with high doses of estradiol. The contradiction here may relate to the different theshold doses for the inputs to the gland since various hormonal inputs affect pineal MAO activity. It seems therefore that estradiol manipulates pineal MAO activity in a dose-dependent, biphasic manner.

1.6.4.2 Effect of estradiol on pineal protein synthesis

Cardinali *et al.*, (1974) observed an increase in the incorporation of radiolabelled amino acids into pineal proteins following the injection of 0,5-3,2µg of estradiol into ovariectomized rats. Estradiol (15nM) induced the synthesis of a specific protein *in vitro*. The properties of this protein were reported in uterus, adenohypophysis and hypothalamus (Mizobe and Kurokawa, 1977). These effects of estradiol on protein synthesis required the integrity of the ganglionic sympathetic neurons since ganglionectomy but not ganglion decentralization, abolished them (Cardinali *et al.*, 1976). It also required continuous interaction of NE with ß-adrenergic receptors, since administration of the ß-blocker propranolol after estradiol administration impaired the ability of the hormone to increase total protein synthesis (Cardinali *et al.*, 1976).

Clementi *et al.*, (1965) reported histological changes indicating depressed pinealocyte function and reduced cytoplasmic RNA following estradiol administration. These authors observed a rise in protein synthesis in the pineal 24 hours after the steroid administration and also found that there were elevated levels of DNA and RNA in pineals 6 hours prior to the increase in protein synthesis.

From these foregoing reports, estradiol does seem to positively influence pineal protein synthesis.

1.6.5 TESTOSTERONE [4-Androsten-17β-ol-3-one]

Testosterone administration has influenced the following pineal metabolic activity and constituents: incorporation of labelled leucine into proteins (Cardinali *et al.*, 1976; Nagle *et al.*, 1975), 5-HT content and turnover (Vacas and Cardinali, 1979), NE turnover rate (Cardinali *et al.*, 1975), HIOMT activities (Nagle *et al.*, 1974) and MAO activities (Urry *et al.*, 1976).

Cardinali *et al.*, (1974 and 1975) have put forward postulations which lacked rigorous proof, on the presence of pineal receptors for testosterone.

One important aspect of the physiological functions of testosterone in the pineal is its aromatization to estradiol. Some workers (Cardinali *et al.*, 1974) believe this takes place prior to testosterone exerting its effect on pineal metabolic activities but this is discounted by other workers (Daya *et al.*, 1985). Evidence for the existence of a pineal site of action for androgens is overwhelming. Nagle *et al.*, (1974) and Cardinali *et al.*, (1974) found that pineals from castrated rats took up radiolabelled testosterone *in vivo* and *in vitro* up to a concentration about half that of the prostate.

1.6.5.1 Effect of testosterone on pineal enzymes

1.6.5.1.1 HIOMT

Houssay and Barceló (1972) administered 30-100μg/day testosterone propionate (TP) for 21 days and found that HIOMT activity in castrated male rats was depressed. The findings by Nagle *et al.*, (1974) that testosterone administered in doses of 0,1-1,0mg per day for 3 days to castrated rats exerted an inductive effect on pineal HIOMT activity whilst pharmacological doses of 5mg/day depressed enzyme activity suggests that testosterone may apparently be exerting a dose-dependent biphasic effect of pineal HIOMT activity. Simultaneous administration of NE reversed the stimulation of HIOMT brought about by TP, and potentiated its inhibition (Nagle *et al.*, 1974).

There are reports about the effect of other androgens on pineal HIOMT activity. Preslock, (1977) found that administration of androstenedione (100μg/day for 18 days) stimulated HIOMT activity in castrated *Cortunix* quail. Benbelbaz-Vega, (1976) also reported that TP administration (10mg/day) depressed pineal HIOMT activity in penguins.

1.6.5.1.2 SNAT

Administration of TP or androstenedione at doses of 100mg/day for 18 days by Preslock, (1977) failed to influence pineal SNAT

activity in castrated *Cortunix* quail. Testosterone administration to castrated male rats or ovariectomized female rats did not produce any significant effect on the responsiveness of pineal adenylate cyclase to NE (Weiss and Crayton, 1970). The findings of Pavlinov and Isachenhov (1979) following the administration of 50μg of various androgens to rats, 2 days after castration and 1 to 6 hours before sacrifice show that the nocturnal rise of pineal SNAT activity was stimulated in 2 to 4 hours. This finding was confirmed by Rudeen and Reiter (1977) who found that administration of subcutaneous pellet of 50μg TP brought about significant nocturnal elevation in pineal SNAT activity in castrated rats relative to approximately that of intact controls.

1.6.5.2 Effect of testosterone on pineal protein synthesis

Nagle *et al.*, (1975) demonstrated that daily subcutaneous administration of TP to castrated rats brought about a 79% increase in pineal protein synthesis. Rats injected with a single dose of 500μg TP at the end of the dark period showed a 150% increase of pineal protein synthesis whereas rats injected at the middle of the light period only showed 54% increase: maxima and minima of pineal testosterone uptake was observed at the same times, indicating that changes in the number of binding sites correlate with modification in the biological activity of the hormone in the pineal cells (Nagle *et al.*, 1975).

The testosterone-induced increase in pineal protein synthesis is

abolished by the administration of propranalol, a ß-adrenergic blocker. This antagonistic effect of propranalol suggest two things:

(i) the adrenergic blocker interrupts the interaction of the neurotransmitter with the cell membrane and thus impairs the effect mediated by an increased neural activity.

(ii) continuous interaction of noradrenaline with postsynaptic receptors is required for the pinealocytes to respond to increased intracellular levels of the gonadal hormone.

1.6.6 PROGESTERONE [4-Pregnen-3,20-dione]

The precise effect of progesterone on pineal metabolic activities have been in doubt for some time now. Neil and Smith (1974), in an assertion that has been widely accepted by many neuroendocrinologists, believed progesterone on its own is incapable in modifying neuroendocrine functions in oophorectomized rats unless estradiol is administered prior or following progesterone injection. Recent reports however indicate that progesterone on its own is able to modulate pineal biochemical functions without concomitant estradiol administration. However, firm conclusions with rigorous proof are hard to come by in view of the fact that the role of endogenous estradiol produced by the adrenal cortex has not been ruled out. At present, there are no reports on progesterone activity in ovariectomized-adrenalectomized rats.

1.6.6.1 Effect of progesterone on pineal enzymes

1.6.6.1.1 HIOMT

Houssay and Barceló (1972) reported progesterone-induced stimulation of HIOMT activity. Yochim and Wallen (1974) demonstrated that induction of pseudopregnancy (and thus of high circulating progesterone levels) caused significant changes of HIOMT rhythm in rats. Chronic progesterone administration to ovariectomized rats inhibited pineal HIOMT activity. This observed inhibition was contrary to the observation that in castrated *Cortunix* quail, progesterone treatment restored depressed levels of pineal HIOMT activity.

1.6.6.1.2 SNAT

There is only one report in the literature on the effect of progesterone on SNAT activity. Preslock (1977) found that in *Cortunix* quail, pineal SNAT did not respond to progesterone treatment.

1.6.6.2 Effect of progesterone on pineal protein synthesis

Cardinali and Vacas (1978b) demonstrated that there was a decrease in pineal protein synthesis following the administration of progesterone to ovariectomized rats. This was regardless whether or not estradiol was simultaneously administered. This may lend support to the observation that

estradiol does not mediate the effect of progesterone on pineal metabolic activities. Similarly, mediation by pituitary hormones on progesterone effects has been ruled out with the findings by Neil and Smith (1974) that progesterone administration is unable itself to modify plasma gonadotropins on prolactin levels. Progesterone appears to be acting directly on the pinealocytes.

1.6.7 METABOLISM OF SEX STEROIDS IN THE PINEAL GLAND

1.6.7.1 Progesterone

Hanukoghi *et al.*, (1977); Luttge and Wallis (1973); Cardinali *et al.*, (1975a) have independently demonstrated that progesterone is actively metabolized from the incubation medium by pineal tissue to 5α-reduced metabolites. Examples are 5α-pregnanedione and 3α-hydroxy-5α-pregnan-20-one.

The pineal gland from ovariectomized rats administered with ^{3}H-progesterone retained more unmetabolized progesterone than in any other CNS areas involved in neuroendocrine regulation. David *et al.*, (1975) also reported that some progestagens including norethynodral and 17α-hydroxyprogesterone were found to accumulate in the pineal gland of rhesus monkeys after intravenous or CSF administration.

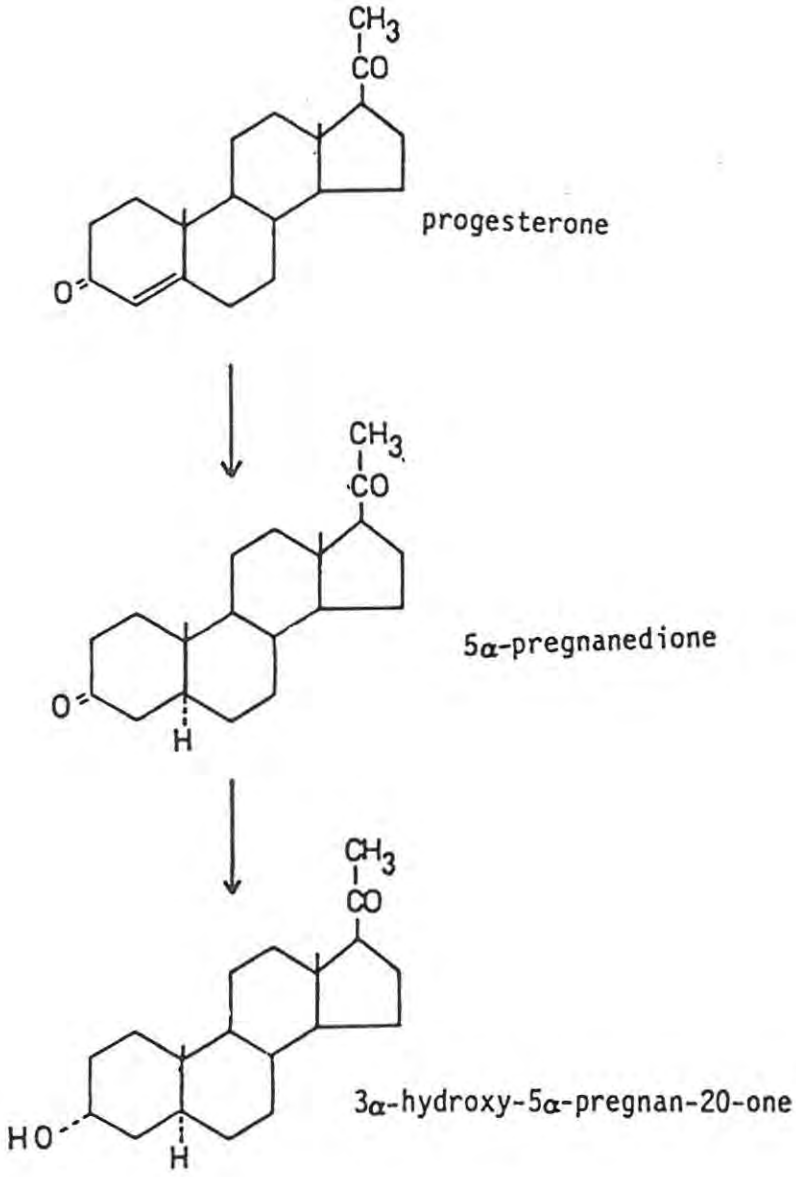


Fig. 10. Metabolism of progesterone in the pineal gland

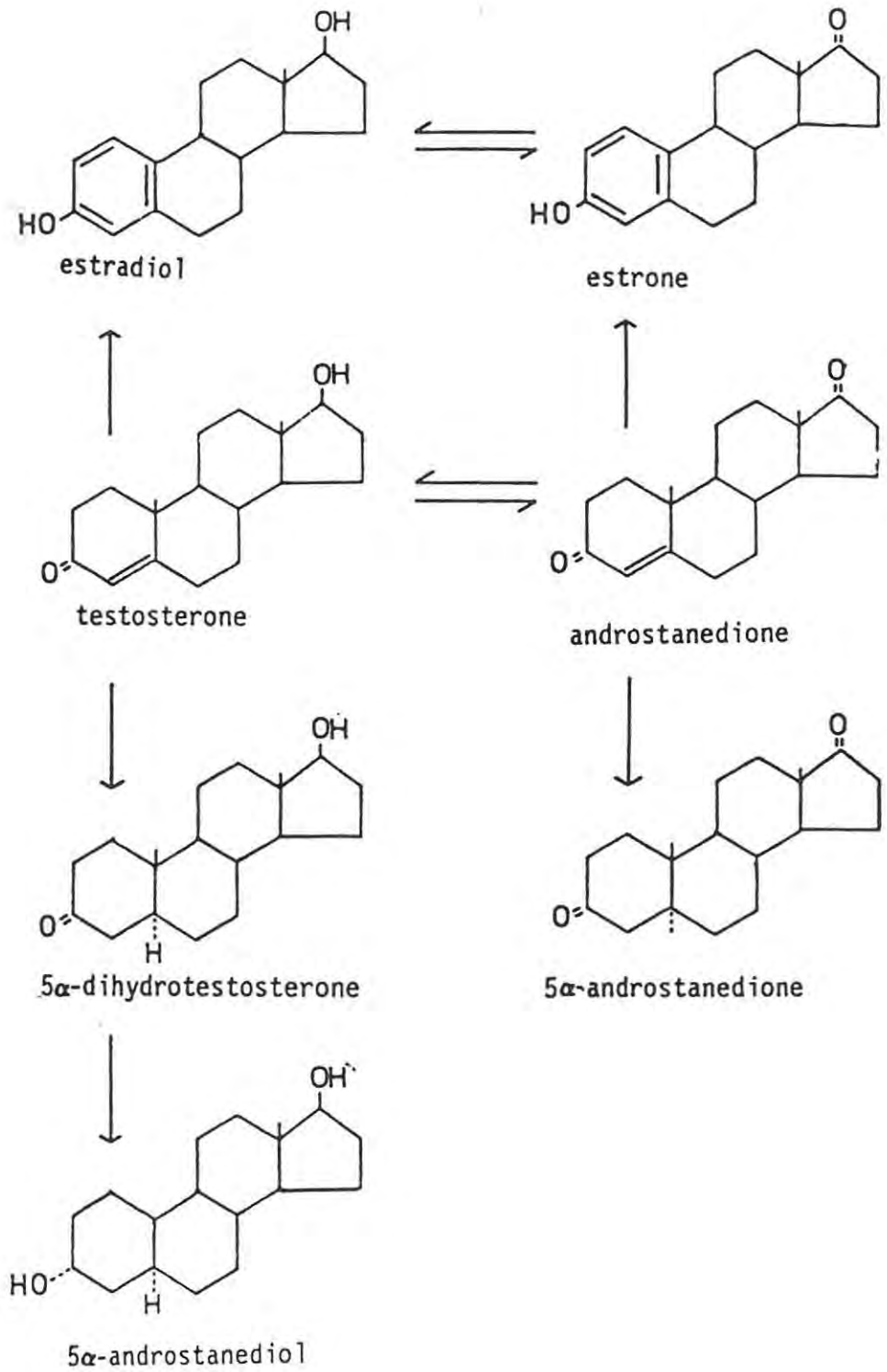


Fig. 11. Metabolism of estradiol and testosterone in the pineal gland

1.6.7.2 Testosterone and estradiol

Cardinali *et al.*, (1974d) reported identical radioactivity levels in the prostate and the pineal gland following the *in vivo* administration or *in vitro* incubation with ^{3}H-testosterone. Cardinali *et al.*, (1974d, 1974e) also reported that ^{3}H-androgen metabolism by pineal cells involves 5α-reduction as well as aromatization to estrogens. The major metabolite is 5α-dihydrotestosterone. Daya *et al.*, (1985) have however shown that testosterone is able to exert its activity without prior conversion to estradiol. ß-estradiol is methylated to the 3-methylether of estradiol by partially purified bovine pineal HIOMT (Weiss *et al.*, 1968).

1.6.8 GONADAL SEX STEROID RECEPTORS IN THE PINEAL GLAND

1.6.8.1 Estradiol receptors

Marks *et al.*, (1972) and Nagle *et al.*, (1972) independently carried out studies to establish the presence of estradiol receptor on the pinealocytes. Nagle *et al.*, (1973) reported that pineal explants obtained from ovariectomized rats took up and retained ^{3}H-estradiol *in vitro* up to 35-fold the concentration in the incubation media and the analysis of cytosol fraction by Cardinali *et al.*, (1975) for high affinity binding sites uncovered the presence of an estradiol-binding protein with K_d in the nanomolar range.

Cardinali *et al.*, (1975) further established that ^{3}H-estradiol binding was displaced by excess estradiol, but not by testosterone, 5α-dihydrotestosterone (DHT) or progesterone and disappeared following incubation with trypsin or iodocetamide.

The pineal estrogen binding protein content is significantly reduced by intraventricular administration of 6-hydroxydopamine and superior cervical ganglionectomy. Sympathetic innervation therefore influences the levels of estradiol binding protein.

By inducing a specific high affinity binding sites in the pineal and uterus following the administration of 2μg of estradiol benzoate for 3 days, Nagle *et al.*, (1973) found that *in vitro* uptake of ^{3}H-estradiol varied proportionally with the quantity of steroid in the medium.

Reports by Notides and Gorski (1966); Katzenellebogen and Gorski (1972) as well as Mizobe and Kurokawa (1978) show that estradiol stimulates the formation of a protein species which resembles the estrogen-induced protein in rat uterus in its electrophoretic mobility. In the rhesus monkey, pineal estraphilic receptors have been characterized physicochemically and appear to be similar to those found in the rat brain (David *et al.*, 1975).

The integrity of the sympathetic input to the pineal gland is required for keeping appropriate levels of electrophilic receptors in the pineal gland (Cardinali *et al.*, 1975). The

interaction of receptor-estradiol complex with nuclear acceptor sites is considered to be the initial step for hormone stimulation of nucleic acid and protein synthesis in the target tissues. By using the ^{3}H-estradiol exchange assay to determine receptor hormone complexes in fractions of rat pineal homogenates, the dissociation constant, dose-response and accumulation curves following estradiol treatment were found comparable to those of the hypothalamus and adenophysis (Cardinali, 1977).

Autoradiographic analysis of rat pineal glands after *in vivo* administration of ^{3}H-estradiol revealed nuclear concentrations of labelled grains in a still undefined population of cells (Stumpf *et al.*, 1976), a finding compatible with the biochemical data on cytoplasmic and nuclear estraphilic receptors. Whether these cells are pinealocytes or glial cells, awaits further investigation (Vernadakis, 1978).

1.6.8.2 Testosterone receptor

Cardinali *et al.*, (1976) and Nagle *et al.*, (1975) demonstrated receptors for testosterone and DHT in the pineal gland. Prior to this, Cardinali *et al.*, (1974c) have demonstrated the binding of androgens in the pineal to cytoplasmic proteins. Pineal glands from castrated rats take up radioactive testosterone *in vivo* and *in vitro* up to a concentration about half that of the prostate (Nagle *et al.*, 1974; Cardinali *et al.*, 1974), previous exposure to androgens *in vivo* significantly enhanced ^{3}H-testosterone uptake *in vitro* (Cardinali *et al.*, 1974).

Saturation analysis of testosterone and DHT binding sites in pineal cytosol indicated a K_d in the nanomolar range (Cardinali *et al.*, 1974), within the physiological concentrations of the hormone expected to reach the pineal from blood stream; binding was specific for androgens.

Liao *et al.*, (1972) reported that the 5α-androstanediol has a low affinity for the receptor similar to that observed in the prostate relative to testosterone and 5α-dihydrotestosterone which had a high affinity. The authors therefore postulated the following order of affinity of the steroids for pineal cytosol binding component: 5α-dihydrotestosterone > testosterone > androstenedione > estradiol > 5α-androstanedione > 5α-androstanediol.

These data suggest the existence of a specific binding for 5α-dihydrotestosterone in the nuclei of pineal cells. This is a similar situation to the prostate gland where 5α-dihydrotestosterone rather than testosterone is bound to nuclear components.

1.6.8.3 Progesterone receptors

Luttge and Wallis (1972) reported the *in vitro* accumulation of ^{3}H-progesterone in several brain regions, the adenohypophysis, the pineal gland and the uterus of ovariectomized rats. Although the nature of radioactivity present in the tissue was not investigated, pineal ^{3}H-progestin uptake was the highest

among the tissues studied. Vacas *et al.*, (1979) reported that a cytosol progesterone receptor in the bovine pineal gland binds specifically with high affinity. In low ionic strength medium, this component exhibits a sediment coefficient similar to that described for the progesterone receptor in the uterus (Vu Hai and Milgrom, 1978), the hypothalamus (Kato and Onuchi, 1977) and the adenohypophysis (Evans *et al.*, 1978; Kato and Onuchi, 1977).

The pineal gland retains bound progesterone in preference to its 5α-reduced metabolite (Karavolas *et al.*, 1978). The peculiar binding characteristics of pineal progesterone-receptor complexes which explain the particular uptake and metabolism in the pineal gland has been reported (Vacas *et al.*, 1979).

Karavolas *et al.*, (1978) suggested that the long retention of unmetabolized progesterone following its administration is a perculiarity of the pineal gland, not observed in hypothalamus, midbrain or pituitary.

1.7 METABOLISM OF TRYPTOPHAN AND THE KYNURENINE HYPOTHESIS

1.7.1 HEPATIC TRYPTOPHAN PYRROLASE [L-TRYPTOPHAN-OXYGEN 2,3-OXIDOREDUCTASE, EC 1.13.11.11]

1.7.1.1 Introduction

Tryptophan pyrrolase is the haem-dependent liver cytosol enzyme that catalyses the oxidative cleavage of the pyrrole ring of L-

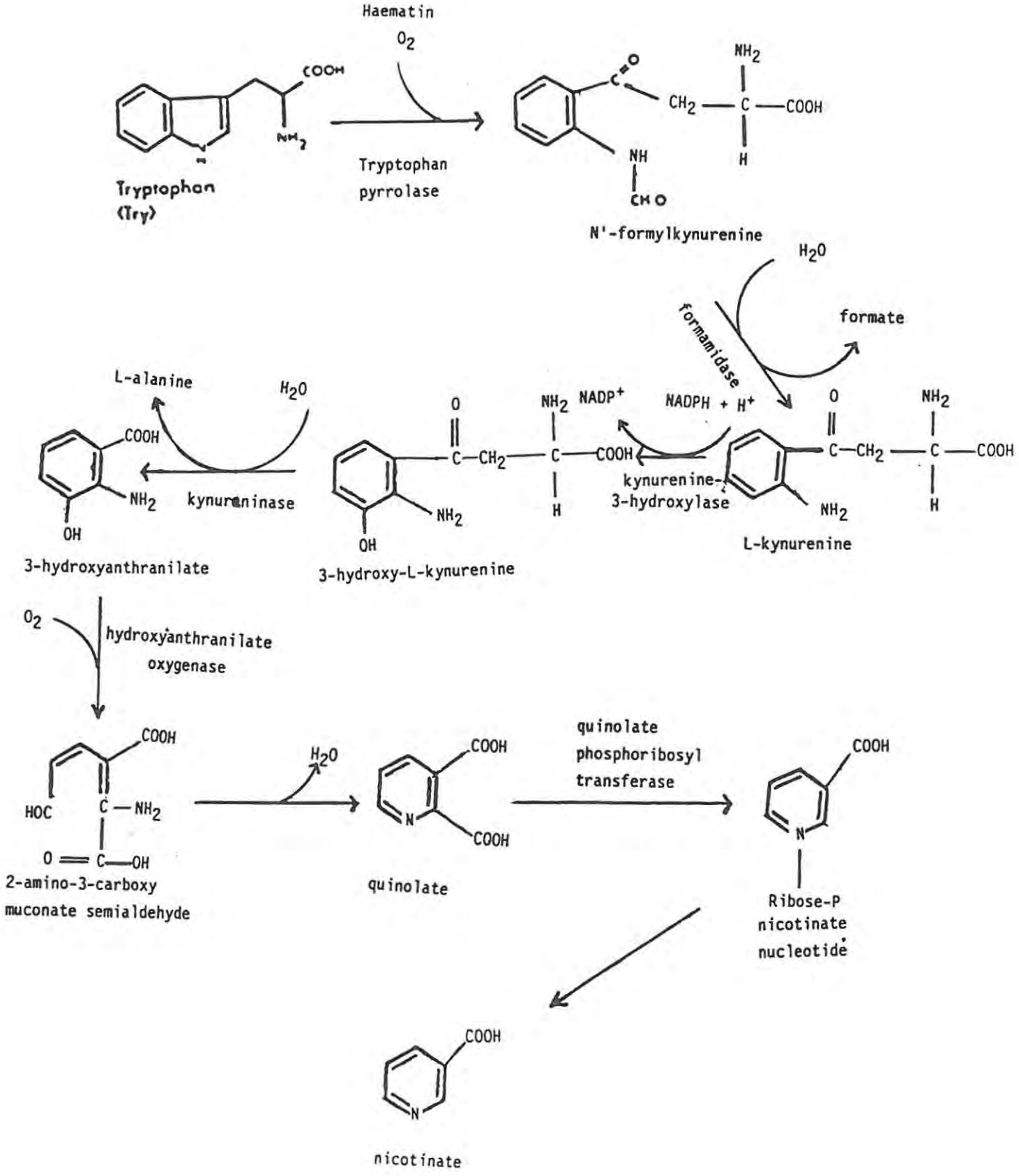


Fig. 12. The Nicotinic Acid pathway

tryptophan to produce N'-formylkynurenine during the initial and rate-limiting step of the kynurenine-nicotinic acid pathway of tryptophan degradation (Young *et al.*, 1978: Young, 1981).

In rat (Feigelson and Greengard, 1961) or human (Altman and Greengard, 1966) liver, but not in livers of certain animal species (Badawy and Evans, 1975), tryptophan pyrrolase exists in two forms. The already active holoenzyme does not require the addition of exogenous haematin for demonstration of its activity whereas the haem-free predominant form or apoenzyme does (Badawy and Evans, 1975).

1.7.1.2 Regulation of Tryptophan pyrrolase Activity

1.7.1.2.1 Activation of tryptophan pyrrolase

The activation of the rat liver enzyme *in vitro* consists of two steps, both of which require the participation of tryptophan. The inactive apoenzyme is first conjugated with haem to form the oxidized (ferrihaem) holoenzyme (Fig.13). This is then reduced to the active form. Although, tryptophan can be replaced by some of its derivatives in the first step, its presence is obligatory for the reduction of the oxidized holoenzyme (Badawy, 1979).

Measurements of both apoenzyme and holoenzyme activities are important, not only to find out which form(s) is inhibited or activated by a particular agent, but also to examine the

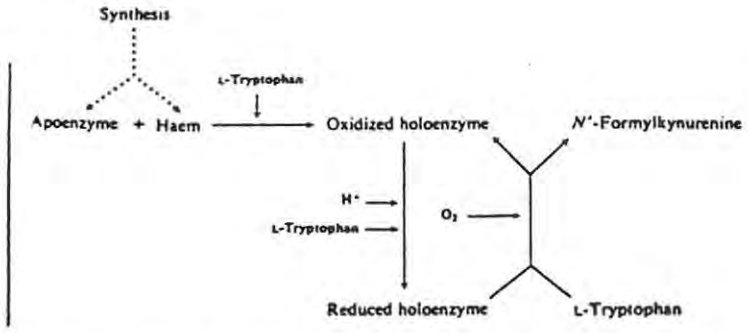


Fig. 13. Activation of rat liver tryptophan pyrrolase *in vitro*

(Badaway, 1979)

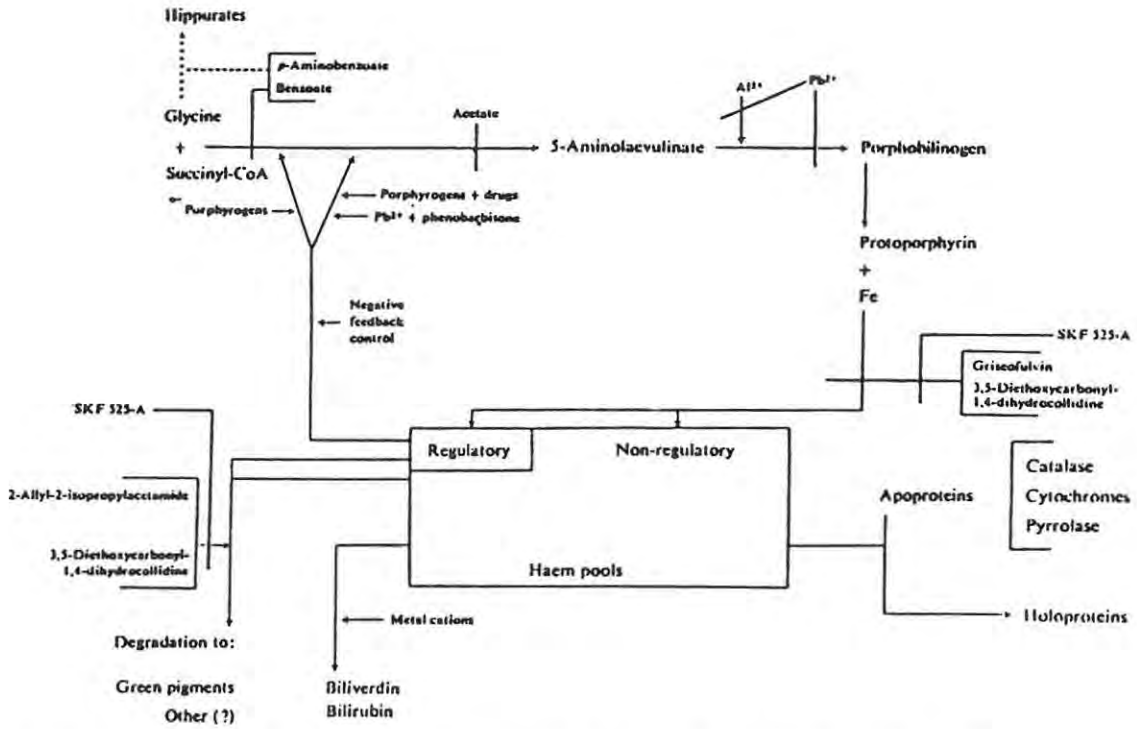


Fig. 14. Interaction between haemoproteins and the haem-biosynthetic and degradative pathways

(Badaway, 1979)

presence or absence of the enzyme. By performing such simultaneous measurements, it has been shown (Badawy and Evans, 1976) that, although both forms are present in the liver of the chicken, mouse, pig, rat, turkey and possibly also man, the apoenzyme is absent from the cat, frog, gerbil, guinea pig, hamster, ox, rabbit and sheep. It is known that species lacking the apoenzyme also do not possess the hormonal induction mechanism and are sensitive to tryptophan toxicity because they metabolize the amino acid largely via the indoleamine routes (Badawy, 1979).

1.7.1.3 Hormonal and substrate mechanisms of tryptophan pyrrolase regulation

By using the catalytic property of the rat liver enzyme, labelled amino acid incorporation into its purified protein and immunological-titration techniques, it has been possible clearly and conclusively to distinguish between these two mechanisms (Schimke *et al.*, 1965; Knox, 1966; Schimke, 1969). The hormonal mechanism involves the induction of synthesis of the apoenzyme, probably by increasing the amount of the pyrrolase mRNA (DeLap and Feigelson, 1978), whereas the substrate mechanism consists of decreased degradation of pre-existing apo-(tryptophan pyrrolase) in the presence of the normal rate of synthesis. It has been suggested (Knox, 1966) that tryptophan stabilizes the enzyme causing an initial activation *in vivo* (Greengard and Feigelson, 1961; Badawy and Evans, 1975). This is unlikely because simple activation (by administration of haematin or its

precursor 5-aminolevulinate) does not stabilize the enzyme (Badawy and Evans, 1975). That stabilization requires the presence of tryptophan rather than changes in pyrrolase activity is suggested by the finding (Badawy and Evans, 1975) that stabilization is achieved by a dose of tryptophan (50mg/kg) that does not enhance or activate the enzyme in fed rats, both under basal conditions and after hormonal induction or cofactor activation.

1.7.1.4 Cofactor mechanism of tryptophan pyrrolase regulation

The saturation of rat-liver apo-(tryptophan pyrrolase) with haem has been shown to be increased after administration of the haem precursor 5-aminolevulinate (Wetterberg *et al.*, 1969; Druyan and Kelly, 1972; Badawy and Evans, 1973b) or haematin (Badawy and Evans, 1975). These two agents therefore resemble tryptophan in activating the apoenzyme. They, however, do not stabilize the pyrrolase and this may explain why the overall enhancement of the enzyme activity by the cofactor mechanism is less strong than that achieved by tryptophan (Badawy, 1979).

1.7.1.5 Distribution of haem in the liver

Liver haem is unequally distributed among mitodonchondrial cytochromes, microsomal cytochromes (cytochromes P-450 and b_5), catalase and cytosolic tryptophan pyrrolase. On the basis of the concentration of these haemoproteins, Marver and Schmid (1972) calculated that, of total liver haem, cytochrome P-450,

catalase and tryptophan pyrrolase utilize 66,16 and 1,4% respectively. The remainder of liver haem is used by the mitochondrial cytochromes, and since these have half-lives of 2 days or longer, it may be suggested that they are unlikely to play an important role(s) in haem utilization under conditions involving rapid changes in haem metabolism. This suggestion is supported by the finding (Druyan and Kelly, 1972) that these cytochromes are not affected by the administration of the haem precursor 5-aminolevulinate or the porphyrogenic (porphyria-producing) drug 2-allyl-2-isopropylacetamide (Badawy, 1989).

1.7.1.6 Disorders of haem and porphyrin metabolism: the porphyrias

The haem-biosynthetic pathway is normally controlled in such a manner that there is no excessive accumulation or excretion of intermediates of the pathway. The porphyrias are a group of conditions (hepatic and erythropoietic) in which the above control is lost and the excretion of porphyrins and their precursors is increased. These conditions affect almost all systems in the body, with abdominal pains and neurological disturbances being the most prominent symptoms.

1.7.1.7 Regulation of haem biosynthesis and the concept of regulatory free-haem pool

Haem is an important component of the functionally vital haemoproteins, and its regulation is therefore of considerable

importance to the functions and integrity of cells, tissues and the body as a whole (Badawy, 1979).

It is now generally accepted that 5-aminolevulinate synthase is the point at which haem regulates its own synthesis by a negative feedback mechanism(s) in livers of chick embryos (Granick, 1966; Granick *et al.*, 1975) and mammals (De Matteis, 1975) including possibly man (Jeelani Dhar *et al.*, 1975). It follows therefore that haem could regulate its synthesis by a dual mechanism: the above negative one when liver haem concentration is increased, and a positive mechanism which involves the removal of the negative one secondary to decreased haem concentration. The mechanism(s) by which haem exerts the negative feedback control mentioned above is not fully understood at present.

Current theories suggest an inhibition of transport of newly-synthesized 5-aminolevulinate synthase from cytosol to mitochondria (in rat liver) (Hayashi *et al.*, 1972), repression of synthase synthesis (Granick *et al.*, 1975) or inhibition of synthase activity (Tait, 1978).

The ability of certain drugs to produce an experimental porphyria in mammals has played an important role in understanding the regulation of haem biosynthesis in the liver. The actions of drugs on mammalian liver 5-aminolevulinate synthase activity could be classified into two types. Firstly, a marked enhancement of synthase activity associated with an

accumulation of porphyrins in the liver is produced after administration of the porphyrogens (porphyria-producing), 2-allyl-2-isopropylacetamide, 3,5-diethoxcarbonyl-1,4-dihydrocollidine and griseofulvin (De Matteis, 1967). By contrast, several lipid-soluble drugs (including phenobarbitone and phenylbutazone) cause a moderate stimulation of synthase activity and an increase in microsomal haem concentration, but not in that of porphyrins (De Matteis, 1973). When, however, one of the lipid-soluble drugs is administered together with a porphyrogen to intact animals (De Matteis and Gibbs, 1972; De Matteis, 1973) or to isolated perfused livers (Bock *et al.*, 1973), it potentiates the effects of the porphyrogen on synthase activity and porphyrin concentration, and De Matteis (1973, 1975) suggested that this potentiation may explain the exacerbation of human hepatic porphyrias by drugs.

The mechanism by which lipid-soluble drugs cause a moderate enhancement of synthase activity is not understood. Satyanarayana Rao *et al.*, (1972) have shown that stabilization of synthase may be involved. By contrast, it is most likely that the masked enhancement of synthase activity by porphyrogens is produced by interference with the negative feedback mechanism involving the occurence of an early depletion of liver haem. It is therefore reasonable to suggest that this haem belongs to the so-called regulatory free-haem pool that has been suggested to be small and rapidly turning over (De Matteis, 1975; Granick *et al.*, 1975). Since such a pool would be too small to be measured directly, the early depletion of haem has been demonstrated by

indirect methods involving the determination of activity or concentration of, or haem utilzation by, the hepatic haemoproteins catalase, cytochrome P-450 and tryptophan pyrrolase. There is considerable evidence (Badawy, 1978) implicating this early depletion of haem in the production of experimental porphyria. This concept in mammals does not apply to avian systems since several drugs known to cause an early depletion of liver haem in mammalian systems are capable of producing porphyria in avians (Granick, 1966; Creighton and Marks, 1972), and this has been suggested (De Matteis, 1973) to be due to the greater sensitivity of avian systems to drugs and also to the liability of their haem-biosynthetic pathway (Badawy, 1989).

1.7.1.8 Comparison of haem utilization by liver haemoproteins

1.7.1.8.1 The haem-saturation ratio

With catalase and cytochrome P-450, the study of haem utilization is complicated by the fact that only the active (holoenzyme) forms can be measured, whereas both the active (holoenzyme) and inactive (apoenzyme) forms of tryptophan pyrrolase can be measured and altered both simultaneously and independently. Moreover, whereas the synthesis of apo-(tryptophan pyrrolase) is not limited by decreased haem availability (Badawy and Evans, 1973b), that of apo-(cytochrome P-450) and apo-(catalase) is limited (Bhat *et al.*, 1977; Yasukochi *et al.*, 1974). These two points together with its

short half-life of 2,3h (Badawy and Evans, 1975) as compared to those of 7-10h and 24-48h for cytochrome P-450 (Marver and Schmid, 1972) and 29h or 2,5 days (Price *et al.*, 1962; Yasukochi *et al.*, 1974) for catalase, render apo-(tryptophan pyrrolase) a more suitable haemoprotein for assessing changes in liver haem concentration.

This assessment is best expressed by the haem-saturation ratio. This is the ratio of holoenzyme/apoenzyme activity, which indicates the extent of the haem saturation of the apoenzyme. The basal ratio (in fed rats) of < 1 is not altered after hormonal induction of the pyrrolase by cortisol administration or by the increased plasma corticosterone concentration during starvation (Badawy and Evans, 1973b; Badawy, 1977b). This is because the rise in the holoenzyme activity is matched by a proportionate increase in that of the total enzyme. This suggests that, under normal conditions of haem metabolism, the liver contains enough haem to saturate newly synthesized apo-(tryptophan pyrrolase), a situation resembling that involving apo-(cytochrome P-450) induction by phenobarbitone and other drugs (De Matteis, 1971), although the latter haemoprotein receives a much greater share of liver haem.

1.7.1.8.2 Factors increasing the haem-saturation ratio

The haem-saturation ratio of tryptophan pyrrolase is increased if only the holoenzyme activity is enhanced or if it is raised relatively greater than that of the total enzyme. This is

achieved by treatments that increase the availability of haem by enhancing its utilization (e.g. phenobarbitone administered in drinking water 24 hrs previously) or its synthesis or concentration (1-2 hrs after administration of Al^{3+}, 5-ALA, tryptophan or haematin) (Badawy and Evans, 1973a, b, 1975; Badawy, 1977a).

Bissell and Hammaker (1977) found that endotoxin administration increases the saturation of apo-(tryptophan pyrrolase) with haem by mechanism involving the dissociation of haem from apo-(cytochrome P-450). With phenobarbitone, it is generally believed that its relatively late (12-24h after administration) effect on cytochrome P-450 is primarily due to the induction of synthesis of the apoenzyme, although there is evidence (De Matteis, 1975) that the drug enhances the utilization by cytochrome P-450 of haem newly synthesized from exogenous precursors and this has also been demonstrated with tryptophan pyrrolase in 5-aminolevulinate-treated rats (Badawy, 1977a). The effects of phenobarbitone on pyrrolase activity and haem utilization (Badawy and Evans 1973a, b; Badawy, 1977a), differ from those on cytochrome P-450 in several respects, the most important of which is that the drug-induced enhancement of haem utilization by tryptophan pyrrolase is pure phenomenon not associated with altered apoenzyme amounts.

1.7.1.8.3 Factors decreasing the haem-saturation ratio

The haem-saturation ratio of tryptophan pyrrolase is decreased

by treatments that limit the availability of haem by increasing destruction, inhibiting its synthesis or enhancing its degradation to bile pigments by stimulating haem oxygenase activity. Under these conditions, the ratio decreases if only the holoenzyme activity is decreased or that of the total enzyme is increased with the former remaining unaltered or being slightly enhanced.

Acetate (inhibitor of the 5-ALA synthase reaction), Pb^{2+} (inhibitor of 5-ALA dehydratase activity), porphyrogens (destroyers of haem and inhibitors of ferrochelatase activity) and substrates of glycine acyltransferase such as benzoate and p-aminobenzoate (diverting glycine away from the 5-ALA synthase step) cause an early decrease in the haem-saturation ratio, whereas metal cations (including Pb^{2+}) produce a late decrease by stimulating haem oxygenase activity (Badawy and Evans, 1973b; Badawy, 1977a). By contrast, catalase has only been reported to respond to the early depletion of haem by porphyrogens (De Matteis, 1978) and to the late one by Co^{2+} (Tephly *et al.*, 1973; Yasukochi *et al.*, 1974) and by repeated administration of phenobarbitone plus the porphyrogen, 3,5-diethylcarbonyl-1,4-dihydrocollidine (Sweeney, 1976).

Cytochrome P-450, on the other hand, responds to the late depletion of haem by metal cations and to the early one by porphyrogens administered alone (Piper and Tephly, 1974; De Matteis 1975; Maines and Kappas, 1976). Moreover whereas early depletion of tryptophan pyrrolase haem caused by 3,5-

diethoxycarbonyl-1,4-dihydrocollidine is potentiated by joint phenylbutazone administration (Badawy, 1976), that of cytochrome P-450 haem is not (De Matteis and Gibbs, 1972).

1.7.1.8.4 Relationship between tryptophan pyrrolase and the regulatory free-haem pool

It is suggested that the regulatory free-haem pool may be present in the cytosol since it is the only route for mitochondria-derived haem to the site(s) of 5-ALA synthase synthesis and/or repression (Yoda and Israels, 1972). The importance of the cytosol is further suggested by the finding *in vitro* (Yoda and Israels, 1972; Israels *et al.*, 1975) that cytosolic proteins, with relatively low- and non-specific-affinity sites for haem are required to contact the mitochondria to facilitate the exit of (newly synthesized) haem into the cytosol.

It has been calculated (Badawy, 1978) that during the early depletion of pyrrolase haem by 3,5-diethoxycarbonyl-1,4-dihydrocollidine plus phenylbutazone, there is a loss of 0,093μM from the cytosol. This haem loss represents the largest depletion of pyrrolase haem under conditions of potentiated experimental porphyria, and is similar to the concentration (0,1μM) suggested by Granick *et al.*, (1975) for free or readily exchangeable haem. It may therefore be suggested that the concentration of the regulatory free-haem pool is similar to, or lower than, the above value. Although, indirect, the above

calculations and the response of pyrrolase to changes in haem metabolism suggest that this enzyme may play an important role in the regulation of haem biosynthesis.

1.7.2 THE INDOLEAMINE HYPOTHESIS

The indoleamine hypothesis of depressive disorders is a variation of the catecholamine hypothesis, which states that depression is associated with a deficiency of brain serotonin (Lapin and Oxenkrug, 1969). Reserpine which depletes brain amines, frequently causes depression (Bunney and Davis, 1965) whereas monoamine oxidase (MAO) inhibitors which raise brain amine levels (Maclean *et al.*, 1965) may alleviate depressive symptoms. The alleviation has been reported to be enhanced by tryptophan (Copper *et al.*, 1967), which is a precursor of the amines 5-HT and tryptamine. The apparent prophylaxis by tryptophan of an annual episode of depression has also been reported (Hertz and Sulman, 1968). These findings suggest an elevation of one or both of the above amines is responsible for the therapeutic effect of MAO inhibitors, and that their insufficiency may be responsible for symptoms of the disease. The effects of reserpine, MAO inhibitors and the tricyclic antidepressants on brain serotonin levels may be of major importance in the aetiology of depressive disorders.

1.7.2.1 Implication of serotonin in depressive disorders

(i) Depressed patients responding to MAO inhibitor drug

ipromazid were found in two studies (Pare and Sandler, 1959; Praag and Leijnse, 1963), though not found in a third (Burgermeister *et al.*, 1963) to have lower initial urinary excretion of the 5-HT metabolite 5-HIAA than those who did not respond.

(ii) Depressed patients have a low content of 5-HIAA in the lumbar cerebrospinal fluid (Ashcroft *et al.*, 1966) which rises on recovery. Although work using animals (Guldberg and Yates, 1968; Eccleston *et al.*, 1968) shows that changes in brain 5-HT are paralleled by changes in brain 5-HIAA in the CSF, the significance of this result is not altogether clear, as lumbar 5-HIAA depends not only on brain 5-HT metabolism but also on 5-HIAA transport within the CSF (Ashcroft *et al.*, 1966). Furthermore, a small group of acute schizophrenics on phenothiazines also had low 5-HIAA.

(iii) There is some direct evidence of low 5-HT (Shaw *et al.*, 1967) or 5-HIAA (Bourne *et al.*, 1968) in the hind-brains of depressive suicides.

(iv) While single doses of 5-hydroxytryptophan, the immediate precursor of 5-HT do not alleviate depression, the remission of a prolonged episode of severe depression by administration of 5-hydroxytryptophan for five days have been reported (Persson and Roos, 1967).

Dewhurst (1968) found that tryptamine can influence mood but this effect is only potentiated by large amounts of tryptophan and MAO inhibitors (Eccleston *et al.*, 1966).

1.7.2.2 Implication of adrenocortical secretion in depression

Abnormally high levels of adrenocortical secretion in the plasma of depressed patients has been reported (Gibbons and McHugh, 1962; Hullin *et al.*, 1967) although some workers (Sachar, 1967; Brooksbank and Coppen, 1967) consider this to be due to hospitalization or residual effects of previous stress, or to be in some way secondary to psychiatric symptoms. However, Bliss *et al.*, (1967) reported that even in healthy subjects exposed to stress, there was an increase in adrenocortical secretion. The following points catalogue the implication of abnormal adrenocortical secretions in depressive disorders.

(i) McClure, (1966), Bridges and Jones, (1966), Doig *et al.*, (1966), Knapp *et al* (1967) all noted that there was elevated plasma corticoid levels in endogenous depression in early mornings - a period of mood disturbances. The suggestion that barbiturates may cause the abnormal adrenocortical secretion (Sachar, 1967) was discounted by Radzialowski and Bousquet (1968) who administered 100mg phenobarbitone per Kg per day for four days, and found no change in plasma corticosterone nor its rhythm in the rat.

(ii) Plasma corticoids are high specifically in the depressed phase (Hullin *et al.*, 1967; Rubin, 1967; Rubin *et al.*, 1968) in manic-depressive disease.

(iii) Depression is frequent in patients with a primary disturbance of pituitary-adrenal function (Trethowan and Cobb, 1952; Cleghorn, 1952).

(iv) Hydrocortisone secretion is not suppressed by dexamethasone in severe depression (Butler and Besser, 1968); this indicates a lack of feedback inhibition of ACTH release which could be responsible for the high hydrocortisone secretion rate found (Gibbons, 1962).

1.7.2.3 Tryptophan and depression

There are reports that L-tryptophan is as effective as imipramine in the management of depression (Broadhurst, 1970; Coppen *et al.*, 1967b) but there are serious doubts about this assertion since no placebo group was included and again since the study was not double-blind. The apparant propylaxis by L-tryptophan of an annual episode of depression has also been reported (Hertz and Sulman, 1968).

Use of tryptophan pyrrolase inhibitors, such as allopurinol and nicotinamide, has been suggested as adjuncts in the treatment of depression (Curzon, 1969). These drugs would protect tryptophan

from excessive degradation along the irreversible pyrrolase-catalysed oxidative kynurenine pathway and thus favour its passage to the brain where it can form serotonin. In a preliminary study, (Chouinard *et al.*, 1977), nicotinamide has been used successfully in combination with L-tryptophan, all the depressed subjects showing significant improvement over a 4-week period.

A study indicating L-tryptophan's ability to potentiate the antidepressive effects of a MAO inhibitor more positively connects indoleamines with depression. This potentiation was initially demonstrated by Copen *et al.*, (1963) and has been confirmed by other workers (Pare, 1963; Glassman and Platman, 1969; Van Praag, 1970). Van Praag (1962), however, had previously been unable to demonstrate this potentiation, either in terms of intensity or of rapidity of the antidepressant effect. Van Praag and Korf (1971a) explaining the variability of the reported results, proposed that there is a serotonin deficient subgroup of depressed patients who respond to L-tryptophan. The combination of L-tryptophan with clomipramine has also been reported to produce a more intensive antidepressive effect than clomipramine alone (Wålinder *et al.*, 1975).

1.7.2.4 Kynurenine Hypothesis

Lapin and Oxenkrug, (1969); Curzon, (1969) and Curzon and Bridges, (1970) have proposed a link between defective metabolism and abnormally high adrenocortical secretion in some

groups of depressed patients.

The mammalian body's supply of tryptophan is usually quite small (Fernstrom and Wurtman, 1974) since it is the least abundant of the essential amino acids in dietary protein. This brings to light the functional significance of tryptophan as a precursor of the neurotransmitter, serotonin. Several reports have detailed out the importance of changes in the availability of tryptophan in the regulation of serotonin synthesis in the brain (Fernstrom and Wurtman, 1971; Gessa and Tagliamonte, 1974; Hanson *et al.*, 1974; Harper and Peters, 1989; Yokogoshi *et al.*, 1987).

Tryptophan is known to be metabolized by several pathways. Less than 3% of dietary tryptophan is converted to serotonin and finally to 5-HIAA, its main excretion product (Fernstrom and Wurtman, 1974). A quantitatively more important route involves the formation of nicotinic acid which is regulated by the liver enzyme, tryptophan pyrrolase, via the kynurenine intermediate.

The activity of liver tryptophan pyrrolase has been reported to be increased by hydroxycorticosteroids (Knox 1951; Knox and Auerbach, 1955; Shan *et al.*, 1968; Curzon, 1969; Young and Sourkes, 1977). This observation led to postulation of the hypothesis that the raised plasma cortisol levels seen in turn, may directly divert tryptophan away from serotonin formation (consequently reducing brain serotonin levels) and, instead increase metabolism along the tryptophan-nicotinic acid pathway

(Curzon, 1969; Curzon and Bridges, 1970). Thus depression is proposed to be associated with a decreased utilization of tryptophan in the formation of serotonin with a consequent increase utilization of tryptophan as a substrate for kynurenine formation.

Determination of brain serotonin levels would give an idea about the relationship between pyrrolase activity and serotonin metabolism. There was a 30% reduction in rat brain serotonin levels (Curzon and Green, 1968) with a concomitant depression in 5-HIAA levels following intraperitoneal administration of 5mg/Kg hydrocortisone. The same authors observed that tryptophan pyrrolase inhibitors (allopurinol and yohimbine) abolished hydrocortisone's effect on serotonin. Again, α-methyltryptophan, which causes pyrrolase activity to increase, also caused a reduction in brain serotonin levels. In other studies, female patients suffering from "endogenous" depression were found to excrete significantly greater amounts of kynurenine and 3-hydroxykynurenine than the female controls (Curzon and Bridges, 1970).

Administration of kynurenine or other tryptophan metabolites formed subsequent to pyrrolase actions causes brain serotonin levels to fall (Curzon and Green, 1968).

Therefore increased formation of these substances due to increased pyrrolase activity may be involved in the mechanism by which brain serotonin levels decrease.

Lapin and Oxenkrug (1969) proposed that the depression of brain serotonin levels may result in weakening of the inhibitory processes on the amygdaloid complex (Fig.15). In man, activation of this area has been reported to increase plasma cortisol levels, presumably by stimulating the anterior pituitary to produce more ACTH. This brings about a vicious circle - low brain serotonin levels leading to activation of amygdaloid complex, increased formation of hydrocorticosteroids and increased activity of tryptophan pyrrolase, which, in turn, would "shunt" the degradation of tryptophan away from serotonin to kynurenine (See Fig.16).

If this hypothesis were true, it would be a matter of attacking the links in the chain in the treatment of depressive disorders. Serotonin precursors could be used in restoring serotonin brain levels. Drugs like imipramine or desipramine which inhibit tryptophan pyrrolase activity (Paracchi, 1967) following chronic administration therefore exert this action as a component of their antidepressive effect. Nicotinamide and allopurinol could be used in the treatment of depression (Curzon, 1969). These drugs would inhibit the degradation of tryptophan along the pyrrolase-catalyzed kynurenine pathway thereby increasing the formation of brain serotonin. Chouinard *et al.*, (1977) reported that the combination of L-tryptophan and nicotinamide has a cumulative antidepressive effect in some depressed subjects.

At present, however, the kynurenine hypothesis remains speculative for the reasons discussed infra.

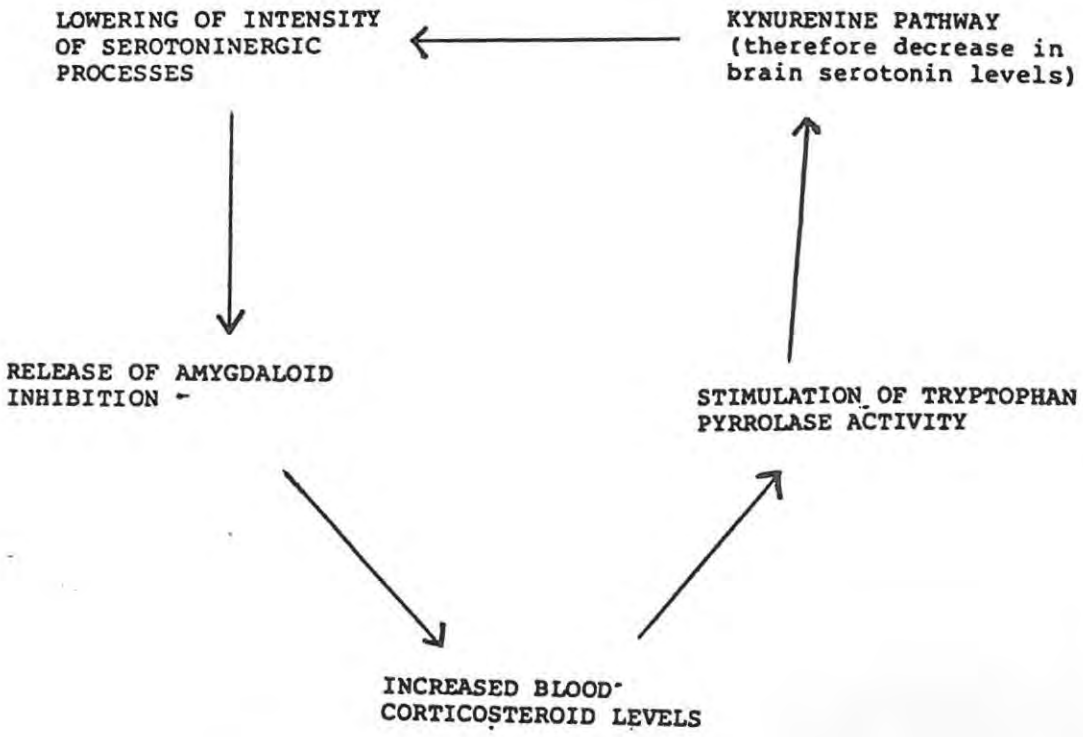


Fig.15 . The Kynurenine Hypothesis and Sequence of Events Proposed by Lapin and Oxenkrug (1969).

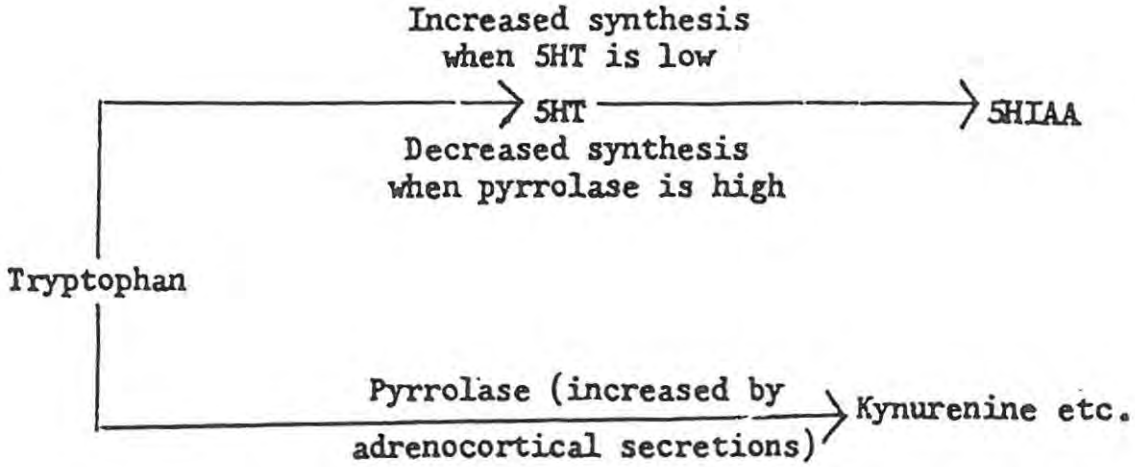


Fig. 16. Biochemical relatioships which may be involved in the regulation of tryptophan metabolism and may be relevant to the depressive illness.

(Curzon, 1969)

(i) It is doubtful if the activation of tryptophan pyrrolase is sufficiently marked to draw a substantial amount of tryptophan away from serotonin synthesis. A drawing off of available tryptophan, however, seems unlikely since the pyrrolase pathway is only one of the important metabolic pathways of tryptophan.

(ii) Van Praag (1977a) has reported a correlation between cortisol and plasma CSF tryptophan has not been studied in depressed patients.

(iii) Normal CSF tryptophan levels have been reported in depressed patients (Ashcroft *et al.*, 1973b).

1.7.2.5 Effect of melatonin on the 5-ALA-induced rise of rat forebrain tryptophan and serotonin

Badawy (1979) and other reports have documented the enhancement of the catabolic conversion of TRP to N'-formylkynurenine during the initial and rate limiting step of the kynurenine-nicotinic acid pathway of TRP degradation following activation of liver tryptophan pyrrolase by either haem or its precursor, 5-ALA.

Daya *et al.*, (1990) however found that if the forebrains of 5-ALA-treated rats were collected after the onset of darkness, the levels of both TRP and 5-HT were significantly higher. This rise in brain indoles coincides with the natural rise in pineal and blood melatonin levels at night, (Reiter, 1988). In studies

undertaken by Daya *et al.*, (1990) to find the link between these two observations, they demonstrated that melatonin administration to rats exposed to light brought about a reversal of the rise in forebrain TRP and 5-HT Melatonin also slightly suppressed forebrain TRP levels in non 5-ALA-treated rats.

The results indicate that melatonin counteracts the 5-ALA-induced rise of rat forebrain tryptophan and serotonin concentrations at night.

1.7.2.6 Motivation For Research

As referred to earlier, sex steroids are known to be associated with depression. Whether the sex steroids induce depression directly or by acting on liver tryptophan pyrrolase is not known since there are no reports in the literature on this topic. The purpose of the present study is to investigate whether a relationship exists between the sex steroids, liver tryptophan pyrrolase and brain indoleamines.

CHAPTER 2

2.1 THE EFFECT OF GONADAL SEX STEROIDS ON HEPATIC TRYPTOPHAN PYRROLASE ACTIVITY

2.1.1 EFFECT OF PARENTERAL ADMINISTRATION OF TESTOSTERONE, ESTRADIOL AND PROGESTERONE ON HEPATIC TRYPTOPHAN PYRROLASE ACTIVITY

2.1.1.1 MATERIALS AND METHODS

2.1.1.1.1 Chemicals

The sex steroids, progesterone and ß-estradiol-3-benzoate and the amino acid L-tryptophan were purchased from Sigma Chemical Company Mo, U.S.A. Testosterone was purchased from Merck, Germany.

Sex steroids solutions in olive oil

1mg/ml solutions of the 3 sex steroids were prepared in olive oil.

0,03M L-tryptophan solution

A 0,03M solution of L-tryptophan was prepared by a slight modification of the method used by Badawy and Evans (1975). A total of 0,612g of L-tryptophan was dissolved in a minimum volume of 2M NaOH and diluted to 40ml with 0,9% NaCl. The pH was then adjusted to 7,3 using 1M HCl. Finally, the solution was made up to 100ml with 0,9% NaCl. This was always prepared

on the day of use.

Sodium phosphate buffer pH 7,0

Sodium phosphate buffer was prepared by dissolving 8g of NaOH and 24g of NaH_2PO_4 separately in 1l of solution and then mixing 300ml and 500ml respectively of the NaOH and NaH_2PO_4 solution to give the final buffer of pH 7,0.

Homogenizing mixture

The homogenizing mixture was prepared by mixing 1,043g KCl and 0,1g NaOH in 100ml of solution. This was always kept ice-cold.

2.1.1.1.2 Animals

Inbred albino Wistar male and female rats were used. The rats which weighed between 200g and 300g were maintained on Epol Rat food and water *ad libitum*. The rats were housed in groups of 6 in plastic cages and kept in an animal room, with temperature at about 20°C and with an automatically regulated lighting system maintaining a 12 hours light/12 hours dark cycle.

2.1.1.1.3 Administration of steroids

2 groups of male rats (6 in each group) were subcutaneously injected with 0,1ml of testosterone (Test Rats) and olive oil (Control Rats) respectively. The animals were injected twice daily at 09h00 and 15h00 for 5 days. Female rats (6 in each test group) were similarly dosed with 0,1ml of progesterone and 0,1ml estradiol. Control rats received doses of olive oil.

2.1.1.2 Determination of Tryptophan Pyrrolase Activity

In the rat liver, circulating tryptophan is degraded through a series of metabolic processes to nicotinic acid. The initial and rate-limiting step of that pathway is the conversion of L-tryptophan to N'-formylkynurenine. This step is catalysed by the haem-dependent oxidoreductase, tryptophan pyrrolase.

The activity of the enzyme was determined by measuring the formation of kynurenine from L-tryptophan (Feigelson and Greengard, 1961).

The rats were sacrificed a day after the last steroid administration. The liver was removed within 30s and then homogenized at 1 100 rev/min in 6,5 volume of 140mM KCl - 2,5mM NaOH homogenizing mixture at 0°C for 1 minute. The liver homogenate was homogenized again, this time using a Wheaton dounce homogenizer with a loose-fitting Teflon pestle. 15ml samples of the liver homogenate were added to a solution made up of 5ml of 0,03M L-tryptophan, 15ml of 0,2M sodium phosphate buffer pH 7,0 and 25ml of distilled water at 0°C.

3ml samples of the assay mixture were incubated at 37°C for 0, 15, 30, 45, 60, 75, 90, 105 minutes with shaking (120 oscillations/min) in stoppered boiling tubes in an atmosphere of 95% O_2 and 5% CO_2. The reaction was stopped at each of the above time-intervals by the addition of 2ml of 0,9M trichloroacetic acid. The flasks and contents were shaken for a further 2

minutes and then filtered on Whatman No.1 filter paper. To 2,5ml of the filtrate, 1,5ml of 0,6M NaOH was added and the kynurenine present was determined by measuring the absorbance on a Bausch and Lomb Spectronic 301.

The activity of tryptophan pyrrolase was calculated from the increase in E_{365} with time during the linear phase. Using a combination of Beer's and Lambert's Laws, $A = Ecl$ with $E = 4\ 540$ *litre/mol.cm*, the amount of kynurenine formed at the end of each time interval was quantitatively determined. Tryptophan pyrrolase activity was expressed in terms of the amount of kynurenine formed in µmole/gram wet weight of liver.

Liver tryptophan pyrrolase exists in two forms (Badawy and Evans, 1974). The already active holoenzyme does not require the addition of exogenous haematin for demonstration of its activity *in vitro* (Feigelson and Greengard, 1961), whereas the haem-free predominant form or the apoenzyme does.

The activity of tryptophan pyrrolase determined in the various experiments therefore applies to the holoenzyme form of the enzyme, since neither haematin nor its precursor 5-aminolevulinic acid was administered to stimulate the apoenzyme.

In the initial and trial run for tryptophan pyrrolase assay, an attempt was made to determine to what degree freezing the liver would have on the activity of the enzyme. The need for this arose out of concern from Badawy's (1974) observation that

guinea-pig liver tryptophan pyrrolase when assayed in fresh homogenates, exists only as the holoenzyme. It does not respond to agents that activate or inhibit rat liver enzyme *in vitro* Only by aging (for 30 min at 5°C) does the guinea-pig liver enzyme develop a requirement for activators or inhibitors.

Tryptophan pyrrolase activity was assayed firstly from livers which have been previously frozen under cryonic (-70°C) conditions and secondly from fresh liver homogenates. The results from the frozen livers were conflicting but those from the fresh homogenates were consistent and reproducible. From then on, only fresh livers were used for enzyme assays.

2.1.1.3 Results

All data are expressed as the mean ± standard error of the mean. The data were analysed by one-way analysis of variance and the statistical significance among the means was determined using the Student-Newman-Keul's Test for 3 or more groups and the student t test for 2 groups. A P value of less than 0,05 was considered statistically significant.

2.1.2 EFFECT OF TESTOSTERONE ADMINISTRATION ON TRYPTOPHAN PYRROLASE ACTIVITY

2.1.2.1 Materials and Methods

Male rats weighing between 200g and 300g were used. 2 groups of 6 rats each were housed in plastic cages. The first group of 6 rats (Test group) were subcutaneously injected with 0,1ml of testosterone solution in olive oil. The injections were carried out twice daily (09h00 and 15h00) for 5 days. The second group (Control group) were similarly injected with the vehicle i.e. olive oil. The rats were sacrificed on the 6th day, the livers rapidly removed, homogenized and the activity of liver tryptophan pyrrolase assayed as described earlier (2.1.1.2).

2.1.2.2 Results

As shown in Table 1 and Fig.17.
From the results, it is evident that for all the time intervals, there was a significant increase in the activity of tryptophan pyrrolase in the livers of the test animals which were dosed with the steroid relative to the controls.

2.1.2.3 Discussion

At present, there are no literature reports on the precise effect of the gonadal sex steroids on hepatic tryptophan pyrrolase activity. It has however been well documented that

tryptophan pyrrolase, the enzyme which catalyses the initial and rate determining step of the oxidative Kynurenine-Nicotinic acid pathway, is dependent on the availability of its haem cofactor, (Bender 1975). Badawy (1987) has shown that when the enzyme is saturated with haem by the administration of the haem precursor 5-aminolevulinic acid (5-ALA) the activity of the enzyme is increased. This brings about increased catabolism of circulating tryptophan and as a consequence reduces tryptophan concentrations for uptake by the brain and thereby reducing brain serotonin levels (Badawy *et al.*, 1987; Daya *et al.*, 1989). Lapin and Oxenkrug (1969) formulated the indolamine theory of affective disorders which associated the deficiency of functionally active serotonin, a neurotransmitter in the brain with depression. The implication here is an agent which raises the level of activity of tryptophan pryrrolase invariably brings about depression. A lot of clinical studies obviously have to be carried out in order to draw the salient link between the sex steroids and depression. Given its strong inductive effect on liver tryptophan pyrrolase, testosterone, if present in concentrations above physiological concentrations may bring about reduced levels of circulating tryptophan.

INCUBATION TIME INTERVAL (MIN)	KYNURENINE CONC. in µmole/g wet weight of liver ± S.E.M.		SIGNIFICANCE students t test
	TEST RATS	CONTROL RATS	
BLANK	0,00	0,00	
15	1,74 ± 0,11	1,64 ± 0,10	N.S.
30	3,95 ± 0,28	3,27 ± 0,27	N.S.
45	6,89 ± 0,48	4,73 ± 0,35	P < 0,05
60	9,23 ± 0,51	6,73 ± 0,43	P < 0,05
75	11,05 ± 0,60	7,82 ± 0,57	P < 0,05
90	13,61 ± 0,69	8,73 ± 0,60	P < 0,05
105	14,98 ± 0,73	9,49 ± 0,61	P < 0,05

TABLE 1. effect of testosterone administration on hepatic tryptophan pyrrolase activity. Test rats were injected twice daily for 5 days. They were sacrificed on the 6th day. (n = 5). Control rats were dosed with the vehicle. Concentration of testosterone was 1mg/ml.

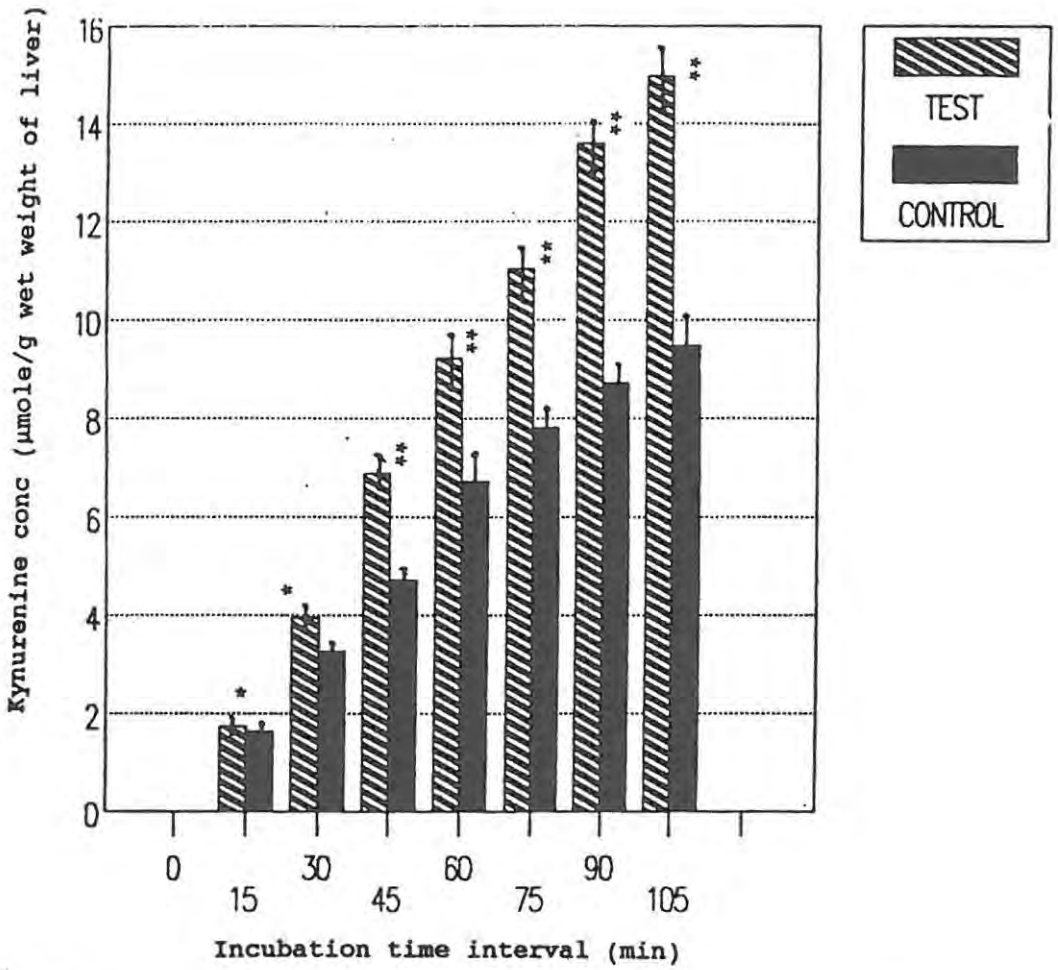


Fig. 17. effect of testosterone administration on hepatic tryptophan pyrrolase activity

* N.S. ** $p < 0.05$

2.1.3 EFFECT OF PROGESTERONE ADMINISTRAITON ON TRYPTOPHAN PYRROLASE ACTIVITY

2.1.3.1 Materials and Methods

Female rats weighing between 200g and 250g were used for this study. 0,1ml of 1mg/ml progesterone solution in olive oil was subcutaneously administered to 6 rats, twice daily for 5 days. The second group of 6 rats were similarly injected with the vehicle. The rats were sacrificed on the 6th day, the livers rapidly removed, homogenized and the activity of liver tryptophan pyrrolase assayed as described previously (2.1.1.2).

2.1.3.2 Results

As shown in Table 2 and Fig.18.
Progesterone administration brought about a significant increase in the activity of tryptophan pyrrolase.

2.1.3.3 Discussion

Progesterone was found to have a positive inductive effect on the activity of hepatic tryptophan pyrrolase.

INCUBATION TIME INTERVAL (MIN)	KYNURENINE CONC. in µmole/g wet weight of liver ± S.E.M.		SIGNIFICANCE students t test
	TEST RATS	CONTROL RATS	
BLANK	0,00	0,00	-
15	1,61 ± 0,15	1,53 ± 0,17	N.S.
30	4,73 ± 0,30	4,97 ± 0,19	N.S.
45	6,81 ± 0,31	4,20 ± 0,32	$P < 0,05$
60	8,84 ± 0,45	6,50 ± 0,51	$P < 0,05$
75	11,12 ± 0,59	6,95 ± 0,58	$P < 0,05$
90	13,07 ± 0,65	7,98 ± 0,61	$P < 0,05$
105	15,71 ± 0,73	10,20 ± 0,68	$P < 0,05$

TABLE 2. effect of progesterone administration on hepatic tryptophan pyrrolase activity. Test rats were injected twice daily for 5 days. They were sacrificed on the 6th day. (n = 5). Control rats were injected with the vehicle.

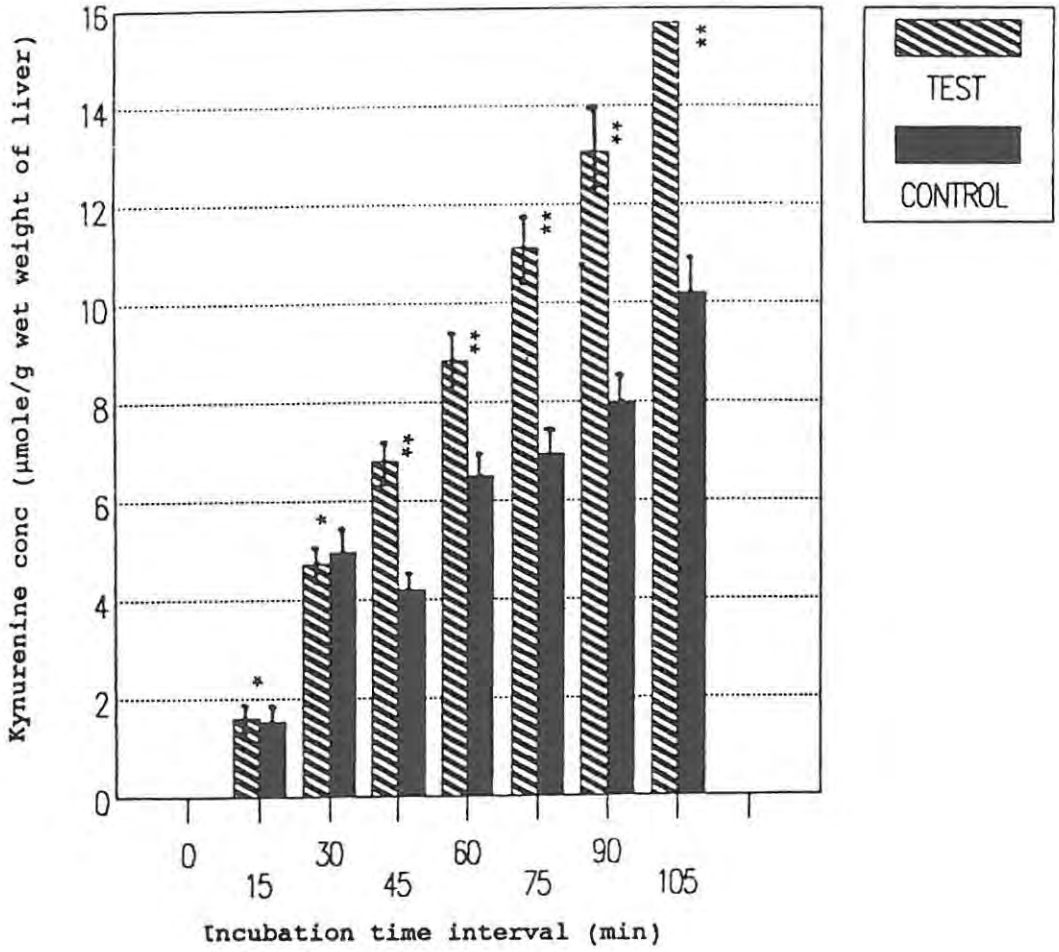


Fig. 18. effect of progesterone administration on hepatic tryptophan pyrrolase activity

* N.S. ** $p < 0.05$

2.1.4 EFFECT OF ESTRADIOL ADMINISTRATION ON TRYPTOPHAN PYRROLASE ACTIVITY

2.1.4.1 Materials and Methods

Female rats weighing between 200g and 250g were used for this study. 0,1ml of 1mg/ml ß-estradiol-3-benzoate solution in olive oil was subcutaneously administered to 6 rats, twice daily for 5 days. The second group of 6 rats were similarly injected with the vehicle. The rats were sacrificed on the 6th day. The livers were rapidly removed, homogenized and the activity of liver tryptophan pyrrolase assayed as described earlier (2.1.1.2).

2.1.4.2 Results

As shown in Table 3 and Fig.19.
ß-estradiol-3-benzoate was found to have a pattern of induction of tryptophan pyrrolase similar to testosterone and progesterone.

2.1.4.3 Discussion

Estradiol in high concentrations may also reduce circulating levels of tryptophan.

INCUBATION TIME INTERVAL (MIN)	KYNURENINE CONC. in μmole/g wet weight of liver ± S.E.M.		SIGNIFICANCE students t test
	TEST RATS	CONTROL RATS	
BLANK	0,00	000	-
15	1,17 ± 0,11	1,23 ± 0,09	N.S.
30	3,10 ± 0,12	3,12 ± 0,11	N.S.
45	5,13 ± 0,38	3,89 ± 0,24	P < 0,05
60	7,98 ± 0,60	4,33 ± 0,38	P < 0,05
75	9,87 ± 0,70	5,11 ± 0,45	P < 0,05
90	11,81 ± 0,78	6,05 ± 0,51	P < 0,05
105	13,27 ± 0,83	7,13 ± 0,67	P < 0,05

TABLE 3. effect of estradiol administration on hepatic tryptophan pyrrolase activity. Test rats were injected twice daily for 5 days. They were sacrificed on the 6th day. (n = 5). Control rats were injected with olive oil.

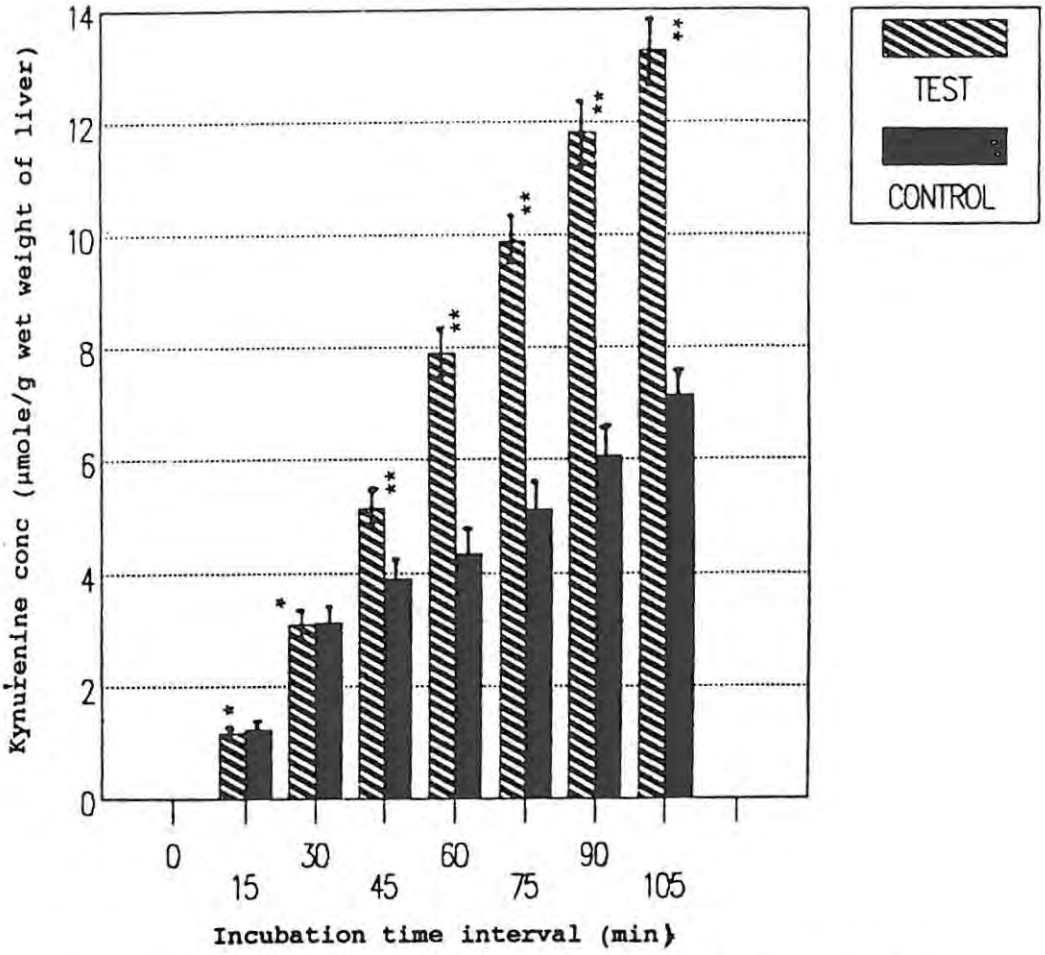


Fig. 19. effect of estradiol administration on hepatic tryptophan pyrrolase activity

* N.S. ** $p < 0.05$

2.2 EFFECT OF TRYPTOPHAN PYRROLASE CONCENTRATION ON KYNURENINE PRODUCTION

2.2.1 MATERIALS AND METHODS

5 male rats weighing 200-300g were used. The rats were exsanguinated and the livers rapidly removed and weighed. The liver was homogenized in 7 vol alkaline KCl on a mechanical homogenizer at 1 100 rev/min for 2 minutes. Table 4 shows the various volumes of buffer, water, tryptophan solution and liver homogenate that were mixed to give a resulting solution that was incubated at 37°C in boiling tubes in an atmosphere of carbogen. The assay mixture was incubated for 60 minutes with oscillations (120/min). The reaction was stopped by the addition of 15% metaphosphoric acid. The assay mixture was then shaken for 5 minutes after which it was filtered. A 3,0ml aliquot of the filtrate was neutralized by 1ml of 1NNaOH solution. The absorbance was then read at 365nm on a Bausch and Lomb Spectronic 301.

2.2.2 RESULTS

Table 5 gives the kynurenine concentrations for the various concentrations of the enzyme. Kynurenine concentrations increased with increasing concentrations of the enzyme. The amount of kynurenine formed for each assay mixture was quantitatively determined by the application of Beer-Lambert Law. The molar extinction coefficient for kynurenine at 365 nm and between pH 6,5 to 7,5 is $4,53 \times 10^3$.

Vol. of Buffer (ml)	Vol.of Water (ml)	Vol. of Tryptophan soln (ml)	Vol. of liver homogenate (ml)
1	0,7	0,3	2
1	1,7	0,3	1
1	2,0	0,3	0,7
1	2,2	0,3	0,5
1	2,4	0,3	0,3
1	2,6	0,3	0,1

TABLE 4. preparation of assay mixture for the quantitative determination of kynurenine at various enzyme concentrations.

Vol. of Buffer (ml)	Vol. of Water (ml)	Vol. of Tryptophan soln (ml)	Vol. of liver homogenate (ml)	Kynurenine concentration in µmole/g wet weight of liver
1	0,7	0,3	2	7,8
1	1,7	0,3	1	4,0
1	2,0	0,3	0,7	3,6
1	2,2	0,3	0,5	3,0
1	2,4	0,3	0,3	2,5
1	2,6	0,3	0,1	2,0

TABLE 5. quantitative determination of kynurenine production with increasing enzyme concentrations.

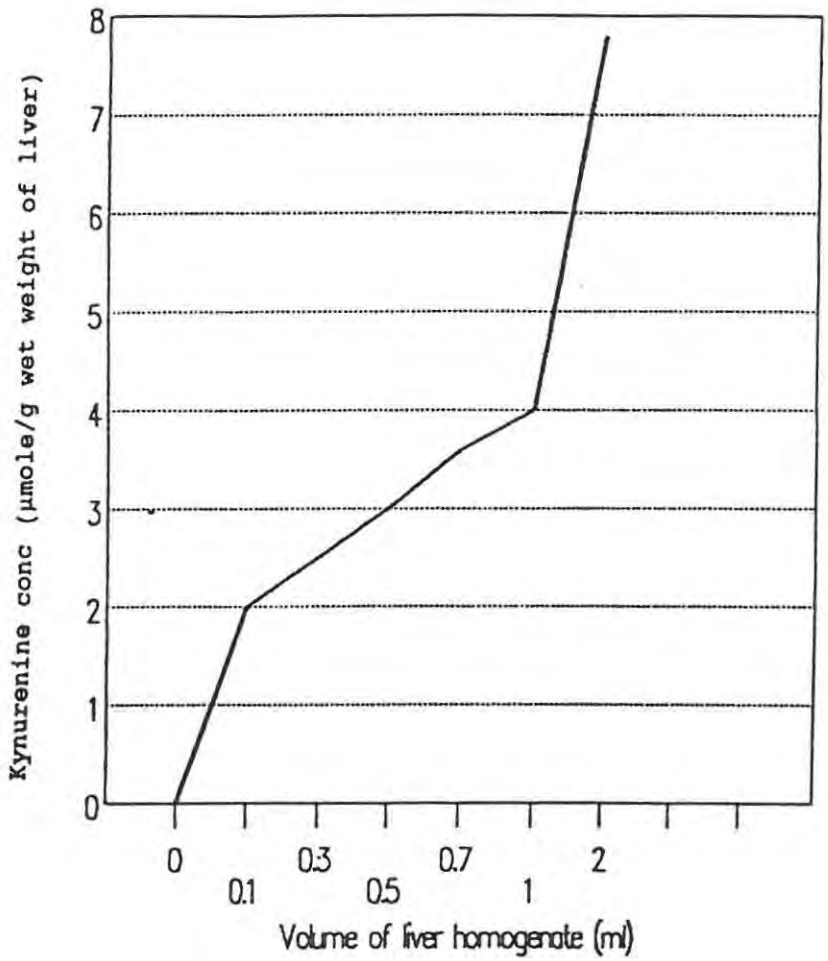


Fig. 20. effect of tryptophan pyrrolase concentration on kynurenine production

2.3 EFFECT OF ORCHIDECTOMY ON HEPATIC TRYPTOPHAN PYRROLASE ACTIVITY

2.3.1 ANIMALS

Male albino rats of the Wistar strain weighing 200-300g were used for this study. The animals were housed under conditions described earlier.

2.3.1.1 Surgery

The surgical procedure used in this study was essentially that used by Daya (MSc dissertation, 1982) and is described infra.

2.3.1.1.1 Anaesthesia

The rat was placed in a dessicator with cotton wool soaked in diethyl ether as the anaesthetic. It was taken out of the dessicator and placed on the operating surface. A 150ml conical flask containing cotton wool soaked in ether was placed about 2cm in front of the rat's nose, with the open mouth of the conical flask directly facing the rat's nose. The surgery was then performed. The rat's respiration was closely monitored during surgery. If the breathing became too weak, the conical flask was moved further away from the rat's nose to help recovery. The colour of the limbs and the tip of the tail gave a good indication of the depth of anaesthesia. A faint pinkness of the limbs and tail was observed during the optimum level of

anaesthesia, optimum in this case meaning a good rate and depth of respiration with a good narcosis. A purple colour was indicative of cyanosis. This method of anaesthesia proved to be successful with a high survival rate and lack of complications. The rats normally recovered consciousness in about 10 minutes after surgery.

2.3.1.1.2 Bilateral Orchidectomy (Castration)

Male rats under anaesthesia were placed in the spread-eagle position and the area around the scrotal sac was moistened with saline. The hair on and around the scrotal sac was shaved.

The shaved area was swabbed with an iodine solution in ethanol. A longitudinal incision about 1cm long was made along the centre line on the scrotal sac to expose the testes. The testes were drawn through the incision by pulling the cauda epididymis with forceps. The testicular blood vessels, vas deferens and spermatic ducts were ligatured distal to the caput epididymis and the testes were excised. The incision was stitched and the rat was allowed to recover.

2.3.1.1.3 Sham-operations

Sham-operations were carried out on the rats for this study and all studies in this dissertation which involved orchidectomy and ovariectomy. The rats used for sham operations went through identical anaesthesia and surgical procedures as those in the

test group except that neither the ovaries nor testes were excised. Unless otherwise stated, all rats which served as controls for orchidectomy and ovariectomy studies were sham-operated.

2.3.2 MATERIALS AND METHODS

A total of 15 male rats weighing 200g-300g, 10 of which had been castrated two weeks earlier were used for this study. The 10 castrated rats were divided into 2 groups of 5 rats each. The first group was subcutaneously dosed with 0,1ml of 1mg/ml testosterone solution, twice daily for 5 days. The second group of castrated rats and the 5 intact rats were subcutaneously injected with the vehicle. The rats were sacrificed by stunning and neck fracture on the 6th day after the injections started. The liver was rapidly removed, homogenized and activity of the tryptophan pyrrolase was assayed as described earlier (2.1.1.2).

2.3.3 RESULTS

As shown in Table 6 and Fig.21.

The activity of tryptophan pyrrolase was highest in the livers of castrated rats which were dosed with testosterone. The enzyme activity was relatively lower in the livers of intact rats. The lowest enzyme activity was found in the livers of castrated rats. For most of the time intervals, the differences between the 3 groups of rats were statistically significant.

2.3.4 DISCUSSION

The activity of tryptophan pyrrolase was highest in the livers of castrated rats dosed with testosterone. The activity of the enzyme in the intact livers was relatively lower. The lowest enzyme activity was in the livers of castrated rats. This observation reinforces the earlier finding (see 2.1.2.3) that testosterone induces tryptophan pyrrolase activity.

By removing the testes of the animals by way of orchidectomy implies the removal of the source of production of testosterone in the body of the animal. A direct consequence of this would be the removal of the inductive effect of testosterone on tryptophan pyrrolase activity. The lowest activity of tryptophan pyrrolase observed in the castrated rats was therefore in conformity with this. The intact rats had circulating testosterone in their bodies which induced tryptophan pyrrolase activity but this activity was still lower than that of the castrated rats which were treated with testosterone. A possible explanation here is that the testosterone administered to the castrated rats were in concentrations far higher than physiological concentrations and this resulted in the highest hepatic tryptophan pyrrolase activity in this particular group.

INCUBATION TIME INTERVAL (MIN)	KYNURENINE CONC. in µmole/g wet weight of liver ± S.E.M.		SIGNIFICANCE
	Castrated rats dosed with testosterone (A)	Castrated rats (B)	Intact rats (C)	A compared to B and C S-N-K test
BLANK	0,00	0,00	0,00	
15	2,13 ± 0,10	1,85 ± 0,09	1,90 ± 0,15	N.S.
30	3,56 ± 0,20	1,90 ± 0,11	3,11 ± 0,20	N.S.
45	6,82 ± 0,83	2,31 ± 0,13	4,35 ± 0,38	$P < 0,05$
60	8,70 ± 0,95	2,80 ± 0,16	5,80 ± 0,50	$P < 0,05$
75	11,03 ± 1,1	3,50 ± 0,19	7,13 ± 0,90	$P < 0,05$
90	14,57 ± 1,5	4,30 ± 0,30	9,73 ± 0,96	$P < 0,05$
105	17,15 ± 1,8	6,80 ± 0,5	11,05 ± 1,3	$P < 0,05$

TABLE 6. effect of castration and testosterone administration on tryptophan pyrrolase activity. A group of castrated rats were dosed with 1mg/ml testosterone twice daily for 5 days. A second group of castrated rats as well as the third group of intact rats were similarly dosed with the vehicle. All the rats were sacrificed on the 6th day.

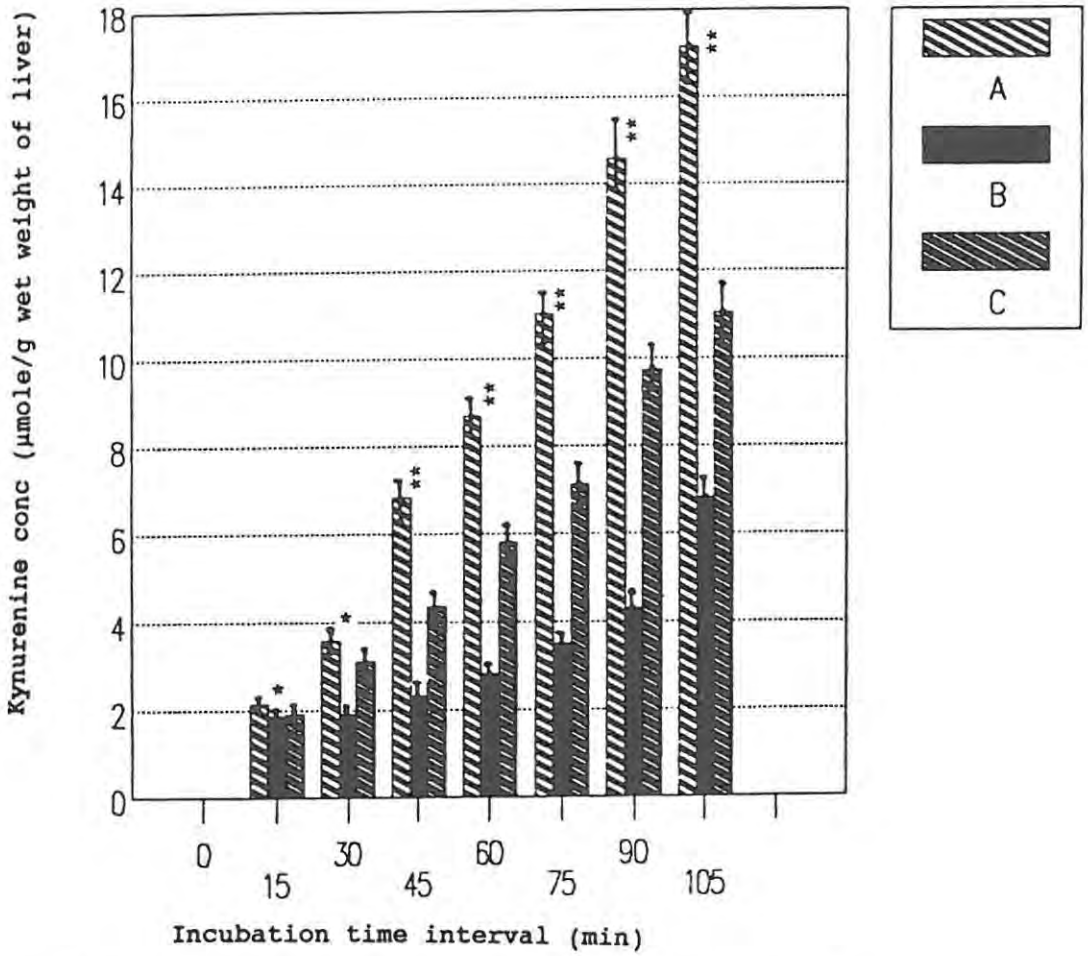


Fig. 21. effect of castration and testosterone administration on liver tryptophan pyrrolase activity

A. castrated rats were dosed with testosterone, twice daily for five days

B. castrated rats were dosed with olive oil

C. intact rats were dosed with olive oil

A compared to B and C * N.S. ** $p < 0.05$

2.4 EFFECT OF OVARIECTOMY ON HEPATIC TRYPTOPHAN PYRROLASE ACTIVITY

2.4.1 ANIMALS

Female albino rats of the Wistar strain weighing 200-250g were used for this study. The rats were housed under conditions described earlier.

2.4.1.1 Surgery

2.4.1.1.1 Anaesthesia

The animals were anaesthesized according to the procedures previously described (2.3.1.1.1).

2.4.1.1.2 Bilateral ovariectomy

After the application of anaesthesia, the rats were placed on their sides on the operating surface and the area of the abdomen on the side, just above the thigh was moistened with saline solution. The hair in this region was cut to expose the skin. The skin was swabbed with a solution of iodine and a 1cm incision was made through the skin exposing the abdominal muscles. An incision was made through the muscles and abdominal wall. The fat surrounding the ovary was immediately visible as it lay just beneath the incision. Using forceps, the fat with the ovary was pulled carefully through the incision. The

ovarian artery, vein and part of the uterine horn were ligatured and the ovary was excised. The incisions were stitched with suture under aseptic conditions. The rat was then turned to the other side and the same procedure was applied to remove the other ovary. The rat was finally allowed to regain consciousness.

2.4.2 MATERIALS AND METHODS

A total of 15 female rats, 10 of which had been ovariectomized two weeks earlier were used for this study. The 10 ovariectomized rats were divided into 2 groups of 5 rats each. 0,1ml of 1mg/ml progesterone solution was subcutaneously administered to the first group twice daily for 5 days. The second group of ovariectomized rats and the 5 intact rats were subcutaneously dosed with the vehicle at the same frequency and for the same number of days. The rats were sacrificed on the 6th day. The livers were rapidly removed, homogenized and the activity of tryptophan pyrrolase was assayed as outlined previously (2.1.1.2).

2.4.3 RESULTS

As shown in Table 7 and Fig.22.

The effect of ovariectomy and progesterone administration on tryptophan pyrrolase activity was identical to the pattern observed with castration and testosterone administration to male rats. The livers of ovariectomized rats dosed with progesterone

exhibited the highest enzyme activity. The enzyme activity was relatively lower in the livers of the intact rats. The lowest enzyme activity was observed in the livers of ovariectomized rats. The differences between the various experimental groups were statistically significant.

2.4.4 DISCUSSION

Presently, there are no literature reports on the effect of ovariectomy and progesterone administration on hepatic tryptophan pyrrolase activity. A possible explanation however of the elevated activity of tryptophan pyrrolase in the livers of ovariectomized rats, dosed with progesterone is that the progesterone administered might be in concentrations higher than physiological i.e. pharmacological. As a result, there would be a strong inductive effect on the activity of the enzyme. The lower enzyme activity in the livers of ovariectomized rats relative to the intact livers is understandable in the light of the fact that the intact rats still have an appreciable level of circulating sex hormones whilst the ovariectomized rats practically have none. This observation on the effect of progesterone on tryptophan pyrrolase actvitiy is consistent with an earlier observation (see 2.1.3.3).

INCUBATION TIME INTERVAL (MIN)	KYNURENINE CONC. in µmole/g wet weight of liver ± S.E.M.			SIGNIFICANCE A compared to B and C
	Ovariectomised rats dosed with testosterone (A)	Ovariectomised rats (B)	Intact rats (C)	S-N-K test
BLANK	0,00	0,00	0,00	
15	1,70 ± 0,15	1,30 ± 0,09	1,04 ± 0,10	N.S.
30	3,85 ± 0,15	3,40 ± 0,23	3,67 ± 0,25	N.S.
45	8,01 ± 0,9	4,47 ± 0,31	5,36 ± 0,38	$P < 0,05$
60	10,59 ± 1,0	5,01 ± 0,36	6,13 ± 0,47	$P < 0,05$
75	13,07 ± 1,1	5,83 ± 0,40	8,52 ± 0,75	$P < 0,05$
90	15,37 ± 1,2	6,30 ± 0,50	10,61 ± 0,90	$P < 0,05$
105	17,89 ± 1,4	7,53 ± 0,67	12,03 ± 1,10	$P < 0,05$

TABLE 7. effect of ovariectomy and progesterone administration on tryptophan pyrrolase activity. The first group of ovariectomized rats were injected with progesterone twice daily for 5 days. The second group of ovariectomized rats as well as the third group of intact rats were similarly injected with olive oil. All the rats were sacrificed on the 6th day.

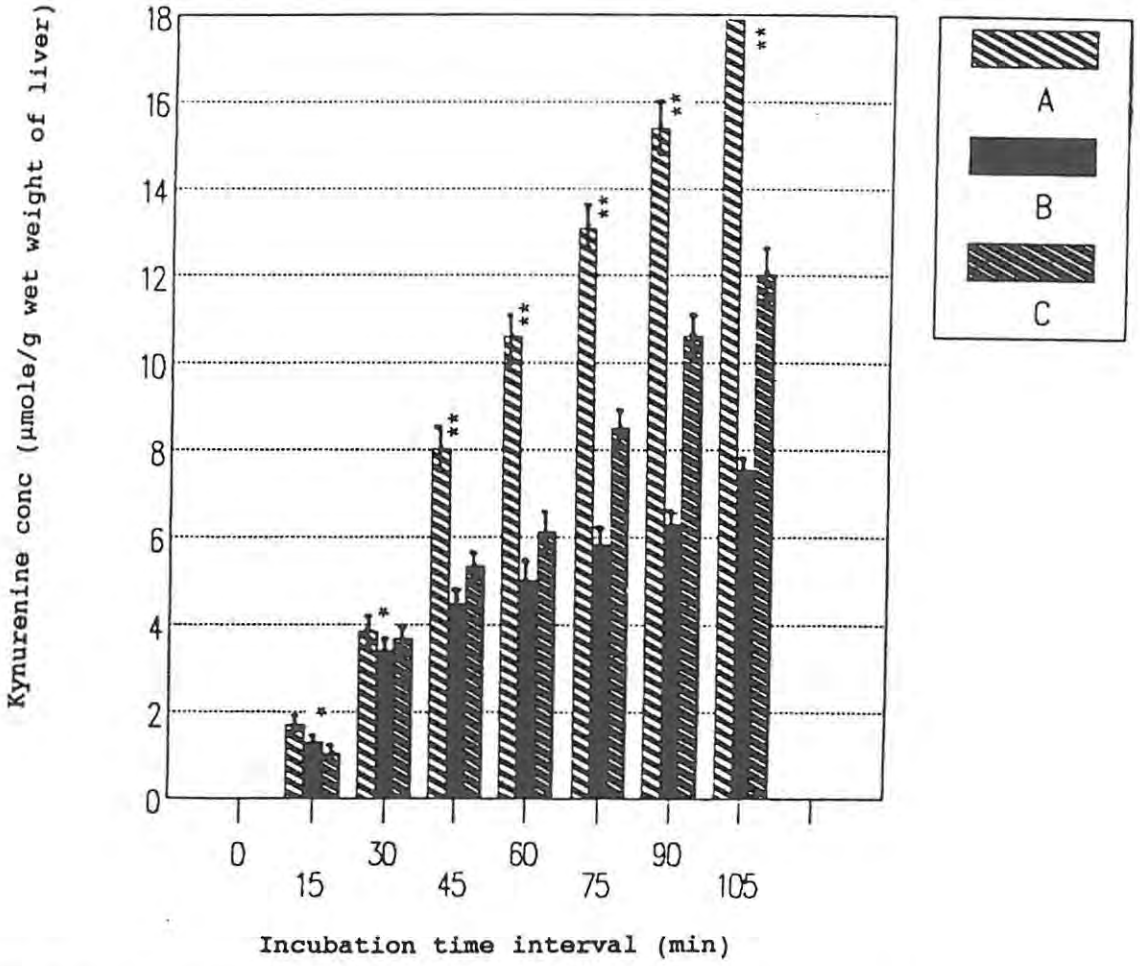


Fig. 22. effect of ovariectomy and progesterone administration on hepatic tryptophan pyrrolase activity

A. ovariectomized rats were injected with progesterone twice daily for five days

B. ovariectomized rats were injected with the vehicle

C. intact rats were injected with the vehicle

A compared to B and C * N.S. ** $p < 0.05$

CHAPTER 3

3.1 ORGAN CULTURE EXPERIMENTS AND SCINTILLOMETRY

Introduction

Strangeways (Strangeways and Fell, 1926) became a pioneer in the use of organ culture when he employed the technique to culture embryonic organ rudiments. The procedure affords the worker the useful advantage of monitoring the effects of drugs on an organ constituent without the complications of organ interactions. Trowell (1959), modified the apparatus and techniques for organ culture so as to keep a number of fully differentiated organs alive *in vitro*, without either growth or differentiation, thus opening the way for many experimental studies (Daya, 1982).

Scintillometry is a very effective technique used in the study of radioisotopic compounds that emit ß-particles. The light-emitting process during scintillation counting of ß-particle emissions occurs as a result of a radioactive sample being placed in a "scintillation cocktail" containing an excitable solvent and one or more fluorescent substances called fluors. Emitted ß-particles contact solvent molecules and transfer some of their energy to these molecules yielding excited solvent molecules. Excited solvent molecules transfer their excitation to other solvent molecules and eventually to a primary fluor molecule which then emits a photon. The photons emitted are then detected by a phototube-photomultiplier (Clark and Switzer,

1977).

3.1.1 MATERIALS AND METHODS

5-Hydroxy [side chain-2-^{14}C] tryptamine creatinine sulphate in aqueous solution containing 2% ethanol was purchased from Amersham International, U.K. The specific activity and radioactive concentration were respectively 53mCi/mmol and 50 μCi/ml.

The BGJb culture medium (Fitton-Jackson modification) with L-glutamine was purchased from Gibco, U.K. The culture medium was fortified with the antibiotics streptomycin (0,1 mg/ml), penicillin (100 units/ml) and amphotericin B (2,5 μ1ml).

The 0,25mm Kieselgel 60 G254 thin layer chromatography plates were purchased from Merck, Germany.

The Van Urks Reagent used for visualizing the spots on the TLC plates was made by adding a solution made up of 2g of 4-dimethylamine-benzaldehyde in 100ml of 25% HCl to 100ml of 90% ethanol.

A solution of the indoleamine standards was prepared by dissolving 1mg of each of the 8 indoleamine standards (5-HT, 5-MT, NAS, MEL, 5-HIAA, 5-MIAA, 5-HTOH, 5-MTOH) in 2,5ml of a solution of 95% ethanol. To this, 2,5ml of a solution of 1% ascorbic acid in 0,1M-HCl was added. The standards were stored

in the dark at -20°C.

Thin layer chromatography: **Solvent A** was prepared by mixing chloroform, methanol and glacial acetic acid in the ratio 93:7:1.

Solvent B was ethyl acetate.

The scintillation cocktail used was Ready-Solv™ MP (multi-purpose), purchased from Beckman, Scotland.

3.1.2 COMPOSITION OF BGJb MEDIUM USED FOR ORGAN CULTURE STUDIES (FITTON-JACKSON MODIFICATION)

The mean tonicity of this medium is 390 milliosmoles.

COMPONENTS	mg/l
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 components	
Calcium lactate	555,00
Glucose	10 000,00
Phenol red	20,00
Sodium acetate	50,00
Amino acids	
L-Alanine	250,00
L-Arginine	175,00
L-Aspartic acid	150,00
L-Cysteine HCl	90,00
Glycine	800,00
L-Histidine	150,00
L-Isoleucine	30,00
L-Leucine	50,00
L-Lysine	240,00

COMPONENTS	mg/l
Amino acids (continued)	
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	
Alpha tocopherol phosphate	1,00
Ascorbic acid	50,00
Biotin	0,20
Calcium panthothenate	0,20
Choline chloride	50,00
Folic Acid	0,20
Inositol	0,20
Nicotinamide	20,00
Para aminobenzoic acid	2,00
Pyridoxal phosphate	0,20
Riboflavin	0,20
Thiamine hydrochloride	4,00
Vitamin B12	0,04
Supplements	
L-Glutamine	200,00
Benzyl penicillin	100,00 units/ml
Streptomycin sulphate	0,1 mg/ml
Amphotericin B	2,5 µg/ml

3.1.3 SEROTONIN METABOLISM IN RAT PINEAL ORGAN CULTURE

3.1.3.1 Introduction

The pineal gland in organ culture is able to metabolize serotonin to its various metabolites. If radiolabelled serotonin is used during incubation, the radioactivity would be manifested in all the indoleamine metabolites. Measurement of the radioactivity on the scintillation counter would give a good indication of the relative levels of the various indoleamine metabolites of radiolabelled serotonin. This technique could therefore be used to determine the effect of various chemical agents on the pineal enzymes responsible for metabolizing serotonin and consequently the effect of these agents on the relative levels of serotonin metabolites. Studies were carried out to determine how serotonin metabolism in rat pineal organ cultures would respond to various manipulations by the gonadal sex steroids.

3.1.3.1.1 Materials and Methods

Five rats were sacrificed and their pineal glands were rapidly removed. Each pineal gland was placed in an incubation tube containing 90μl of BGJb culture medium. 10μl of (side chain-2-^{14}C)-serotonin creatinine sulphate was then added. The incubation tubes were gassed with carbogen (95% O_2; 5% CO_2). The incubation tubes were firmly covered and the pineals were incubated in the dark at 37°C for 24 hours. The reaction was

stopped at the end of 24 hours by removing the pineal gland from the culture medium. A 10μl aliquot of the culture medium was twice spotted on the TLC plate measuring 10cm x 10cm. A gentle stream of nitrogen gas was used to dry the spot after each spotting of the culture medium. Finally, standards of all the serotonin metabolites under study were spotted on top of the culture medium spot, once again allowing a gentle flow of nitrogen gas to dry the spots. Drying the spots with nitrogen gas prevents atmospheric oxidation of the metabolites.

After spotting, the TLC plates were placed in TLC tanks containing TLC solvent A. The solvent was allowed to move up the TLC plate, reaching a height of 7cm. The plate was then removed from the tank, dried by a gentle flow of nitrogen and then placed back in the same solvent to run in the same direction. After the solvent front reached a height of 7cm, the TLC plate was taken out again, dried under nitrogen, turned 90° and then placed in the second TLC tank containing TLC solvent B. All the runs were performed in total darkness by placing a light proof box over the TLC tanks (Klein and Notides, 1969).

The ethyl acetate was evaporated off with a gentle stream of nitrogen gas and the spots on the plates were visualized by spraying with Van Urks reagent. The plates were then placed in an oven at 60°C for 20 min to allow for the development of the spots. After development, the spots were scraped off the TLC plate into scintillation vials. 1ml of 95% ethanol was added to the scrapings in the vials followed by 3ml of scintillation

cocktail. The radioactivity of the various metabolites was measured on a Beckman LS 3801 Scintillation counter. In preliminary studies, individual standards were chromatographed in this manner to identify the location of all the various metabolites.

3.1.3.1.2 Results

Blanks were run in exactly the same way except that no tissue was included. The blank values were subtracted from the results before expressing results. The results were expressed as the d.p.m. per 10μl of culture medium.

3.1.3.1.3 Discussion

(See Fig.23)

The standards of the indoleamines were spotted on the same spot as the culture medium. The essence of this was to enable visualization of the spots on the plate since the concentrations of the various indoleamines in the culture medium were so small they may not be detected with the Van Urks reagent. There was an appreciable level of separation of all the metabolites. 5-HT and 5-MT however remained at the origin, where the medium was spotted. The levels of radioactivity counted in all the metabolites were detectable.

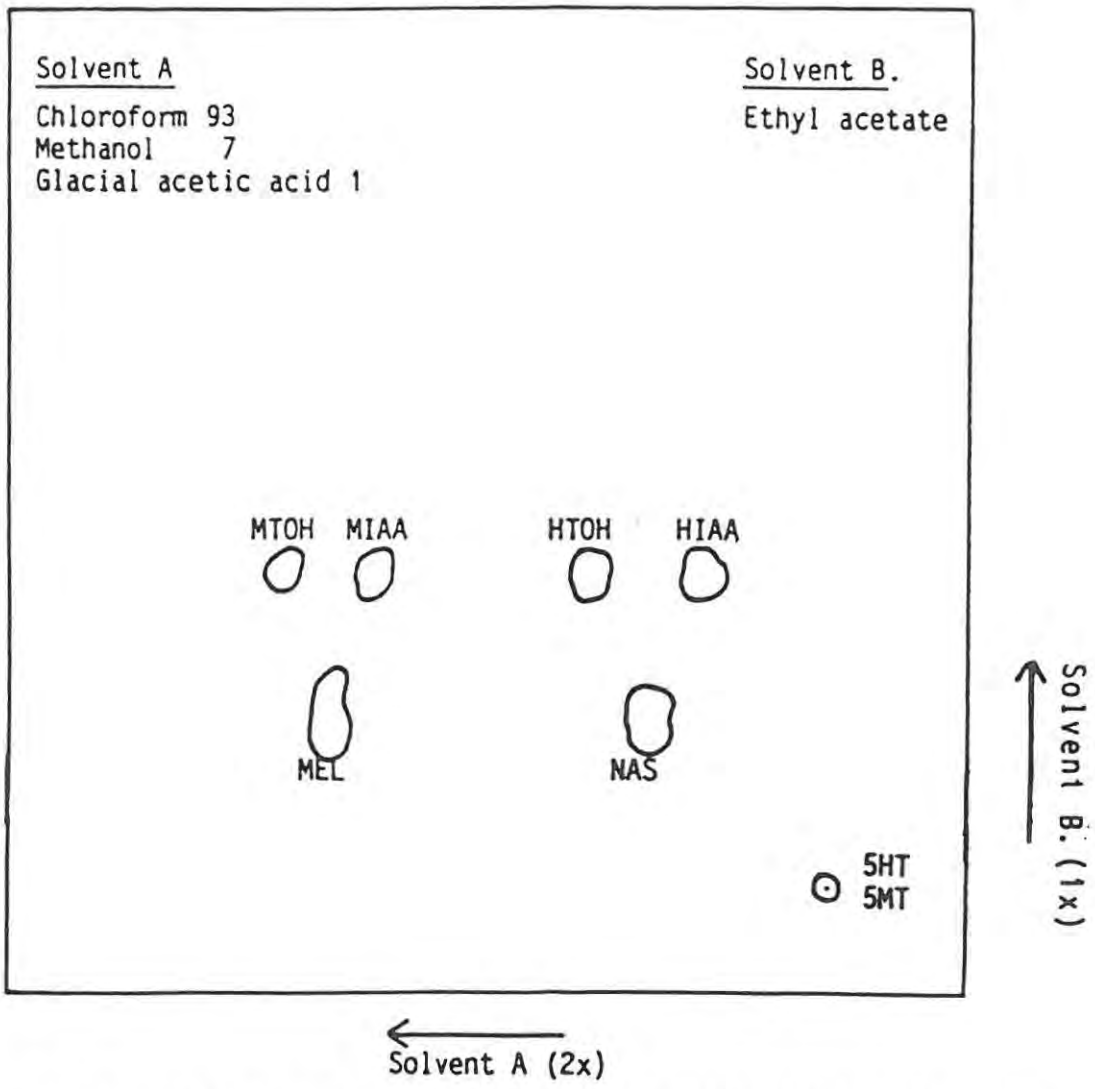


Fig 23. Tracing of chromatographic separation of pineal indole compounds.

5HT	=	Serotonin
5MT	=	5-Methoxytryptamine
NAS	=	N-acetylserotonin
MEL	=	Melatonin
MTOH	=	Methoxytryptophol
MIAA	=	Methoxyindoleacetic acid
HTOH	=	Hydroxytryptophol
HIAA	=	Hydroxyindoleacetic acid.

(Daya, 1982)

3.1.3.2 Effects of Testosterone on the Metabolism of ^{14}C-Serotonin by Organ Cultures of Pineals from Castrated Male Rats

3.1.3.2.1 Introduction

Since the sex organs, the gonads, are responsible for producing the sex hormones in the animals, it became logical to look at the effect of orchidectomy and ovariectomy and the direct influence of testosterone, progesterone and estradiol in the culture medium on ^{14}C-serotonin metabolism by the rat pineals in organ culture.

3.1.3.2.2 Materials and Methods

A stock solution of testosterone was made up in 95% ethanol. This was appropriately diluted to produce a final conc. of 10nM in the organ culture medium. A total of 15 male rats weighing between 200g and 300g, 10 of which had been castrated two weeks earlier, were used for this study. The castrated rats were divided into 2 groups of 5 rats each. After sacrifice, the pineals of the first group of castrated rats were cultured in the presence of radiolabelled serotonin and 10nM testosterone. The pineals of the second group of castrated rats and the intact rats were cultured in the presence of radiolabelled serotonin and the vehicle. After 24 hours, the incubation was terminated and 10μl aliquots of the media were chromotographed in duplicate and the radioactivity of the various metabolites was measured as

described earlier (3.1.3.1.1).

3.1.3.2.3 Results

As shown in Table 8 and Fig.24.
The levels of MEL, HIAA, MIAA and HTOH were significantly higher in the pineal cultures from castrated rats incubated in the presence of testosterone relative to pineal cultures from castrated and intact rats which were incubated with the vehicle i.e. diluted 95% ethanol. MTOH levels were expectedly lower in the latter group compared to the former group.

3.1.3.2.4 Discussion

The levels of various indoleamine metabolites of serotonin in organ cultures would depend to a large extent on the activities of the various pineal enzymes responsible for synthesizing these indoleamines. The results indicate that, there was a significant increase in the levels of MEL, HIAA, MIAA and HTOH in the pineal cultures from castrated rats incubated with testosterone compared to the pineal cultures from castrated and intact rats which were incubated with the vehicle. The higher levels of 5-MT and MIAA were consistent with the observation (Nagle *et al.*, 1974) that, castration exerts inhibitory effects on pineal HIOMT activities of male rats which are opposite to the effects observed after testosterone administration in low doses. The higher levels of 5-MT and MIAA in the testosterone-treated pineal cultures might therefore be a direct consequence

of the increased activity of HIOMT. The elevated levels of HIAA and HTOH in the testosterone-treated pineal cultures relative to the other groups might suggest that testosterone might exert a possible modulatory effect on the activities of the two pineal enzymes; aldehyde dehydrogenase and alcohol dehydrogenase.

The observation of higher levels of MEL in the testosterone-treated pineal cultures compared to the other groups was consistent with the finding by Karasek *et al.*, (1978) that castration can bring about a decrease in pineal concentration of cAMP. SNAT is stimulated by pineal cAMP, potentiating its activity of synthesizing NAS and MEL from serotonin. Therefore, any effect that brings about reduction in the concentration of cAMP would most certainly exert a consequent reduction in the activity of SNAT. The low levels of NAS observed in the testosterone-treated pineal cultures were contrary to the established observations in the literature. The results of the experiment show that testosterone can influence most of the pineal enzymes responsible for synthesizing the various indoleamines in pineal organ cultures and as a result influence the various levels of the indoles during organ culture.

The precise mechanism of testosterone on pineal metabolic activity is yet to be elucidated since present literature reports are conflicting. Cardinali *et al.*, (1974b) have reported that the rat pineal can aromatize radiolabelled testosterone into estrone and estradiol *in vivo* and *in vitro*. Preslock (1975), further observed that, although these

conversion rates of testosterone into androgens and estrogens are relatively low, it is possible that testosterone is converted into these metabolites within the pineal prior to exerting biological acitivity. This is not however consistent with Daya's (1985) observation that the stimulatory activity of testosterone is not dependent on prior conversion of testosterone to estradiol. Further work is required to clarify this position.

SEROTONIN METABOLITE	DPM READINGS/10µl culture medium ± S.E.M. (A) Intact rat pineals	(B) Castrated rat pineals	Significance student t test	(C) Castrated rat pineals incubated with Testosterone	Significance compared to B students t test
NAS	553,7 ± 40	703,3 ± 38	P < 0,05	657,9 ± 50	N.S.
MEL	357,1 ± 10	372,2 ± 15	N.S.	1 113,5 ± 67	P < 0,01
HIAA	13 237,3 ± 200	13 561,1 ± 300	P < 0,01	16 656,1 ± 250	P < 0,05
MIAA	563,3 ± 50	497,8 ± 25	P < 0,05	703,9 ± 37	P < 0,05
HTOH	3 609,1 ± 100	4 303,3 ± 100	P < 0,05	6 307,4 ± 250	P < 0,05
MTOH	1 273,5 ± 150	857,6 ± 50	P < 0,05	903,7 ± 57	N.S.
5HT, 5MT	80 231,6 ± 1 500	50 300,1 ± 1 405	P < 0,01	90 531,0 ± 1 750	P < 0,005

TABLE 8. effect of orchidectomy and the direct influence of testosterone on radiolabelled serotonin metabolism in rat pineal organ cultures. Pineals were cultured for 24 hours in the presence of radiolabelled serotonin and ethanol (A and B) and in the presence of radiolabelled serotonin and 10nM testosterone (C). (n = 4)

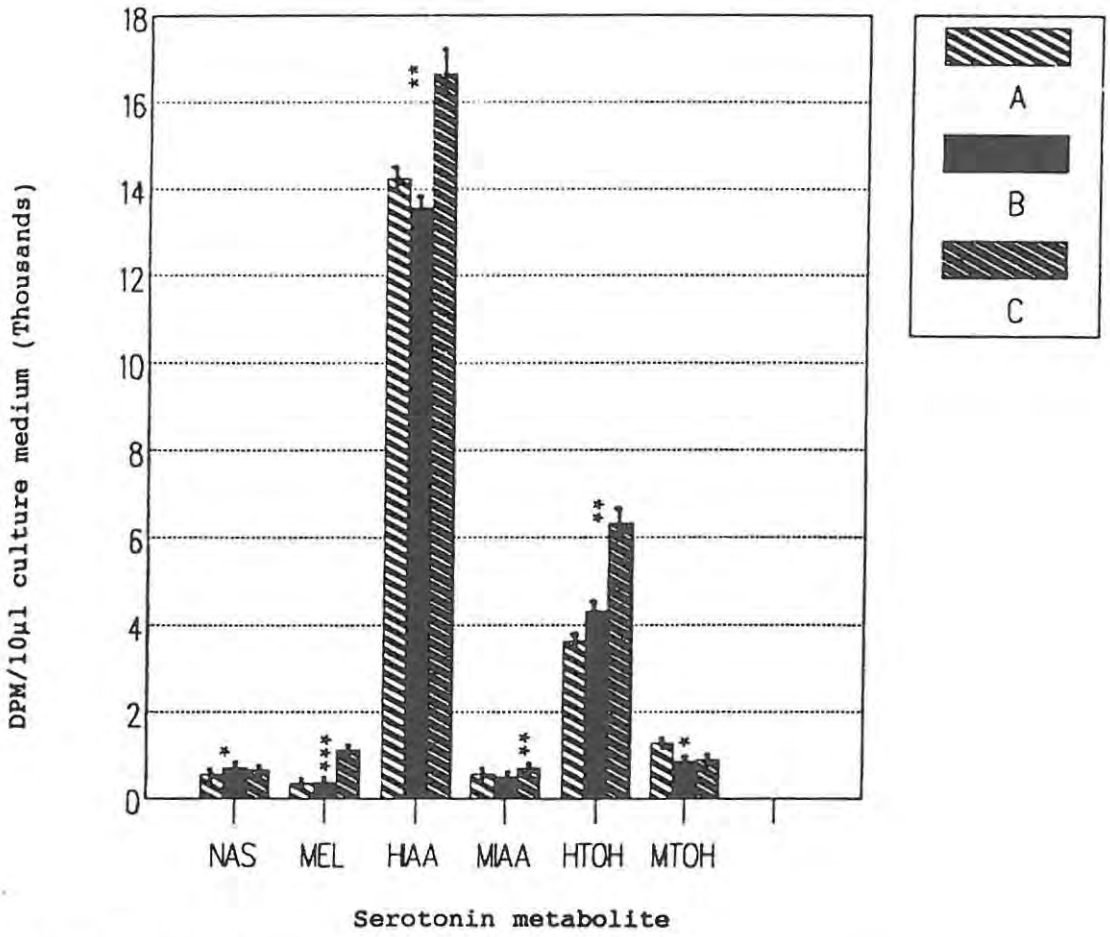


Fig. 24. effect of orchidectomy and direct influence of testosterone on radiolabelled serotonin metabolism in rat pineal organ cultures

A. pineals from intact rats were incubated with the vehicle

B. pineals from castrated rats were incubated with the vehicle

C. pineals from castrated rats were incubated with testosterone

C compared to B * N.S. *p < 0.05 **p < 0.01

3.1.3.3 Effect of Progesterone on the Metabolism of ^{14}C-Serotonin by Organ Cultures of Pineals from Ovariectomized Female Rats

3.1.3.3.1 Materials and Methods

A solution of progesterone in 95% ethanol was prepared and diluted to give a final concentration of 10nM in the culture medium. Fifteen female rats, weighing 200-250g, 10 of which had been ovariectomized two weeks earlier were used for this study. The ovariectomized rats were divided into two groups of 5 rats each. After sacrifice, the pineals of the first group of ovariectomized rats were incubated with ^{14}C-serotonin in the presence of 10nM progesterone. The pineals of the second group of ovariectomized rats and intact rats were incubated with radiolabelled serotonin and the vehicle. After 24 hours of incubation, 10μl aliquots of the media were chromotographed and the radioactivity of the various metabolites were determined as described earlier (3.1.3.1.1).

3.1.3.3.2 Results

As shown in Table 9 and Fig.25.

3.1.3.3.3 Discussion

Presently, there are no literature reports on the effect of ovariectomy and the direct influence of progesterone on the

metabolism of radiolabelled serotonin by female rat pineal cultures. Daya (1982) has observed that low doses of progesterone administered chronically stimulates HIOMT activity whereas higher doses administered chronically inhibit HIOMT activity. However, the progesterone used for this study was administered in pharmacological doses.

From the results however, there was a significant increase in the levels of MEL, HIAA and HTOH in the ovariectomized rat pineal cultures incubated with progesterone compared to the ovariectomized rat pineal cultures which were incubated with the vehicle. It appears that, at physiological concentrations, the sex hormone has a stimulatory role in the melatonin synthesis pathway.

SEROTONIN METABOLITE	DPM READINGS/10µl culture medium ± S.E.M. (A) Intact rat pineals	(B) Ovariectomized rat pineals	Significance student t test	(C) Ovariectomized rat pineals incubated with Progesterone	Significance compared to B students t test
NAS	203,6 ± 5	250,3 ± 9	P < 0,05	97,3 ± 4	P < 0,05
MEL	135,7 ± 6	99,5 ± 4	P < 0,05	150,7 ± 6	P < 0,05
HIAA	20 564,9 ± 145	10 516,8 ± 103	P < 0,01	25 013,3 ± 170	P < 0,005
MIAA	300,1 ± 11	385,8 ± 10	N.S.	360,7 ± 15	N.S.
HTOH	3 951,7 ± 95	3 261,3 ± 56	P < 0,05	4 307,7 ± 81	P < 0,01
MTOH	457,3 ± 15	497,9 ± 11	N.S.	487,6 ± 20	N.S.
5HT, 5MT	37 307,6 ± 900	50 016,0 ± 800	P < 0,005	40 103,5 ± 1 003	P < 0,05

TABLE 9. effect of ovariectomy and the direct influence of progesterone on radiolabelled serotonin metabolism in rat pineal organ cultures. Pineals were cultured in the presence of radiolabelled serotonin and ethanol (A and B) and in the presence of radiolabelled serotonin and 10nM progesterone.

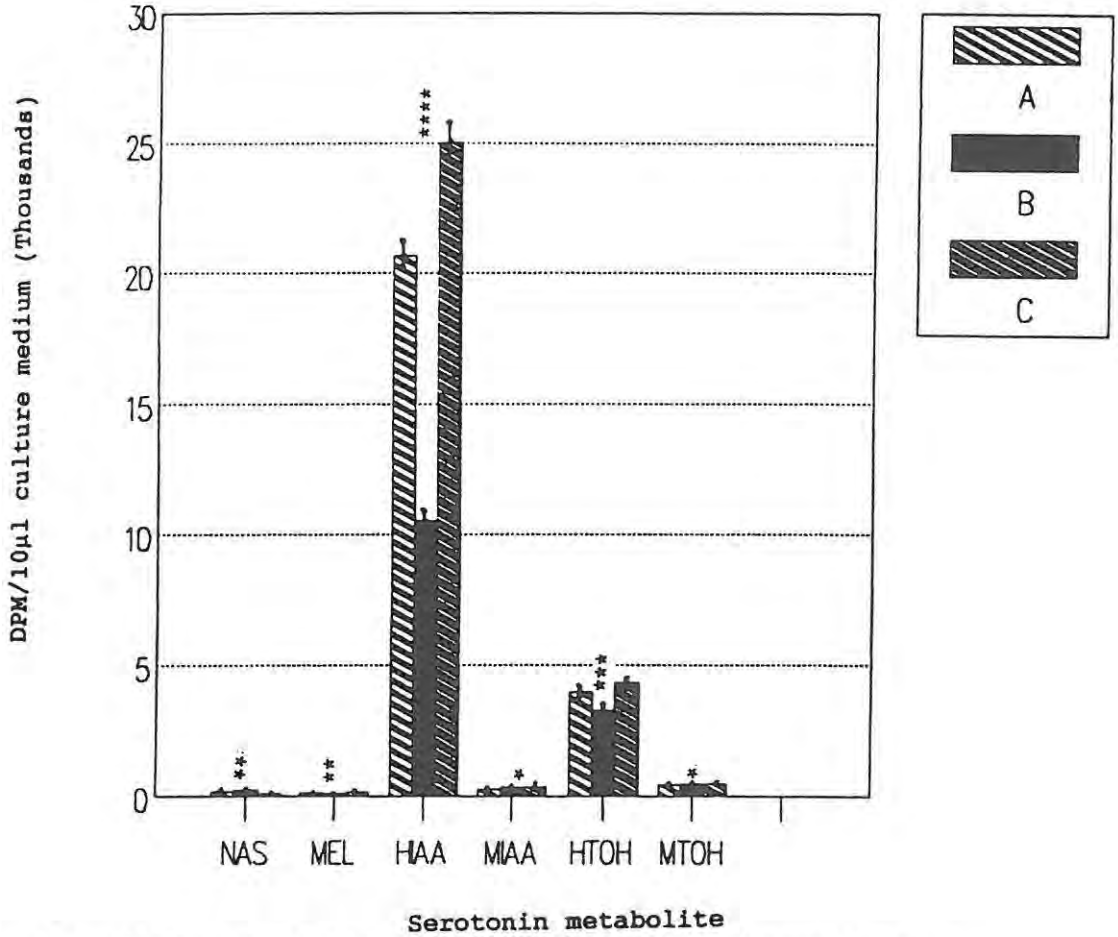


Fig. 25. effect of ovariectomy and the direct influence of progesterone on radiolabelled serotonin metabolism in rat pineal organ cultures

A. pineals from intact rats were incubated with the vehicle

B. pineals from ovariectomized rats were incubated with the vehicle

C. pineals from ovariectomized rats were incubated with progesterone

C compared to B * N.S. ** $p < 0.05$ *** $p < 0.01$ **** $p < 0.005$

3.1.3.4 Effect of Estradiol on the Metabolism of ^{14}C-Serotonin by Organ Cultures of Pineals from Ovariectomized Female Rats

3.1.3.4.1 Materials and Methods

A stock solution of ß-estradiol-3-benzoate in 95% ethanol was prepared and this was diluted to give a final concentration of 10nM in the culture medium. Fifteen female rats weighing between 200g and 250g, 10 of which had been ovariectomized two weeks earlier were used for this study. The ovariectomized rats were divided into two groups of 5 rats each. The pineals of the first group of ovariectomized rats were incubated with radiolabelled serotonin and 10nM ß-estradiol-3-benzoate after sacrifice. The pineals of the second group of ovariectomized rats and intact rats were incubated with radiolabelled serotonin and the vehicle. After 24 hours of incubation, 10µl aliquots of the media were chromatographed and the radioactivity of the various serotonin metabolites were determined as described earlier (3.1.3.1.1).

3.1.3.4.2 Results

As shown in Table 10 and Fig.26.
Estradiol appeared to have significantly enhanced HIOMT activity.

3.1.3.4.3 Discussion

In recent years, evidence has accumulated which indicates the pineal gland may be a target organ of steroid hormones. The pineal gland has been shown, both *in vivo* and *in vitro* to take up and retain estradiol (Marks *et al.*, 1972; Nagle *et al.*, 1972; Nagle *et al.*, 1973; Carinalli *et al.*, 1975). It has also been reported that pineal cells contain cytosol as well as nuclear components capable of binding estradiol (Mark *et al.*, 1972; Cardinali *et al.*, 1975), suggesting the possibility that the mode of action of estradiol in the pineal conforms to the general scheme of steroid hormone action accepted in other target organs, particularly in the uterus (Mizobe and Kurokawa, 1976; Jensen *et al.*, 1971; O'Malley *et al.*, 1974).

There was a significant increase in the MEL levels with a concomitant decrease in NAS levels in the ovariectomized rat pineal cultures incubated with estradiol relative to ovariectomized rat pineals incubated with the vehicle.

This observation suggested estradiol might have potentiated the activity of HIOMT but not SNAT. The stimulatory effect of estradiol on HIOMT was also manifested in the significant increase in the levels of MIAA in the cultures incubated with estradiol compared to cultures incubated with the vehicle. However, depressed levels of MTOH was observed in the pineal cultures incubated with estradiol. This observation might lend support to the hypothesis of the presence of different HIOMT

enzymes in the pineal. The stimulatory effect of estradiol on pineal HIOMT is in accord with the observations by Mizobe and Kurokawa (1976) that estradiol significantly increased HIOMT activity in a dose-dependent manner within the concentration range from 0,1 to 15nM. This is the range of physiological concentration of estradiol in the ovarian venous blood of rats (Hori *et al.*, 1979).

Ovariectomy depressed HIOMT activity in female rats as has been observed by Cardinali (1974). Cardinali *et al.*, (1974b) also observed a decrease in pineal protein synthesis in ovariectomized female rats. The consequence of this might be a reduced synthesis in HIOMT protein and hence a reduced HIOMT activity.

The higher levels of NAS in the pineal cultures of ovariectomized rats incubated with the vehicle relative to the pineal cultures of ovariectomized rats incubated with estradiol were in agreement with the observation that estradiol has no stimulatory effect on pineal SNAT (Illnerová, 1975); (Preslock, 1974 and 1975). Estradiol might have however played a modulatory role in the activities of aldehyde dehydrogenase and alcohol dehydrogenase in view of the significant increases in the levels of HIAA and HTOH in estradiol-treated cultures but not in the vehicle-treated cultures.

It appears quite surprising that estradiol has no modulatory effect on SNAT activity in view of Preslock's (1977) observation

that testosterone is converted into estradiol before exerting biological activity. A possible explanation here is that there may be slight structural differences between endogenous estradiol metabolized from testosterone and exogenous estradiol. SNAT may be sensitive to these differences.

SEROTONIN METABOLITE	DPM READINGS/10µl culture medium ± S.E.M. (A) Intact rat pineals	(B) Ovariectomized rat pineals	Significance student t test	(C) Ovariectomized rat pineals incubated with estradiol	Significance compared to B students t test
NAS	75,4 ± 3	103,7 ± 5	P < 0,05	83,1 ± 4	P < 0,05
MEL	250,3 ± 10	123,3 ± 6	P < 0,005	257,8 ± 9	P < 0,005
HIAA	15 601,7 ± 201	14 351,7 ± 105	P < 0,05	17 532,9 ± 150	P < 0,005
MIAA	431,7 ± 30	280,3 ± 15	P < 0,001	582,3 ± 21	P < 0,001
HTOH	3 998,1 ± 251	2 561,1 ± 207	P < 0,05	3 864,7 ± 273	P < 0,05
MTOH	653,7 ± 25	957,3 ± 25	P < 0,05	589,9 ± 10	P < 0,05
5HT, 5MT	70 512,5 ± 859	39 561,3 ± 657	P < 0,005	68 512,3 ± 963	P < 0,05

TABLE 10. effect of ovariectomy and the direct influence of estradiol on radiolabelled serotonin metabolism in rat pineal organ cultures. Pineals were cultured in the presence of radiolabelled serotonin and ethanol (A and B) and in the presence of radiolabelled serotonin and 10nM estradiol (C).

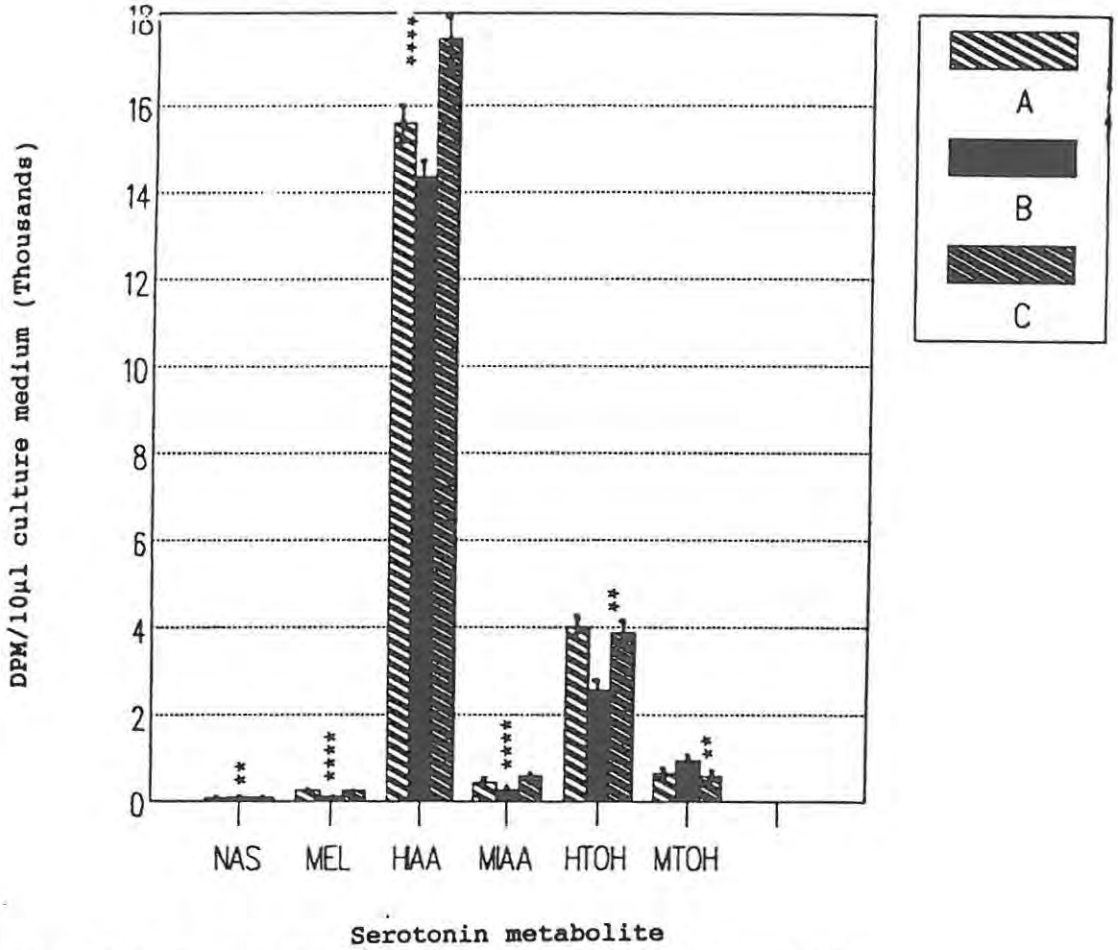


Fig. 26. effect of ovariectomy and the direct influence of estradiol on radiolabelled serotonin metabolism in rat pineal organ cultures

A. pineals from intact rats were incubated with the vehicle

B. pineals from ovariectomized rats were incubated with the vehicle

C. pineals from ovariectomized rats were incubated with estradiol

C compared to B $^{*}p < 0.05$ $^{****}p < 0.005$

3.1.3.5 Effect of Testosterone on Nocturnal Levels of ^{14}C-Serotonin Metabolites in Organ Cultures of Pineals from Castrated Male Rats

3.1.3.5.1 Introduction

The pineal gland is the site at which light:dark information is translated, or transduced, into a chemical messenger. The pineal gland, in essence, is the intermediary between the external photoperiod (perceived through the eye) and the internal milieu (Reiter, 1991). The eyes communicate with the pineal by a neuronal route which includes the retinohypothalamic tract, the suprachiasmatic nuclei and the pre- and postganglionic fibres of the peripheral sympathetic nervous system (Reiter, 1981).

With the onset of darkness, the sympathetic nerves innervating the pineal gland are stimulated. This results in the activation of adenylate cyclase which converts ATP to cAMP. cAMP in turn stimulates SNAT and HIOMT, the two pineal enzymes responsible for converting serotonin into melatonin. Consequently, the rat pineal concentration of serotonin and melatonin follow an inverse relationship (Romero, 1976; Relkin, 1976; Quay, 1974; Wolstenholme, 1971; Wurtman *et al.*, 1968).

3.1.3.5.2 Materials and Methods

Male rats with a mass of 200g-300g, 10 of which had been

castrated two weeks earlier were used for this study.

Six hours prior to sacrifice, the rats were housed in cupboards in a dark room secured from all incoming light. The room was kept lit with a dim red light. Red light does not influence the pineal enzymatic activities. The rats were sacrificed at 23h00 and the pineals rapidly removed. Five pineals from the castrated group were incubated with 10nM testosterone. The pineals from the five remaining castrated rats and the 5 pineals from the intact rats were incubated with the vehicle.

The incubation was stopped after 24 hours and 10μl aliquots of the various media were chromatographed. The radioactivity of the various serotonin metabolites were determined as described earlier.

3.1.3.5.3 Results

As shown in Table 11 and Fig.27.
The diurnal variation in pineal MEL synthesis was evident when these results were compared to those of the daytime study (3.1.3.2.3). Castration seemingly reduced the nocturnal rise in SNAT activity. Testosterone appeared to have restored the reduced nocturnal activity of SNAT.

3.1.3.5.4 Discussion

The relative levels of radioactivity of the various indoleamines

were indicative of their levels in the medium after ^{14}C-Serotonin metabolism. Comparing the results from this study to the results obtained from the daytime study (see Table 8), it is evident that there was a diurnal variation in MEL levels in relation to the photoperiod which came about as a consequence of the already established diurnal variation in SNAT activity (from literature reports). The nocturnal levels of MEL were signficantly higher than the levels in the daytime study. This is in agreement with previous reports (Sheving *et al.*, 1961; Okada, 1971; Fernstrom and Wurtman, 1971). Axelrod (1974) has observed that melatonin synthetic and secretory activity of the pineal gland is determined primarily by the release of norepinephrine from postganglionic sympathetic nerve endings that terminate in the gland (Kappers, 1960). The release of norepinephrine is strictly associated with darkness when the suprachiasmatic nuclei are relieved of the inhibitory signal from the eyes due to interaction of photons with the retinas (Reiter, 1991). Klein and Weller, (1970), Deguchi and Axelrod, (1972) have also reported the diurnal variations in SNAT activity.

The results also indicate that castration of the rats appeared to have resulted in a decrease of the nighttime elevation of SNAT activity. Castration has been shown (Karasek *et al.*, 1978) to reduce the pineal concentration of cAMP. This consequently reduces SNAT activity since cAMP plays an essential stimulatory role on the activity of SNAT. Pavlinov and Isachenkov (1979) as well as Rudeen and Reiter (1980) have published similar

observations about reduction in the nocturnal rise of SNAT activity in castrated male rats. Their results also show that testosterone replacement restored the reduced nocturnal activity of SNAT. This shows that testosterone could regulate the activity of SNAT.

SEROTONIN METABOLITE	DPM READINGS/10μl culture medium ± S.E.M. (A) Intact rat pineals	(B) Castrated rat pineals	Significance student t test	(C) Castrated rat pineals incubated with testosterone	Significance compared to B students t test
NAS	1 505,3 ± 97	1 023,7 ± 67	P < 0,05	2 073,7 ± 123	P < 0,005
MEL	1 793,5 ± 106	1 209,3 ± 53	P < 0,01	2 204,3 ± 140	P < 0,005
HIAA	18 644,7 ± 500	7 361,3 ± 381	P < 0,05	12 644,1 ± 603	P < 0,05
MIAA	165,4 ± 13	91,8 ± 6	P < 0,05	252,9 ± 7	P < 0,05
HTOH	4 108,4 ± 375	1 495,2 ± 105	P < 0,005	4 773,7 ± 250	P < 0,01
MTOH	91,3 ± 6	104,1 ± 7	N.S.	110,3 ± 6	N.S.
5HT, 5MT	30 149,3 ± 1 200	54 488,3 ± 1 501	P < 0,05	70 626,7 ± 1 803	P < 0,005

TABLE 11. effect of testosterone on nocturnal levels of ^{14}C-serotonin metabolites in organ cultures of pineals from castrated male rats. Pineals were cultured in the presence of radiolabelled serotonin and ethanol (A and B) and in the presence of radiolabelled serotonin and 10nM testosterone (C).

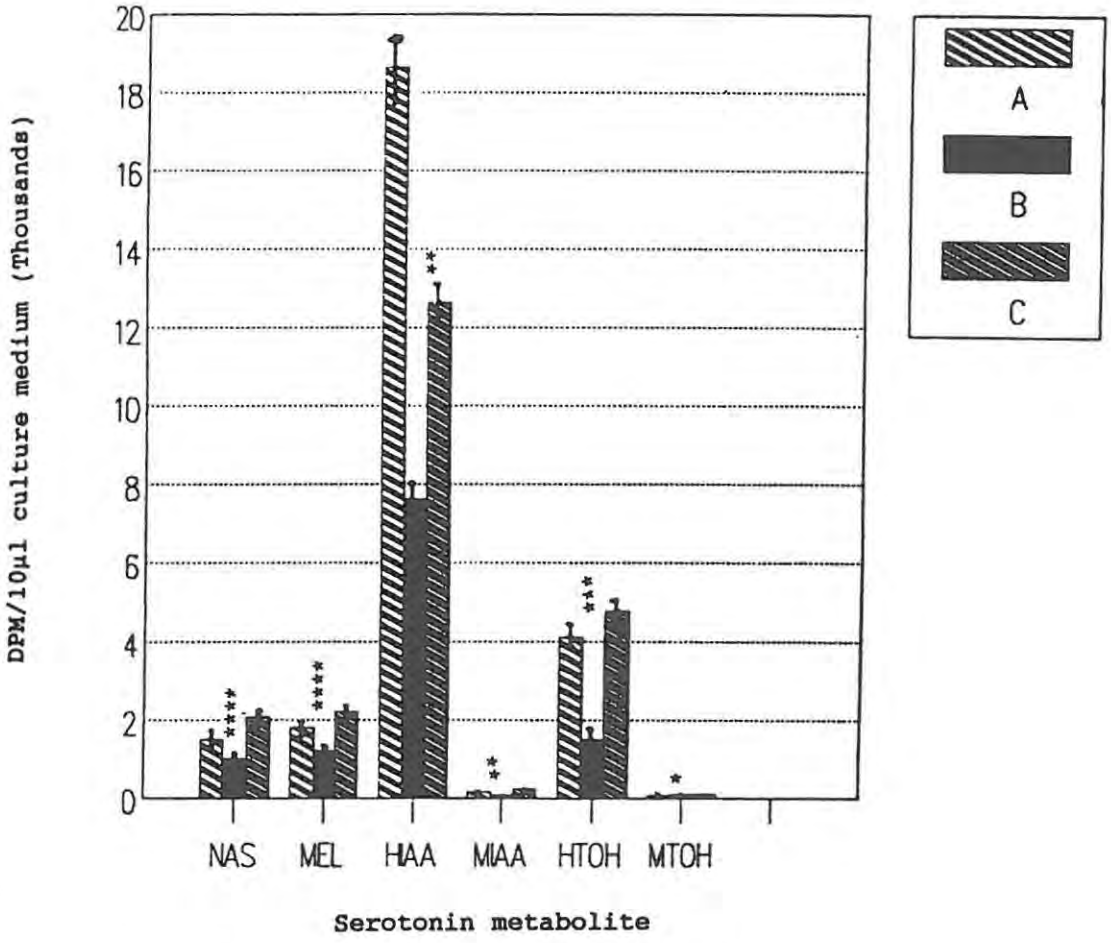


Fig. 27 effect of testosterone on nocturnal levels of ^{14}C-serotonin metabolites in organ cultures of pineals from castrated rats

A. pineals from intact rats were incubated with the vehicle

B. pineals from castrated rats were incubated with the vehicle

C. pineals from castrated rats were incubated with testosterone

C compared to B * N.S. *p < 0.05 **p < 0.01 ***p < 0.005

3.1.3.6 Effect of Estradiol on Nocturnal Levels of ^{14}C-Serotonin Metabolites in Organ Cultures of Pineals from Ovariectomized Female Rats

3.1.3.6.1 Materials and Methods

Fifteen female rats with a mass of 200-250g, 10 of which had been ovariectomized two weeks earlier were used for this study. The rats were kept in the dark before sacrifice as already described in 3.1.3.5.2. At 23h00, the rats were killed and the pineals were rapidly removed. Five pineals from the ovariectomized group were cultured with 10nM estradiol. The pineals from the five remaining ovariectomized rats as well as those from the 5 intact rats were incubated with the vehicle.

The incubation was stopped after 24 hours and 10μl aliquots of the various media were chromatographed. The radioactivity of the various serotonin metabolites was determined as described earlier (3.1.3.1.1).

3.1.3.6.2 Results

As shown in Table 12 and Fig.28.
The nocturnal levels of MEL were higher than in the daytime study. There was no significant difference between MEL levels in cultures of ovariectomized rats incubated with estradiol relative to those incubated with the vehicle.

3.1.3.6.3 Discussion

The nocturnal levels of MEL were significantly higher than the levels during daytime. This is consistent with literature reports (Sheving *et al.*, 1961; Okada, 1971). At present, however, there are no reports in the literature on the effect of estradiol and ovariectomy on the nighttime metabolism of radiolabelled serotonin.

Comparing MEL levels in the pineal cultures of ovariectomized rats incubated with estradiol to the MEL levels of the cultures of ovariectomized rats incubated with the vehicle, there were no statistically significant differences. This probably shows that estradiol did not exert any modulatory effect on SNAT activity. However, the significant increase in MEL levels in the pineal cultures of intact rats compared to the other two groups shows clearly that ovariectomy causes a depression in MEL synthesis.

SEROTONIN METABOLITE	DPM READINGS/10µl culture medium ± S.E.M. (A) Intact rat pineals	(B) Ovariectomized rat pineals	Significance student t test	(C) Ovariectomized rat pineals incubated with estradiol	Significance compared to B students t test
NAS	80,3 ± 5	97,6 ± 6	N.S.	79,3 ± 6	N.S.
MEL	1 593,2 ± 76	186,7 ± 11	P < 0,005	193,7 ± 8	N.S.
HIAA	13 061,1 ± 185	12 983,7 ± 102	N.S.	12 501,5 ± 120	N.S.
MIAA	681,7 ± 53	482,9 ± 41	P < 0,005	547,3 ± 25	N.S.
HTOH	2 547,1 ± 153	2 337,6 ± 125	N.S.	2 010,1 ± 102	N.S.
MTOH	447,6 ± 31	586,3 ± 38	P < 0,05	431,8 ± 26	P < 0,05
5HT, 5MT	60 101,6 ± 603	56,703,7 ± 567	P < 0,05	59 567 ± 593	N.S.

TABLE 12. effect of estradiol on nocturnal levels of ^{14}C-serotonin metabolism in rat pineal organ cultures of pineals from ovariectomized female rats. Pineals were cultured in the presence of radiolabelled serotonin and ethanol (A and B) and in the presence of radiolabelled serotonin and 10nM estradiol (C).

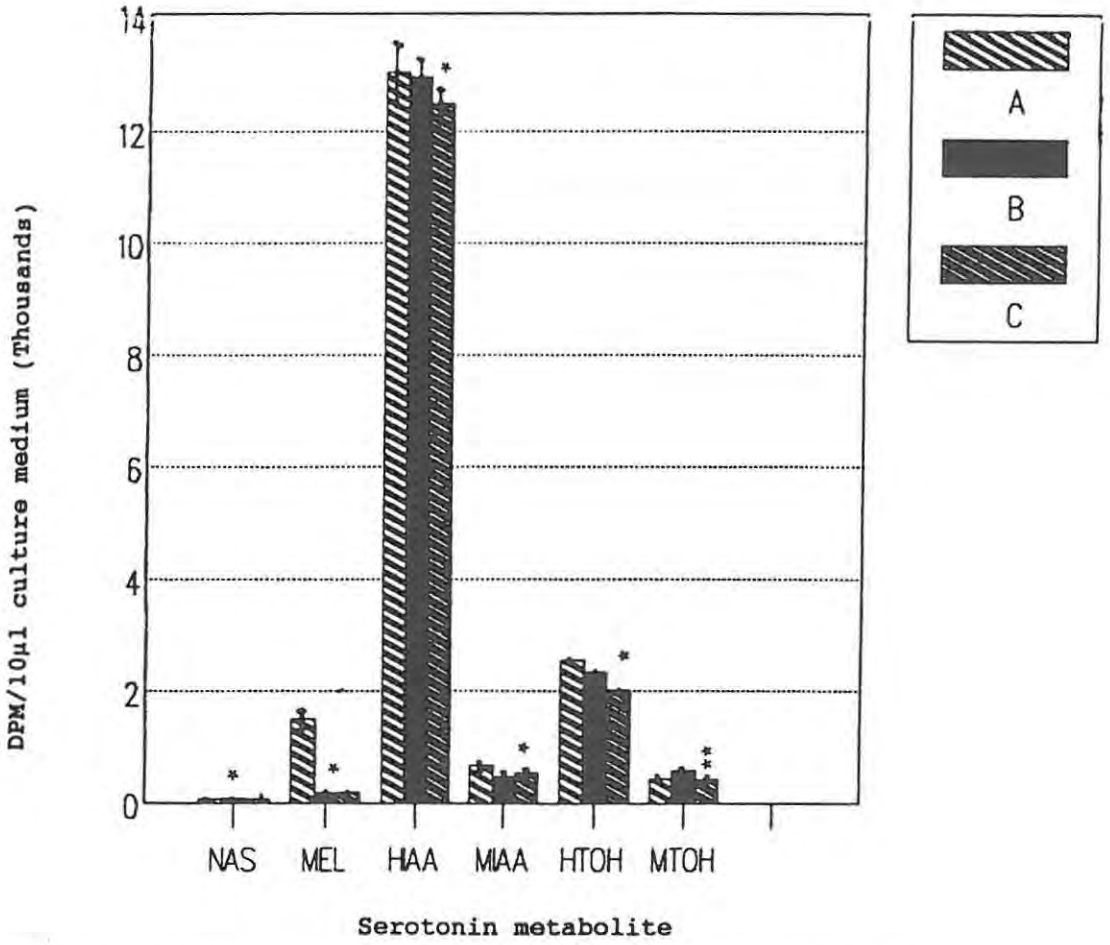


Fig. 28. effect of estradiol on nocturnal levels of ^{14}C-serotonin metabolites in organ cultures of pineals from ovariectomized female rats

A. pineals from intact rats were incubated with the vehicle

B. pineals from ovariectomized rats were incubated with the vehicle

C. pineals from ovariectomized rats were incubated with estradiol

C compared to B * N.S. ** $p < 0.05$

3.1.3.7 Cumulative Effect of Testosterone and Isoprenaline on ^{14}C-Serotonin Metabolism by Rat Pineals in Organ Culture

3.1.3.7.1 Introduction

Wurtman *et al.*, (1971) have established through organ culture studies that the noradrenaline-induced stimulation of pineal synthesis of ^{14}C-melatonin and ^{14}C-serotonin is inhibited by propranolol, a ß-adrenergic blocker and not by phenoxybenzamine, an α-adrenergic blocker. This observation implied that the stimulation mechanism involved ß-receptors. Prior to this Kappers (1960) had established that the pineal was innervated by the sympathetic nerves. The neurotransmitter was identified as noradrenaline (Wolfe *et al.*, 1962; Pellegrino de Iraldi and Zieher, 1966). Bäckström (1977), using selective $ß_1$ and $ß_2$ agonists and antagonists in rat pineal organ cultures, showed that the adrenoreceptors in the rat pineal respond similarly to the $ß_1$ adrenoreceptor subgroup. The physiological dispositions of isoprenaline, a ß-agonist are identical to noradrenaline. Isoprenaline could therefore stimulate the ß-receptors and this stimulation could lead to the activation of adenylate cyclase (Weiss and Costa, 1967; Weiss, 1971; Weiss and Strada, 1972). Cyclic AMP in turn activates SNAT and HIOMT, the two pineal enzymes responsible for converting serotonin to melatonin. Isoprenaline would therefore be expected to enhance melatonin synthesis in the pineal gland. Since testosterone exerts a stimulatory effect on SNAT activity, this study was carried out

to observe the cumulative effect of isoprenaline and testosterone on melatonin synthesis by rat pineals in organ cultures.

3.1.3.7.2 Materials and Methods

Isoprenaline was purchased from Sigma Chemical Company, Mo, USA. Fifteen male rats weighing between 200 and 300g were used for this investigation. The rats were sacrificed and the pineals removed. The pineals of the first group of 5 rats were incubated in BGJb medium with testosterone and isoprenaline solutions each with a final concentration of 10nM. The second group of five pineals were incubated with testosterone and the third group of pineals were incubated in the presence of the vehicle.

After 24 hours, 10μl aliquots of the media were chromotographed on TLC plates as described earlier. The radioactivity was then read on the scintillation counter.

3.1.3.7.3 Results

As shown in Table 13 and Fig.29.
The results show that testosterone potentiated isoprenaline effect on MEL synthesis.

3.1.3.7.4 Discussion

Isoprenaline and testosterone together brought about a cumulative elevation in the levels of MEL in the culture medium which was significant compared to MEL levels in the pineal cultures incubated with either testosterone or the vehicle. This is consistent with the fact that isoprenaline, a ß-agonist stimulates pineal SNAT via cAMP. Testosterone therefore amplified the isoprenaline response on the enhanced synthesis of melatonin.

DPM readings/10μl culture medium
± S.E.M.

MEL LEVELS	Pineals incubated with Testosterone and Isoprenaline (A)	Pineals incubated with Testosterone (B)	Significance student t test	Pineals incubated with Ethanol (C)	Significance compared to A students t test
MEL LEVELS	3 457,3 ± 106	883,1 ± 43	P < 0,01	396,7 ± 18	P < 0,005

TABLE 13. Cumulative effect of testosterone and isoprenaline on melatonin levels in rat pineals in organ culture. Pineals were incubated with testosterone and isoprenaline (A), testosterone alone (B) and ethanol (C) all in the presence of radiolabelled serotonin.

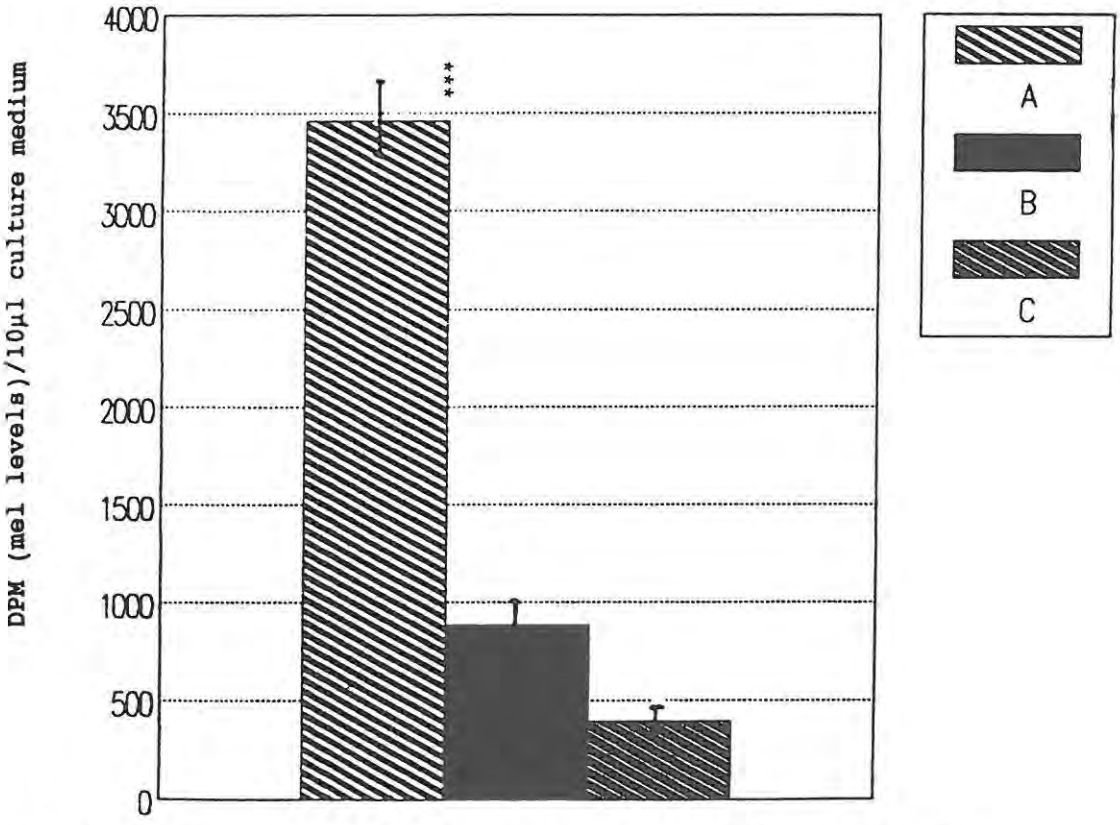


Fig. 29. cumulative effect of testosterone and isoprenaline on melatonin synthesis in rat pineals in organ cultures

A. pineals were incubated with testosterone and isoprenaline

B. pineals were incubated with testosterone

C. pineals were incubated with the vehicle

A compared to B $\overset{***}{*}$ $p < 0.01$

CHAPTER 4

4.1 EFFECT OF GONADAL SEX STEROIDS ON L-[^{14}C]-TRYPTOPHAN UPTAKE BY RAT CORTICAL SYNAPTOSOMES

4.1.1 INTRODUCTION

The circadian variation in the concentration of serotonin in the rat brain has been well established in numerous literature reports (Sheving *et al.*, 1968; Okada, 1971; Fernstrom and Wurtman, 1971). Plasma concentrations of L-tryptophan have also been shown to vary throughout the light-dark cycle (Fernstrom and Wurtman, 1971; Dam *et al.*, 1984), although a correlation between plasma 'free' L-tryp and brain serotonin concentrations has not been readily discernable (Morgan *et al.*, 1975). Beyond the blood brain barrier, however, L-tryp levels seem to correlate more closely with those of 5-HT and 5-HIAA (Morgan *et al.*, 1975). It is believed that tryptophan hydroxylase is the rate-limiting step in the synthesis of 5-HT because it is unsaturated with respect to L-tryp (McGeer *et al.*, 1968).

Therefore the intra-synaptic concentrations of L-tryp would be expected to influence the rate of synthesis of serotonin. Thus the mechanism of uptake of L-tryp into nerve endings appear to be of prime importance in the study of the regulation of serotonin biosynthesis (Loizou and Redfern, 1988).

Cortical synaptosomes are metabolically active, membrane-

enclosed presynaptic nerve terminals resulting from 'pinching off' the nerve terminals at the point of connection with the axon, and are ideal for studying the neural synthesis of neurotransmitters such as serotonin.

4.1.2 MATERIALS AND METHODS

4.1.2.1 Animals

Male and Female rats of the Wistar strain, weighing between 200 and 300g were used in the course of this study. The rats were given food and water *ad libitum* and they were subjected to a 12 hours light : 12 hours dark cycle.

4.1.2.2 Buffers and Solutions

(i) Tris-HCl buffer pH 7,2

This was prepared by adding 250ml of 0,2M-Tris (24,2g Tris/ 1 000ml) to 211ml of 0,2N-HCl (7,3g HCl/1 000ml). The volume was then made up to 800ml. The pH of the resulting solution was adjusted to 7,2 by the addition of 0,1NHCl. Distilled water was added to make up for a final volume of 1 000ml. This was kept refrigerated.

(ii) 0,32M-Sucrose solution in Tris-HCl buffer, pH 7,2

This was prepared by dissolving 10,9g of sucrose in 100ml of Tris-HCl buffer. The solution was always kept ice-cold.

(iii) Krebs-Bicarbonate buffer pH 7 (for maintaining rat brain synaptosomes).

This was prepared by dissolving the following chemicals in 1 000ml of distilled water.

Chemical	Concentration (mM)	Mass in g per 1 000ml solution
NaCl	11,84	0,692
KCl	4,70	0,350
$MgSO_4.7H_2O$	1,10	0,271
KH_2PO_4	1,20	0,163
$NaHCO_3$	25	2,100
glucose	11,10	2,000
$CaCl_2.2H_2O$	2,50	0,368

This buffer was always prepared on the day of use. All the chemicals were purchased from the British Drug House, England.

(iv) Protein Assay Solutions

Solution A: 1% copper sulphate solution. Prepared by dissolving 1,0g $CuSO_4.5H_2O$ in 100ml distilled water.

Solution B: 2% sodium tartrate solution. Prepared by dissolving 2,0g sodium tartrate in 100ml distilled water.

Solution C: 2% sodium carbonate in 0,1MNaOH. Prepared by dissolving 2.0g Na_2CO_3 and 0,4g NaOH in 100ml distilled water.

Solution D: This was prepared by mixing, in order, 1ml solution A, 1ml solution B and 98ml of solution C. This was always prepared before use (Clark and Switzer, 1977).

Solution E: Folin-Ciocalteau reagent.

(v) Protein standard Solution

Prepared by dissolving 10mg of Bovine Serum Albumin in 10ml of distilled water.

(vi) Steroid hormones

1mg/ml solutions of testosterone, progesterone and ß-estradiol-3-benzoate were prepared in olive oil.

(vii) Radioactive L-[methylene-^{14}C]-Tryptophan

was purchased from Amersham, England. The radiolabelled tryptophan had a specific activity of 54μCi/mmole and a radioactive concentration of 50μCi/ml.

(viii) Scintillation cocktail

Ready - solv™ MP (Multi-purpose) was purchased from Beckman, Switzerland.

(ix) 'Cold' Tryptophan solutions

This was prepared according to the procedure outlined in 2.1.1.2.

4.1.2.3 Preparation of synaptosomes

After sacrifice and decapitation, the brain was removed and placed on a piece of filter paper in a Petri dish soaked with Krebs-bicarbonate buffer which was kept cold by placing on ice,

to prevent degradation. The two frontal cerebral hemispheres were dissected free, weighed and placed in enough ice cold 0,32M sucrose in Tris-HCl buffer, pH 7,2 to give a 10% w/v homogenate (Loizou and Redfern, 1988).

The cortex was homogenized in a Wheaton glass mortar with a Teflon pestle until a homogenous suspension, free from all visible cellular matter was obtained. The glass mortar containing the brain tissue was kept on ice during the homogenization procedure. The homogenate was then centrifuged at 7 000 r.p.m. and 4°C for 10 minutes in a Hettich Universal K2S centrifuge with an angle head motor (catalog number 1375) to sediment out nuclei and cellular debris.

The supernatant was recovered in pre-weighed centrifuge tubes and centrifuged at 25 000 r.p.m. and 4°C for 30 min in a Beckman L8-80M ultracentrifuge with a 70lTl rotor to sediment the synaptosomes. The supernatant from this centrifugation was discardered and the final pellet resuspended in Krebs-bicarbonate buffer to give a 10% w/v suspension.

4.1.2.4 Measurement of tryptophan uptake by synaptosomes

The procedure began with the addition of 200µl of synaptosomal suspension to 600µl of Krebs-bicarbonate buffer in incubation tubes. After allowing 10 min for temperature equilibration, the assay was started by adding 200µl of three typtophan solutions, containing 0,2µCi L-[methylene-^{14}C]-tryptophan and sufficient

'cold' L-tryptophan to give a final concentration of 0,01; 0,05 and 0,1mM. This was done in triplicate. The final reaction mixtures were shaken continuously during the 5 minutes incubation period. The reaction was terminated by separating the synaptosomes from the incubation medium by filtration through a Whatman GF/C 2,5cm glass microfibre filters which were presoaked with Krebs-bicarbonate and housed in 12-place Millipore sampling manifold. The retained synaptosomes were then washed three times with 2,0ml aliquots of Krebs-bicarbonate buffer. The filters with the washed synaptosomes were placed in scintillation vials to which was added 4,5ml scintillation cocktail. The filters were allowed to stand overnight in the dark to ensure synaptosomal rupture and release of the accumulated L-[^{14}C]-tryptophan to minimize quenching by synaptosomal membranes (Loizou and Redfern, 1988). The radioactivity was then read on a Beckman LS3801 scintillation counter.

4.1.2.5 Protein determination in synaptosome preparations

The protein in each synaptosome preparation was determined by a slight modification of Folin-Lowry method. 1,2ml of appropriately diluted protein sample was mixed with 6ml of solution D and left to stand for 10min at room temperature. 0,3ml of solution E was then added, and the resulting solution was immediately mixed. The solution was allowed to stand for 30 min at room temperature after which the absorbance was read on a Bausch and Lomb Spectronic 301 at 500nm. Following the same

procedure and using Bovine Serum Albumin, a standard curve was drawn.

4.1.3 EFFECT OF TESTOSTERONE ON L-[^{14}C]-TRYPTOPHAN UPTAKE BY RAT CORTICAL SYNAPTOSOMES

4.1.3.1 Materials and Methods

10 male rats weighing between 200g and 300g were used for this study. The 5 rats in the test group were dosed with 0,1ml testosterone, twice daily for 5 days. The 5 rats in the control group were similarly dosed with the vehicle. On the 6th day, the rats were sacrificed, decapitated and the brains were removed. Procedures for the preparation of the synaptosomes and L-[^{14}C]-tryptophan uptake as outlined in 4.1.2.3 were followed.

4.1.3.2 Results

As shown in Table 14 and Fig.30.

The results show that testosterone significantly elevated the uptake levels of L-[^{14}C]-tryptophan in the synaptosomal preparations. The results also do indicate that there was an increase in the uptake of radiolabelled L-tryptophan with an increase in the concentration of mixed 'hot' and 'cold' L-tryptophan solution from 0,01mM to 0,05mM. However, there was a decrease in the uptake of radiolabelled L-tryptophan by the synaptosomes at the highest concentration of 0,1mM. This pattern of change in the uptake levels was observed in the test

group as well as in the control group.

4.1.3.3 Discussion

At present, there are no literature reports on the effect of testosterone on the uptake of L-tryptophan by the brain synaptosomes. However, it is evident from the results that testosterone signficantly enhanced the uptake of L-tryptophan by the synaptosomal preparations. This may be a compensatory mechanism effected by testosterone to make up for the reduced levels of circulating tryptophan - a consequence of the induction of liver tryptophan pyrrolase by testosterone.

The non-sepcific binding was reduced by washing the tryp-receptor complex after collection on a filter with aliquots of Krebs-bicarbonate solution.

From a concentration of 0,01mM to 0,05mM, there was a significant enhancement of "hot" L-tryptophan uptake by the synaptosomes in the Test as well as in the Control groups. A possible explanation here is, more of the "cold" L-tryp was involved in non-specific binding allowing the vesicles to specifically bind to the "hot" L-tryp. The decrease in "hot" L-tryp uptake by the synaptosomes in the highest L-tryp concentration of 0,10mM in both experimental groups suggest that the "cold" L-tryp at that concentration would have saturated the non-specific binding sites of the synaptosomal preparations and subsequently have bound to the specific sites, thus effectively

reducing the binding levels of "hot" L-tryp.

Concentration of mixed "hot" and "cold" L-tryp soln (mM)	DPM readings/mg protein ± S.E.M.		significance
	Test group	Control group	students t test
0,01	7 905,3 ± 205	5 370,31 ± 167	P < 0,01
0,05	11 498,7 ± 403	5 697,1 ± 157	P < 0,01
0,10	6 067,2 ± 357	4 411,5 ± 190	P < 0,05

TABLE 14. effect of testosterone on L-[^{14}C]-tryptophan uptake by rat cortical synaptosomes. Rats were dosed with 0,1ml testosterone twice daily for 5 days. They were sacrificed on the 6th day and the brains assayed for tryptophan uptake. Control rats were dosed with the vehicle.

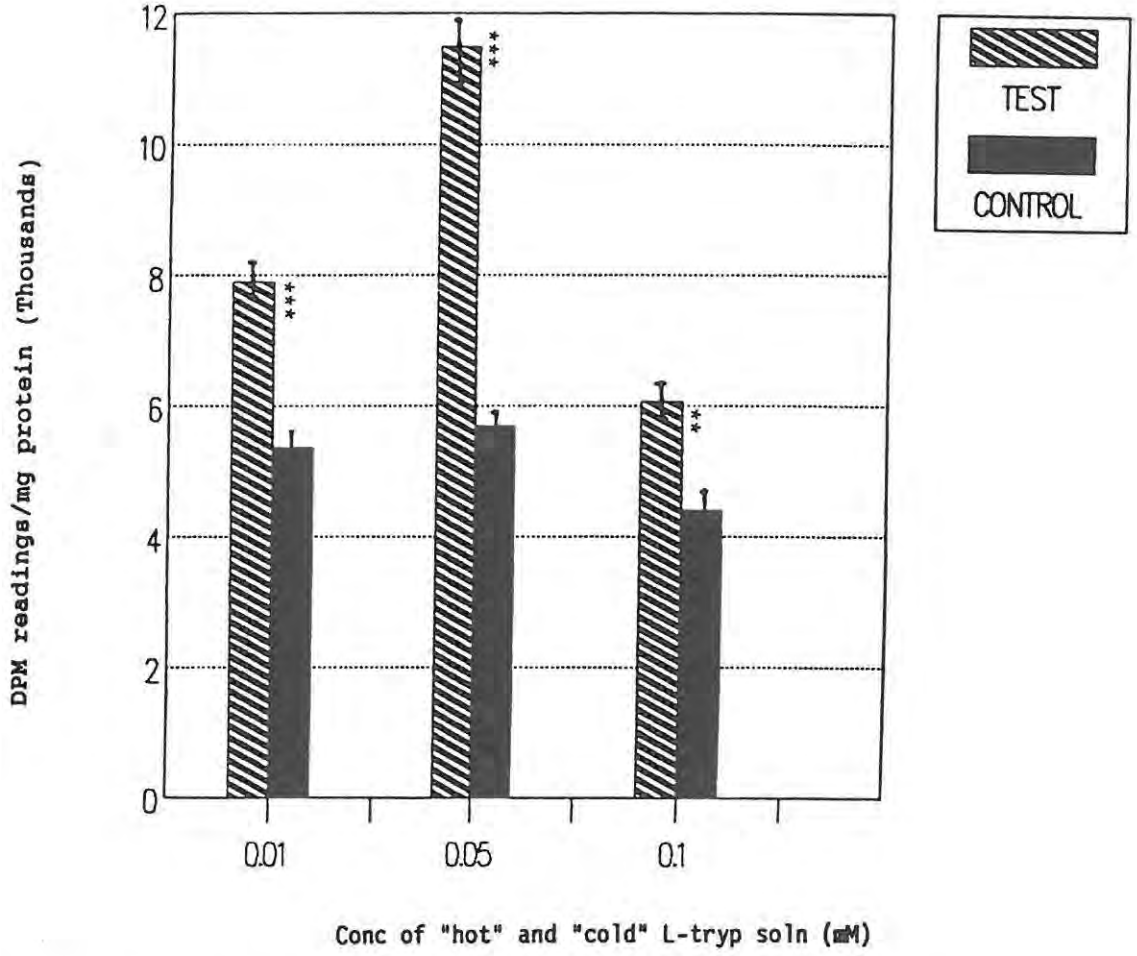


Fig. 30 effect of testosterone on radiolabelled tryptophan uptake by rat cortical synaptosomes

** $p < 0.05$ *** $p < 0.01$

4.1.4 EFFECT OF ESTRADIOL AND PROGESTERONE ON L-[^{14}C]-TRYPTOPHAN UPTAKE BY RAT CORTICAL SYNAPTOSOMES

4.1.4.1 Materials and Methods

Twenty female rats weighing between 200 and 250g were used for this study. Five rats each in the Test and Control groups were respectively dosed with 0,1ml progesterone or estradiol, and olive oil twice daily for 5 days. The rats were sacrificed on the 6th day and the uptake of L-[^{14}C]-tryptophan in the brains was determined as described in 4.1.2.3.

4.1.4.2 Results

As shown in Tables 15 and 16; Figs. 31 and 32.
Progesterone and estradiol, like testosterone significantly raised the uptake levels of L-[^{14}C]-tryptophan in the synaptosomal preparations.

4.1.4.3 Discussion

As outlined in 4.1.3.3., the elevated levels of L-[^{14}C]-tryptophan uptake by the synaptosomes from the brains of the rats dosed with the sex steroids relative to the controls may be a compensatory mechanism effected by the sex steroids to make up for the depressed levels of circulating tryptophan - a consequence of the inductive effect of the sex steroids on hepatic tryptophan pyrrolase activity.

Concentration of mixed "hot" and "cold" L-tryp soln (mM)	DPM readings/mg protein ± S.E.M. Test group	Control group	significance students t test
0,01	8 169,5 ± 304	5 420,1 ± 153	P < 0,01
0,05	12 970,3 ± 351	7 271,8 ± 257	P < 0,01
0,10	6 125,2 ± 157	4 467,7 ± 103	P < 0,05

TABLE 15. effect of progesterone on L-[^{14}C]-tryptophan uptake by rat cortical synaptosomes. Rats were dosed with 0,1ml progesterone twice daily for 5 days. They were sacrificed on the 6th day and the brains assayed for tryptophan uptake. Control rats were injected with olive oil.

Concentration of mixed "hot" and "cold" L-tryp soln (mM)	DPM readings/mg protein ± S.E.M. Test group	Control group	significance students t test
0,01	5 180,7 ± 97	5 420,8 ± 120	N.S.
0,05	13 135,3 ± 303	7 271,7 ± 187	P < 0,01
0,10	8 859,1 ± 236	4 467,3 ± 121	P < 0,01

TABLE 16. effect of estradiol on L-[^{14}C]-tryptophan uptake by rat cortical synaptosomes. Rats were injected with 0,1ml estradiol twice daily for 5 days. They were sacrificed on the 6th day and the brains assayed for tryptophan uptake. Control rats were dosed with olive oil.

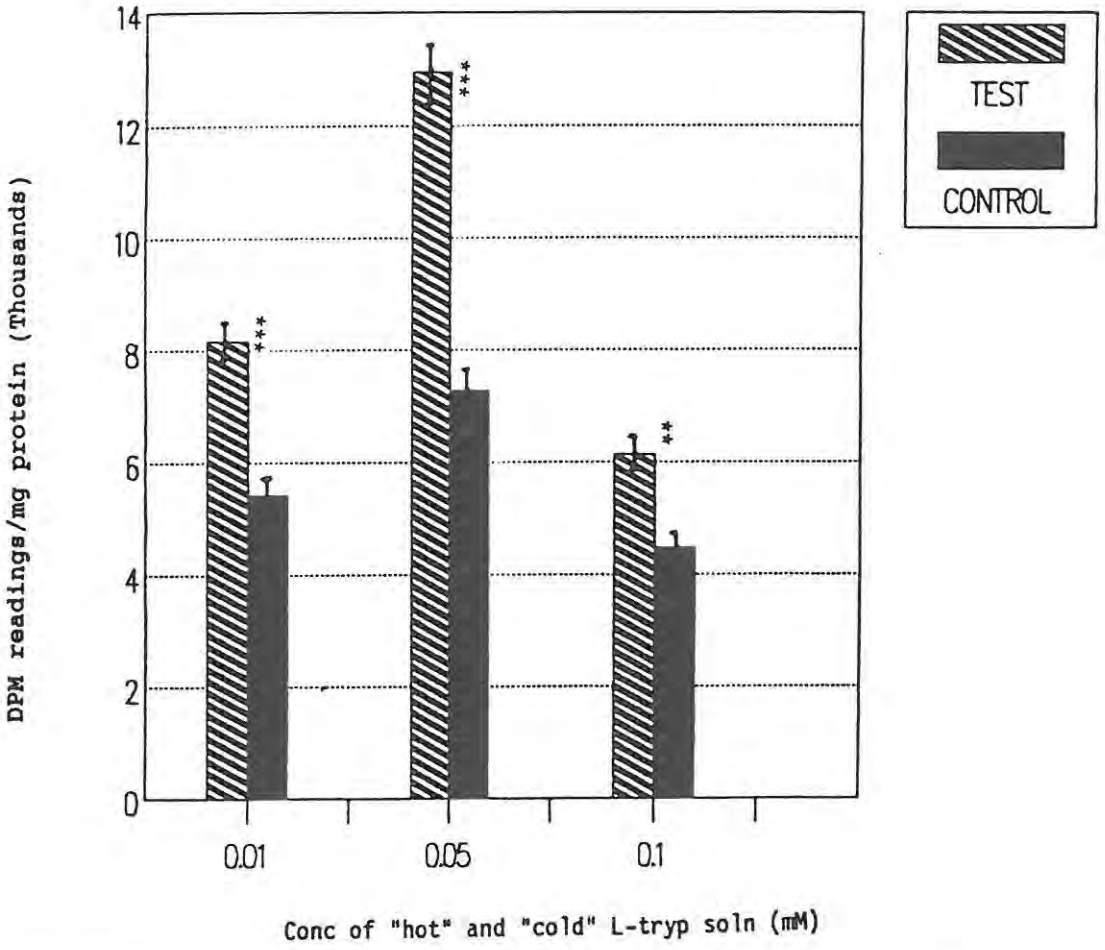


Fig. 31. effect of progesterone on radiolabelled tryptophan uptake by rat cortical synaptosomes

$^{**}p < 0.05$ $^{***}p < 0.01$

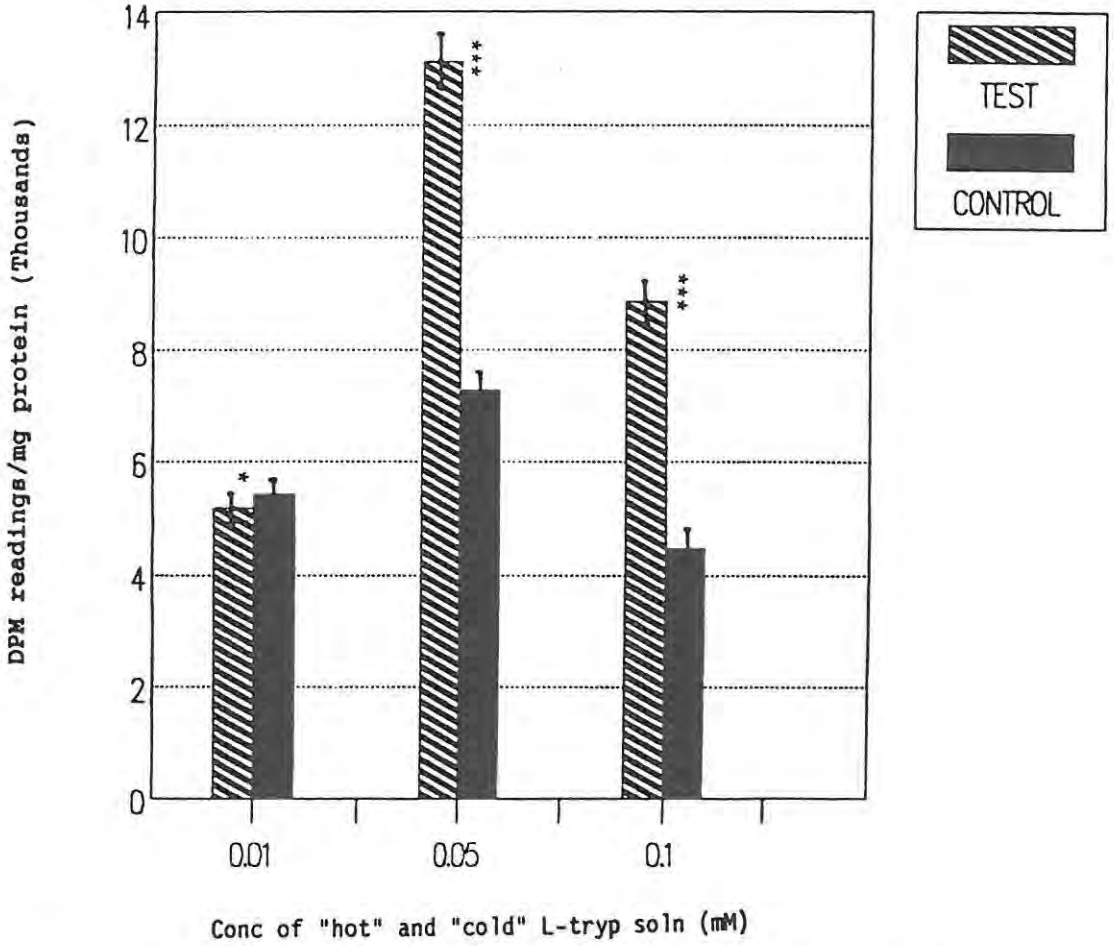


Fig. 32. effect of estradiol on radiolabelled tryptophan uptake by rat cortical synaptosomes

*N.S. $^{***}p < 0.01$

CHAPTER 5

5.1 CONCLUSION

5.1.1 INTRODUCTION

The primary objective of this work was to investigate firstly, the effect of the gonadal sex steroids on hepatic tryptophan pyrrolase activity; secondly the physiological implications of this effect on circulating levels of tryptophan; thirdly, the biochemical manifestations of these two effects on the biosynthesis of various indoles in the pineal gland of castrated male rats and ovariectomized female rats; and finally to observe how the brain uptake of L-tryptophan would be affected by the various sex steroids.

5.1.2 HEPATIC TRYPTOPHAN PYRROLASE ACTIVITY

Kynurenine concentration was quantitatively determined and this was plotted as a function of time in the linear phase for all the three sex steroids. There was a significant inductive effect by the steroids on liver tryptophan pyrrolase activity. The concentrations of the sex steroids administered were pharmacological rather than physiological and this might explain their inductive actions. Relative to testosterone and estradiol, progesterone appears to exert the highest level of induction of tryptophan pyrrolase activity.

The biochemical implication involves the following possible mechanism: In pharmacological concentrations, the sex steroids could stimulate liver tryptophan pyrrolase, "shunting" the brain and pineal metabolism of L-tryptophan in favour of the oxidative kynurenine-nicotinic acid metabolic pathway. The consequence is reduced levels of circulating tryptophan and reduced serotonin production in the brain. Deficiency of functionally-active brain serotonin has been implicated in certain depressive disorders (Lapin and Oxenkrug, 1969).

Ovariectomy and castration significantly brought about depressed levels of liver kynurenine relative to intact controls as well as castrated and ovariectomized rats which were dosed with the appropriate steroid. The highest kynurenine concentrations and therefore the highest enzyme activity were found in castrated and ovariectomized rats dosed with the steroid. This reinforces the induction concept of liver tryptophan pyrrolase by the gonadal sex steroids.

5.1.3 METABOLISM OF ^{14}C-SEROTONIN BY RAT PINEAL ORGAN CULTURES

5.1.3.1 Introduction

The technique of organ culture was used to observe the direct effects of various chemical agents on ^{14}C-serotonin metabolism by the rat pineal gland. Much of the work reported in the literature on this aspect of pineal biochemistry has been done

mainly on the two pineal enzymes, SNAT and HIOMT. However, this study investigated the relative levels of the various indole metabolites of ^{14}C-serotonin under different experimental conditions of organ culture. SNAT and HIOMT are the dominant pineal enzymes involved in the biosynthesis of the various indoles in the pineal gland and therefore the relative levels of the indoles in any particular experimental condition of organ culture would be a reflection of the relative activities of these pineal enzymes.

5.1.3.2 Effect of gonadal sex steroids on ^{14}C-serotonin metabolism

5.1.3.2.1 Testosterone

The predominant indole found in all the organ culture studies was 5-HIAA. Castration of male rats resulted in depressed levels of MEL, HIAA, MIAA and HTOH relative to intact controls as well as castrated animals whose pineals were incubated with testosterone. Castration of male rats have been found to

(i) exert inhibitory effects on pineal HIOMT activity (Nagle *et al.*, 1974),

(ii) cause a decrease in pineal concentration of cAMP (Karasek *et al.*, 1978).

The finding that the levels of the various indoles in the pineals from castrated rats which were incubated with 10nM testosterone were higher relative to the other two groups

suggests that testosterone might play a modulatory role in the activities of HIOMT and SNAT.

Daya (1985) observed that the stimulatory activity of testosterone is not dependent on prior conversion of testosterone to estradiol, suggesting a direct action of testosterone. In contrast, Cardinali *et al.*, (1974) found that the rat pineal can aromatize radiolabelled testosterone into estradiol prior to exerting biological activity. The concentration of testosterone used in this study was in the physiological concentration range. It was therefore not possible to determine what effect pharmacological concentrations of testosterone would have on SNAT. Testosterone was the only steroid capable of probably influencing SNAT activity in this study.

5.1.3.2.2 Progesterone

The levels of MEL, HIAA and HTOH in the ovariectomized rat pineal cultures incubated with progesterone were significantly higher than the pineal cultures incubated with the vehicle. This suggests that at physiological concentrations, progesterone has a stimulatory role in the melatonin synthesis pathway.

Reports on the effects of progesterone on ^{14}C-serotonin metabolism in pineal organ cultures are few. Daya (1982) observed a biphasic dose-dependent effect of progesterone on HIOMT activity.

5.1.3.2.3 Estradiol

The results here show a significant increase in the MEL levels with a concomitant decrease in NAS levels in the ovariectomized rat pineal cultures incubated with estradiol relative to ovariectomized rat pineals incubated with the vehicle as well as the intact controls. Estradiol might probably have potentiated HIOMT activity but not SNAT activity. The concentration of estradiol used was in the physiological range; however, Mizobe and Kurokawa, (1976) have established that estradiol influences HIOMT activity in a biphasic dose-dependent manner.

That estradiol has no modulatory effect on SNAT activity lends support to Daya's (1985) observation that testosterone does not depend on prior conversion to estradiol before exerting biological activity since testosterone was found in Daya's (1982) study to stimulate SNAT activity.

5.1.4 EFFECT OF GONADAL SEX STEROIDS ON THE NIGHTTIME METABOLISM OF ^{14}C-SEROTONIN BY RAT PINEAL ORGAN CULTURES

5.1.4.1 Testosterone

Comparing these nighttime results to the daytime study, the levels of MEL were significantly higher in the former study. The diurnal variation in pineal melatonin synthesis was therefore evident. From the relative levels of indoles in the

various experimental groups, castration resulted in a decrease in the nocturnal elevation of MEL levels. Castration therefore possibly depresses SNAT nocturnal activity. Testosterone replacement restored the reduced nocturnal MEL levels.

5.1.4.2 Estradiol

Ovariectomy brought about reduced MEL synthesis comparing the various experimental groups. There were signficantly higher MEL levels in the pineal cultures of intact rats compared to the pineals from ovariectomized rats which were incubated with estradiol as well as pineals from ovariectomized rats which were incubated with the vehicle.

In this study, estradiol, once again, was found to have no modulatory effect on the melatonin synthesis pathway.

5.1.5 CUMULATIVE EFFECT OF TESTOSTERONE AND ISOPRENALINE ON ^{14}C-SEROTONIN METABOLISM IN ORGAN CULTURE

Isoprenaline, a ß-adrenergic agonist, like norepinephrine can stimulate cAMP formation via the ß-adrenergic mechanism for enhanced production of pineal melatonin. In this study, testosterone was found to potentiate the isoprenaline response.

5.1.6 EFFECT OF GONADAL SEX STEROIDS ON L-[^{14}C]-TRYPTOPHAN UPTAKE BY RAT CORTICAL SYNAPTOSOMES

All three sex steroids used in this study significantly enhanced the uptake of L-tryptophan by the cortical synaptosomes. This may be interpreted as a compensatory mechanism effected by the sex steroids to make up for the reduced levels of circulating tryptophan - a consequence of the induction of liver tryptophan pyrrolase activity by the gonadal sex steroids.

5.1.7 GENERAL

The gonadal sex steroids significantly influenced the daytime as well as the nighttime levels of various indoles in the course of the metabolism of ^{14}C-serotonin by rat pineal organ cultures. Considering the relative indole levels, it becomes evident that the sex steroids exerted modulatory effects on HIOMT activity. In case of SNAT, it was only testosterone which might have possibly stimulated this enzyme bringing about enhanced synthesis of melatonin.

Testosterone would therefore appear to play certain biochemically vital roles in the regulation of pineal function.

There have been numerous reports on the effect of pineal function on regulation of the gonads but very little information on the regulation of pineal function by the sex steroids is available. From the results, it does appear that the sex

steroids have salient modulatory roles in the regulation of pineal biochemistry. The pineal gland therefore appears to be a target hormone for the sex gland.

CHAPTER 6

SUMMARY

CHAPTER 1

A comprehensive overview of the location, anatomy, morphology, circadian rhythms and various biochemical interactions between the pineal gland and the gonads (mediated by the gonadal sex hormones and melatonin, a putative hormone) was discussed. Also discussed was the biochemical significance of the regulatory roles played by the sex steroids on liver enzyme, tryptophan pyrrolase, and the relevance of these roles in certain clinical disorders.

CHAPTER 2

The effects of testosterone, estradiol and progesterone on liver tryptophan pyrrolase activity were investigated. All the three sex steroids significantly brought about induction in the enzyme activity with progesterone demonstrating the highest level of induction. The concentration of all the sex steroids used was above the physiological range.

Ovariectomy and castration significantly brought about depression in tryptophan pyrrolase activity.

CHAPTER 3

The effects of the gonadal sex steroids on the levels of various indoleamine metabolites in the metabolism of ^{14}C-serotonin by pineals from ovariectomized and castrated rat organ cultures during the daytime as well as the nighttime were investigated.

The nocturnal levels of MEL were relatively higher in the two time groups, a finding consistent with previous reports on the circadian variation of melatonin synthesis. The testosterone-treated cultures were found to have higher levels of the various indoles, particularly MEL, MIAA and HTOH relative to the other cultures treated with the vehicle. This strongly suggested a stimulatory effect of testosterone on SNAT and HIOMT, the two prominent enzymes in the indoles synthesis pathways.

Comparing the relative indole levels in the various cultures, it does appear that estradiol and progesterone positively influenced HIOMT activity even though they probably failed to modulate SNAT activity. Since only physiological concentrations of these hormones were used in these studies, it would not be possible to comment on how the activities of the two pineal enzymes would respond to very high (pharmacological) or very low steroid concentrations.

Castration and ovariectomy were found to depress the levels of the various indole metabolites. However, ovariectomy and treatment with estradiol and progesterone did not affect MEL

levels in the various cultures. Castration depressed the nocturnal elevation in MEL levels. Testosterone replacement restored these levels.

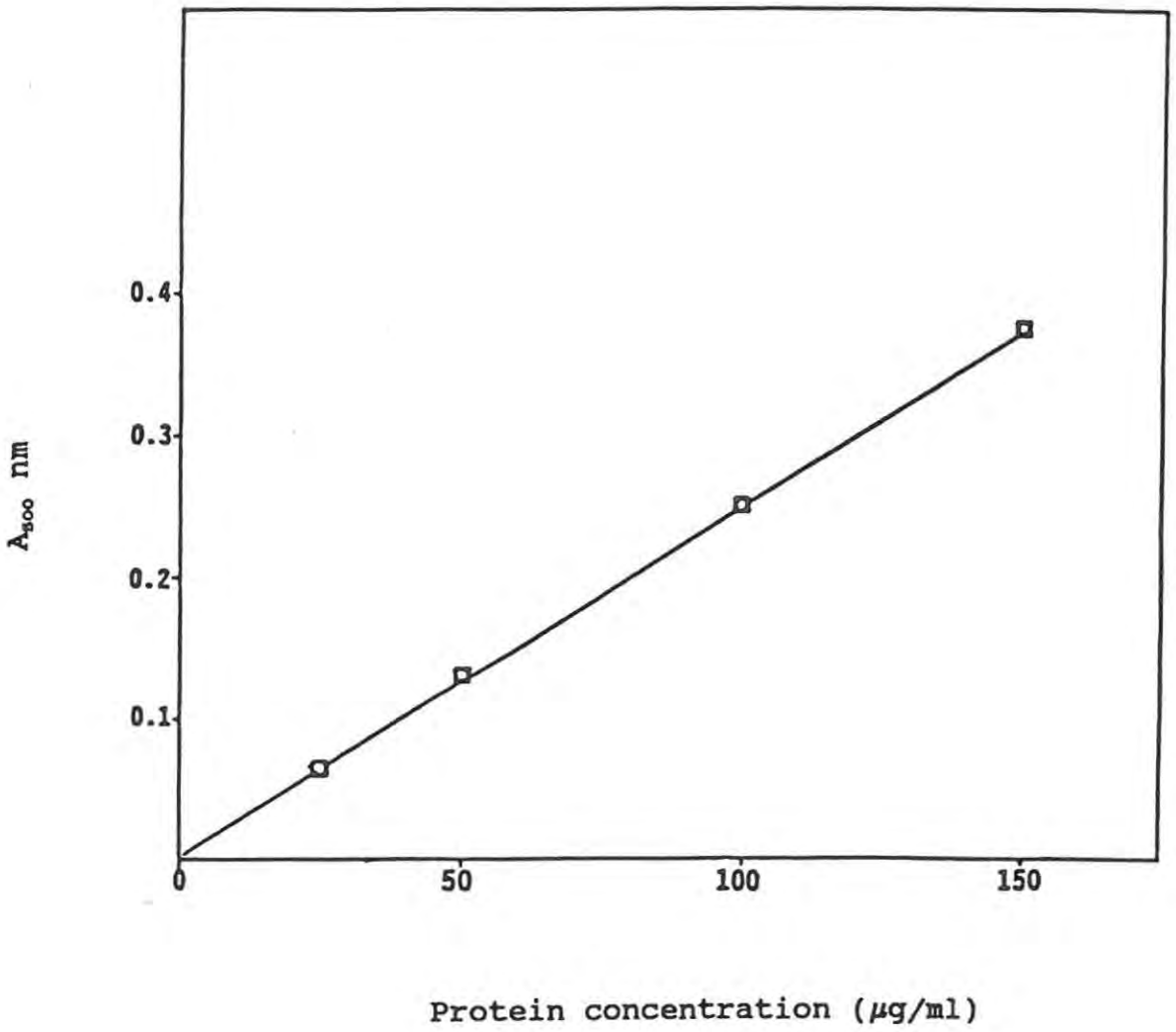
The cumulative effect of testosterone and isoprenaline on MEL levels in rat pineals in organ cultures was also investigated. MEL levels in cultures incubated with isoprenaline and testosterone together were significantly higher than those in cultures incubated with testosterone alone or with the vehicle. This suggested a potentiation of the isoprenaline response by testosterone.

CHAPTER 4

The influence of the gonadal sex steroids on the uptake of radiolabelled tryptophan by rat cortical synaptosomes was investigated. This study involved using three solutions of mixed "hot" and "cold" tryptophan of different concentrations. There was a significant increase in tryptophan uptake in the incubation media of all three sex hormones as well as controls from a concentration of 0,01mM to 0,05mM but the uptake was found to decrease from 0,05mM to 0,1mM in all the experimental groups.

The results also suggest that the gonadal sex steroids significantly enhanced the level of tryptophan uptake by the cortical synaptosomal preparations, relative to the control group. As explained previously, this may be a compensatory

mechanism effected by the sex steroids to make up for the reduced levels of circulating tryptophan - a consequence of induction of liver tryptophan pyrrolase activity by the gonadal sex steroids.



Appendix 1. Protein standard curve.

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