THE INFLUENCE OF SEX STEROIDS ON PINEAL ENZYMES

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LIST OF ABBREVIATIONS USED

AMP	Adenosine Monophosphate
ACTH	Adrenocorticotropic Hormone
CAST	Castrated
Ci	Curie
CNS	Central Nervous System
CSF	Cerebrospinal Fluid
c.p.m.	Counts per minute
DNA	Deoxyribonucleic Acid
d.p.m.	Disintegrations per minute
ESCR	External Standard Channels Ratio
FSH	Follicle-stimulating Hormone
FSH-RF	Follicle-stimulating Hormone Releasing Factor
GABA	Gamma Amino Butyric Acid
GH	Growth Hormone
GMP	Guanosine Monophosphate
HCL	Hydrochloric Acid
H1OMT	Hydroxyindole-O-methyltransferase
HIAA	5-Hydroxyindoleacetic Acid
HTOH	5-Hydroxytryptophol
5-HT	Serotonin
5-HTP	5-Hydroxytryptamine
i.p.	intraperitoneal
i.v.	intravenous
L-Dopa	L-Dihydroxyphenylalanine
LH	Luteinizing Hormone
LH-RH	Luteinizing Hormone Releasing Hormone
MAO	Monoamine Oxidase
MEL	Melatonin

MEM	Minimum Essential Medium
MIAA	5-Methoxyindole Acetic Acid
MTOH	5-Methoxytryptophol
MT	5-Methoxytryptamine
NA	Noradrenaline
NAS	N-Acetylserotonin
OVX	Ovariectomized
RNA	Ribonucleic Acid
S.I.F.	Small Intensely Fluorescent
SNAT	Serotonin-N-Acetyltransferase
T3	Triiodothyronine
TLC	Thin-Layer Chromatography
TRH	Thyrotropin Releasing Hormone
TSH	Thyroid Stimulating Hormone.

Omissions

BM	Body Mass
CVS	Cardio Vascular System

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A B S T R A C T

The influence of the gonadal sex steroids namely, estradiol, progesterone and testosterone on the two major enzymes responsible for the synthesis of melatonin in the pineal gland was investigated.
These enzymes are Serotonin-N-acetyltransferase (SNAT) and Hydroxyindole-O-methyltransferase (H1OMT).

Testosterone was found to be the only sex steroid capable of influencing SNAT activity whereas all three of the sex steroids were found to influence H1OMT activity in a bi-phasic dose-dependent manner.

The influence of these sex steroids on radiolabeled serotonin metabolism by pineals in organ culture was also investigated.

Ovariectomy, castration and the sex steroids were all found to alter the pattern of the radiolabeled serotonin metabolism by these pineal glands in organ culture.

CHAPTER 1

LITERATURE REVIEW

1.1 HISTORY OF PINEAL RESEARCH

The history of pineal research can be divided into three periods.

1.1.1 The First Period

Herophilos (325 - 280 B.C.), a famous anatomist at the University of Alexandria in Egypt, was the first to discover the pineal organ in man. He regarded the pineal as a tap, regulating the flow of the spirit from the third to the fourth ventricle. According to Galen ($\pm$ 130 B.C.), the pineal was just a "gland" like other glands, filling the gap between branching vessels and supporting them. The true function of glands was still quite unknown. It was Galen who termed the pineal organ "soma konoeides" because the organ resembles the cone of a pine tree.

Berengario da Capri (1470 - 1530) was the first to examine the pineal gland of man more carefully. Vessal (1555) described the topography and consistency of the pineal gland but did not say much about its possible function. Descartes (1596 - 1650) is cited as stating that the pineal gland is the seat of the soul. At this time the problem of the localization of the soul was very much in the mind of both philosophers and scientists.

The seat of the soul and other related theories haunted neuroscience and delayed progress in this field for more than 20 centuries.

1.1.2 The Second Period

The beginning of the second era in pineal research is

characterized by a renewed and more systematic interest in pineal comparative anatomy, histology and embryology.

De Graaf (1886) and Korschelt and Spencer (1905) reported that phylogenetic regression of the pineal was evident. Studnicka (1905) stated that phylogenetically, the pineal develops from a photoreceptor organ into a complicated gland having an enigmatical function.

Following the discovery of endocrine glands by Claude Bernard (1813 - 1878) and Brown-Sequard (1817 - 1894), the interest of physiologists and clinicians became more intense. At the end of the 19th century the mammalian pineal gland was already considered a serious candidate for hormone production.

Heubner (1898) first described a boy suffering from a pinealoma and showing signs of precocious puberty. Berblinger (1920) and Engel (1936) were quite convinced that the pineal gland showed inhibitory activity on gonadal development, being of the opinion that its extract was antigonadotropic.

Bargman (1943), after dealing with the microscopic anatomy and histology of the pineal, stressed the necessity of investigations on the influence of light on the pineal.

1.1.3 The Third Period

In the third period, history of pineal research blends with present research. Pinealogy has reached adulthood.

1.1.4 "Gland" or Neuroendocrine Transducer?

For several years, numerous researchers directed their efforts toward delineating the glandular characteristics of the pineal, only to reach a very disappointing conclusion. The pineal

could not be termed a gland because it did not satisfy the operational criteria for an endocrine organ.

Traditionally, three criteria must be satisfied to demonstrate that a particular structure is a gland :

1) that removal produces a noticeable change elsewhere in the body.
2) that injection of an extract of the gland or of its products reverses this change and re-establishes the original function, and
3) that if the organ is transplanted to another anatomic site, it functions normally and thus keeps the body from developing the syndrome associated with its removal.

This traditional definition of a gland was too restrictive for the pineal in that it failed to encompass the concept of the neuroendocrine transducer i.e. a cell that secretes its own hormone in response to a neurotransmitter released locally.

The role of the pineal cells appear to be to convert such a neural input (noradrenaline released from its sympathetic neurons) into a hormonal output (Melatonin). Hence the term neuroendocrine transducer seems to be more appropriate.

1.2 PINEAL ANATOMY

1.2.1 Location

The pineal gland of the rat is superficially situated in the rat brain, just rostral to the cerebellum and between the occipital poles of the cerebral hemispheres. Dorsally, it is covered by the confluence of the superior sagittal and transverse sinuses.

1.2.2 The pineal stalk

In the albino rat, the pineal keeps its connection with the commisural region by means of a very slender pineal stalk. During development, the stalk is formed by fusing and growing out of the anterior and posterior epiphyseal peduncles originating from the most proximal parts of the rostro-dorsal and caudo-ventral walls of the original epiphyseal evagination.

Topographically, the stalk can be divided into three parts, namely, a proximal part, a midpart and a distal part. The proximal part lies between the habenular and caudal commissures. The midpart is often extremely thin. The distal part is the longest of the three structures and is situated between both the rostral and caudal collicles.

1.2.3 Blood Supply

The pineal has an independent arterial supply consisting of up to four branches of the posterior cerebral artery. The capillary network is at least as dense as in the cerebral cortex, and the venous drainage consists of 12 to 16 short veins, all draining into the great cerebral vein and thereafter via the confluens sinuum immediately in the systemic venous circulation (Hodde 1979).

1.2.4 Nervous innervation.

Generally, the pineal has two types of innervation, (1) a pinealofugal (afferent) and a pinealopetal (efferent) one. Pinealofugal innervation is exclusively observed in non-mammalian pineals containing functional photoreceptor cells.

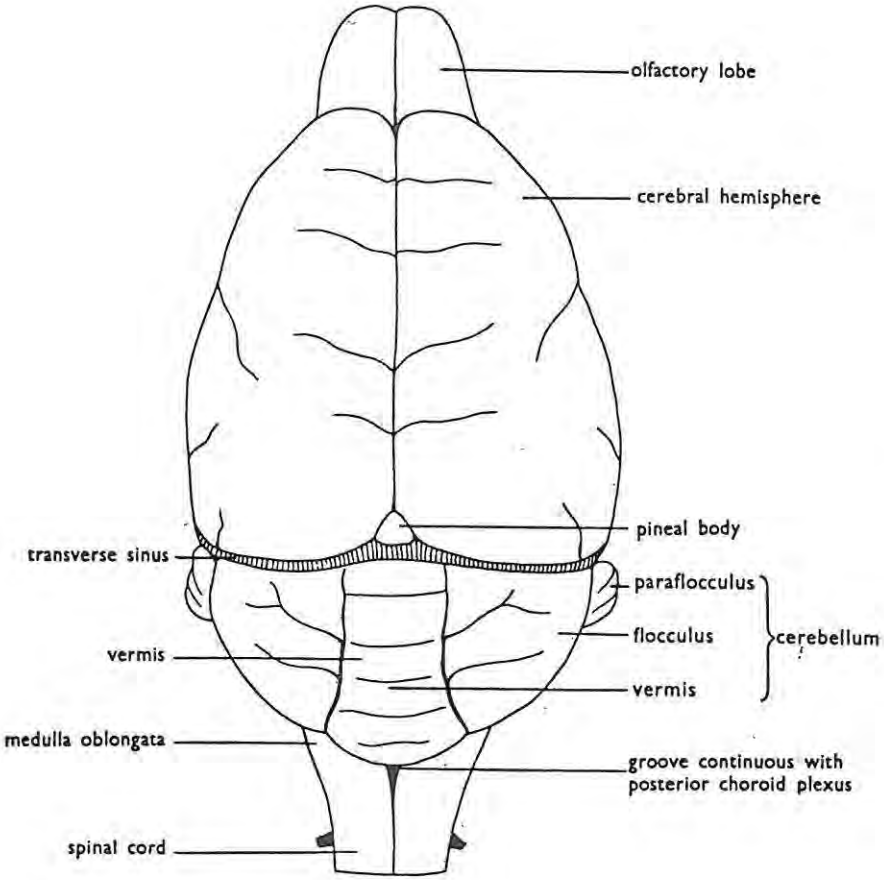

FIG. 1. Dorsal view of the rat brain.

(Rowett, 1968)

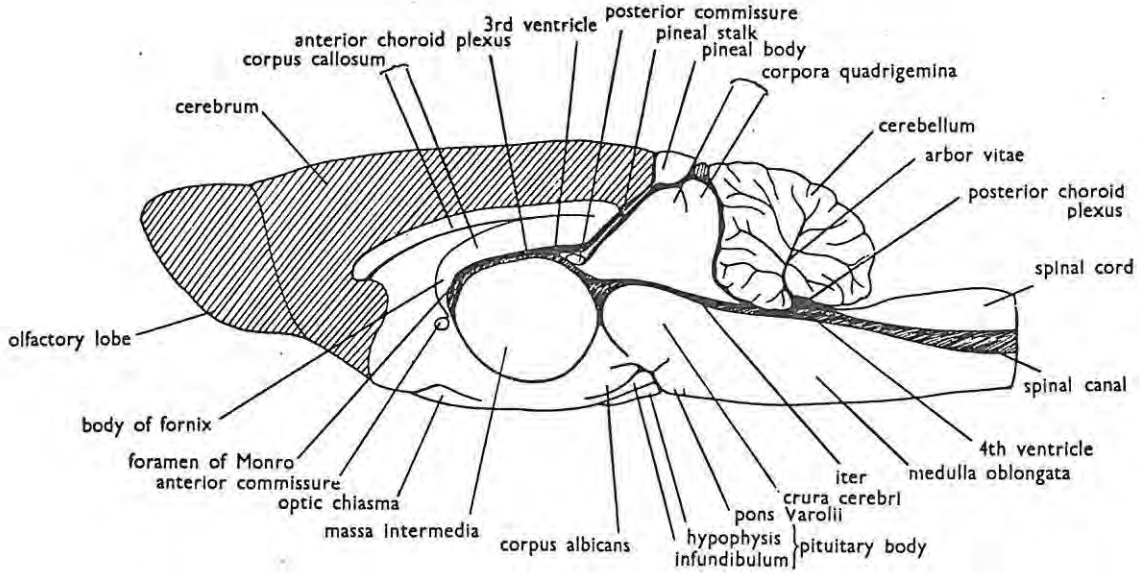

FIG. 2. Median sagittal view of the rat brain.

(Rowett, 1968)

The mammalian pineal gland is richly innervated by pinealopetal orthosympathetic fibres which originate in the superior cervical ganglia and reach the organ by way of the nervii conarii and along pineal vessels (Kappers, 1960). The importance of this sympathetic pineal innervation has been demonstrated to be of primary importance for the pineal secretory function in mammals. Wolfe et al., 1962 using autoradiography, showed that the neurotransmitter involved here, is noradrenaline.

Kenny, 1961; Romijn, 1973a,b; 1975, demonstrated the presence of a parasympathetic pinealopetal innervation next to a sympathetic innervation in some mammals. Their function so far remains unknown. The terminals of both these types of fibres do not form true synaptic contacts with the pinealocytes, but end freely in the parenchyma.

Aberrant commissural fibres often form hairpin loops in the proximal part of the pineal (Kappers, 1960). Recent electrophysiological investigations (Dafny et al.,1975; McClung and Dafny, 1975, Dafny, 1977; Cleck 1979) reveal that stimuli originating in parts of the limbic system of the brain run to the pineal via the medullary striae, the habenular nuclei and the pineal stalk.

Fairly recently, indirect histophysiological, histochemical and biochemical evidence for the presence of extrahypothalamic neurosecretory fibres in the pineal gland has been obtained in cattle and sheep (Lukaszyk and Reiter, 1975), the rat (Pévet, et al., 1978, 1979) and the monkey (Lukaszyk and Reiter, 1974). The function of such pineal neurosecretory fibres is unknown.

1.3 THE PINEAL CELLS

Different categories of cells in the mammalian pineal gland have been described by many authors. There is still a lot of confusion concerning the nomenclature of the pineal parenchymal cell categories. Most authors agree that the major part of the pineal parenchyma is composed of pinealocytes. However, different opinions exist as to the number of populations of pinealocytes present. Besides pinealocytes, another category of parenchymal cells which differ morphologically from pinealocytes has been described by Wolfe (1965). These cells are usually referred to as interstitial cells, dark cells, glial cells etc.

A common feature of pineal cells in the first category of cells, normally referred to as pinealocytes, is the formation of granular vesicles. The second category of pineal cells differ from the first in their morphological aspect. They are located close to the perivascular space and do not have granular vesicles (Pévet, 1977). According to Upson et al. (1976), these granular vesicles contain the antigonadotropic hormone(s).

1.3.1 Ultrastructure of the Pineal Cell

1.3.1.1 Cytoplasm

Individual ribosomes and clusters of 4 to 12 ribosomes are scattered throughout the cytoplasm and do not form any association with 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. The epiphyseal endoplasmic reticulum lacks the crisp tubular profiles, compactness and the pattern of branching characteristic of an agranular endoplasmic reticulum.

The cytoplasm contains numerous lipid inclusions, 1 to 4 u in width and ranging from globular to scalloped forms.

Mitochondria in epiphyseal cells are moderately abundant. Milofsky (1958) observed "gaint" mitochondria in epiphyseal cells. These are up to 4 or 5 μ long.

Vernate bodies, consisting of 3 to 20 irregular lamelliform convolutions surrounding a central zone of ill-defined granular material in which variable numbers of glycogen particles, lipid droplets, mitochondria, vesicles and grumose granules are present. It appears that attenuated mitochondria are involved directly in the morphogenesis of vernate bodies.

Synaptic ribbons occur singly or in clusters of 2 to 8 in the perikarya and polar processes of these cells, usually situated just beneath the plasmalemma.

The presence of a microtubular bouquet is the most striking component of the epiphyseal cell. 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 micropinocytosis. Grumose globules, containing fine membranous and granular matter distributed evenly in a dense amorphous matrix are present but usually sparsely distributed.

1.3.1.2 Nucleus

The nuclear envelope 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.

1.4 PINEAL BIOCHEMISTRY

1.4.1 Melatonin

In 1917, McCord and Allen discovered that extracts from bovine pineals contained a substance which lightened the skin colour of amphibians. In 1957, Lerner and coworkers set out to isolate the substance which caused this effect. A year later (Lerner et al. 1958) succeeded in isolating the substance which they demonstrated to be a methoxyindole. This was identified to be N-acetyl-5-methoxytryptamine (Lerner et al., 1959; Lerner and Case, 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).

1.4.1.1 Tissue Distribution

Its tissue distribution is unique in that at any one time, most of the melatonin present in a mammal is located within its pineal gland. It is a very lipophillic substance and hence its access to the CNS is not hindered by a blood brain barrier.

Melatonin disappears from the whole mouse in several metabolic phases. In the first ten minutes after injection, there is a rapid decrease in total body ^{3}H-melatonin, with a half-life of about 2 minutes (Kopin et al., 1961). After 40 minutes, the rate of disappearance of melatonin is much slower, corresponding to a half-life of 35 minutes.

One hour after ^{3}H-melatonin is injected i.v. into the cat, it concentrates in the pineal gland, parts of the eye and the ovary. Other endocrine tissues, peripheral nerves and the sympathetic nervous chain also selectively take up labelled melatonin, but to a smaller 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).

Peripheral nerves contain endogenous melatonin (Barchas and Lerner, 1964) but cannot synthesize this indole (Axelrod and Weissbach, 1961). Since nerves can take up melatonin from the circulation, this is taken as indirect evidence that the pineal secretes melatonin into the blood stream. Anton-Tay and Wurtman (1969) showed that melatonin concentrates a hundred times more in the brain if injected into the CVS. Recently, Kopp et al.,(1980) demonstrated the presence of melatonin in the human brain. The melatonin level in the brain was fifty times lower than that of the pineal gland.

Reppert and Klein, (1978) found that melatonin is rapidly transferred from the maternal circulation into lactating mammary tissue and found in the stomachs of the suckling rats from where it is absorbed.

Bubenik et al. (1978) demonstrated the presence of melatonin in rat retina and Harderian gland. This was confirmed by Pang et al., (1980).

During the dark period, melatonin is detectable in rat and human plasma (Lynch and Wurtman, 1972; Arendt, 1977). This is the case in nocturnal and diurnal animals.

About 60 - 80% of the hormone in the plasma is bound to serum albumin (Cardinali et al, 1972).

1.4.1.2 Biosynthesis of Melatonin and other Pineal Indoles

1.4.1.2.1 Melatonin

The biosynthesis of melatonin commences with the uptake of circulating tryptophan by the parenchymal cells of the pineal gland. A major portion of this is 5-hydroxylated to 5-hydroxy-tryptophan. This conversion is catalysed by tryptophan hydroxylase (Lovenberg et al., 1967; Jequier et al., 1969) which may differ from the one in the brain (Nakamura et al., 1965). It requires the presence of oxygen, ferrous iron and a reduced pteridine cofactor (Lovenberg et al., 1962; Snyder and Axelrod, 1964). The 5-hydroxytryptophan is then decarboxylated to form serotonin (Lovenberg et al, 1962; Snyder and Axelrod, 1964), which is found in very high concentrations in the pineal (Giarman and Day, 1959). The enzyme involved here is the pyridoxal dependent aromatic -L-amino acid decarboxylase (Snyder and Axelrod, 1964a). This enzyme has a concentration in the pineal greater than in any other tissues examined (Snyder and Axelrod 1964b). The next step involves the acetylation of serotonin to form N-acetylserotonin by the enzyme serotonin-N-acetyl transferase (SNAT), acetyl CoA being the donor of the acetyl group (Weissbach et al.,1960). Finally, the N-acetylserotonin is converted to melatonin by o-methylation in the 5-position. This step is catalysed by the enzyme, hydroxyindole-o-methyl-transferase (HIOMT) (Axelrod and Weissbach, 1960; 1961; Cardinali and Wurtman,1972; Axelrod and Lauber, 1968).

1.4.1.2.2. Synthesis of other Pineal Indoles (See Fig. 3)
Serotonin is also oxidized 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.

1.4.1.3 Neural Control of Pineal Indole Biosynthesis
The mammalian pineal gland receives most or all of its innervation via postganglionic sympathetic neurons, which originate in the superior cervical ganglia (Kappers, 1960) and terminate near the pineal parenchymal cells and blood vessels (Milofsky 1957). The density of the nerve terminals contain relatively large quantities of serotonin (Giarman and Day, 1959) in addition to noradrenaline. This phenomenon could possibly reflect competition for noradrenaline storage sites between catecholamine and serotonin molecules, which are present in very high concentrations within neighbouring pineal parenchymal cells (Owman, 1965). Rat pineal cells can concentrate another presumed transmitter, γ-aminobutyric acid (GABA), in vitro (Schon et al.,1975).

The effects of noradrenaline on pineal metabolism have been most thoroughly studied using a rat pineal organ culture system in which individual pineals are incubated for up to 48 hours

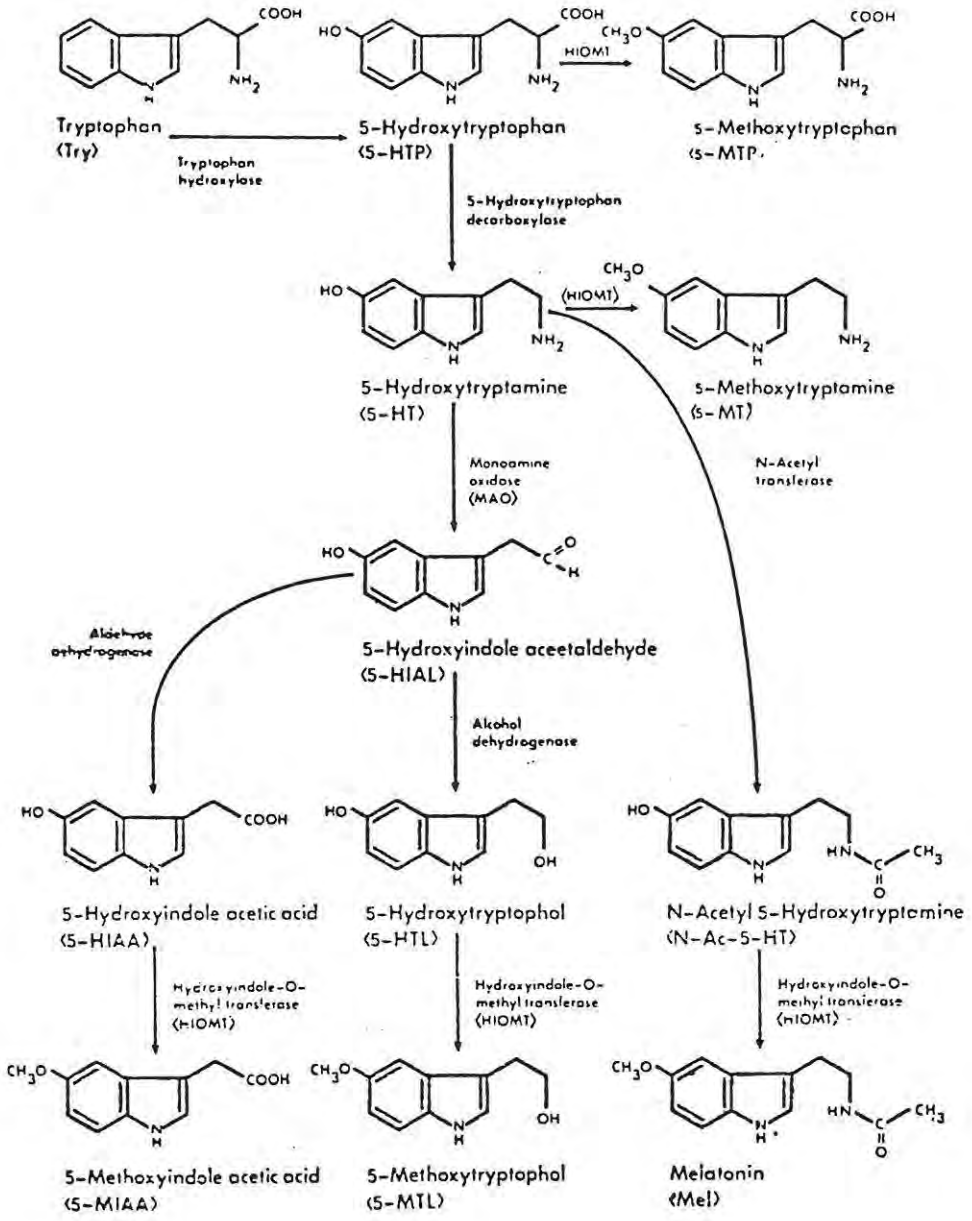

Fig.3. The indole metabolism in the pineal gland.

with synthetic media containing isotopically labelled tryptophan (Klein 1969) and (Shein and Wurman, 1969). The pineals take up the tryptophan readily and incorporate it into proteins (Wurtman et al.,1969) or metabolize it to 5-hydroxy and 5-methoxyindoles (Axelrod et al.,1969). Noradrenaline accelerates the synthesis of serotonin and melatonin in cultured pineals by a process involving the α-adrenergic system (Wurtman et al. 1971 ; Deguchi and Axelrod, 1972). The evidence that cyclic AMP participates in the control of melatonin synthesis is threefold:-

1) Noradrenaline activates adenyl cyclase in pineal homogenates (Weiss and Costa, 1967; Uzunov and Weiss, 1971; Strada and Weiss, 1974).
2) Dibutyryl cyclic AMP, but not cyclic AMP itself, accelerates melatonin synthesis from radioactive tryptophan in cultured pineals (Shein and Wurtman 1969; Berg and Klein, 1971).
3) Dibutyryl cyclic AMP was found in some studies, to enhance pineal SNAT activity (Deguchi and Axelrod, 1972; Klein et al., 1970).

Wurtman et al., (1968) outlined the pathway along which light reaches the pineal as follows :-
Light → retina → inferior accessory optic tract → medial forebrain bundle → preganglionic sympathetic nerves → superior cervical ganglion → postganglionic sympathetic nerves → pineal gland.

The onset of darkness activates the sympathetic nerves to the pineal (Taylor and Wilson, 1970) causing major increases in the activities of SNAT and H1OMT, the two enzymes that catalyse the conversion of serotonin to melatonin (Wurtman et al.,1963;

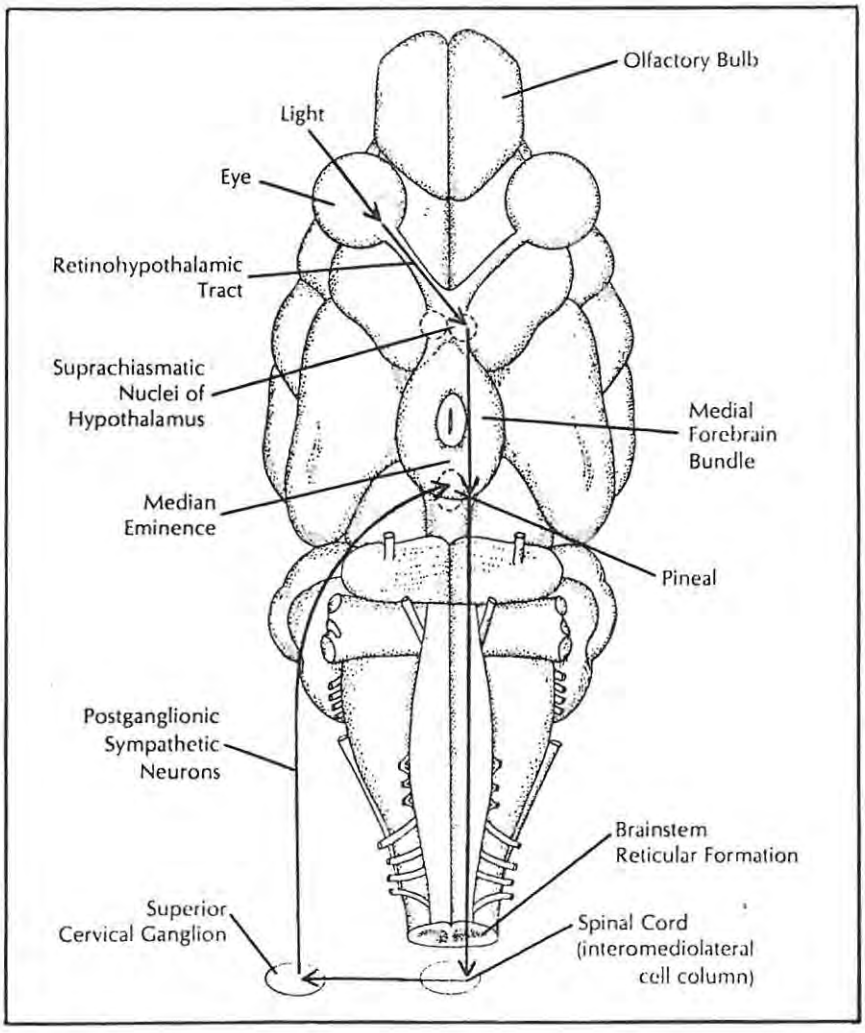

FIG. 4 . Neural pathways to the pineal gland.

(Wurtman, 1980)

Klein and Weller, 1970).

The transmission of information about light from the retina to the pineal utilizes at least two pathways. One involves fibres that run in the optic nerves to the optic chiasm and then cross, becoming the inferior accessory optic tract. The other involves fibres that run directly from the retina to the suprachiasmatic nuclei of the hypothalamus. In both cases, numerous additional neurons and synapses are needed to bring the information to the superior cervical ganglia of the sympathetic nervous system, the source of the noradrenaline-releasing terminals in the pineal gland (Wurtman, 1980).

Continuous darkness and blinding, abolish the circadian changes in enzyme activity, while the same effect is produced by pineal denervation (Wurtman et al., 1964; Wurtman and Axelrod, 1965a; Axelrod et al.,1965).

Levy et al., (1980) showed that bright artificial light suppressed nocturnal secretion of melatonin in six normal human subjects. Using different degrees of brightness of lighting, they established that the human response to light is qualitatively similar to that of other mammals.

1.4.1.4 Melatonin binding sites in the Pineal Gland.

Vacas and Cardinali (1980) demonstrated the presence of melatonin binding sites in bovine pineal glands using a rapid filtration procedure. Specific binding was thermolabile and decreased following incubation with trypsin. The binding was pH dependent with maximum binding at physiological pH.

They tested various melatonin analogues for their ability to inhibit ^{3}H-melatonin binding and showed these to be inhibitory

in the following order :- 6-hydroxymelatonin > 5-hydroxy-tryptophan > 5-methoxyindole acetic acid > 5-hydroxy-indole acetic acid > tryptamine > 5-methoxytryptophol > 5-methoxy-tryptamine > 5-hydroxytryptophol > 2-methyl indole > serotonin > N-acetyl serotonin.
These authors propose that melatonin receptors may be present in the pineal gland.

1.4.1.5 The Pineal Adrenoreceptor

After it had been established that the pineal was sympathetically innervated (Kappers, 1960) and that noradrenaline was the neurotransmitter (Wolfe et al.,1962; Pellegrino de Iraldi and Zieher, 1966), attempts were made to classify the pineal adrenoreceptor.

Wurtman et al., (1971) using pineal organ cultures showed that the noradrenaline-induced stimulation of pineal synthesis of ^{14}C-melatonin and ^{14}C-serotonin is inhibited by propranolol, a β-adrenergic blocker, and not by phenoxybenzamine, an α-adrenergic blocker. This observation indicated that the mechanism of stimulation involved interaction with "classic" β -receptors.

Bäckström, (1977) using the selective β_1 and β_2 agonists and antagonists in rat pineal organ cultures, showed that the adrenoreceptors in the rat pineal respond similarly to the β_1 adrenoreceptor subgroup.

Stimulation of the β-receptor activates adenyl cyclase (Weiss and Costa, 1967; Weiss, 1971; Weiss and Strada, 1972). Experiments of Zatz and O'Dea (1976) proved that the effect of cyclic AMP on SNAT activity is mediated by a protein kinase. This implies that SNAT activity depends on phosphorylation

by a protein kinase.

1.4.1.5.1 The phenomenon of supersensitivity and subsensitivity of the β -adrenergic receptor in the pineal gland.

The responsiveness of a pineal cell is dependant on the previous exposure of the β-adrenergic receptor of the cell to the neurotransmitter, noradrenaline. When the noradrenergic activity is abolished by denervation or reduced by reserpine, decentralization or by continuous lighting, the sensitivity of the β-adrenergic receptors in the pineal with respect to the response of cyclic AMP or SNAT in greatly enhanced. About 10% of the concentration of the neurotransmitter can achieve the same magnitude of induction of SNAT in the sensitized pineals when compared to the concentrations required by the desensitized pineals. The dose-response curve of SNAT induction and cyclic AMP elevation is shifted to the left by depletion of neurotransmitter. The maximum responsiveness, however, does not change. Conversely, if the number of catecholamine molecules reacting with the β-adrenergic receptor is increased for a period of time, the pineal adrenoreceptor becomes less responsive to its agonist i.e. larger amounts of catecholamine are necessary to produce the same degree of increase in SNAT activity. The dose-response curve of SNAT induction is shifted to the right by repeated administration of agnoist (Deguchi and Axlerod, 1973). This phenomenon could be due to either a change in the conformation of the β-receptor or a change in the number of available receptors. This phenomenon occurs diurnally as the amount of catecholamine varies in relation to the photo-period (Romero and Axelrod,1974).

Romero and Axelrod, (1975) showed that in the case of supersensitivity, there was a lag period prior to the induction of SNAT. However, in the case of subsensitivity (which results in reduced SNAT activity) induction is far more rapid in onset. This implies that a precursor is necessary before final production of enzyme protein.

Experiments carried out by Kebabian et al.,(1975) and Romero et al.,(1975), indicate that the phenomenon of super- and subsensitivity can be attributed to an increase or decrease in the number of β-adrenergic receptors available.

Results obtained by Wilkonson (1978), indicate that the sensitivity of pineal β-receptors appears to be dependent upon calcium ions, possibly by controlling enzyme induction at an intracellular site beyond the β-receptor.

1.4.2. Effect of Drugs on Pineal Indole Synthesis

1.4.2.1. Serotonin

The rate at which rat pineals synthesize serotonin can be increased by adding 5-hydroxytryptophan to, or elevating the tryptophan concentration of the culture medium (Shein et al., 1967; Wurman et al., 1967), although it has been reported that high tryptophan concentrations can inhibit total tryptophan hydroxylation in cultured pineal glands (Bensinger et al., 1974). L-Noradrenaline lowers the serotonin content of cultured pineals, possibly by increasing the conversion of the indole to N-acetylserotonin (Klein et al.,1973). This effect is blocked by propranolol (Wurtman et al.,1971). Mescaline increases the conversion of ^{14}C tryptophan to ^{14}C serotonin by cultured pineals, an effect that is not mimicked by LSD or psilocybin (Shein et al.,1971).

In vivo, pineal serotonin levels are increased by injecting tryptophan (Snyder and Axelrod 1965; Zweig and Axelrod, 1969; Deguchi and Barchas, 1972) or 5-hydroxytryptophan (Snyder and Axelrod 1965), and decreased by p-chlorophenylalanine (pCPA) (Deguchi and Barchas, 1972), an inhibitor of both tryptophan hydroxylase and catechol synthesis (Wurtman et al 1974), or by R04 - 4602 (Hyyppa et al.,1971), an inhibitor of aromatic L-amino acid decarboxylase. The daily rhythm in pineal serotonin content is disrupted by administering reserpine or the MAO inhibitors to rats (Snyder and Axelrod, 1965; Snyder et al., 1967), resulting in a failure of pineal serotonin levels to decline nocturnally.

N-methyl-3-piperidyl benzoate, an anticholinergic agent, exerts similar effects (Merritt and Sulkowski 1969). The administration of noradrenaline, dopamine (Zweig and Axelrod, 1969), or actinomycin D (Snyder et al 1967) blocks the daytime increase in pineal serotonin. Serotonin uptake by pinealoctyes is partially sodium dependent (Ducis and Distefand, 1980).

1.4.2.2 Melatonin

A number of compounds bearing a structural similarity to L-noradrenaline i.e. D-noradrenaline, L-adrenaline, dopamine, tyramine, octopamine, tryptamine (Axelrod et al 1969) as well as amphetamine (Bäckström and Wetterberg, 1973) and the MAO inhibitors, Catron (Axelrod et al 1969) and harmine (Klein and Rowe, 1970), increase the rate at which cultured rat pineals synthesize ^{14}C-melatonin from ^{14}C-tryptophan. This conversion is not affected by morphine (Shein et al 1970) and decreased by cycloheximide, a protein synthesis inhibitor (Axelrod et al 1969). Propranolol and other β-adrenergic blockers but not phenoxybenzamine can block the noradrenaline induced increase

in melatonin synthesis in cultured rat pineals (Wurtman et al.,1971). Neither of these receptor blocking agents affect the increase in melatonin synthesis caused by dibutyryl cyclic AMP (Wurtman et al.,1971), however, cycloheximide and actinomycin D block this effect (Berg and Klein, 1971).

1.4.2.3 Pineal cyclic AMP, cyclic GMP and Adenyl cyclase

Weiss and Costa (1967) showed that L-noradrenaline activates adenyl cyclase in pineal homogenates. This response is reduced during proestrus (Weiss and Crayton, 1970; Davis, 1978). The synthesis of cyclic AMP is also accelerated by this catecholamine in both homogenates (Weiss and Costa, 1968) and in cultured rat pineals (Weiss 1969). This effect of L-nor - adrenaline is blocked by propranolol (Klein et al.,1978) and dichloroisoprenaline and is inhibited to a certain extent by trifluperazine and chlorpromazine but is not affected by the alpha lytics, phentolamine and phenoxybenzamine (Weiss and Costs 1968; Uzunov and Weiss, 1971). The relative potencies of noradrenaline and isoprenaline on pineal cyclic AMP levels appear to be dose-dependent (Auerbach et al.,1981). The noradrenaline induced activation of cyclic AMP is potentiated by introducing theophylline, a phosphodiesterase inhibitor to the culture medium (Strada et al.,1972). Deguchi (1973) showed that the level of cyclic AMP in rats injected i.v. with isoprenaline rose and declined to the initial level after 15 minutes (Deguchi and Axelrod, 1972). In rat pineal organ cultures, the efflux of both cyclic AMP and cyclic GMP is blocked by probenecid. Desmethylimipramine blocks the stimulated accumulation and efflux of cyclic GMP (O'Dea et al.,1978). Chloragen increases adenyl cyclase and cyclic AMP levels and decreases cyclic GMP levles in pineal organ cultures (Minneman, 1977). Human chorionic gonadotropin administration to rats,

decreases pineal cyclic AMP levels (Karasek and Karasek, 1978).

1.4.2.4. Pineal Phospholipids

The rate at which cultured pineal glands incorporate inorganic ^{32}P into phosphatidyl inositol (PI) (Muraki, 1972), monophosphoinositide (Basinka et al.,1973), and phosphatidyl glycerol (PG) (Eichberg et al.,1973) increases with concentrations of L-Noradrenaline ranging from 1 to 300 u M and decreases the synthesis of phosphatidylethanolamine (Basinka et al.,1973). D-noradrenaline, L-adrenaline, dopamine, tyramine, octopamine (Eichberg et al.,1973)as well as phenylephrine and propranolol (Hauser et al.,1974) accelerate synthesis of pineal PI and PG. The synthesis of monophosphoinositide is accelerated by acetylcholine and physostigmine or by serotonin. It is also interesting to note that the alpha lytic, phenoxybenzamine has been reported to have the phospholipid stimulatory effects of noradrenaline and phenylephrine but not of propranolol (Hauser et al.,1974). This indicates that phospholipid stimulation in the pineal takes place via two separate systems. Dibucaine, a local anaesthetic, accelerates the degradation of phospholipids in the pineal (Eichberg and Hauser, 1974).

Nijjar et al.,(1980) provided evidence against dopaminergic and further support for alpha adrenergic receptor involvement in the pineal phosphatidylinositol effect using dopamine and alpha adrenergic agonists.

1.4.2.5 Pineal Morphology

Wolfe et al.,(1962) showed that the pineal noradrenaline is stored in granulated vesicles of postganglionic sympathetic neurons. The administration of reserpine decreases this granularity whereas iproniazid administration increases it (Pellegrino de Iraldi and De Robertis, 1963). Smith and

Kappers, (1975) showed that pCPA administration decreases the serotonin content of pineal cells. The parenchymal serotonin is depleted by α-methyltyrosine whereas the neural serotonin is depleted when tryptophan hydroxylase is inhibited by α-propyldopacetamide (Falck et al.,1966).

The drugs, metaraminol (Bertler et al.,1964),desmethylimipramine (Jaim-Etcheverry and Zieher, 1971), 6-hydroxydopamine (Eranko, 1971), tyramine (Pellegrino de Iraldi and Suburo, 1972) and anti-nerve growth factor (Schott et al.,1970) cause morphologic changes in pineals compatible with increased biosynthetic activity (Karasek, 1974).

1.4.2.6. Pineal enzymes

1.4.2.6.1 Tryptophan hydroxylase

This enzyme is inhibited by pCPA in vivo and in vitro when added to organ cultures of rat pineals (Bensinger et al.,1974; Lovenberg et al.,1967, Deguchi and Barchas 1972a).

1.4.2.6.2. H1OMT (Hydroxyindole-O-methyltransferase).

Substituted N-benzoyltryptamines and N-phenylacetyltryptamines (Ho et al.,1971), haloperidol; Hartley et al.,1972; Ho et al., 1973), fluphenazine, GABA (Hartley et al.,1972), and oxypertine (Ho et al.,1973), have been shown to inhibit H1OMT activity in vitro but not in vivo. In organ culture, rat pineal H1OMT activity has been shown to increase after the addition of 10^{-5}M noradrenaline (Klein 1969). Dimethyltryptamine increases the O-methylation of N-acetylserotonin when added to pineal homogenates (Hartley and Smith, 1973). The administration of L-dopa to rats has been shown to increase H1OMT activity (Lynch et al.,1973). Three possible mechanisms of action of L-dopa could be: either a release of noradrenaline from the

pineal sympathetic nerve endings or the conversion of L-dopa to dopamine which then acts agonistically or the conversion of L-dopa to noradrenaline.

Pteridines have also been shown to have an effect on H1OMT activity. The investigations of Cremer-Bartels et al.,(1975) and Cremer-Bartels and Hollwich (1978) on the influence of 2.4.7-triamino-6-phenylpteridine on retinal and pineal H1OMT and the presence of pteridines in the pineal demonstrated by Ebels et al.,(1979) suggest that pteridines may regulate H1OMT activity in the pineal. Ebels et al.,1979 showed that reduced neopterin stimulates H1OMT activities for the substrates 5-hydroxytryptophan (5-HTP), 5-hydroxytryptamine (5-HT) and 5-hydroxyindole-acetic acid (5-HIAA) during the night. They found that H1OMT activities for the combination N-acetylserotonin (NAS) / 5-hydroxytryptophol (5-HTOH) are shifted to a later moment in the dark period whereas H1OMT activities for the substrates 5HTP, 5-HIAA and NAS/ 5-HTOH are shifted towards an earlier period.

1.4.2.6.3. SNAT (Serotonin-N-acetyltransferase).

The administration of L-dopa increases the activity of pineal SNAT (Deguchi and Axelrod, 1972) possibly by the same mechanism discussed above for H1OMT.

SNAT acitivity of cultured rat pineals is increased by the addition of noradrenaline (Klein et al.,1971; Deguchi and Axelrod, 1973a; Klein and Weller 1973). Deguchi (1973) showed that isoprenaline injected i.v. to rats results in an increase in SNAT activity one hour later with a decline to the initial level after 5 hours. Prior treatment with propranolol 3 hours before the experiment blocks this increase in

SNAT activity while propranolol administration during maximal levels of SNAT produces a rapid decline in SNAT activity indicating that high SNAT activity requires continuous stimulation of the β-adrenergic receptors of pineal cells. Cyclic AMP appears to be involved in the translation process in formation of new enzyme molecules as actinomycin-D has no immediate effect on SNAT induction (Deguchi and Axelrod, 1972). Zatz et al.,(1976) suggest that the lag period prior to the increase in levels of SNAT could be ascribed to a lack of mRNA which first has to be synthesized during the lag period.

Klein et al.,(1970) observed an increase in SNAT activity after adding dibutyryl cyclic AMP to pineal cultures. Adrenaline, L-dopa, octopamine (Klein and Weller, 1970), isoprenaline, theophylline and the MAO inhibitors pargyline and Catron (Deguchi and Axelrod, 1972) increase SNAT activity in pineal organ cultures. The local anaesthetics, cocaine and procaine also increase SNAT ctivity in pineal cultures. This effect is mediated by the release of noradrenaline from sympathetic nerve terminals (Holz et al.,1974). The fall in pineal serotonin content followed by an increase in SNAT activity can be blocked by giving rats pargyline (an MAO inhibitor). Pargyline also first decreases and then increases SNAT activity in vivo if given during the light period of a diurnal cycle (Illnerova, 1974).

Lynch et al.,(1973a); Sampson, (1975), have shown that melatonin synthesis is increased by stress and hypoglycaemia. Lynch et al.,(1973a), have shown that rats immobilized for several hours or made hypoglycaemic with insulin have elevated levels of both melatonin and pineal SNAT. These increases are brought about due to release of catecholamines from the

sympathetic nerve terminals and the adrenal medulla and can be blocked by propranolol.

1.5. THE GONADAL SEX STEROIDS AND THE PINEAL GLAND.

1.5.1. Effect on pineal morphology

Boya et al., (1980) injected chicks with sex steroids and showed histochemically and ultrastructurally from posthatching to 90 days of age, that the pineal gland shows signs of early maturity. These authors suggest that this precocious maturity could be a reaction of the pineal gland to early high levels of sex hormones circulating in the blood.

1.5.2. Effect on pineal adenyl cyclase

Weiss and Crayton, (1970) showed that noradrenaline activates the pineal adenyl cyclase of male rats to a greater extent than that of female rats. The pineal adenyl cyclase system of ovariectomized rats was found to be more responsive to activation by noradrenaline. The chronic administration of estradiol but not of progesterone, inhibits the noradrenaline and sodium fluoride activated adenyl cyclase system, but does not affect the basal level of this enzyme. Testosterone has no effect on this system.

These authors postulated that estrogens could act (i) on pinealocytes by directly inhibiting the synthesis of the enzyme or its affinity for noradrenaline or (ii) indirectly via the nervous system at any point between the retinal receptors and the post ganglionic sympathetic fibres to the pineal.

1.5.3. Effect on pineal SNAT activity

1.5.3.1. Estradiol

In the pineal gland, noradrenaline acts via the adenyl cyclase

system to increase the level of SNAT (Klein and Weller, 1970; Klein et al.,1971).

If noradrenaline functions through adenyl cyclase to increase the SNAT level, then theoretically the administration of estradiol should inhibit the rise in the SNAT level after the administration of catecholamines. This was investigated by Illnerova (1975) who found that ovariectomized rats treated with a daily dose of estradiol 10mg/100g BM for 3 days did not show any significant change in pineal SNAT during the night when the enzyme is activated, nor during the day after the administration of isoprenaline. Illnerova described two possible mechanisms for the inability of estradiol to decrease SNAT activation by catecholamines :-

i) either previous activation of adenyl cyclase is not necessary for the activation of SNAT by catecholamines or :-

ii) the response of the adenyl cyclase system is unchanged after the administration of estradiol.

Preslock, (1974) and (1975) reported that estradiol benzoate had no effect on pineal SNAT activity in castrated and intact female Coturnix quail when administered chronically for 18 days.

1.5.3.2. Androgens

Preslock, (1974a) and (1975) reported that testosterone proprionate and androstenedione had no apparent effect on pineal SNAT activity when administered at doses of 100 µg per day for 18 days to castrated male Coturnix quail.

Pavlinov and Isachenkov (1979) investigated the effects of the androgens viz. testosterone, 5α-dihydrotestosterone, 3α - 17β androstanediol and 3β - 17β androstanediol on the nocturnal elevation of SNAT activity in the pineal gland of

castrated male rats. They injected rats with 50ug of the various androgens 2 days after castration 1 to 6 hours before sacrifice. They showed that castration weakened the nocturnal peaks of the SNAT activity by 30%. A single androgen administration stimulated the nocturnal rise of enzyme activity in 2 to 4 hours. The physiological activity of the androgens were found to be distributed in the following way:- 5 α-dihydrotestosterone, 3α-17 β - androstanediol, testosterone. The β epimer had no activity. Also recently, Rudeen and Reiter (1980) examined pineal SNAT activity in intact rats, castrated rats, and in rats that had been castrated and had received a subcutaneous pellet of 50 mg testosterone proprionate. Castration resulted in significantly depressed nocturnal levels of pineal SNAT in comparison to that of intact rats, confirming the results of Pavlinov and Isachenkov (1979) mentioned above. Testosterone administration restored plasma LH levels to normal values in castrated rats but did not induce the nocturnal pineal enzyme activity to levels found in intact rats. It is possible that the dose of testosterone these authors used was too high.

1.5.3.3. Progesterone

As yet there are no reports on the effects of progesterone on pineal SNAT activity.

1.5.4. Pineal SNAT activity during the estrus cycle.

Cardinali and Vacas (1978) showed that SNAT activity did not show modifications as a function of the stage of the estrus cycle, nocturnally or diurnally. Cardinali et al.,(1974c), reported the absence of modifications in SNAT activity during the estrus cycle in ewes.

In a recent study, Shivers and Yochim (1979), showed that pineal SNAT activity determined 3 times a day throughout the estrus cycle in rats exposed to a lighting schedule of 6 hrs light; 18 hrs dark or 14 hrs light; 10 hrs dark, did not differ significantly in either photoperiod examined.

These data suggest that SNAT does not regulate changes found in pineal melatonin content during the estrus cycle, (Quay, 1964).

1.5.5. Effect of gonadal steroids on pineal protein synthesis.

1.5.5.1 Estradiol

Using adult female rats, Clementi et al.,(1965),found histological changes indicating depressed pinealocyte function and reduced cytoplasmic RNA following estradiol administration. The rat pineal protein level was found to increase 24 hours after administration of the dose of estradiol. These authors also showed that the levels of DNA and RNA in the pineals increase 6 hours prior to the increase in protein levels indicating an increase in protein synthesis. This increase is due to a positive feedback of gonadotropin (Nir et al., 1970).

Depression of melatonin and protein synthesis follows ovariectomy in rats. Conversely, low doses of estradiol increase both rates of synthesis to about normal levels, while higher doses of estradiol depress pineal function (Cardinali et al., 1974b and 1975d).

Nir et al.,(1970), investigated the influence of 17-β-estradiol on pineal metabolism by measuring RNA, DNA and protein content of pineal glands from immature female rats. They injected these rats with a single dose of estradiol (10 µg) and assayed the pineal content of RNA, DNA and protein,15, 18 and 24 hours

after dosing. 18 hours after injection these authors detected an elevation in pineal RNA and DNA while an elevation in total protein was only detected at 24 hours. These authors also demonstrated that chronic administration of estradiol (5 µg for 7 days), had no effect on pineal RNA, DNA or protein. They postulated that the above increase in protein synthesis in immature female rats was due to increased LH secretion by the pituitary. However, this has not been verified using hypophysectomized rats.

Cardinali et al.,(1976), showed that ovariectomized rats subjected to bilateral superior cervical ganglionectomy and then treated with estradiol, do not show an increase in pineal protein synthesis. These authors also showed that estradiol treatment augments labeled amino acid incorporation into proteins in rats whose preganglionic connections between the superior cervical ganglia and the spinal cord are interrupted. They also demonstrated that the administration of propranolol after estradiol injection impairs the increase in protein synthesis brought about by estradiol.

In pineal organ cultures, estradiol produces an increase in DNA-dependent RNA polymerase activity. This effect is blocked by clomiphene citrate (a competitive inhibitor of estradiol) and RNA synthesis inhibitors (Mizobe and Kurokawa, 1976).

1.5.5.2 Progesterone

The administration of progesterone to ovariectomized rats decreases pineal protein synthesis regardless of whether estradiol is simultaneously administered or not (Cardinali and Vacas, 1978). Since progesterone administration is unable by itself to modify plasma gonadotropins or prolactin levels

(Neill and Smith, 1974), these results are best interpreted in terms of direct effects of progesterone on pineal cells rather than mediated via pituitary hormones.

1.5.5.3 Testosterone

Castration of male rats depresses melatonin and protein synthesis. Conversely, low doses of testosterone increase the synthesis of melatonin and proteins in the pineal while higher doses of this steroid depress pineal function (Cardinali 1974b and 1975b).

Male castrated rats subjected to bilateral superior cervical ganglionectomy and then treated with testosterone do not show an increase in pineal protein synthesis. Testosterone treatment augments labeled amino acid incorporation into proteins in rats whose preganglionic connections between the superior cervical ganglia and the spinal cord have been interrupted. The administration of propranolol after treatment with testosterone, impairs the increase in pineal protein synthesis brought about by testosterone (Cardinali et al.,1976).

The antagonistic effect of propranolol on the enhanced pineal protein synthesis brought about by estradiol or testosterone administration can be interpreted in two ways : (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.

From the foregoing discussion it is evident that the sex steroids require an intact neural pathway to act on the

pinealocytes.

1.5.6 Metabolism of sex steroids in the pineal.

1.5.6.1 Progesterone

Active metabolism of progesterone takes place after incubation of pineal explants with the labeled steroid in vitro. Progesterone is taken up by tissue from the incubation medium (Luttge and Wallis, 1973; Cardinali et al.,1975a) and is converted to 5 α-reduced metabolites, i.e. 5 α-pregnanedione and 3 α-hydroxy- 5 α pregnane - 20 - one. These findings have been confirmed by Hanukoglu et al.,(1977) In addition, 20 α -hydroxysteriod dehydrogenase activity was detected. Some other progestagens including norethynodrel and 17 α-hydroxy-progesterone were found to accumulate in the pineal gland of rhesus monkey following i.v. or CSF administration (David et al., 1975). Ovariectomized rats injected with ^{3}H-progesterone retain more unmetabolised progesterone in the pineal gland than in any other CNS areas involved in neuroendocrine regulation.

1.5.6.2. Testosterone and estradiol

The pineal glands of castrated rats incubated in vitro with ^{3}H-testosterone take up and retain ^{3}H-radioactivity up to a tissue concentration 18-fold that present in the incubation medium. Following injection in vivo or incubation in vitro with ^{3}H-testosterone, the radioactivity in the pineal does not differ significantly from that recovered from the prostate, a known target for androgens (Cardinali et al.,1974d). ^{3}H-androgen metabolism by pineal cells comprises 5 α- and 17- β - reduction, as well as aromatization to estrogens (Cardinali et al.,1974d; 1974e). 5 α - dihydrotestosterone

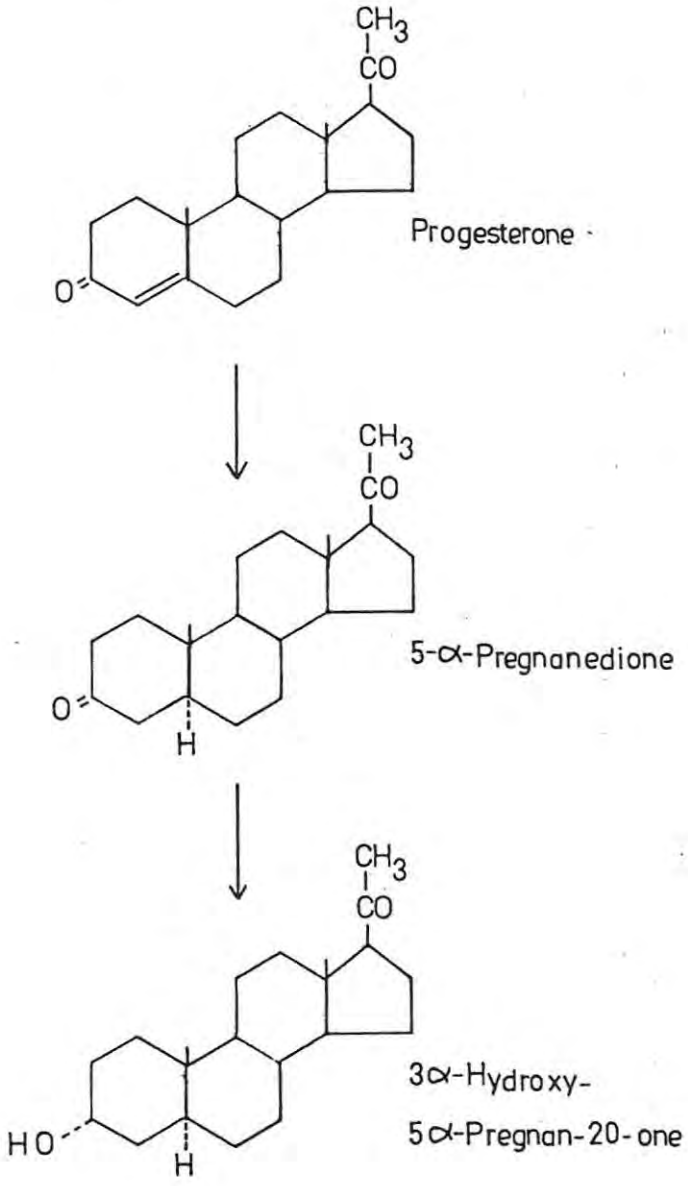

Fig. 5. Metabolism of progesterone in the pineal gland.

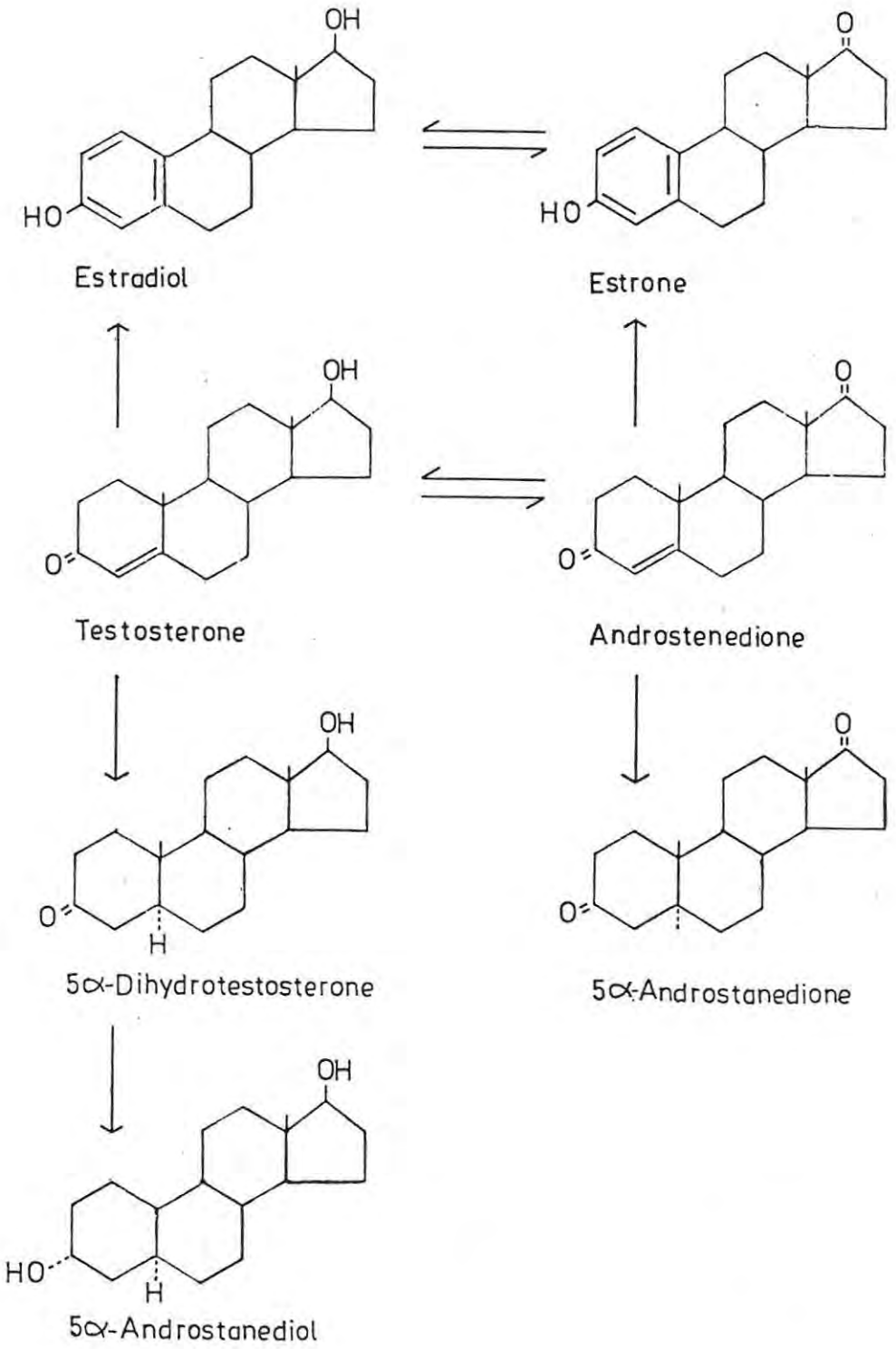

Fig. 6. Metabolism of estradiol and testosterone in the pineal gland.

is the major metabolite recovered from pineal cell nuclei. These data support the view that the pineal gland resembles other brain areas involved in gonadotropin regulation as far as the metabolism of testosterone is concerned. To what extent testosterone effects on pineal function require prior conversion to estrogens has yet to be demonstrated. Estradiol is converted to estrone in the pineal gland. Weiss et al., (1978) showed that 17 β -estradiol is methylated to the3-methyl-ether of estradiol by partially purified bovine pineal H1OMT.

1.5.7 Gonadal sex steroid receptors in the pineal gland.

1.5.7.1 Estradiol receptor

Marks et al.,(1972) examined various brain regions of ovariectomized rats for the presence of soluble estradiol binding protein. They found the pineal gland to have a much higher content of estradiol binding protein than other brain regions, close to that found for the adenohypophysis. Intraventricular administration of 6-hydroxydopamine significantly reduces and superior cervical ganglionectomy reduces the pineal estrogen binding protein content. From these data it appears that the level of this estradiol binding protein is influenced by pineal sympathetic innervation.

Subsequent to this, Nagle et al.,(1973)demonstrated that the in vitro uptake of radiolabelled estradiol by the rat pineal is proportional to the amount of steroid present in the medium. Pineal homogenates bind radiolabelled estradiol to high affinity, low capacity cytosol receptors similar to that of the rat uterus. These specific high affinity binding sites in the pineal and uterus can be induced in ovariectomized rats by daily administration of 2 ug of estradiol benzoate for 3 days.

In a recent study, Mizobe and Kurokawa (1978), using rat pineal organ cultures, showed that estradiol induces formation of a protein species with an approximate molecular mass of 97 000 which is distinct from the H1OMT protein per se, and resembles the estrogen-induced protein reported for the rat uterus (Notides and Garski, 1966; Katzenellenbogen and Gorski, 1972) in its electrophoretic mobility.
A cytoplasmic protein that binds estradiol selectively with a high affinity and binding site concentration of 50 f moles per mg of protein has been found to be present in pinealocytes and does not differ physicochemically from estrophillic receptors of estrogen target tissues (Cardinali et al., 1975). In the rhesus monkey, pineal estrophillic receptors have been characterized physicochemically and appear to be similar to those found in the rat pineal (David et al., 1975).

The interaction of receptor estrogen complex with nuclear acceptor sites is thought to be the initial step for hormone stimulation of nucleic acid and protein synthesis (Clark and Peck, 1976). By using the ^{3}H-estradiol exchange assay to determine receptor hormone complexes in nuclear fractions of pineal homogenates, the dissociation constant and dose-response and accumulation curves following estradiol were found to be comparable to those of hypothalamus and adenohypophysis (Cardinali 1977). Maximal amounts of pineal receptor estrogen complexes have been detected in proestrus, coinciding with the peak values of plasma estradiol by the same author. Using autoradiographic analysis of rat pineal glands after in vivo administration of ^{3}H-estradiol, Stumpf et al.,(1976) showed nuclear concentration of labeled grains in pineal cells.

1.5.7.2 Progesterone receptor

In a recent publication, Vacas et al, (1979) characterized a cytosol progesterone receptor in the bovine pineal gland. The experiments show that the bovine pineal gland contains a cytosol protein which binds specifically and 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).

Vacas et al.,(1979) proposed that the perculiar binding characteristics of pineal progesterone-receptor complexes might account for the particular uptake and metabolism of progesterone in the pineal gland, where progesterone rather than its 5∝-reduced metabolites is retained (Karavolas et al., 1978).

High affinity binding of progesterone to pineal cytosol receptors provides a molecular basis for the existence of a direct modification of pineal function by progesterone.

1.5.7.3 Testosterone receptor

Cardinali et al, (1974c) demonstrated the binding of androgens in the pineal to cytoplasmic proteins. Nuclei-free fractions of rat pineal homogenates incubated with ^{3}H-testosterone have a high affinity, low capacity binding to the supernatant. The 5∝-androstanediol has a low affinity for the receptor similar to that observed in the prostate

(Liao et al.,1972) in comparison to testosterone and 5 ∝ -dihydrotestosterone which had a high affinity. The androgen binding components in the bovine pineal were shown to be proteins which possibly require sulphydryl groups for activity. These authors proposed the following order of affinity of the steroids for the pineal cytosol binding component: 5 ∝ -dihydrotestosterone > testosterone > androstenedione > estradiol > 5 ∝ -androstanedione > 5 ∝ -androstanediol.

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

1.5.8 Effect of gonadal sex steroids on pineal H1OMT Activity.

1.5.8.1 H1OMT Activity during the estrus cycle.

Wurtman et al.,(1965) found that rat pineal H1OMT activity varies two-fold during the estrus cycle, being greatest during diestrus and lowest during proestrus and estrus in mature cycling rats.

In contrast to this, Cardinali et al (1974b) observed that rat pineal H1OMT activity was highest during estrus. These authors hypothesised that the increased levels in plasma estradiol occuring at the day of proestrus results in enhancement of H1OMT activity 24 hours later, coinciding with the peak of vaginal cornification induced by estrogens.

1.5.8.2 Effect of estradiol on H1OMT activity.

Subsequent to the report by Wurtman et al., (1965) that pineal H1OMT activity varies during the estrus cycle, Alexander et al., (1970) found that when female rats were ovariectomized at 28 days of age followed by sacrifice at 53 days, pineal H1OMT activity was greater than in unoperated controls. They repeated the above experiment and administered 50 ug of estradiol benzoate at days 50 and 52, with sacrifice at 53 days of age. This lowered H1OMT activity to that of intact controls. Ovariectomy at 37 days of age followed by sacrifice at 53 days had no effect on H1OMT activity compared to unoperated controls. This led these authors to suggest that the inhibitory effect of the ovary upon pineal H1OMT activity may decrease with age in maturing rats.

It was generally accepted that estradiol lowered pineal H1OMT activity (Wurtman et al.,1965; Alexander et al.,1970), until Houssay and Barcelo (1972) demonstrated that in ovariectomized rats injected daily with 20 ug estradiol benzoate for 25 days, pineal H1OMT activity increased significantly accompanied by a decrease in pineal mass.

Nagle et al.,(1972) showed that administration of estradiol (2,5 µg every 12 hours for 3 days) significantly reduced H1OMT activity compared to controls. In contrast, Cardinali et al., (1974b) showed that rats castrated 3 weeks earlier exhibited significantly lower pineal H1OMT activity than control rats in estrus. Low dose estradiol administration to ovariectomized rats increased pineal H1OMT activity.

Wallen and Yochim (1974) demonstrated that ovariectomy significantly decreased H1OMT activity below levels found during

the estrus cycle but did not affect the characteristic rhythmicity of the enzyme. They administered daily doses of estrone (10 µg) to rats ovariectomized during metestrus and found that the H1OMT level was restored to that of the controls but that the rhythmicity of the enzyme was distorted due to estrogen replacement therapy. They showed that (i) the rhythmicity of the pineal gland as related to the estrus cycle is independent of the ovaries and (ii) there is a "feedback relationship" between ovarian function and the mean rate of pineal H1OMT activity.

Preslock (1974a and 1975) demonstrated a stimulation of pineal H1OMT activity in ovariectomized Coturnix quail receiving a daily dose of estradiol benzoate (100 µg per day) for 18 days. The dose used here is much higher than doses which are stimulatory in rats but might however be "physiological" in this specie since these avians when sexually mature are under constant estrogen stimulation for the high rate of egg production.

Mizobe and Kurokawa (1976) cultured pineal glands from female rats which had been ovariectomized 2 weeks prior to sacrifice. They reported that H1OMT activity increases slightly during the first 2 hours of incubation and then returns to the baseline. Addition of estradiol (15nM) to the culture medium caused an 80% elevation in H1OMT activity which was maintained for a further 3 hours but was no longer apparent at the 24th hour of incubation. They showed the increase in H1OMT activity to be dose-dependent in the range 0.1 to 15nM which is the physiological concentration range of estradiol in the ovarian venous blood of rats (Hori et al.,1968). The estradiol induced increase of pineal H1OMT activity is abolished

by cycloheximide and puromycin (protein synthesis inhibitors) indicating that the increased H1OMT level is possibly due to de novo synthesis of H1OMT protein.

A possible explanation for the conflicting reports may involve the dose of estradiol used. It is likely that lower doses of estradiol, which may be within a physiological range, apparently stimulate pineal H1OMT, while higher doses, which may exceed the physiological level, appear to be inhibitory.

1.5.8.3 Effect of progesterone on H1OMT activity.

Houssay and Barcelo (1972) demonstrated that progesterone administration to ovariectomized rats at a dose of 200 µg / day produced a significant decrease in pineal H1OMT but did not affect pineal mass. The concurrent administration of progesterone and estradiol benzoate also decreased H1OMT activity to below that of intact controls but not as effectively as with the administration of progesterone alone. Administration of progesterone in doses of 20 µg/day or 100 µg/day for 25 days also showed an inhibitory effect on H1OMT activity.

Wallen and Yochim (1974) showed that H1OMT activity is depressed during pseudo-pregnancy in female rats, a period when progesterone levels are high.

In contrast to studies on the rat, Preslock (1974b and 1975) demonstrated that progesterone restored H1OMT activity of castrated Coturnix quail to levels similar to those of intact controls.

Progesterone addition to rat pineal organ cultures inhibits the release of melatonin (Wilkonson and Arendt, 1977).

It is possible that progesterone may exert different effects in rats and quail suggesting species differences between mammals and avians in the response of pineal H1OMT to progesterone.

1.5.8.4 Effect of testosterone on H1OMT activity.

Nagle et al.,(1974) reported that pineal and retinal H1OMT activity decreases 24 to 72 hours after castration. The administration of testosterone proprionate (0.1-1 mg /day stimulated pineal H1OMT activity in castrated male rats while higher doses inhibit pineal H1OMT activity. Doses ranging from 0.1 to 5 mg /day increase retinal H1OMT activity. The simultaneous administration of noradrenaline (250-400 µg) partially reverses the stimulation of pineal H1OMT activity caused by 0.5 mg/day of testosterone proprionate, and potentiates the inhibition induced by 5 mg/day of testosterone proprionate. Noradrenaline reverses the testosterone induced stimulation of retinal H1OMT.

From this report it appears that antagonism exists between testosterone and noradrenaline for induction of H1OMT activity.

In Coturnix quail, Preslock (1974a; 1975) demonstrated a stimulation of pineal H1OMT activity in castrated males receiving androgens in a dose of 100 µg /day for 18 days. Both testosterone proprionate and androstenedione increase H1OMT activity in castrates to levels approximating that of controls.

Pavilnov and Isachenov (1979) showed that the peaks of the nocturnal rise in pineal H1OMT activity are depressed by 40% in castrated male rats. A single androgen administration of 50 µg to rats 2 days after castration and 1-6 hours before sacrifice stimulates the nocturnal rise of the enzyme in 2-4 hours.

1.5.9. Possible sites for sex steroid action on the pineal.

1.5.9.1. Endocrine -neural transducing processes in the sympathetic pathway to the pineal gland.

The administration of testosterone, FSH (follicle stimulating hormone) or LH (lutenizing hormone) to castrated rats and the changes in the synthesis and metabolism of noradrenaline in the superior cervical ganglia after estradiol, testosterone, gonadotropin or prolactin treatment of rats modify noradrenaline turnover in pineal nerve endings. Theoretically, the hormones may act on the sympathetic pathway:-

(i) at the level of pineal nerve endings, directly or through a local feedback loop involving the pinealocytes.
(ii) at the level of the ganglionic perikarya.
(iii) at any point of the multisynaptic descending pathway connecting photoreceptors with the superior cervical ganglia (Cardinali and Vacas, 1978).

A possible ganglionic site of action for hormones has been suggested from (Cardinali, 1979) :-

(i) The effects of testosterone and estradiol on pineal protein synthesis and from the effects of gonadotropins on pineal serotonin metabolism.
(ii) Some of the effects of estradiol in ganglia (changes in tyrosine hydroxylase and dopamine- hydroxylase activities) persist after decentralization of the ganglia.
(iii) High affinity binding sites for estradiol have been detected in cytosol and nuclear fractions of homogenates prepared from ganglia, indicating that estrophillic receptors are present in the ganglionic perikarya (Cardinali, 1979).

However, there is no conclusive proof of a link between hormone binding sites and effects of estradiol in the superior cervical ganglion. Theoretically, estradiol may act at the level of the ganglionic neurones or on any other cell component of the ganglion, eg. small intensely fluorescent interneurones (S.I.F.).

1.5.9.2. Interaction between neural and hormonal signals. Noradrenaline which is released from pineal nerve endings induces the synthesis of pineal receptor proteins for estrogens and androgens. Studies carried out on pineal organ cultures reveal that high affinity specific hormone binding to cytoplasmic and nuclear components become severely depressed in chronically ganglionectomized rats and that at the same time, cytoplasmic receptor-estradiol or receptor-5α- dihydrotestosterone complexes become undetectable (Cardinali and Vacas, 1978). Receptors do not disappear totally from denervated pineal glands since significant translocation of receptor-estradiol complexes from the cytoplasm to the nucleus occurs in ganglionectomized rats. The administration of isoprenaline to ganglionectomized rats, restores receptor sites for estrogens and androgens in pineal cells. Pretreatment with propranolol impairs the noradrenaline effects on hormone receptors in ganglionectomized rats.

Reinduction of hormone binding sites by noradrenaline in ganglionectomized rats which are expected to have fully degenerated nerve endings suggests that the steriod receptors are localized in the pineal cells rather than in nerve endings.

Conclusive evidence in this respect comes from the autoradiographic localization of labelled estrogens and androgens

in pineal cell nuclei (Stumpf and Sar, 1977).

1.6 Gonadotropins and pineal enzyme activity

Little information is currently available on the regulation of pineal SNAT and H1OMT by pituitary gonadotropins. H1OMT activity of rats which have been hypophysectomized at the age of 28 days and sacrificed at the age of 53 days is substantially lower than that of controls. If however, the rats are hypophysectomized at the age of 37 days and sacrificed at the age of 53 days, H1OMT activity is not affected, implying that the stimulatory effect of the pituitary upon H1OMT may decrease with age (Alexander et al.,1970). A decrease in H1OMT activity of hypophysectomized male rats was demonstrated by Urry et al.,(1972 and 1976). However, the administration of human chorionic gonadotropin (HCG) did not restore pineal H1OMT activity to that of intact controls. Preslock, (1975) showed that hypophysectomy of adult female Coturnix quail had no effect upon pineal H1OMT activity. The administration of HCG to intact or hypophysectomized quail had no effect on pineal H1OMT or SNAT activity.

Sandrock (1980) showed that hypophysectomy decreases H1OMT activity while dexamethasone administration restores enzyme levels to control values.

Antonov et al.,(1977) showed that i.v. administration of synthetic LH releasing hormone (LH-RH) to infantile as well as sexually mature rats of both sexes increases pineal H1OMT activity for 5 hours after administration.

The effect of pituitary hormones on pineal enzyme activity could be mediated by a direct effect on the pineal or possibly indirectly, by altering the secretion of gonadal steroids.

1.7 Environmental illumination and the pineal gland.

1.7.1. Effect of light on pineal mass.

Fiske et al.,(1960) were the first to show that the structure and function of the mammalian pineal gland are related to environmental illumination. They showed that pineal mass decreases by 25% in rats kept in continuous light for 6-25 weeks. Rats kept in darkness for the same period did not show any alteration in pineal mass. These results were confirmed by Wurtman et al., (1961) and Quay (1961). Subsequent to this, it was shown that even short periods of continuous illumination of 1 to 2 days could produce a significant decrease in pineal mass. (Wurtman et al.,1963a). Axelrod et al., (1965) demonstrated that the mass of pineals in rats varied with a 24 hour cycle, being lowest at the end of the daily light period. This effect is abolished by bilateral orbital enucleation (blinding) (Snyder et al.,1965).

1.7.2. Non-retinal pathway of light to the pineal gland of newborn rats.

Zweig et al.,(1966) provided evidence for the existence of a non-retinal pathway of light to the pineal gland in 12 day old rats, involving the head. Hooding of 12 day old blinded rats abolishes the effects of light on the pineal gland. This pathway of light probably controls physiological processes in the pineal before the eyes are fully developed.

1.7.3. Effect of light on pineal SNAT activity.

Klein and Weller, (1970) showed that pineal SNAT undergoes a 24 hour diurnal rhythm which is 180^{o} out of phase with the rhythms of serotonin and aromatic-L - amino acid decarboxylase (Brownstein et al.,1973). SNAT activity increases 20 to 50 fold at night in darkness (Klein and Weller, 1970; Deguchi

and Axelrod, 1972.) When rats are maintained in continuous darkness or they are blinded, the rhythmic change in SNAT activity persists, indicating that in the absence of light, SNAT activity is driven by an endogenous "biological clock" (Klein and Weller, 1970; Deguchi and Axelrod, 1972). This circadian rhythm is suppressed by constant illumination and by bilateral superior cervical ganglionectomy (Klein et al., 1971). This circadian rhythm can be inverted by reversing the environmental lighting (Nir et al., 1974). The circadian rhythm in SNAT activity appears 6 days after birth (Zweig et al., 1966 ; Ellison et al.,1972) at the time when sympathetic innervation to the pineal begins (Hakanson et al.,1967). Deguchi (1975) demonstrated that the circadian rhythm of SNAT activity in rat pineal glands develops even when infant rats are born and raised in continuous darkness or in constant light. This rhythm persists in pups when their mother is coupled in darkness and maintained in darkness during pregnancy and after the pups were born. These results indicate that the "biological clock" that regulates the circadian rhythm in SNAT activity is generated in the CNS, independant of environmental illumination but is modified by environmental illumination.

Minneman et al.,(1974) demonstrated a dose-response relationship between intensity of ambient lighting and pineal SNAT activity. They showed that as little as 0,5 μ Watts/cm^2 caused a greater than 50% suppression of SNAT activity. Maximum inhibition was obtained with 15 μ Watts/cm^2. The intensity range within which environmental illumination controls pineal function lies between full moonlight (0,03-0,05 μ Watts/cm^2) and full sunlight (50 000 μ Watts/cm^2).

Shivers and Yochim, (1979) showed that :-

(i) light entrains the endogenous rhythm in SNAT activity such that the peaks occur around the midpoint of the dark phase of the photoperiod.

(ii) increasing the proportion of light/day, alters the amplitude in SNAT activity in a parabolic manner.

(iii) increasing the proportion of light/day does not change the duration of time that SNAT activity is elevated, except in extremely long light conditions. One minute exposure of rats to light is sufficient to initiate the fall in SNAT activity during darkness (Illnerova et al.,1979). Administration of isoprenaline at the end of such a light exposure, completely blocks the decline in SNAT activity. The response of SNAT to a one minute light exposure is a function of the time of the night when the pulse is applied (Illnerova and Vanecek, 1979). The exposure of rats to this light pulse at night might cause complex changes in the SNAT rhythm (Vanecek and Illnerova, 1979).

These effects of light on pineal SNAT activity can be attributed to the inhibitory effect of light on the sympathetic activity of nerves to the pineal gland (Klein, 1973).

1.7.4 Effect of light on HIOMT activity.

1.7.4.1. Properties of HIOMT

Axelrod and Weissbach, (1960) demonstrated the presence of hydroxyindole-O-methyltransferase (HIOMT) in bovine pineal glands. This enzyme is capable of transferring the methyl group of S-adenosyl-methionine to the hydroxy group of N-acetyl-serotonin.

Later, the same authors (Axelrod and Weissbach, 1961) purified

H1OMT from bovine pineal glands and reported its properties. They showed that unlike catechol-O-methyltransferase, this enzyme had no requirement for a metal. The optimum enzyme activity occurs between pH 7.5 and 8.3. N-acetylserotonin proved to be the best substrate. However, other 5-hydroxy-indoleamines,serotonin and its N-methylated derivatives, as well as 5-hydroxyindole acetic acid are also O-methylated, but at a much reduced rate. They also demonstrated the presence of this enzyme in pineal glands of the monkey,rat and quail. The electrophoretic mobility of quail H1OMT is faster than that of cattle. The enzyme also differs markedly in heat stability and Km values in these two species (Axelrod and Vesell, 1970).

Deguchi and Barchas, (1971) showed that S-adenosyl-homocysteine, the product which results when S-adenosylmethionine donates its methyl group, is a potent inhibitor of H1OMT activity and binds to H1OMT with a higher affinity than S-adenosylmethionine.

Jackson and Lovenberg, (1971) showed that H1OMT has a molecular mass of between 76 000 and 78 000, but also occurs as higher molecular mass aggregates. They also demonstrated that the enzyme exists as two differently charged molecular species which are identical in all other chemical and physical properties and that the enzyme has two subunits with a monomer molecular mass of about 39 000. In the pineal, H1OMT is a major protein, constituting about 4% of the soluble proteins in the gland.

Cardinali and Wurtman, (1972) demonstrated the presence of H1OMT in the rat pineal, retina and Harderian gland. They found that Mg^{++} enhances H1OMT activity in the Harderian

gland but does not affect H1OMT activity in the pineal and retina. These authors concluded that pineal and retinal H1OMT enzymes were very similar but differed from that in the Harderian gland in requirements for storage, pH optima and metal ions.

1.7.4.2 Effect of light on H1OMT rhythm

There is a diurnal fluctuation in pineal H1OMT activity. H1OMT activity increases1,6 to 3-fold during the night and drops during the day (Axelrod et al.,1965). Light suppresses the activity of this enzyme (Wurtman et al.,1963 b). The nocturnal rise of this enzyme is abolished by bilateral superior cervical ganglionectomy (Axelrod et al., 1965).

Vollrath et al., (1974), demonstrated the existence in male and female rats, of a 7day rhythmic activity of pineal H1OMT. In both sexes, pineal H1OMT activity was found to be highest on Saturdays and lowest on Thursdays.

Cardinali et al.,(1972) exposed rats which were housed in darkness for 7 days, to green, blue or yellow light and observed the time-related decreases in pineal H1OMT activity. They showed that green light has the most pronounced effect in decreasing H1OMT activity. Red light was found to have no effect.

Yochim and Wallen (1974) showed that although H1OMT activity rises and remains high in rats placed in continuous darkness (Wurtman and Axelrod, 1965), an estrus cycle pattern of H1OMT activity remains. The presence of such a rhythm in the dark suggests an endogenous aspect of the rhythm. They described the pattern of enzyme activity as the interaction

of two component rhythms with slightly different periodicities whose characters are endogenous, synchronized with the estrus cycle, yet modified by light. Wallen and Yochim (1974) described these rhythms mathematically by a mixed wave equation. All these effects can be accounted for by changes in the numerical values of the non-periodic terms of the mixed wave equation.

Pevet et al (1980) found that rat pineal H1OMT activity involved in the synthesis of melatonin is high at the end of the dark period and at the middle of the light period. The pineal H1OMT activity concerned with the production of 5-methoxytryptophol is only observed during the light period. Comparing the peaks, these authors conclude that the pineal produces more 5-methoxytryptophol than melatonin. These authors suggest that different H1OMT enzymes are involved in the synthesis of 5-methoxytryptophol and melatonin. Balemans et al., (1980) found that in rat pineals and Harderian glands, a larger amount of 5-methoxytryptophol than melatonin is synthesized, confirming the proposal by Pevet et al.,(1980).

Weiss, (1968) showed in contradiction to other reports, that noradrenaline inhibits H1OMT activity. This observation is in agreement with the results of studies by Browstein and Heller (1968), who found a decrease in H1OMT activity after stimulation of the sympathetic nerves innervating the pineal gland. According to Wurtman (1969), acetylcholine is involved in the increase in H1OMT activity. The influence of a parasympathetic agent is observed only during darkness, suggesting that acetylcholine cancels the inhibition of noradrenaline which shows a maximal concentration in darkness. Recently, muscarinic receptors have been identified in rat and sheep

pineal glands (Taylor et al., 1980).

Jackson and Lovenberg (1971), speculate that the diurnal variation observed in the activity of H1OMT might be due to an association-dissociation phenomenon while Yang and Neff (1976) showed that the diurnal variation is due to an alteration of enzyme molecule numbers rather than a change in enzyme kinetics.

1.8 Other rhythms in the pineal gland.

1.8.1. Seasonal variations in H1OMT activity

Balemans et al.,(1980b), determined the H1OMT activities involved in the synthesis of several 5-methoxyindoles during the night in April, June, October and January in pineal glands of 21 day old male rats. They observed high H1OMT activity for the synthesis of melatonin / 5-MTOH in the months of January and April and low activity in June and October. The activity maxima coincided with peaks of activity found for the synthesis of 5-methoxytryptophan. The synthesis of melatonin occured only in June and October whereas synthesis of 5-MIAA occured only in January. These experiments were conducted in the northern hemisphere.

Smith, et al.,(1981) determined H1OMT activity in postmortem human pineal glands. They observed an annual bimodal rhythm in H1OMT activity with maximal values occuring in January and July and minimum values in March and October.

1.8.2. Noradrenaline rhythm

The observation that pineal SNAT and H1OMT undergo diurnal 24 hour rhythms led researchers to suspect that there might also be a 24 hour fluctuation in its content of the sympathetic

neurotransmitter, noradrenaline. This catecholamine is present in the pineal in high concentrations (Pellegrino de Iraldi and Zieher 1966; Wurtman and Axelrod 1966).

Wurtman and Axelrod (1966) found that the noradrenaline concentration in pineals is threefold higher at night than during the day if rats are kept under diurnal lighting cycles. This rhythm is abolished by blinding, continuous darkness or continuous illumination (Wurtman et al.,1967). Brownstein and Axelrod (1974) demonstrated that pineal noradrenaline turnover is more than twice as rapid during the night as during the day, confirming the results of Wurtman and Axelrod (1966). In contradiction to Wurtman et al.,(1967), Brownstein and Axelrod (1974) found that this rhythm persists in blinded animals indicating that the rhythm is endogenous.
The increase in pineal noradrenaline content at night could be the result of enhanced synthesis, decreased liberation, or decreased enzymatic destruction of the catecholamine. Brownstein and Axelrod (1974) suggest that the 24 hour rhythm in pineal noradrenaline turnover probably reflects diurnal variations in the release of the neurotransmitter. Pineal noradrenaline content declines by 12 hours and virtually disappears by 24 hours after bilateral superior cervical ganglionectomy in rats (Morgan and Hansen, 1978).

1.8.3 Melatonin rhythm

All factors affecting SNAT and HIOMT are likely to affect melatonin synthesis since these enzymes are involved in the biosynthetic pathway for melatonin synthesis. Hence, like SNAT and HIOMT, melatonin synthesis undergoes a diurnal rhythm, reaching levels of 7 to 10 times greater at night than during the day (Quay, 1964) and (Lynch, 1971). This rhythm has been

shown to persist in constant darkness (Ralph et al, 1971), indicating that the rhythm is endogenous but modified by light (Illnerova et al., 1978). In human volunteers Lynch et al., (1975) showed that urinary content of melatonin is several times higher between 23h00 and 07h00 than between 15h00 and 23h00. Serum levels of melatonin are several fold higher at night than during the day in human subjects (Arendt, 1977). A diurnal rhythm in plasma melatonin concentration of rhesus monkeys has been demonstrated by Jenkins et al.,(1980), with high plasma melatonin levels during the dark phase of the photoperiod. Hedlund et al., (1977) showed that the level of melatonin in the cerebrospinal fluid of calves increases 17-fold at night in comparison to daytime levels.

Pang et al.,(1980) found that there is a diurnal rhythm in rat retinal melatonin content indicating that melatonin may possibly regulate the diurnal rhythm of eye pigmentation in vertebrates.

Wetterberg et al (1976) reported a melatonin rhythm during the human female menstrual cycle. However, Jenkin et al., (1980) did not detect any statistically significant differences in plasma melatonin levels during the 28 day menstrual cycle in the rhesus monkey. Arendt, (1978) reported a seasonal change of melatonin levels in blood of human subjects. Alteration in the phase of melatonin rhythm has been reported for persons travelling across longitude (Wetterberg, 1978), in patients with hypothalamic lesions (Wetterberg, 1979) and in manic -depressive disease (Jimmerson et al 1977; Lewy et al, 1978).

1.8.4. Serotonin rhythm

The mammalian pineal gland is extremely rich in serotonin content. Quay, (1963) demonstrated that pineal serotonin content undergoes a diurnal circadian rhythm. It is highest during the day and lowest at night (opposite to the melatonin rhythm) in nocturnal and diurnal animals. Snyder et al., (1964a and 1965b) showed that this rhythm persists in continuous darkness and in blinded animals, implying that the rhythm is endogenous. Neither hypophysectomy, ovariectomy, adrenalectomy or thyroidectomy affects the 24 hour serotonin rhythm, indicating that this rhythm is not generated by circulating hormones (Wurtman et al., 1964b). This rhythm has been shown to be under sympathetic nervous control (Fiske 1964; Snyder 1964b and 1965b). The rhythm can be reversed by reversing the environmental illumination (Snyder and Axelrod, 1966). Resynchronization to the new schedule requires about 6 days. Wragg et al.,(1968) observed a significant diurnal rhythm in pineal serotonin content among rats aged 8, 15 or 20 days, even following bilateral superior cervical ganglionectomy. No rhythm could be demonstrated in denervated pineals from 60-day old rats. These results suggest that prior to weaning, this rhythm may result from oscillations that originate within the pineal itself. In rats, this rhythm is present at the 6th day of age (Zweig et al., 1966) prior to complete innervation of the pineal (Machado et al., 1968). Brammer (1979) confirmed the results of Wragg et al.,(1968) by determining serotonin synthesis in rat pineal organ cultures. Pineals from 11 to 15 day old rats show a persistent daily rhythm in serotonin synthesis in organ culture. This rhythm is not found in cultured pineals of 23 to 25 day old rats even though both groups exhibit an in vivo

rhythm. It is also interesting to note that the persistence of timing ability *in vitro* by cultured bird pineal glands is very similar to that observed in immature rats (Binkley, 1978).

The circadian rhythm of serotonin in the pineal can be explained by two factors, both of which are influenced by light:-

(i) Light causes a marked increase in aromatic-L-amino acid decarboxylase activity (Snyder *et al.*, 1964a) which is the serotonin forming enzyme (Snyder and Axelrod, 1964a). This enzyme is under sympathetic nervous control (Snyder *et al.*,1965a) and undergoes a circadian rhythm in phase with the serotonin rhythm.

(ii) SNAT, which catalyses the conversion of serotonin to N -acetylserotonin also undergoes a diurnal rhythm (discussed earlier). This rhythm is 180^{o} out of phase with the rhythms of serotonin and aromatic-L-amino acid decarboxylase (Brownstein *et al.*, 1973).

As a result of (i), Serotonin levels are high during the day and (ii), low during the night.

Bovine pineal serotonin content undergoes a circannual rhythm with high values at the winter solistices and lowest values at the summer solistices. The serotonin peaks correspond to a decreased fertility in these cattle during the winter months (Philo and Reiter, 1980).

Walker and Timiras (1980) found that testosterone administration as a single dose to female rats on day 3 of life depresses pineal serotonin levels and abolishes the circadian serotonin rhythm up to 20 days of age.

1.8.5. Taurine rhythm

Taurine, which is believed to be a modulator of membrane

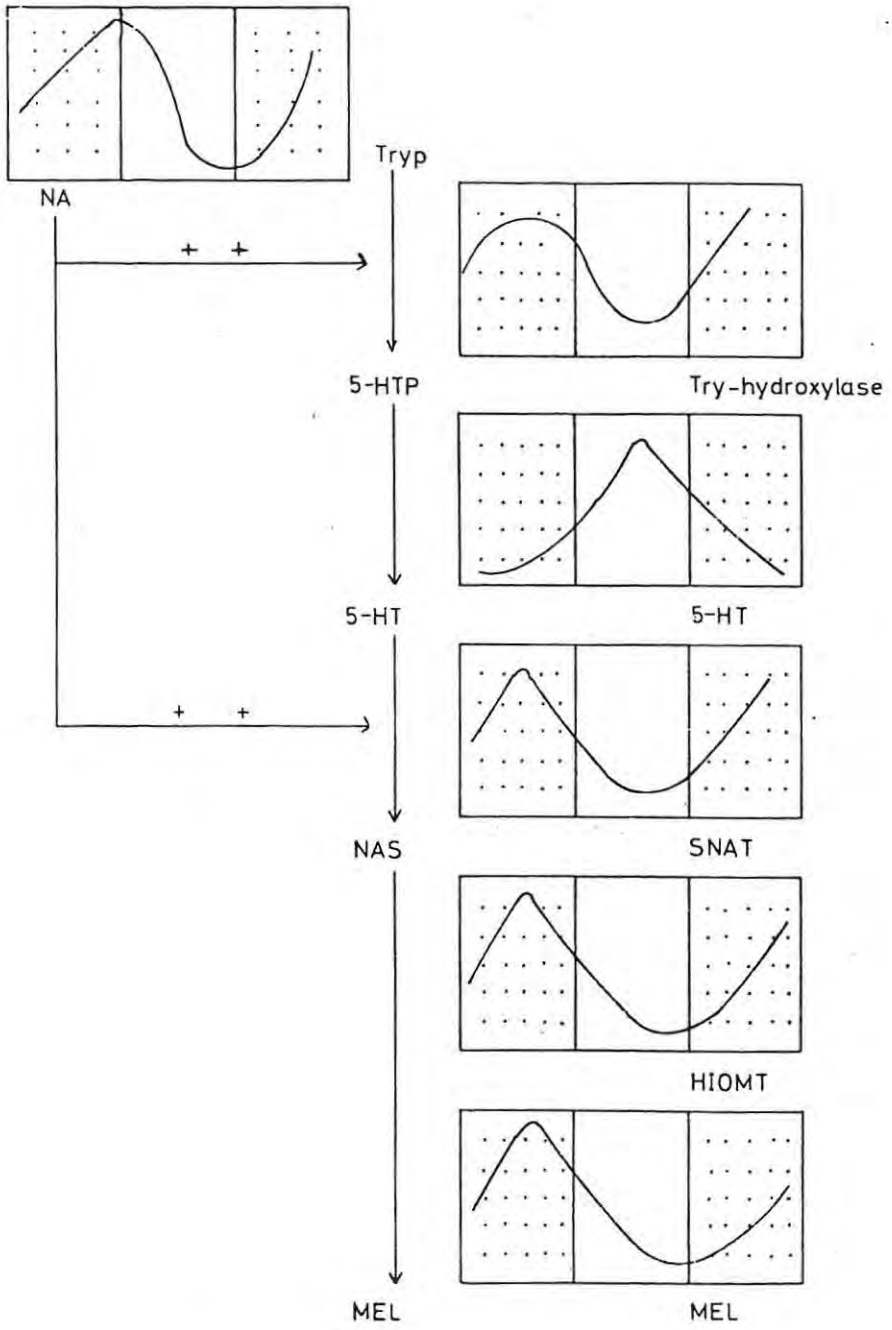

Fig.7. Scheme of day/night rhythms of noradrenaline (NA), tryptophan hydroxylase, serotonin (5-HT), SNAT, H1OMT and melatonin (MEL), and the influence of noradrenaline on tryptophan hydroxylase and SNAT.

excitability in muscle and a neuroinhibitory transmitter in the CNS, is present in relatively large quantities in the retina and the pineal gland (Grosso et al., 1978). These authors reported that there is a circadian taurine rhythm in the mature rat pineal gland. The maximum taurine concentration occurs at the midpoint of the light period and the minimum concentration occurs at the beginning of the dark period. Bilateral superior cervical ganglionectomy lowers pineal taurine levels, indicating that the taurine rhythm may be sympathetically regulated. Blinding has no effect on this rhythm.

Rat pineals in organ culture are capable of synthesizing radiolabeled taurine from radiolabeled cystine in the culture medium (Ebels et al., 1980).

1.8.6. Synaptic ribbon rhythm

King and Dougherty (1980) found that there is a circadian rhythm in synaptic ribbon numbers in rat pinealocytes from day 1 of age to day 10. Recently Hewing,(1980) showed that the pinealoctyes of hamsters deprived of light for 5 weeks have elevated numbers of synaptic ribbons. Exposure of these animals to light results in a marked drop in the number of synaptic ribbons.

1.9. Endocrine effects of melatonin and other pineal compounds.

1.9.1. Effect on the pituitary gland.

1.9.1.1. Gonadotropin secretion

In 1965, Moszkowska (1965) confirmed that melatonin administration reduces ovarian mass and also showed that repeated

melatonin administration delays vaginal opening. Chu et al.,(1964) and McIsaac et al., (1964) showed that melatonin administration decreases the incidence of estrus vaginal smears in rats and mice exposed to normal lighting conditions, and therefore probably interferes with ovulation. Doses of melatonin that delay puberty and decrease ovarian mass also increase LH content in the pituitary glands of rats, implying that melatonin suppresses LH secretion (Adams et al., 1965). Exogenous melatonin directly inhibits spontaneous ovulation (Collu et al, 1971). Methoxytryptophol, another methoxy-indole has an even more pronounced effect in reducing the frequency of positive estrus smears (McIsaac et al., 1964) than melatonin (Wurtman et al, 1963).

Median eminence implants of melatonin suppresses the post-castration-induced rise in serum LH levels (Fraschini 1968 and 1968a). 5-MTOH implanted directly into the pituitary gland has no effect on pituitary FSH stores, whereas serotonin seems to increase them (Fraschini, 1969). These results indicate that 5-MTOH and serotonin may possibly control FSH secretion, possibly by activation of special receptors in the median eminence. However, serotonin and 5-MTOH median eminence implants do not modify pituitary stores of LH, while melatonin and 5-hydroxytryptophol (5-HTOH) significantly reduce pituitary content of this gonadotropin. The implantation of melatonin in the cerebral cortex or in the anterior pituitary itself, has no effect on pituitary stores of LH (Fraschini and Martini 1970). The results obtained by Fraschini and Martini, (1970) with 5 HTOH are in variance to results obtained by Farrel et al., (1966) who showed that this compound has no effect on the endocrine system.

This is probably due to the fact that in Farrels experiments, the 5-HTOH was administered systemically. From these results, it appears that melatonin and 5-HTOH inhibit LH secretion, while serotonin and 5-MTOH inhibit FSH secretion. The effects of the pineal indoles and methoxyindoles seem to be exerted through specific brain receptors rather than directly on pituitary cells. Kamberi et al., (1970) showed that LH release by melatonin and serotonin is not due to a direct action on the anterior pituitary. Melatonin injected i.v. causes a reduction in serum LH concentration, supporting the view that the indoles and methoxyindoles act indirectly via the hypothalamic-hypophyseal complex.

The ability of melatonin to suppress the castration-induced rise in serum LH was observed by Roche et al., (1970) and Reiter, (1973). Ovariectomy increases pituitary and plasma levels of gonadotropins. Low doses of melatonin increase this effect while higher doses do not (Trakulrungsi et al., 1979) in hamsters. Low doses of melatonin administered (1-100 ug) to sexually immature rats, decreases LH spontaneous activity and decreases pituitary LH content (Syutkin et al., 1980).

Alonso et al., (1978) showed that the pituitary content and concentration of LH and FSH are increased by pinealectomy in both intact and castrated rats, suggesting that the pineal gland exerts an inhibitory action on the hypothalamic-pituitary axis that seems to be independent of the testicular function.

At variance with these results, Damian et al., (1978)showed that pinealectomy in rats does not influence the daily pituitary LH content and reverses the hypophyseal FSH values in the

dark period. The intact rats show maximum values at midnight while pinealectomized rats exhibit minimum values at midnight. During the last four days of pregnancy in rats, pinealectomized rats have higher LH levels than sham-operated controls (Nir et al., 1979).

Serotonin has been shown to inhibit the synthesis of hypothalamic releasing factors i.e. FSH-releasing factor (FSH-RF) in vitro (Moszkowska et al., 1973). Serotonin administration has also been shown to cause ovarian hypertrophy (Kamberi et al., 1971). It is possible that this is due to inhibition of FSH-RF and LH-RH. Melatonin can act directly on the neonatal pituitary gland and suppress the response to LH-RH, which in turn suppresses pituitary LH release (Martin and Klein, 1976; Martin et al., 1977). The rat pineal content of LH-RH displays a circadian variation with maximum levels at 6 pm. and minimum levels at 6 am. (Antonov, 1977). The pineal gland and the superior cervical ganglia accumulate i.v. injected radiolabeled LH-RH to a greater extent than other brain areas. It accumulates in the pinealocytes and its accumulation is not modified by denervation. It is, possible that the pineal gland is a target organ for LH RH (Trentini et al., 1980)

Moszkowska et al., (1971) isolated two active fractions from an extract of sheep pineal powder.

They referred to these fractions as fraction F_2 and F_3. F_3 has a molecular mass of less than 700 and is free of melatonin and 5-MTOH. This fraction acts directly on the pituitary to interfere with FSH secretion whereas F_2 contains indoleaminergic material and acts directly on nerve structures involved with pituitary regulation. Recently, Benson (1980)

detected the presence of taurine, closely associated with fractions possessing antigonadotropic activity in bovine pineal glands. Damian et al., (1980) demonstrated the presence of a pineal polypeptide capable of causing a much greater fall in serum gonadotropin levels than that caused by melatonin.

It is interesting to note that Damian et al.,(1978) demonstrated the presence of a gonadotropin, resembling LH in bovine pineal glands. The concentration of this gonadotropin in the pineal exceeds the LH concentration in the blood 555 times and that of the CSF, 6,8 times.

1.9.1.2. Growth Hormone (GH)

As early as 1959, Malm et al.,(1959) observed an increase in body mass of pinealectomized rats. Later, Wurtman et al., (1968b) and later Sorrentino et al.,(1972) showed that pineal stimulation by darkness retards growth. Relkin,(1972) reported a decrease in pituitary and plasma GH levels and retarded body growth in animals exposed to constant darkness. This effect is counteracted by pinealectomy. Sham-operated rats display a diurnal rhythm in growth hormone release, with reduced release during the dark. Pinealectomy reduces the level of GH during the day but not during the night (Ronnelkeiv and McCann, (1978). It appears therefore that melatonin has an inhibitory role on GH secretory mechanisms. Collu et al., (1972) showed that serotonin enhances GH release. Gram quantities of melatonin also reduce the rise in plasma GH among humans made hypoglycaemic with insulin (Smythe and Lazarus 1974).

Smythe and Lazarus (1973) proposed that melatonin and serotonin act antagonistically on GH release and that melatonin can act as a competitive inhibitor at serotonin receptor sites.

1.9.1.3. Prolactin

Pinealectomy results in a 13% decline in milk yield during the second half of the nursing period in rats (Nir et al., 1968). Blind rats are deficient in milk and prolactin production (Reiter, 1972). Kamberi et al., (1971) reported that injection of serotonin or melatonin into the third ventricle of rats, increases serum prolactin levels. Intravenous administration of 5-hydroxytryptophan (5-HTP) or melatonin increases serum prolactin levels in rats, while serotonin is without effect (Lu and Meites, 1973). These authors also showed that the diurnal changes in prolactin secretion run parallel to those of pineal activity. Relkin, (1972b) found an increase in pituitary and decrease in plasma prolactin levels in pinealectomized male rats, implying an inhibition of prolactin release by pinealectomy.

The exposure of rats to short photoperiods flattens the diurnal prolactin rhythm. Pinealectomy lowers prolactin levels but does not affect the diurnal rhythm. Neurtralization of plasma melatonin and N-acetylserotonin causes a significant reduction in prolactin levels although the diurnal rhythm persists. The diurnal prolactin rhythm was also observed by Willoughby (1980), with high levels during the dark period. Pinealectomy slightly delays the fall in the mean prolactin concentration which normally occurs in the

light period (Niles et al., 1977).

Sackman et al., (1977) showed that daily melatonin injections (25 µg for 50 days) depresses prolactin levels in intact hamsters whereas in pinealectomized hamsters, the same treatment fails to alter prolactin levels. In intact animals, 5-MTOH and N-acetylserotonin slightly depress prolactin levels, these effects being negated by pinealectomy. Daily afternoon injections of melatonin to anosmic male rats results in depressed serum prolactin levels (Blask et al., 1980).

Hanew, et al., (1980) investigated the effects of various pineal compounds on prolactin secretion in cultured rat pituitary clonal cells. These cells only produce prolactin when grown in Ham's F-10 medium. They showed that 5-hydroxytryptophan, serotonin and N-acetylserotonin have no direct effect on prolactin secretion from these cells. In contrast, melatonin clearly enhances prolactin secretion from these cells. Arginine vasotocin, a well known pineal polypeptide with antigonadotropic properties, also stimulates prolactin secretion by these cells, but in low concentrations only. Higher concentrations inhibit secretion.

From these studies it can be concluded that the pineal gland has a modulatory effect on prolactin release.

1.9.2 Adrenal glands

1.9.2.1. Aldosterone secretion

Farrel (1959) claimed to have discovered in pineal extracts, a compound which specifically causes the release of aldosterone. This substance, he termed, adrenoglomerulotropin.

Farrel (1960) separated another factor from pineal glands which inhibits aldosterone secretion. Subsequent to this, Farrel and McIsaac (1960) claimed that the chemical identity of the pineal adrenoglomerulotropin is 1-methyl-6-methoxy-1,2,4,4 - tetrahydro-2-carboline whose structure is not very different from that of melatonin.

Fabre et al.,(1965) proposed that the inhibitor of aldosterone secretion which Farrell had reported may possibly be ubiquinone.

Jouan (1963) and Jouan and Samperez (1964) reported that any aldosterone stimulating activity present in extracts of mammalian pineals results from their high concentration of serotonin. The infusion of angiotensin II induces an increase in the serotonin contents of pineal, hypothalamus and brain stem in dogs. The data point to the possible existence of positive and negative feedback relationships between the brain isorenin- angiotensin system and serotonin metabolism in the pineal gland (Haulica et al.,1980).

Haulica et al., (1979) identified a renin-like enzyme in the adult human pineal gland.

Gromova et al.,(1967a and 1967b) reported that acute administration of melatonin significantly reduces aldosterone production, while hypophysectomy abolishes this effect.

Machado and Da Silva (1963) observed that rats injected with a rat pineal extract conserve sodium more efficiently than controls. Paradoxically, Tanner and Hungerford (1962) reported that pinealectomy causes rats to retain more sodium than sham-operated controls. Kinson et al.,(1967) reported an approximately three fold increase in aldosterone secretion levels one month after pinealectomy.

At present, it appears as if the pineal is involved in aldosterone secretion but no conclusions can be drawn at this stage.

1.9.2.2 Glucocorticoid secretion

Pinealectomized rats have been shown to secrete an increased amount of corticosterone (Kinson and Singer, 1967). Barrel and Lapwood (1978) found that the rise in cortisol secretion during the morning hours in sham-operated rams is absent in pinealectomized rams. These studies indicate that the pineal gland exerts a tonic influence on adrenocorticotropin (ACTH) secretion.

The administration of purified or synthetic pineal principles has also provided conflicting results. Barchas et al., (1969) reported that in rats, acute or chronic subcutaneous administration of melatonin does not modify plasma levels of corticosterone, ACTH or pituitary stores of ACTH. However, Fraschini et al., (1968) found that intrahypothalamic implants of melatonin cause a significant reduction of adrenal mass in adult castrated male rats. The acute administration of melatonin significantly increases corticosterone production in intact rats (Gromova et al.,1967a). De Prospo and Hurley (1971) injected melatonin into the lateral cerebral ventricles of female rats and showed that melatonin failed to depress the pituitary-adrenal axis.

In contrast, Motta et al., (1971) showed that after i.v. injection of a high dose of melatonin (165 ug/rat), the levels of plasma corticosterone in rats under resting conditions are significantly reduced. Both 5-MTOH and 5-HTOH are able to mimic the effect of melatonin when given i.v. in sufficient amounts.

Daily i.p. melatonin administration for one week stimulates adrenal 5 -∝- reductase activity in ovariectomized and hypophysectomized rats (Ogle and Kitay, 1977). These same authors, (1978) incubated tissue slices and homogenates of rat adrenal glands in the presence of 45n moles/ml of melatonin or serotonin. They found that melatonin stimulates 5 -∝ -reductase activity, confirming the earlier results. The secretion rates of dihydrocorticosterone and tetrahydrocorticosterone increase with a concomitant decline in secretion of corticosterone. Serotonin does not alter any of these parameters of adrenal steroidogenesis. Medhi and Sandor (1977) demonstrated that melatonin inhibits the transformation of radiolabeled progesterone to cortisol and aldosterone in a dose-dependent manner in vitro when bovine adrenal cortex is used. These authors also showed that when bovine adrenal microsomal preparation is used, melatonin inhibits 17- and 21-hydroxylation and concluded that melatonin might possibily be a non-competitive inhibitor of the microsomal 17- and 21-hydroxylases.

Recently, Komissarenko et al., (1979) isolated an epiphyseal factor from bovine pineal glands which is capable of suppressing corticosteroidogenesis in rats.

From the reports it seems possible that the pineal gland might be directly involved in modulating corticosteroidogenesis.

1.9.3 Thyroid gland

Aron, (1960) described an activation of the histological character of the thyroid gland in pinealectomized turtles.

Scêpovic, (1963) demonstrated an increase in uptake of thyroidal ^{131}I in pinealectomized rats. The administration of pineal extract causes a decrease in both thyroid mass and ^{131}I

accumulation by the thyroid gland. Furthermore, the development of thiouracil-induced goiter is inhibited by the administration of pineal extract (De Luca et al., 1961). Melatonin administration has been shown to produce similar effects (Baschieri et al., 1963). The thyroxine secretory rate is decreased by about 20% in the melatonin-treated rat (Singh and Turner, 1962). In contrast, Thiéblot et al., (1966) found that melatonin administered to prepuberal rats produces marked hypertrophy of the thyroid gland. Relkin (1972c) reported that pinealectomy in rats at the age of weaning changes the activity of the pituitary-thyroid axis for a short period after the operation. When TRH and melatonin were administered concurrently, the same author (Relkin, 1978) found that the inhibitory effect of the pineal in prepuberal male rats is exerted at the level of the hypothalamic secretion of TRH. Chronic melatonin treatment has been reported to decrease pituitary levels and increase plasma levels of thyrotropic hormone (Panda and Turner, 1968). In blinded hamsters, pinealectomy reverses the depression of the free thyroxine index (Vriend et al., 1977), suggesting a pineal inhibition of thyroid function. The same author (Vriend and Reiter, 1977) showed that melatonin decreases the free thyroxine index in hamsters. Recently, Brammer et al., carried out extensive studies from which they concluded that there is no evidence of any significant interactions between the pineal gland and the hypothalamic-pituitary-thyroid axis. Also recently, Gordon et al., (1980) found that chronic melatonin administration to weaning rats, increases basal TSH and radiolabeled iodine uptake by the thyroid. In contrast to other reports, these authors found that melatonin increases the thyroid gland size and thryroxine content of the thyroid. Optic nerve

section increases serum TSH and triiodothyronine (T3) levels, the latter being reversed by pinealectomy. Vriend et al., (1980) demonstrated the presence of a thyrotropin releasing hormone (TSH) inhibitor in the rat pineal gland.
From these studies it appears that the pineal gland could influence thyroid function either by central effects or by direct action on the thyroid gland.

1.9.3.1. Effect of thyroid hormones on pineal function

The only report in the literature on this is by Nir and Hirschmann, (1978). In rat pineal organ cultures, these authors showed that TSH has no effect on tryptophan metabolism. However, thyroxine at a dose of 2,5 µg increases the formation of melatonin and N-acetylserotonin from tryptophan. T3 produces increases in concentrations of all pineal indoleamines. Larger doses of thyroxine or T3 do not produce any of the changes observed at the lower doses. These results suggest a direct positive feedback between the thyroid and pineal glands.

1.9.4 The pineal gland

Rats exposed to constant darkness for 14 days or administration of melatonin to rats, induces changes in pineal ultrastructure indicative of increased pineal activity (Freire and Cardinali, 1975). Ribosomes, procentrioles, microtubules, nucleoli and golgi apparatus all increase in number. These authors also observed increases in microtubular protein content, SNAT and H1OMT activities.

Further support for increased pineal activity after the administration of exogenous melatonin has been put forward by Barratt et al.,(1977), who showed that melatonin has varied

effects on the fine structure of the hamster pineal gland. The pinealocyte nuclear characteristics of melatonin-treated hamsters are, smaller average diameter, less polymorphism and more heterochromatin as well as reductions in the amount of smooth endoplasmic reticulum and lipid moieties. There is an increase in the number of large mitochondria, membrane whorls and dense-cored secretory vesicles in the pinealocytes indicating enhanced pineal gland activity.

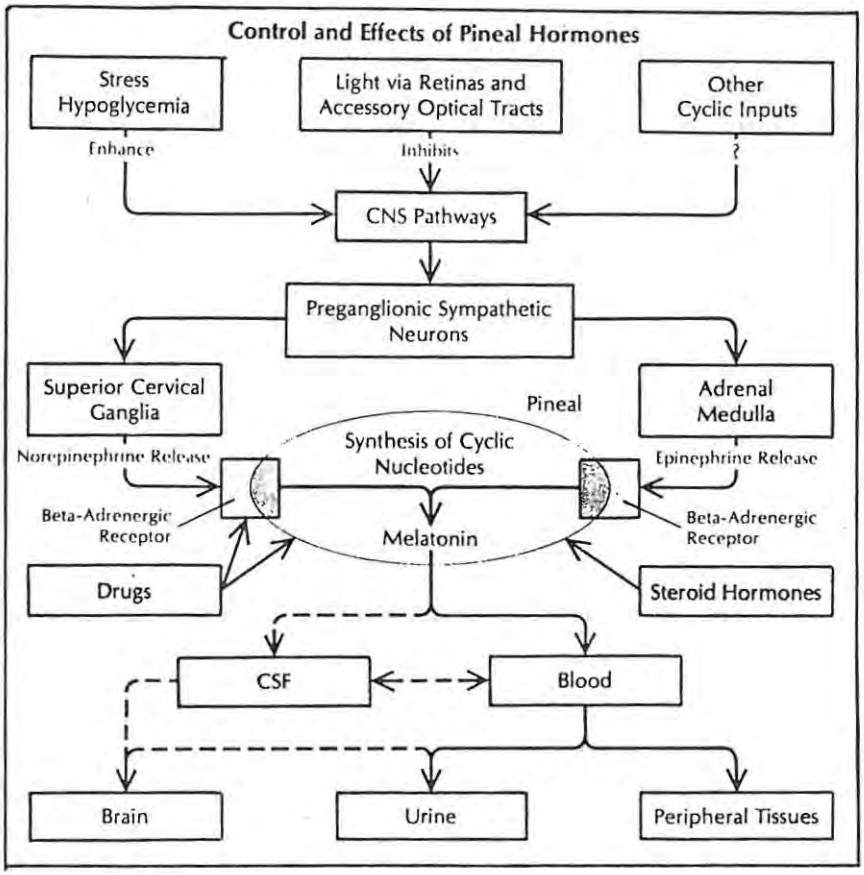

FIG. 8 . Control and effects of pineal hormones. Diagram showing the areas of action of hormones on the pineal and of pineal hormones on other areas.

(Wurtman, 1980)

CHAPTER 2

2.1 THE EFFECT OF GONADAL SEX STEROIDS ON PINEAL SNAT AND H1OMT ACTIVITY.

2.1.1 MATERIALS AND METHODS

2.1.1.1 Animals

Albino rats of the Wistar strain were used throughout this study. All rats used, were housed individually in plastic cages with food and water ad lib. The animal room was kept at approximately 20°C and had a light/dark cycle of 12 hours light / 12 hours dark controlled by a time switch. The light intensity during the light phase was approximately 300 Watts/cm^2 provided by cool white fluorescent tubes.

2.1.1.2. Dosing of animals.

All the steroids used for dosing animals throughout this study, were dissolved in olive oil and kept in amber bottles. The animals were dosed with 0,1 ml of olive oil containing the steroid (for test animals) or plain olive oil (controls) subcutaneously in the region of the thigh.

2.1.1.3 Surgery

2.1.1.3.1. Anaesthesia

Surgical procedures were carried out initially, using pentobarbitone anaesthesia. An i.p. dose of 65 mg / kg of body mass was used. Very often rats did not reach satisfactory anaesthesia. This effect appeared to differ from one day to the next. After surgery the rats took approximately 3 hours to recover from the anaesthetic. The biggest problem encountered using this anaesthetic was that about 80% of the rats developed respiratory problems during anaesthesia and eventually died. A few of these animals survived after

i.p. administration of nikethamide, a respiratory stimulant and by using a respirator. It was decided to abandon the use of pentobarbitone since the survival rate was very poor and time spent attending to the animals which survived was excessive. Ether anaesthesia was subsequently employed. Rats were put into a dessicator with cotton wool soaked in ether. As soon as the rats were asleep, they were taken out of the dessicator and placed on the operating surface. A 25 ml bottle containing cotton wool soaked in ether was placed about 1-2 cm in front of the rat's nose, with the open mouth of the bottle facing the rats nose. The surgery was then performed. The bottle was removed when surgery had been completed. The rats then recovered in about 10 minutes. During surgery, if the respiration became too weak, the bottle was moved further away from the rat's nose. 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. A few rats which stopped breathing due to excessive inhalation of ether, were placed on a respirator for about one minute after which they regained their ability to respire and recovered uneventfully. This method of anaesthesia was used wherever surgery was performed because of the high survival rate, ease of administration with the ability of observing the depth of anaesthesia and complete lack of complications.

2.1.1.3.2. Bilateral ovariectomy

Female rats that had been separated from males at the pre-pubertal stage were ovariectomized in adulthood. Ovariectomies were performed at least two weeks prior to dosing of the animals.

After 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 this incision. With the aid of a forceps, the fat with the ovary and part of the uterus were pulled carefully through the incision. The ovarian artery, vein and part of the uterus just below the fallopian tube were ligatured and the ovary was excised. The incisions were stitched with suture. The same procedure was performed on the opposite side to remove the other ovary.

Ovariectomy by ventral incision proved to be awkward as well as exhibiting high mortality and was thus not suitable.

2.1.1.3.3. Bilateral orchidectomy (castration)

The term "castrated" is used by some authors to mean ovariectomy or orchidectomy whereas others prefer to use it as meaning orchidectomy, as will be used throughout this study.
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

was cut and the area was swabbed with an iodine solution. A longitudinal incision, 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 a 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 rats were allowed to regain consciousness.

2.1.1.3.4. Sham-operations.

These were performed in exactly the same manner as described in 2.1.1.3.2 and 2.1.1.3.3 with the exception that no ligatures or excisions were made.

2.1.1.5 Monitoring of estrus cycles.

A piece of cotton wool was wrapped around the end of an orange stick to form a bud. This was inserted into the vagina and turned clockwise once, and then withdrawn. The bud was then allowed to touch a few drops of water on a microscope slide. A positive estrus smear was indicated by a white precipitous layer of cornified cells on the slide.

2.1.1.6 Thin-Layer chromatography (TLC)

Throughout this study, wherever TLC was used, glass plates were coated to a thickness of 0,25 mm using Kieselgel G60 (Merck). The slurry which consisted of 30g Kieselgel + 65 ml of water was sufficient to coat five 20 x 20 cm plates simultaneously.

2.1.1.7 Enzyme assays

2.1.1.7.1 H1OMT Assay

The assay technique used for the determination of H1OMT activity was developed by Axelrod and Weissbach (1961) and modified by Morton (personal communication). The assay is based on the transfer of a radioactive (^{14}C) - methyl group from the methyl donor, s-adenosyl-L - (methyl -^{14}C) methionine to the substrate, N-acetylserotonin to form radioactive melatonin (N-acetyl-5-(methoxy-^{14}C) tryptamine). In the pineal gland, H1OMT O-methylates N-acetylserotonin to form melatonin. By adding radioactive s-adenosyl methionine to pineal homogenates incubated with N-acetylserotonin, radioactive melatonin is formed which can be extracted and measured by liquid scintillometry. The amount of radioactive melatonin formed can be used as an indication of H1OMT activity.

Rats were killed swiftly by neck fracture using a metal rod. The animals were decapitated and a pair of scissors was used to make an incision through the bone on either side from the foramen magnum to near the orbit. The top of the skull was removed with forceps and the pineal gland was removed rapidly. In many cases, the pineal gland was found to adhere to the top of the skull. Individual pineals were homogenized in 145µl of cold 0,05 M phosphate buffer using a teflon pestle. Homogenization was performed by hand, until all visible tissue was homogenized.

To this homogenate, 9 µl of 7×10^{-4} M N-acetylserotonin was added followed by 6µl (0,03 µCi) of s-adenosyl-L - (methyl-^{14}C) - methionine (specific activity 0,4-0,5 mCi/mMol).

(The radiochemical Centre, Amersham). Both of these were found to be chromatographically pure using TLC. The homogenate was then incubated for 1 hour at 42°C. A blank from which the tissue was omitted was also incubated. After one hour of incubation, the reaction was stopped by the addition of an equal volume (160 ul) of 0,2 M borate buffer, pH10 to each tube. Following this, 3 ml of toluene-isoamyl alcohol (97:3) was added and the tubes were vortexed for two minutes. The tubes were then centrifuged for ten minutes at 3 000 r.p.m. The clear organic layer from each tube was aspirated into clean tubes. From each tube, 2 ml of the organic layer was added to 6 ml of Instagel(R) (Packard Instrument Company) in glass scintillation vials. Each vial was counted for its radioactivity in a Beckman LS3150T liquid scintillation counter for 20 minutes. Quenching was corrected by using the external standard channels ratio (ESCR) method of quench correction. This was used to convert counts per minute (c.p.m.) to disintegrations per minute (d.p.m.). The d.p.m. values were then converted to picomoles of product formed per gland per hour.

2.1.1.7.2. SNAT Assay

The assay technique used for determination of SNAT activity is that developed by Deguchi and Axelrod (1972) and modified by Morton (personal communication). The assay is based the acetylation of tryptamine with (1-^{14}C) acetyl-coenzyme A by SNAT to form (^{14}C) - acetyltryptamine. By adding (^{14}C) acetyl-coenzyme A to pineal homogenates and incubating with tryptamine, radioactive acetyltryptamine is formed which can be extracted into an organic solvent and measured

by liquid scintillometry. The amount of radioactive acetyltryptamine formed can be used as an indication of SNAT activity.

Rats were killed by neck fracture and pineal glands were removed as described previously. Individual pineal glands were homogenized in 200 µl of 0,05 M phosphate buffer, pH 6,5 containing tryptamine hydrochloride ($4,5 \times 10^{-3}$ M). To this, 50 µl of (1-^{14}C) - acetyl - coenzyme A (0,1 mCi) with a specific activity of 5,55 mCi/mMol was added followed by the addition of 50 µl of 0,1M phosphate buffer, pH 6,5. The tubes were incubated at 20^{o}C for 3 hours. The reaction was terminated by the addition of 300 µl of 0,2M borate buffer, pH10. Following this, 3 ml of toluene-isoamyl alcohol (97:3) was added to the tubes and the tubes were vortexed on a rotamixer for 2 minutes. The tubes were then centrifuged for 10 minutes at 3 000 r.p.m. The organic layers were aspirated into clean tubes. Two ml of the aspirate from each tube was added to 6 ml of Instagel$^{(R)}$ in glass scintillation vials. Each vial was counted for 20 minutes and c.p.m. were converted to d.p.m. as described in 2.1.1.7.1 The d.p.m. values were converted to picomoles of (^{14}C)-N-acetyltryptamine formed per gland per hour (Deguchi and Axlerod, 1972). Blanks in which tissue was omitted were also incubated. The blank values were subtracted from the assay values before expressing the results. The results were not expressed as pM/mg/hr as weighing of the pineals proved to be difficult due to evaporation of the buffer in which they were weighed.

2.1.1.8 Significance

Throughout this study, the Students-t-test was used to evaluate significance.

2.1.2. H1OMT activity during the estrus cycle.

2.1.2.1 Materials and Methods

Female rats with an approximate mass of 180 - 200 g were housed individually as described earlier. Estrus cycles were monitored daily between 09h00 and 10h00. Only those rats showing at least two consecutive regular estrus cycles were used. Rats in the same stage of the estrus cycle were killed and the pineal H1OMT activity was determined immediately (n=8 for each stage of the cycle).

2.1.2.2. Results (See Table 1 and Fig. 9)

H1OMT activity was found to be approximately two fold higher during estrus than in proestrus. During diestrus and metestrus, H1OMT activity levels were lower than that found in estrus but higher than the proestrus levels. No statistically significant difference could be found between diestrus and metestrus.

2.1.2.3 Discussion

These results are in agreement with that of Cardinali et al., (1974b) who also found H1OMT activity to be higher during estrus, but in disagreement with results obtained by Wurtman et al.,(1965)who showed that H1OMT activity is lowest during estrus. However, Wurtman et al.,also found that H1OMT activity was low in proestrus. It is possible that the

Stage of Cycle	H1OMT Activity pM Melatonin formed/gland/hr ± S.E.M.	Significance
Proestrus	212,1 ± 28 •	P<0,001
Estrus	440,5 ± 39 *	P<0,001
Diestrus	322,2 ± 15 *•	P<0,001
Metestrus	307,3 ± 22 *•	P<0,001

TABLE 1. Variation in rat pineal H1OMT activity during the estrus cycle. (n=4).

* Significantly different from proestrus.

• Significantly different from estrus.

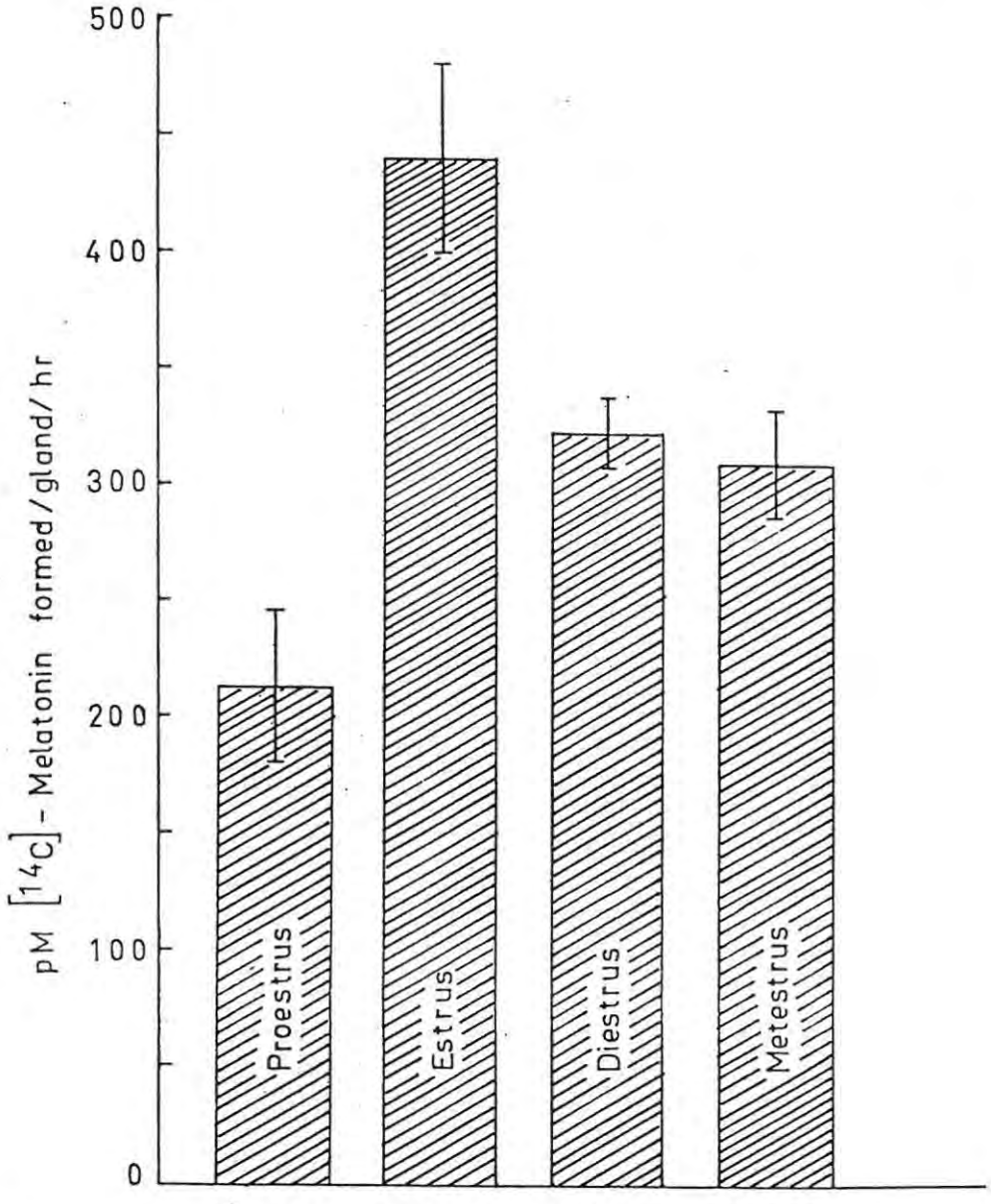

Fig. 9.HIOMT activity during the estrus cycle.

high levels of plasma estradiol during proestrus suppress H1OMT activity (Wurtman, 1980) or that these high levels of estradiol during proestrus induce an increase in H1OMT activity 24 hours later, in estrus.

2.1.3. Effect of ovariectomy and estradiol on H1OMT Activity

2.1.3.1. Materials and methods

Female rats with a mass of 200-230 g were used. Sham-operated rats were killed during the estrus phase of the estrus cycle and pineal H1OMT activity was determined immediately. Ovariectomized rats were injected subcutaneously with estradiol benzoate in doses ranging from 0 - 10 µg/day for 3 days. The rats were dosed at 09h00. At 09h00 on the morning of the 4th day, the rats were killed (n=4 at each dosage level) and H1OMT activity was determined immediately. The sham-operated rats and ovariectomized rats which received no estradiol, were injected with the vehicle only (0,1 ml olive oil). The experiment was repeated and the results were expressed as a mean of the two experiments.

2.1.3.2. Results (See Table 2 and Fig. 10)

Pineal H1OMT activity in ovariectomized rats was found to be significantly lower compared to that in the sham-operated rats in estrus. Estradiol benzoate, in doses of 0,01, 0,1 and 1 µg/day for 3 days restored H1OMT activity in the ovariectomized rats to levels approximating, and even above levels found in the sham-operated rats. Estradiol benzoate, at a dose of 10 µg / day for 3 days suppressed H1OMT activity in the ovariectomized rats to levels below that found in

Surgery	Dose of Estradiol benzoate µg/day for 3 days	H1OMT Activity pM [^{14}C]-Melatonin formed/gland/hr ± S.E.M.	Significance
Sham operated	0	500,4 ± 41	P<0,001
OVX	0	348,1 ± 33	P<0,001
OVX	0,01	515,2 ± 52	P<0,001
OVX	0,1	592,4 ± 72	P<0,001
OVX	1	588,1 ± 74	P<0,001
OVX	10	142,6 ± 15	P<0,001

TABLE 2. Effect of ovariectomy and estradiol benzoate administration to ovariectomised rats on rat pineal H1OMT activity. Rats were dosed with either estradiol benzoate or olive oil, 0,1 ml (vehicle) at 9 a.m. for 3 days and sacrificed at 9 a.m. on the fourth day. The experiment was carried out in duplicate.(n=4). Sham operated rats were sacrificed inthe estrus phase of the estrus cycle.

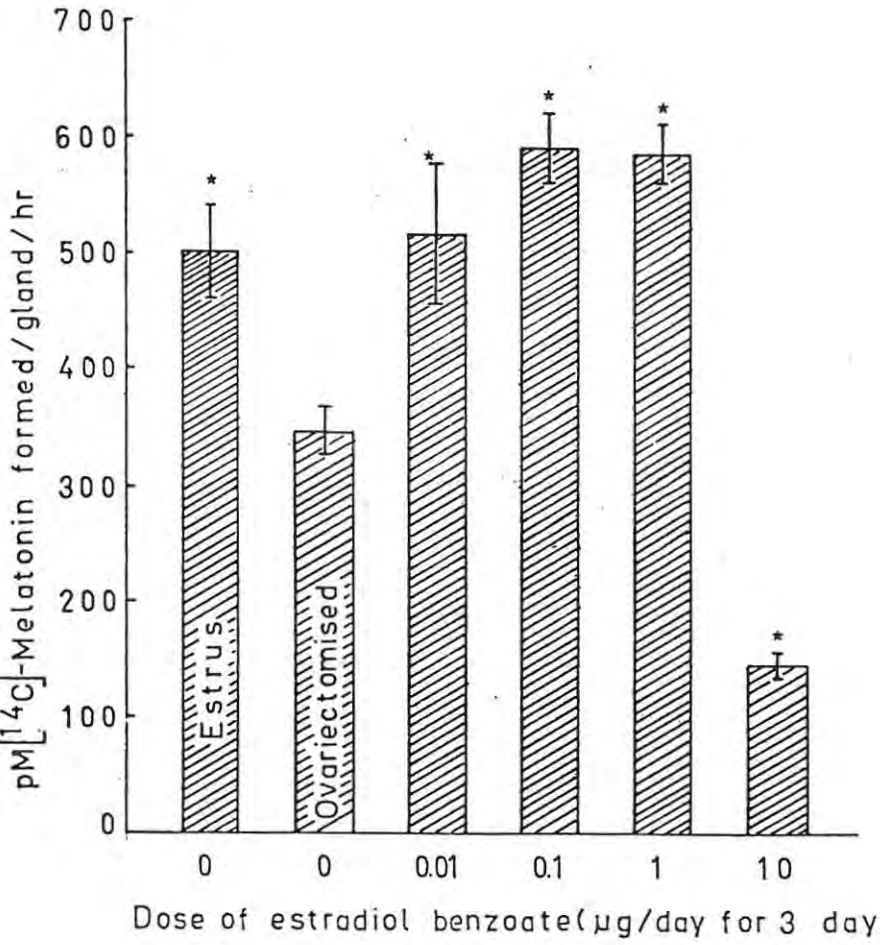

Fig. 10. Effect of ovariectomy and estradiol benzoate administration to ovariectomised rats on rat pineal HIOMT activity. (n=4).

* $P < 0,001$.

the control ovariectomized rats.

2.1.3.3 Discussion

The reduction of H1OMT activity in ovariectomized rats is in agreement with the reports by Cardinali (1974) and Wallen and Yochim (1974). This reduction in H1OMT activity could possibily be due to a decreased pineal protein synthesis observed in ovariectomized rats (Cardinali et al., 1974b) which could possibily result in a decreased synthesis in H1OMT protein. Assuming this explanation to be correct, the stimulatory effects of low doses of estradiol observed in this study can be accounted for, since low doses of estradiol have been shown to increase pineal protein synthesis in ovariectomized rats, while high doses of estradiol have been shown to inhibit pineal protein synthesis (Cardinali et al., 1974b and 1975d). This could possibly explain the inhibitory effects of a higher dose of estradiol benzoate (10 µg / day for 3 days) on H1OMT activity in ovariectomized rats.

It appears therefore, that estradiol has a biphasic dose-dependent effect on pineal H1OMT activity.

2.1.4 Effect of progesterone on pineal H1OMT activity

2.1.4.1 Materials and methods

Female rats with a mass of 220 - 240 g which had been OVX two weeks earlier, were used. The rats (n=4 at each dosage level) were injected at 09h00 with progesterone in doses of 0; 0,01; 0,1; 1; 10; 100 and 1 000 µg / day for 3 days. The rats were killed in the morning (09h00) of the fourth day and pineal H1OMT activity was determined immediately.

The control group of rats were injected with the vehicle only (0,1 ml olive oil / day for 3 days). The experiment was repeated and the results are expressed as the mean of the results of the two experiments.

2.1.4.2. Results (See Table 3 and Fig. 11).

Although all doses of progesterone increased H1OMT activity a statistically significant elevation in H1OMT activity was only observed in groups of rats dosed with 10, 100 and 1 000 µg progesterone / day for 3 days.

2.1.4.3. Discussion

From this study, it appears that acute progesterone administration increases pineal H1OMT activity. Except for Preslock (1974a and 1975) who showed that acute progesterone administration to castrated quails elevated pineal H1OMT activity to that of controls, there are no reports as yet on the effects of acute progesterone administration on pineal H1OMT activity in the literature.

However, Cardinali and Vacas (1978) showed the acute administration of progesterone decreases pineal protein synthesis in a dose-dependent manner. Since a decrease in pineal protein synthesis would not explain the results obtained in this study nor the results obtained by Preslock, another explanation has to be sought. It is possible that progesterone affects the association-dissociation phenomenon of the H1OMT subunits described by Jackson and Lovenberg,(1971).

Surgery	Dose of Progesterone µg/day for 3 days	H1OMT Activity. pM [14C]-Melatonin formed/gland/hr. ± S.E.M.	Significance
OVX	0	380,8 ± 56	- -
OVX	0,01	501,5 ± 120	N.S.
OVX	0,1	553,0 ± 128	N.S.
OVX	1	552,2 ± 100	N.S.
OVX	10	520,0 ± 61	P<0,01
OVX	100	550,6 ± 70	P<0,005
OVX	1000	513,1 ± 47	P<0,005

TABLE 3. Effect of progesterone administration to ovariectomised rats on pineal H1OMT activity. Rats were dosed at 9 a.m. for 3 days* and sacrificed at 9 a.m. on the fourth day. The experiment was carried out in duplicate (n=4)

* with either progesterone or 0,1 ml olive oil (vehicle).

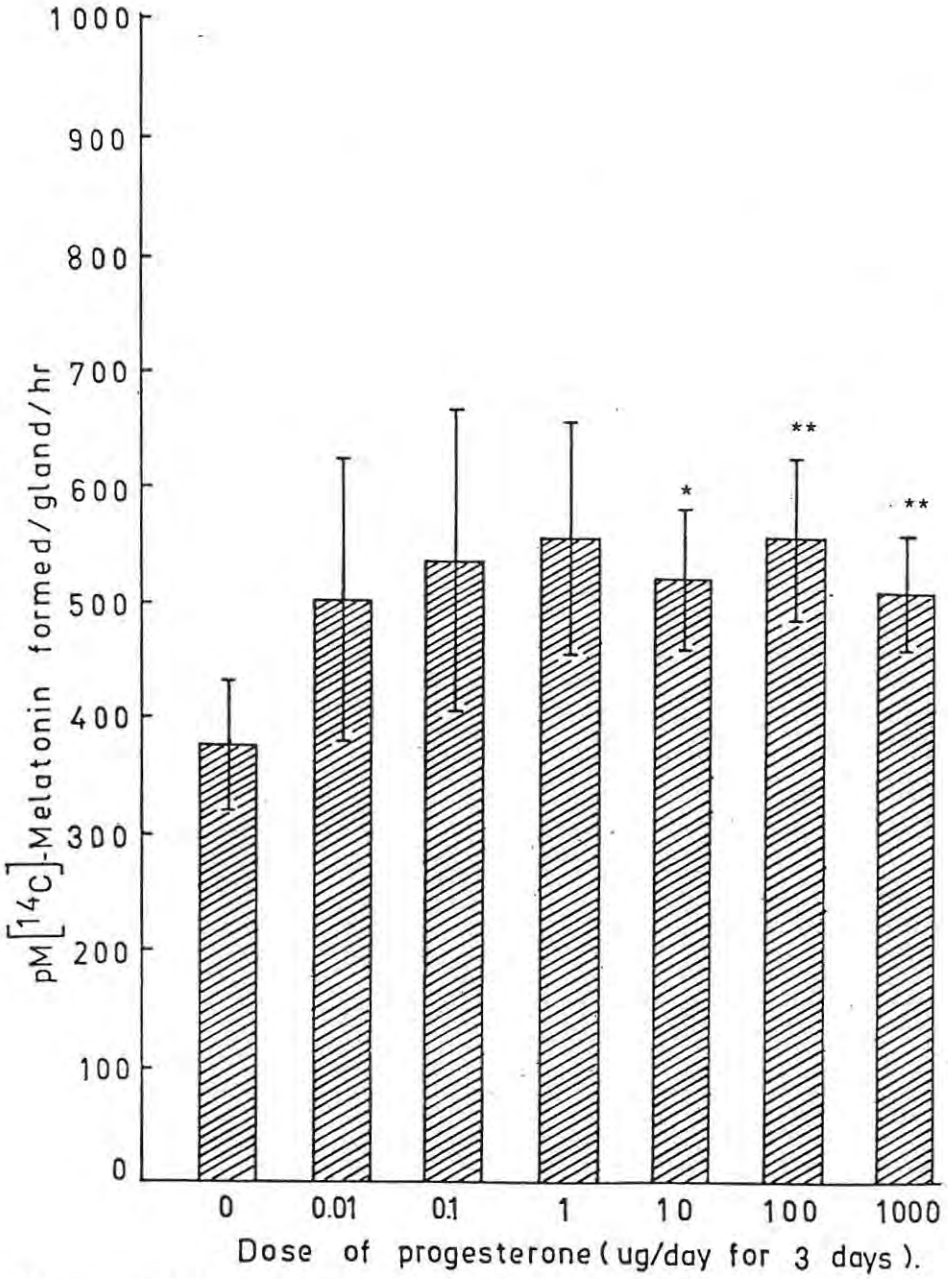

Fig. 11. Effect of progesterone administration to ovariectomised rats on rat pineal H1OMT activity. (N=4).
* <P 0,01
** <P 0,005

2.1.5 Effect of chronic progesterone administration on pineal H1OMT activity

2.1.5.1 Materials and Methods

Female rats with a mass of 220 - 240 g and which had been ovariectomized two weeks earlier were used. The rats (n=4 per dosage level) were injected at 09h00 with progesterone at doses of 10 or 100 µg / day for 30 days. At 09h00 on the 31st day, the rats were killed and pineal H1OMT activity was determined immediately. The control ovariectomized rats were injected with the vehicle only (0,1 ml olive oil / day for 30 days). The experiment was run in duplicate and the results are expressed as a mean of the results of the experiments.

2.1.5.2 Results (See Table 4 and Fig. 12).

The rats which received progesterone at a dose of 10 µg /day for 30 days had a statistically significant elevation of pineal H1OMT whereas those rats which received progesterone at 100 µg/day for 30 days had a statistically significant depression of H1OMT activity compared to the control rats.

2.1.5.3 Discussion

From these results, it appears that low doses of progesterone administered chronically stimulate H1OMT activity whereas higher doses administered chronically inhibit H1OMT activity. The latter part of this statement is in agreement with the report by Houssay and Barcelo (1972) who demonstrated that progesterone administration to rats in a dose of 200 µg/day for 25 days, significantly depresses H1OMT activity.

Surgery	Dose of progesterone µg/day for 30 days	H1OMT Activity pM [^{14}C]-Melatonin formed/gland/hr ± S.E.M.	Significance
OVX	0	360,2 ± 70	-
OVX	10	536,1 ± 68	P<0,005
OVX	100	139,6 ± 29	P<0,001

TABLE 4. Effect of chronic progesterone administration to ovariectomised rats on rat pineal H1OMT activity. Rats were dosed with either progesterone or 0,1 ml olive oil (vehicle) at 9 a.m. for 30 days and sacrificed at 9 a.m. on the 31st day. The experiment was carried out in duplicate.(n=4).

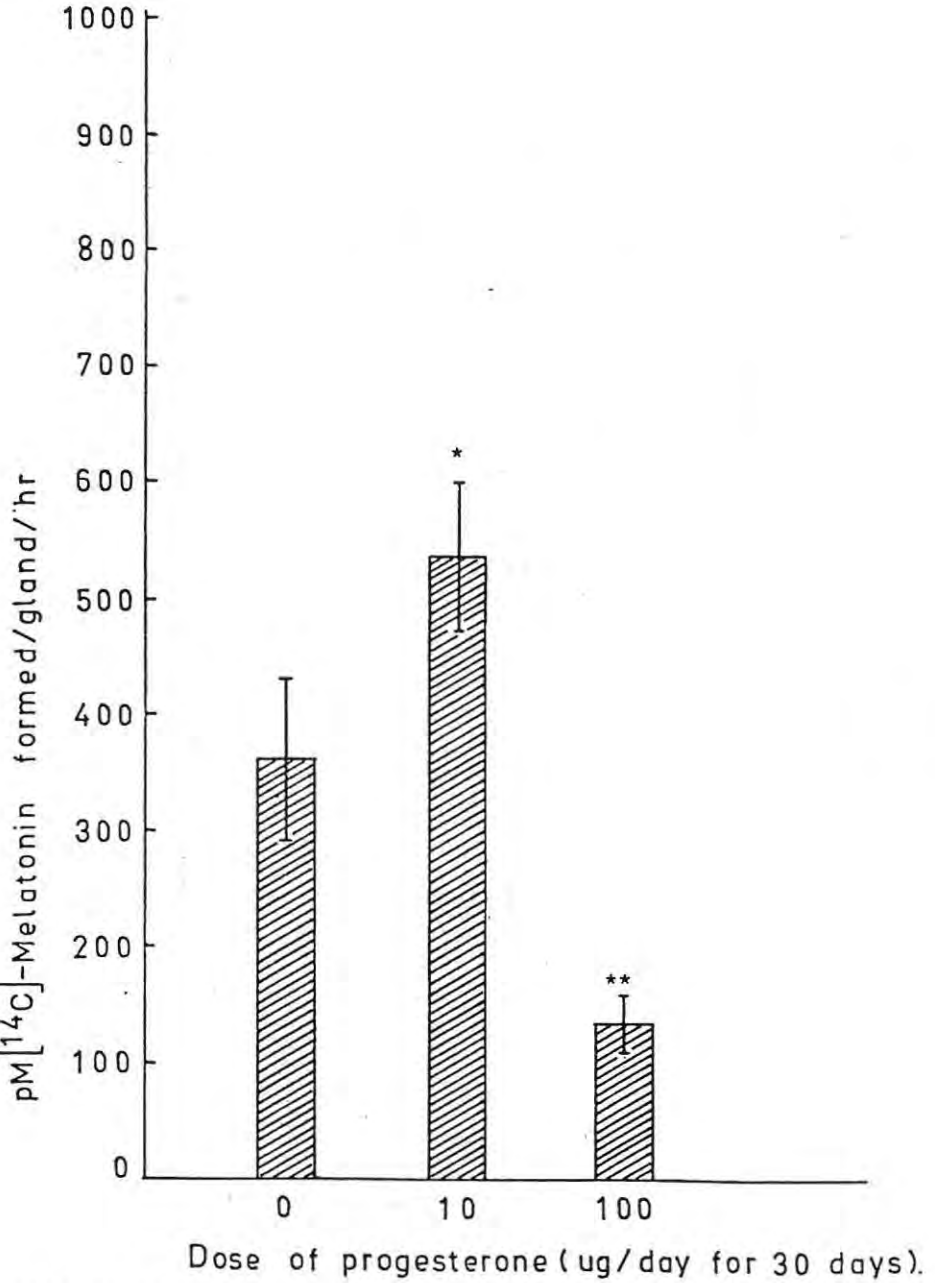

Fig. 12. Effect of chronic progesterone administration to ovariectomised rats on rat pineal H1OMT activity. (n=4).

* $P < 0,005$

** $P < 0,001$

2.1.6 Effect of testosterone on pineal H1OMT activity.

2.1.6.1 Materials and Methods

Male rats with a mass of 230 - 250 g were used. A total of 4 rats per dosage level were used. Sham-operations and castrations were performed two weeks prior to this study. Castrated rats were injected with testosterone proprionate at doses of 0,01; 0,1; 1; 10; 100; 1 000 or 10 000 µg/day for 3 days at 09h00. The sham-operated and castrated controls were injected with the vehicle only (0,1 ml olive oil / day for 3 days). The rats were sacrificed at 09h00 on the 4th day and pineal H1OMT activity was determined immediately. The experiment was repeated and the results are expressed as the mean of the results of the two experiments.

2.1.6.2 Results (See Table 5 and Fig. 13).

H1OMT activity in the control castrated rats was approximately half that of the sham-operated vehicle treated rats. The castrated rats treated with testosterone proprionate ranging from 0,01 to 1 000 µg / day for 3 days exhibited dose-dependent increases in H1OMT activity. The elevation of H1OMT activity induced by testosterone proprionate at doses of 1; 10; 100 and 1 000 µg / day for 3 days approximated the levels observed in the sham-operated rats. However, the dose at 10 000 µg / day for 3 days depressed H1OMT activity to levles approximating those of the castrated controls.

2.1.6.3 Discussion

From the results it can be concluded that castration reduces

Surgery	Dose of Testosterone proprionate µg/day for 3 days	HIOMT Activity pM [$^{14}_{C}$]-Melatonin formed/gland/hr ± S.E.M.	Significance
Sham operated	0	788,2 ± 62	P<0,001
CAST	0	337,5 ± 23	
CAST	0,01	581,6 ± 40	P<0,001
CAST	0,1	578,7 ± 46	P<0,001
CAST	1	745,8 ± 58	P<0,001
CAST	10	764,2 ± 67	P<0,001
CAST	100	781,2 ± 69	P<0,001
CAST	1000	802,1 ± 76	P<0,001
CAST	10000	352,1 ± 24	N.S.

TABLE 5. Effect of castration and testosterone proprionate administration to castrated rats on pineal HIOMT activity.
Rats were injected with testosterone proprionate or 0,1 ml olive oil (vehicle) for 3 days at 9 a.m. and sacrificed at 9 a.m. on the fourth day. The experiment was carried out in duplicate. (n=4).

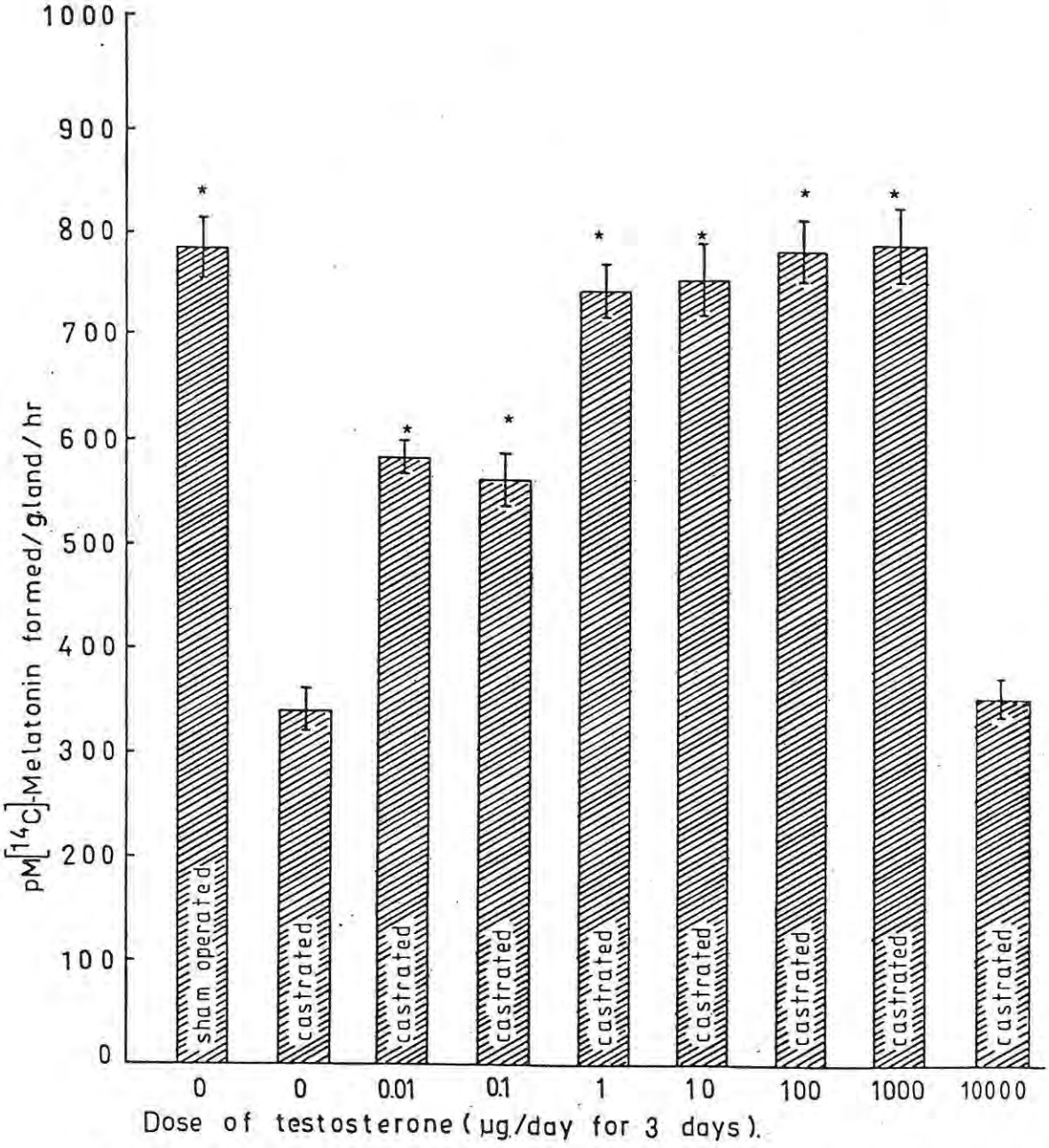

Fig. 13. Effect of castration and testosterone proprionate administration to castrated rats on rat pineal HIOMT activity. (n=4). $P < 0,001$

H1OMT activity. This confirms the report by Nagle et al (1974), that castration reduces pineal H1OMT activity. High doses of testosterone (10 000 µg / day for 3 days) inhibit H1OMT activity whereas lower doses stimulate H1OMT activity indicating that testosterone has a biphasic dose-dependent effect on H1OMT activity. A similar relationship was observed by Nagle et al.,(1974). If pineal protein synthesis regulates H1OMT levels then it is possible that the relationship observed above can be explained by results obtained by Cardinali (1974b and 1975d) who reported that low doses of testosterone stimulate pineal protein synthesis whereas high doses depress pineal function.

2.1.7 Effect of ovariectomy and estradiol benzoate administration on the basal and nocturnal levels of pineal SNAT activity.

2.1.7.1 Materials and Methods

Female rats with a mass of 200 - 220 g which had been ovariectomized two weeks previously were used. In the case of the basal enzyme activity, this was determined at 09h00 during the light phase of the photoperiod. The nocturnal level of the enzyme was measured in the middle of the dark phase of the photoperiod. Ovariectomized rats were dosed at, 09h00 with 1; 10 or 100 µg estradiol benzoate / day for 3 days (n=4 rats per dosage level). The sham-operated intact controls and ovariectomized controls were injected with the vehicle only (0,1 ml olive oil / day for 3 days). The rats were sacrificed at 09h00 or midnight on the 4th day and SNAT activity was determined by the method described earlier. The experiment was repeated and the results were expressed as the mean of the results of the two experiments.

2.1.7.2 Results (See Table 6 and Fig. 14).

In all the groups, the nocturnal level of SNAT was approximately fifty times higher than the basal levels of this enzyme. Neither ovariectomy nor estradiol benzoate administration affected the basal or nocturnal levels of SNAT significantly.

2.1.7.3 Discussion

The diurnal variation in SNAT activity with high levels during the dark phase and low levels during the light phase confirmed the reports of several authors in this field (Klein and Weller, 1970; Deguchi and Axelrod, 1972). The lack of effect of estradiol on pineal SNAT activity indicates that this steroid probably does not modulate melatonin synthesis by affecting pineal SNAT. Other reports of the lack of effect of estradiol on pineal SNAT activity were published by Illnerova (1975) and Preslock (1974 and 1975). It is strange that the high doses of estradiol used in this study did not suppress the nocturnal SNAT activity by suppressing protein synthesis (Cardinali et al 1974b and 1975d). The rise in SNAT activity has been shown to be blocked by cycloheximide, a protein synthesis inhibitor (Deguchi and Axelrod, 1972a). It is possible that the high doses of estradiol do not inhibit protein synthesis for the formation of SNAT molecules.

SURGERY	SHAM OPERATED	OVX	OVX	OVX	OVX	TIME OF DOSING AND OF SACRIFICE	SIGNIFICANCE
Dose of Estradiol Benzoate µg/day for 3 days	0	0	1	10	100		
Snat activity. pM [^{14}C]-N-Acetyl tryptamine formed per gland/hr ± S.E.M.	22,0 ± 5	27,0 ± 1	23,1 ± 5	26,1 ± 1	23,5 ± 2	9 a.m.	N.S.
	759,1 ± 57	752,2 ± 57	730,1 ± 62	709,1 ± 71	754,2 ± 68	Midnight	N.S.
	24,0 ± 2	25,3 ± 3	24,2 ± 3	22,5 ± 3	25,1 ± 2	9 a.m.	N.S.
Significance between basal and nocturnal SNAT levels in same dosage groups.	P<0,001	P<0,001	P<0,001	P<0,001	P<0,001		

TABLE 6. Effect of ovariectomy and estradiol benzoate administration to ovariectomised rats on the basal and nocturnal levels of rat pineal SNAT. Rats were injected with either estradiol benzoate or 0,1 ml olive oil (vehicle) at 9 a.m. or midnight for 3 days and sacrificed at 9 a.m. or at midnight on the 4th day. The experiment was carried out in duplicate. (n=4).

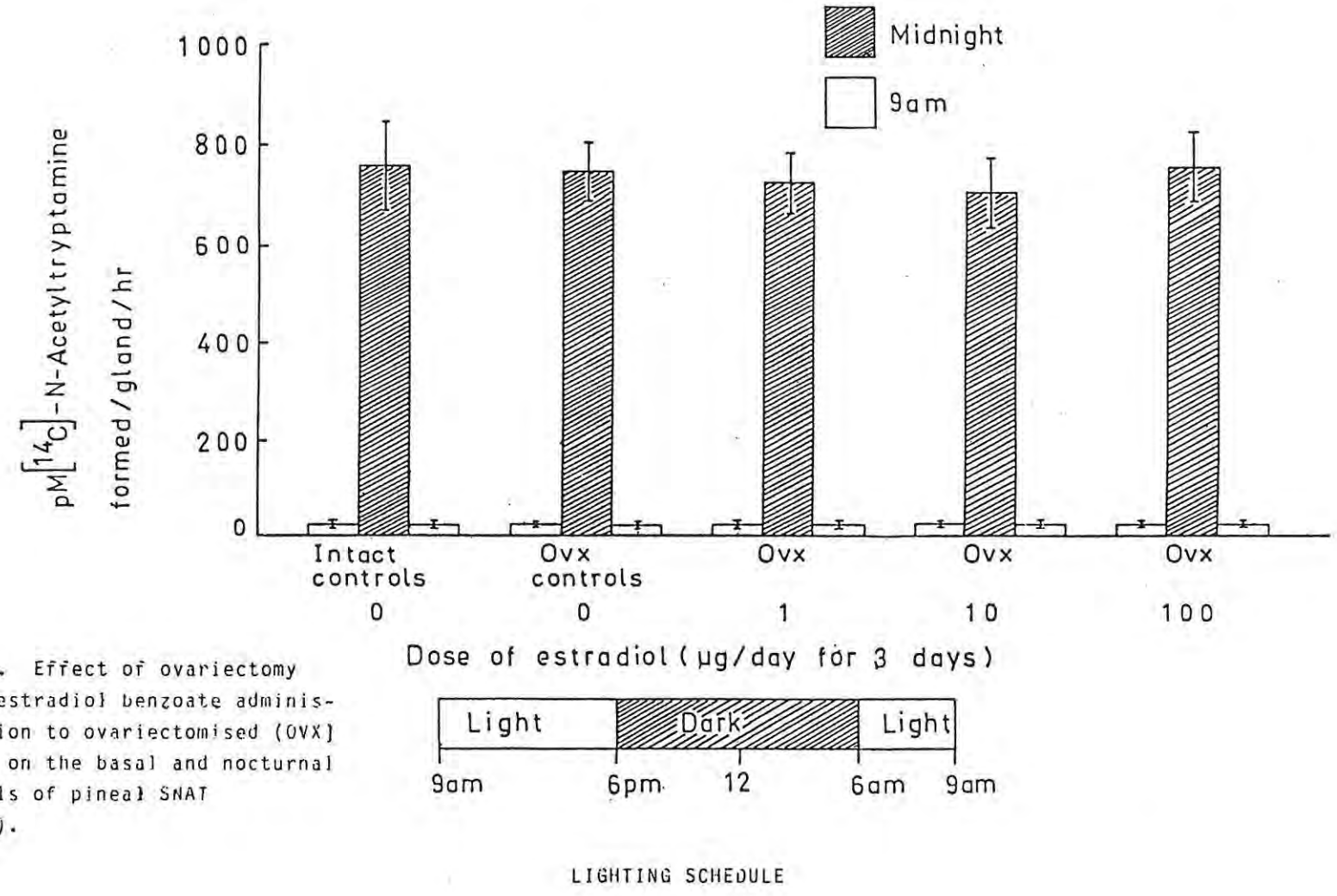

Fig. 14. Effect of ovariectomy and estradiol benzoate administration to ovariectomised (OVX) rats on the basal and nocturnal levels of pineal SNAT (n=4).

2.1.8 The effect of progesterone administration on the basal and nocturnal levels of pineal SNAT activity.

2.1.8.1 Materials and Methods

Female rats with a mass of 200 - 220 g which had been ovariectomized two weeks previously were used. Again, the basal enzyme activity was determined at 09h00 during the light phase and the nocturnal level of the enzyme was determined at midnight. The ovariectomized rats were dosed at 09h00 with 1; 10 or 100 µg of progesterone / day for 3 days (n=4 rats per dosage level). The rats were sacrificed at 09h00 or at midnight on the 4th day and SNAT activity was determined. The experiment was repeated and the results were expressed as the mean of the results of the two experiments.

2.1.8.2 Results (See Table 7 and Fig. 15)

Progesterone administration in the doses used, did not affect the basal or the nocturnal levels of SNAT significantly.

2.1.8.3 Discussion

Progesterone, like estradiol does not affect pineal SNAT activity and therefore probably does not modulate melatonin production by affecting SNAT. Although progesterone has been shown to decrease protein synthesis in the pineals of ovariectomized rats (Cardinali and Vacas, 1978), it does not seem to affect the rise in SNAT activity which depends on synthesis of SNAT molecules.

SURGERY	SHAM OPERATED	OVX	OVX	OVX	OVX	TIME OF DOSING AND OF SACRIFICE	SIGNIFICANCE
Dose of Progesterone (single dose) µg/day for 3 days	0	0	1	10	100		
SNAT activity.	22,9 ± 5	27,0 ± 1	25,8 ± 4	29,2 ± 4	27,5 ± 3	9 a.m.	N.S.
pM [^{14}C] N-acetyl tryptamine formed per gland/hr	759,1 ± 60	752,0 ± 57	690,2 ± 60	718,0 ± 68	681,7 ± 50	Midnight	N.S.
	24,0 ± 2	25,3 ± 3	27,3 ± 3	26,2 ± 3	23,6 ± 3	9 a.m.	N.S.
Significance between basal and nocturnal SNAT levels in same dosage groups	P<0,001	P<0,001	P<0,001	P<0,001	P<0,001		

TABLE 7. Effect of ovariectomy and progesterone administration to ovariectomised rats on the basal and nocturnal levels of rat pineal SNAT. Rats were injected with either Progesterone or 0,1 ml olive oil at 9 a.m. or midnight for 3 days and sacrificed at midnight on the 4th day or at 9 a.m. on the 4th day. The experiment was carried out in duplicate. (n=4).

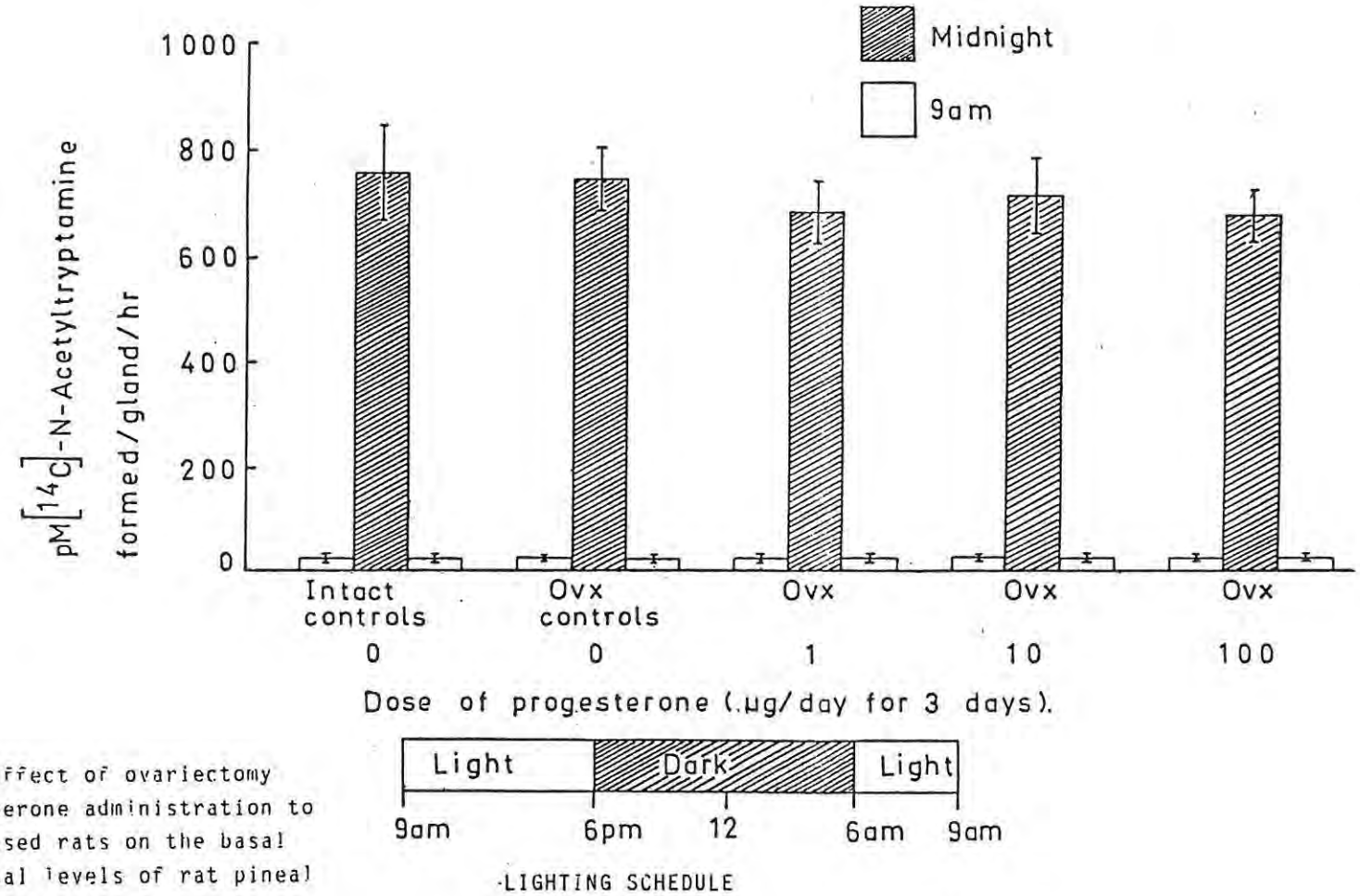

Fig.15. Effect of ovariectomy and progesterone administration to ovariectomised rats on the basal and nocturnal levels of rat pineal SNAT.
(n=4)

2.1.9. The effect of testosterone on the basal and nocturnal levels of pineal SNAT activity.

2.1.9.1. Materials and Methods

Male rats with a mass of 230 - 250 g which has been castrated two weeks previously were used. All the rats were injected at 09h00 (n=4 rats per dosage level). The sham-operated intact controls and the castrated controls were injected with the vehicle only i.e. 0,1 ml olive oil / day for 3 days. Castrated rats treated with testosterone received a 100 µg dose of testosterone proprionate daily for 3 days. Groups of rats were sacrificed at 09h00 on the fourth day to determine the basal enzyme activity and other groups at midnight, to determine the nocturnal enzyme activity. The experiment was repeated and the results are expressed as the mean of the results of the two experiments.

2.1.9.2 Results (See Table 8 and Fig. 16)

There was a very significant decrease in the nocturnal rise of SNAT activity in the vehicle-treated castrated rats compared to the sham-operated intact controls. Besides this decrease in the nocturnal rise, there was also a statistically significant elevation of the basal level of SNAT activity in the vehicle treated castrated rats compared to the controls.

The rats which received testosterone had a statistically significant depression of the basal level of the enzyme and the nocturnal rise of the enzyme in these rats was restored to levels close to those of the controls.

2.1.9.3 Discussion

The depression of the nocturnal rise of pineal SNAT activity

in these castrated male rats could possibily be explained by the observation by Karasek et al.,(1978), that castration in male rats results in a decrease in the pineal concentration of cyclic AMP. Since SNAT is dependent on cyclic AMP for its activation, it can be expected that a reduction in cyclic AMP levels in the pineal gland will result in a reduction in SNAT activity. The elevation of the basal levels of SNAT in these castrated rats is more difficult to explain. One explanation could be that there might be a phase shift in the diurnal rhythm, possibly to the right. This could explain why the nocturnal levels are lower and the basal levels higher.

Recently, two reports have also been published where the authors have observed a similar decrease in the nocturnal rise of pineal SNAT activity in castrated male rats (Pavlinov and Isachenkov, 1979; Rudeen and Reiter, 1980).

In this study, testosterone restored the nocturnal rise of the enzyme to that of the controls but depressed the basal levels indicating testosterone does influence SNAT activity. Testosterone appears to be essential for the maintenance of the normal diurnal rhythm pattern. It is possible that testosterone somehow affects the release or synthesis of noradrenaline by the sympathetic nerves of the pineal gland to bring about these observed effects.

SURGERY	SHAM OPERATED	CAST	—	CAST	SIGNIFICANCE COMPARED TO CAST CONTROL	TIME OF DOSING AND OF SACRIFICE
Dose of testosterone proporionate µg/day for 3 days	0	0	—	100		
			SIGNIFICANCE			
SNAT activity pM [^{14}C]-N-Acetyl tryptamine formed per gland/hr ± S.E.M.	31,6 ± 2	38,5 ± 0,7	P < 0,001	17,5 ± 1	P < 0,001	9 A.M.
	1081,5 ± 120	401,4 ± 51	P < 0,001	997,8 ± 51	P < 0,001	Midnight
	30,0 ± 2	36,2 ± 2	P < 0,005	13,7 ± 4	P < 0,001	9 A.M.
Significance between basal and nocturnal SNAT levels in same dosage groups.	P < 0,001	p < 0,001	—	P < 0,001		

TABLE 8. Effect of castration and testosterone administration to castrated rats on the basal and nocturnal levels of SNAT. Rats were injected with either testosterone proprionate or 0,1 ml olive oil (vehicle) at 9 a.m. or midnight for 3 days and sacrificed at 9 a.m. or midnight on the 4th day. The experiment was carried out in duplicate (n = 4).

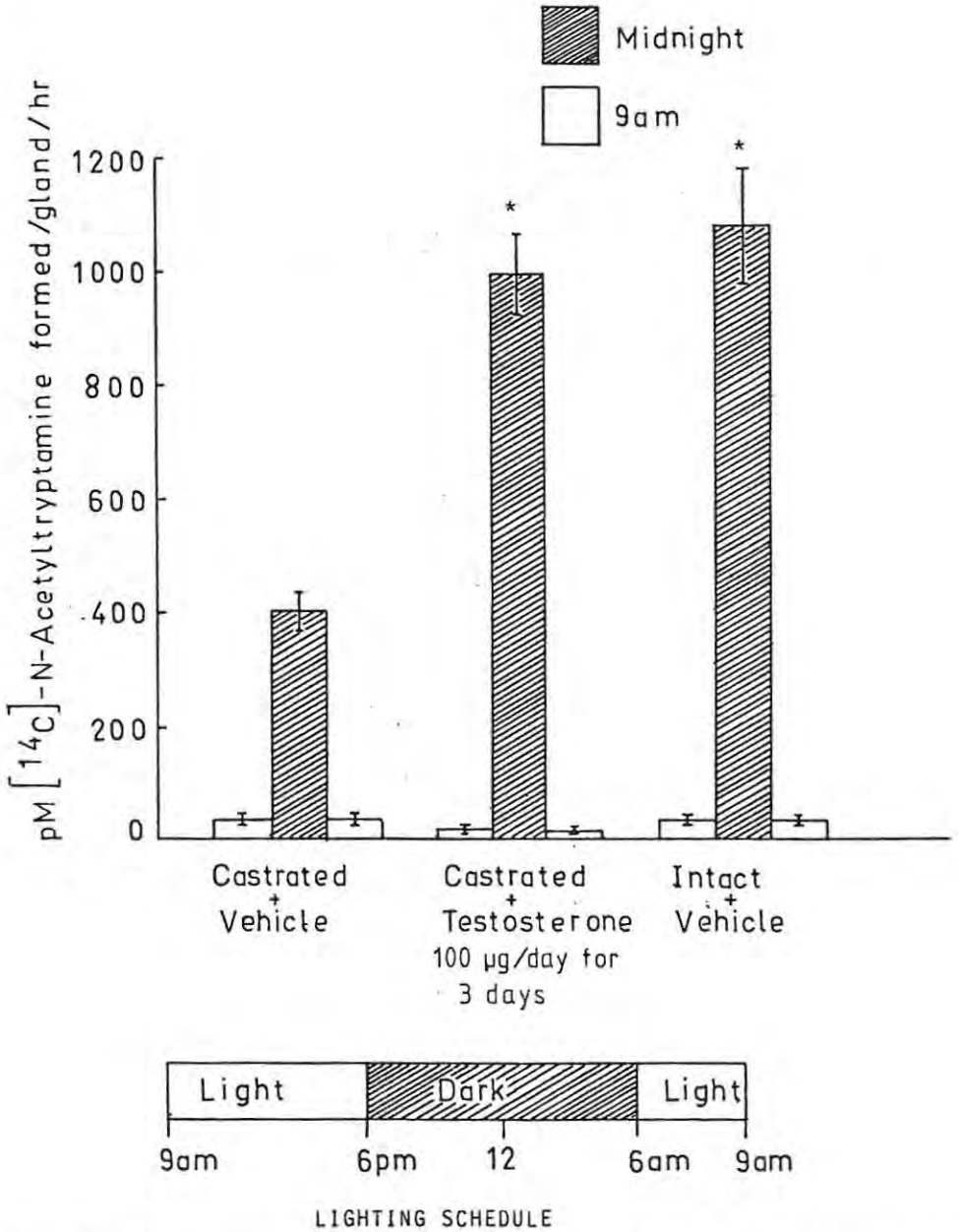

Fig. 16. Effect of castration (CAST) and testosterone administration to castrated rats on the basal and nocturnal levels of rat pineal SNAT. (n=4).

* $P < 0,001$

CHAPTER 3

TISSUE AND ORGAN CULTURE EXPERIMENTS

3.1 Tissue Culture

3.1.1 Introduction

Tissue and organ culture studies are extremely useful in determining the effects of drugs on cell or organ constituents with the advantage of the absence of complexities of organ interaction.

3.1.2 Materials and Methods

3.1.2.1. Culture Medium

Eagles minimum essential medium (MEM) with Earle's salts, glutamine and non-essential amino acids was obtained commercially in powdered form in packets sufficient to make one litre of medium.

3.1.2.2 Preparation of medium

The contents of a packet of medium were emptied into 950 ml of double-distilled deionized water at room temperature, and stirred gently until dissolved. 2,2 g of sodium bicarbonate ($NaHCO_3$) was added and allowed to dissolve. The volume was brought up to one litre with double-distilled, deionized water. The pH of the medium was reduced to 0,3 pH units below the desired final working pH i.e. 7,2 using IN HCl. This was done because the pH was found to rise by 0,3 pH units after filtration. After this, the medium was supplemented with penicillin to a final concentration 0,06 µg/ml and streptomycin sulphate to a final

concentration of 0,1 µg/ml. The medium was sterilized by positive pressure membrane filtration using nitrogen. The pore size of the filter was 0,2 µm . The medium was then asceptically transferred and stored in sterile 200 ml medical flats. The medium was then supplemented with a sterile solution of Amphotericin B (Fungizone $^{(R)}$) to a final concentration of 2,5 µg/ml. This was added after filtration in order to prevent the filter from becoming clogged. Finally, the medium was supplemented with 10% foetal calf serum (virus and mycoplasma screened). At least once per week, a sample of medium was tested for possible microbial contamination. The medium was stored refrigerated.

3.1.2.3 Explant Cultures

Albino rats of the Wistar strain were killed as described previously and their pineals were removed aseptically. The pineal glands were bisected and aseptically placed on the inner ventral surface of 25cm^2 (30 ml) Corning$^{(R)}$ tissue culture flasks. 5ml of the culture medium was pippetted into each flask and the flasks were allowed to stand on their sides for thirty minutes to allow the tissue to adhere to the wall of the flask. After this, the flasks were allowed to rest on their ventral surfaces, allowing the medium to cover the explants. The caps of the flasks were loosened and the flasks were placed on a rack in a Formica Scientific CO_2 incubator at 37^oC with an atmosphere of 5% CO_2 : 95% air and a humidity of 95%. The medium was

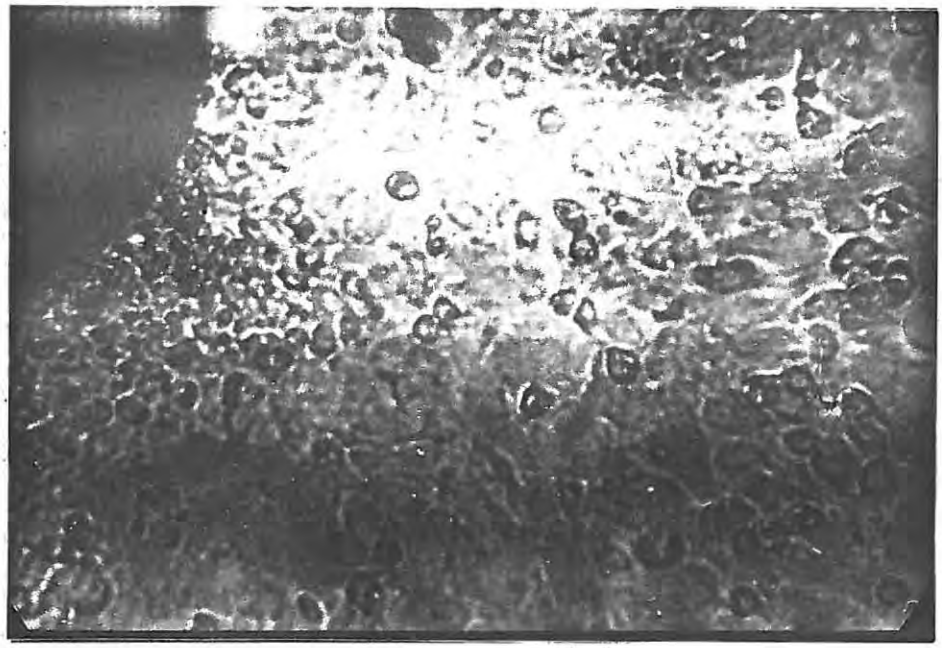

Fig. 17. Pineal cells after 6 days in culture. Cells can be seen growing outwards from the explant. (100X)

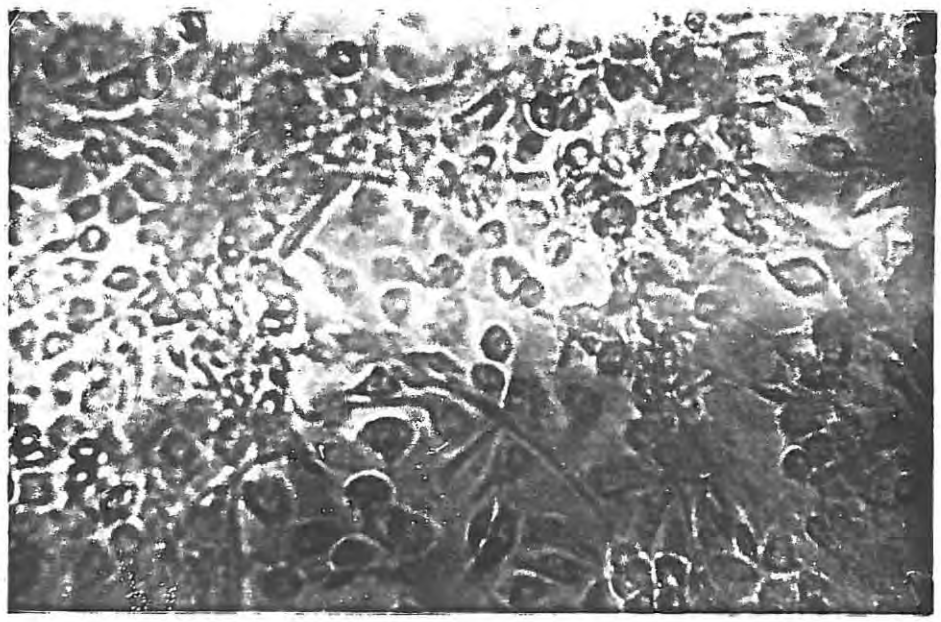

Fig. 18. Pineal cells after 10 days in culture. Pineal cells can be seen aggregating to form cell nests (100X).

replenished every two days. Exhausted medium was acidified before being discarded in order to hydrolyse the antibiotics.

3.1.3 Results (See Fig. 17 and 18).

All explants showed signs of growth after two days in culture. The pineal cells could be seen in different shapes. Some were round, long and others appeared to be elongated with irregular shapes. The cells formed a network after six days. After ten days in culture, pinealoctyes aggregated to form cell nests.

3.1.4 Discussion

The technique proved to be extremely successful. However, research using this could unfortunately not be continued due to the fact that the use of an inverted phase contrast microscope, which was found to be essential for this work, was not available.

3.1.5

COMPOSITION OF EAGLE'S MEM USED FOR TISSUE CULTURE STUDY

COMPONENTS	mg/L
Inorganic Salts	
Calcium chloride (anhyd.)	200.00
Potassium chloride	400.00
Magnesium sulphate (anhyd.)	97.72
Sodium chloride	6 800.00
Dihydrogen sodium orthophosphate	140.00
Other Components	
Glucose	1 000.00
Phenol red	10.00
Amino Acids	
L - Alanine	8.9
L - Arginine. HCl	126.00
L - Asparagine. H_2O	15.00
L - Aspartic acid	13.30
L - Cystine. 2HCl	31.29
L - Glutamic acid	14.70
L - Glutamine	292.00
Glycine	7.50
L - Histidine HCl. H_2O	42.00
L - Isoleucine	52.50
L - Leucine	52.40
L - Lysine HCl	72.50
L - Methionine	15.00
L - Phenylalanine	32.00

L - Proline	11.50
L - Serine	10.50
L - Threonine	48.00
L - Tryptophane	10.00
L - Tyrosine (Disodium salt)	52.10
L - Valine	46.00

VITAMINS

D - Ca pantothenate	1.00
Choline chloride	1.00
Folic acid	1.00
i -Inositol	2.00
Nicotinamide	1.00
Pyridoxal HCl	1.00
Riboflavin	0.10
Thiamine HCl	1.00

SUPPLEMENTS

Sodium bicarbonate	2,2 g/L
Benzyl Penicillin	0,06 mg/ml
Streptomycin sulphate	0,1 mg/ml
Amphotericin B	2,5 µg /ml
Foetal calf serum	100 ml/L

3.2. Organ culture

Introduction

Organ culture originated in the Strangeways Laboratory more than fifty years ago (Strangeways and Fell, 1926; Fell and Robinson, 1929). At that time, the method was used chiefly for the culture of embryonic organ rudiments. Subsequently, Trowell (1959) very successfully adapted and modified the apparatus and techniques for organ culture so as to keep a number of fully differentiated organs or parts thereof alive *in vitro* without either growth or dedifferentiation, thus opening the way for many experimental studies.

In this study, an attempt was made to simplify the method developed by Trowell (1959).

3.2.1 Materials and Methods

Organ culture dishes sufficient to hold 400 µl of culture medium were made by cutting 2 ml amber glass ampoules about 1 cm from the base. Lips were made on these vessels to enable easy handling with forceps. A small stainless steel gauze (0,5mm) rack was made to fit into the culture vessels so that when 400 µl of medium was added to the vessels, the medium only just touched the bottom surface of the gauze rack. A number of these stainless steel grids were made. These grids were boiled briefly in 10% sodium bicarbonate, rinsed and then immersed in pure nitric acid overnight and then rinsed thoroughly again, dried and sterilised at 120°C for 2 hours. The culture vessels were washed with detergent, rinsed and then immersed in pure nitric acid overnight after which they were rinsed well with double-distilled deionized water, dried and

and sterilized at 120°C for 2 hours.

Pineal glands were removed under aseptic conditions after sacrificing rats and cultured using 400 µl of culture medium according to the methods outlined below. The culture vessels were then placed in sterile medical flats (200 ml) containing a piece of sterile filter paper which was saturated with sterilized water. The purpose of this moist filter paper was to ensure a humid atmosphere within the culture bottle. The medical flats were placed on their flat sides with the filter paper and culture vessel on the ventral flat side of the bottle. The bottle was immediately gassed with carbogen (95% O_2 : 5% CO_2), the lid was screwed on tightly and the bottle was placed in an incubator at 37°C. After 24 hours, the pineal glands were removed and H1OMT activity was determined.

In order to find a suitable method of culturing pineal glands, the following variations were tried using H1OMT activity as a marker.

(i) Pineal glands were placed on the stainless steel gauze racks which were then placed in culture vessels containing 400 µl of either Eagles MEM or BGJb medium (Fitton-Jackson modification). BGJb medium was originally developed by Biggers (1961) who used it for the culture of tibiae and it was modified by Dr Fitton-Jackson (unpublished observations).

In this method of culture the pineal glands were 'fed' with medium from beneath while the major surface of the

(continued on p.116)

3.2.1.1

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 7 H_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
L - Methionine	50.00
L - Phenylalanine	50.00
L - Proline	400.00
L - Serine	200.00
L - Threonine	75.00
L - Tryptophane	40.00
DL - Valine	65.00

Vitamins

Alpha tocopherol phosphate	1.00
Ascorbic acid	50.00
Biotin	0.20
Calcium pantothenate	0.20
Choline chloride	50.00
Folic Acid	0.20
Inositol	0.20
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 mg/L
Benzyl Penicillin	100 units/ ml
Streptomycin sulphate	0,1 mg/ ml
Amphotericin B	2,5 µg/ ml

pineals was exposed to the atmosphere of carbogen.
(ii) Pineal glands were cultured as described above with BGJb medium in the dark and in the presence of white light.
(iii) Pineal glands were submerged in the 400µl of BGJb medium and cultured as described above but without the gauze.
(iv) Pineal glands were cultured using BGJb medium as described above but without the gauze. Instead, the pineal glands were placed on pieces of lens paper which were then floated on the medium.

The sterile BGJb medium was supplemented with L-glutamine 200 mg/ℓ, Benzyl penicillin 0,06 mg / ml, Streptomycin sulphate 0,1 mg / ml and Amphotericin B 2,5 µg / ml.
The Eagles MEM was prepared in the same manner described in 3.1 except that for the organ culture studies, the foetal calf serum was omitted as in the case of BGJb medium. The reason for the omission of foetal calf serum is that this is usually added where growth of the cells is required and since these organ culture experiments were not performed for longer periods than 24 hours, it was decided not to include this in the medium. Another reason for not using the foetal calf serum was the possibility of the serum containing gonadal sex steroids.

3.2.2 Results

(i) Pineals cultured in BGJb medium exhibited HIOMT activities which approximated levels found in fresh pineals whereas those cultured using Eagles MEM had low HIOMT activities.
(ii) Pineals cultured in the presence of light had significantly lower levels of HIOMT compared to those cultured in the dark. Light decreased HIOMT activity after 4 hours in

culture.

(iii) Pineal glands cultured while submerged in the medium had very low H1OMT activity.

(iv) Pineal glands cultured on lens paper also had low H1OMT activities.

3.2.3 Discussion

From the results it was obvious that the technique used in (i), section 3.2.2 using BGJb medium was the best of the methods tried. The other methods tried probably caused anoxia (submerging) or possibly toxic effects (lens paper) in the pineals. It was interesting to note that light had an effect on H1OMT activity. It is possible that the pineal in the adult rat responds similarly to a non-retinal pathway of light as do pineals of newborn rats (Zweig et al., 1966).

For all further studies, pineals were cultured in the dark. The incubator used did not allow light to enter when the front door was shut.

3.2.4 Effect of 10nM estradiol on H1OMT activity in pineal organ cultures.

3.2.4.1 Materials and Methods

Female rats with a mass of 180 - 200 g and which had been ovariectomized two weeks previously were used. A total of four rats were used per result. Each point was the mean of the four values.

3.2.4.1.1. Controls.

Rats were sacrificed and the pineal glands were removed using aseptic techniques and cultured on stainless steel gauze as described in (i), section 3.2.2 using BGJb medium. One pineal gland was cultured per culture vessel. The pineal glands were cultured from 0 - 24 hours in the presence of 4×10^{-4}M ethanol (vehicle) which was added to 390 µl of the medium, as 10 µl of a 1 : 10 000 dilution of 95% ethanol. Pineal H1OMT activity was determined at various times between 0 and 24 hours in these cultured pineal glands. For pineals cultured for 0 hours, the pineal glands were dipped into the culture medium, rinsed with saline and H1OMT activity was determined.

3.2.4.1.2 Test

Pineal glands were cultured as above but in the presence of 10nM estradiol. This concentration of estradiol is within the physiological range in the female rat (Hori et al., 1968). The estradiol solution was made up as follows :-
A stock solution of 17-β-estradiol 4mM was made up in 95% ethanol. This was diluted 10 000 times and used as a working solution and 10 µl of this was added to 390 µl of culture medium in the culture vessel to produce a final concentration of 10nM estradiol. Pineal H1OMT activity was determined at various times between 2 and 24 hours. Because of the endogenous rise in H1OMT activity observed at 2 hours in culture in the control group, it was decided to introduce pineal glands of the test group to estradiol only from 2 hours of culture. From 0 to 2 hours, these pineal glands were cultured in the same manner as those of the control

Surgery	Hours in Culture	H1OMT Activity pM $[^{14}C]$-Melatonin formed/gland/hr ± S.E.M.		Significance
		Control	10nM estradiol	
OVX	0	256,6 ± 19	-	-
OVX	1	276,0 ± 40	-	-
OVX	2	310,5 ± 18	Estradiol added	-
OVX	3	234,0 ± 22	307,5 ± 28	P<0,005
OVX	4	272,0 ± 26	282,1 ± 25	N.S.
OVX	5	186,2 ± 23	228,5 ± 8	P<0,01
OVX	6	204,9 ± 17	242,1 ± 14	P<0,01
OVX	7	224,5 ± 20	391,2 ± 26	P<0,001
OVX	9	200,0 ± 17	393,5 ± 28	P<0,001
OVX	24	258,1 ± 26	947,1 ± 103	P<0,001

Table 9. Effect of 10nM estradiol on H1OMT activity in rat pineal organ cultures from 2 to 24 hrs. Pineals from OVX rats were cultured for 2 hrs in the absence of estradiol and then for further hours in the absence and presence of 10nM estradiol. The experiment was carried out in duplicate. (n = 4).

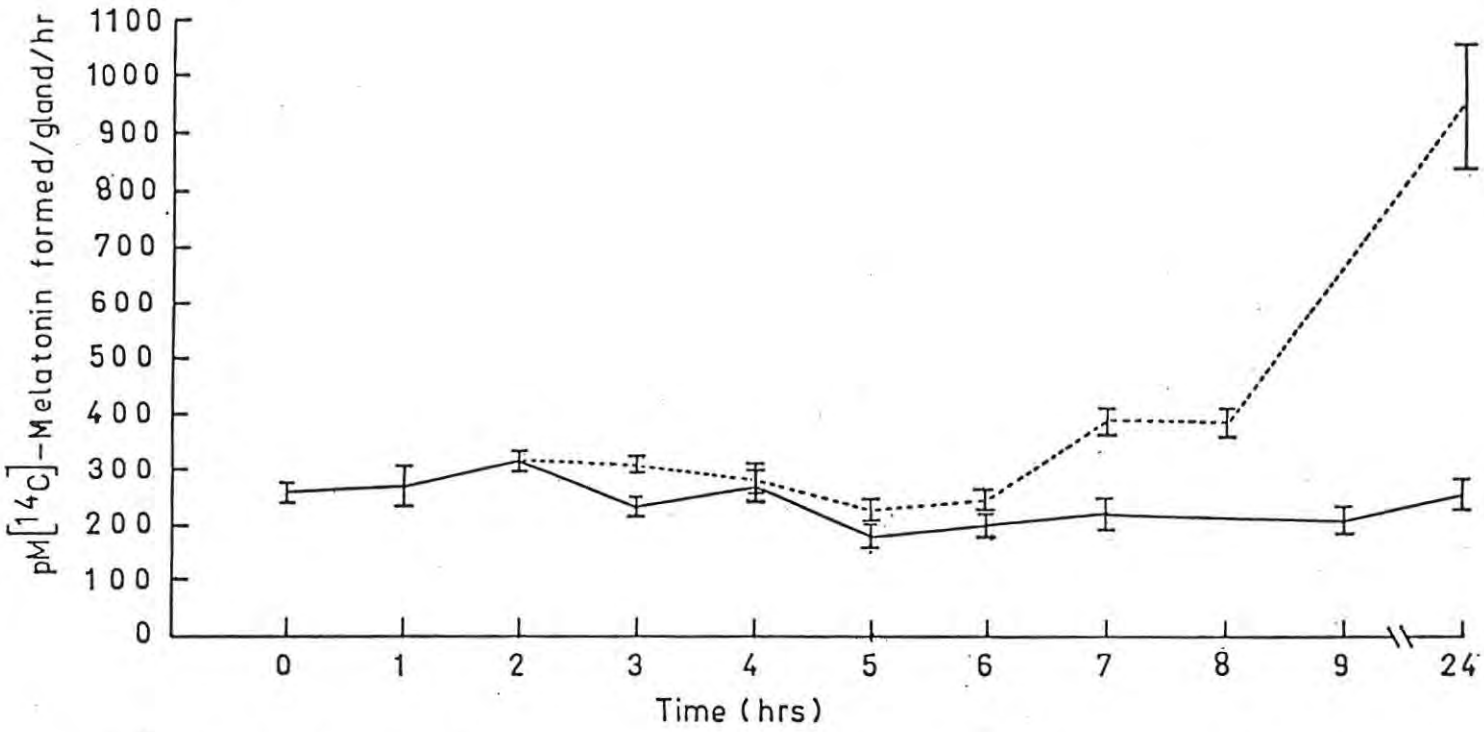

Fig.19. HIOMT activity of pineal glands cultured in the absence(———) and presence(----------) of 10nM estradiol, from 0 – 24hrs.

group. At 2 hours, these pineal glands were transferred to vessels containing estradiol in the culture medium.

3.2.4.2 Results (See Table 9 and Fig. 19)

The pineal glands cultured in the presence of estradiol had very significantly higher levels of H1OMT activity from about 7 hours in culture, rising to levels three times higher than the starting levels at 24 hours in culture. The control group had very small variations in H1OMT activity during the 24 hours of culture.

3.2.4.3 Discussion

From this study it can be concluded that estradiol at the concentration used, increases pineal H1OMT activity from 7 to 24 hours later. It is possible therefore that the high levels of estradiol during proestrus in rats results in the high levels of H1OMT activity observed during estrus (Chapter 2), 24 hours later.

3.2.5 Dose-response relationship of estradiol concentration versus H1OMT activity

3.2.5.1 Materials and Methods

Pineal glands (n=4 per result expressed as a mean of the 4 results) from female rats, mass : 180 - 200 g and ovariectomized 2 weeks previously were cultured for two hours in the presence of $4x10^{-4}$M ethanol (vehicle) and for a further 22 hours in the presence of different concentrations of estradiol ranging from 0 - 1 000 nM. At the end of the 24 hour culture period, the pineal glands were rinsed with saline and H1OMT activity was determined. Pineal glands not incubated with estradiol, were incubated with the

Surgery	Estradiol Concentration (nM)	H1OMT Activity pM [^{14}C]-Melatonin formed/gland/hr ± S.E.M.	Significance
OVX	0	225,1 ± 15	-
OVX	0,1	265,3 ± 30	P<0.05
OVX	1	353,0 ± 22	P<0,001
OVX	10	815,6 ± 70	P<0,001
OVX	100	611,4 ± 48	P<0,001
OVX	1000	162,2 ± 11	P<0,01

TABLE 10. Dose-response relationship of estradiol concentration versus H1OMT activity in rat pineal organ cultures. Pineals from OVX rats were cultured in the absence of estradiol for two hours and for a further 22 hrs in the presence or absence of estradiol, in duplicate (N=4). Rats were ovariectomised 2 weeks prior to sacrifice.

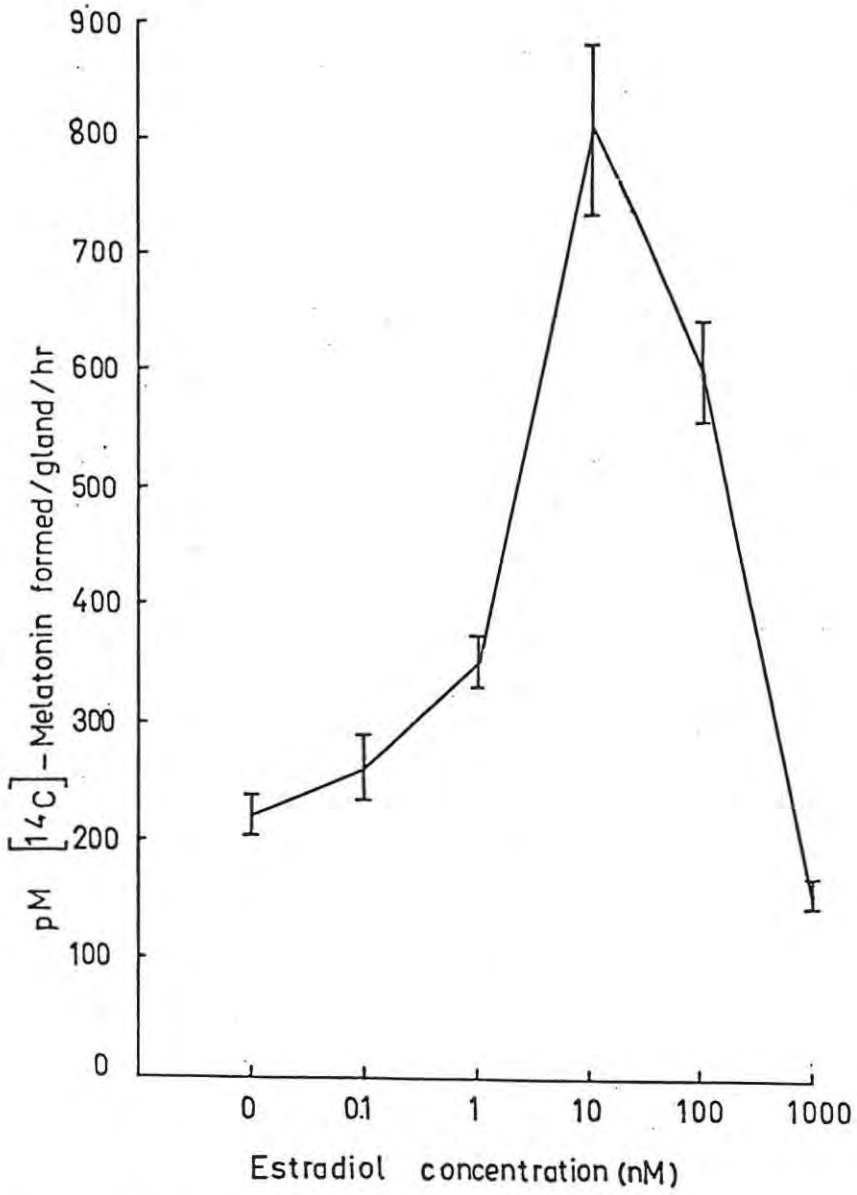

Fig. 20. Dose-response relationship of estradiol concentration versus HIOMT activity in rat pineals. Pineals from ovariectomised rats were cultured for 2 hrs in the absence of estradiol and for a further 22 hrs in the presence of estradiol (n=4)

vehicle.

3.2.5.2 Results (See Table 10 and Fig. 20)

A dose-response relationship was observed. Estradiol in concentrations of 1, 10 and 100 nM increased pineal H1OMT significantly compared to pineals not incubated with estradiol (controls). A concentration of estradiol at 10nM caused maximal stimulation of H1OMT activity. Estradiol at a concentration of 1 000 nM depressed H1OMT activity to levels below that observed in the controls.

3.2.5.3 Discussion

These results show a definite biphasic dose-dependent effect on H1OMT activity with the lower doses being stimulatory and higher doses inhibitory. This effect is similar to that observed in the in vivo experiments discussed in Chapter 2. The modulatory effect of estradiol on melatonin synthesis is therefore probably exerted at the level of H1OMT.

3.2.6 Serotonin metabolism studies in rat pineal organ culture experiments

3.2.6.1 Basic technique

3.2.6.1.1 Introduction

If the effects of individual drugs on the metabolism of ^{14}C-serotonin by pineals in organ culture could be determined, much more information would be obtained. The levels of individual metabolites formed by pineal enzymes could be studied and any effect of drugs on the enzymes would be reflected by the level of the metabolites. This

hypothesis was studied.

3.2.6.1.2 Materials and Methods

Essentially the technique involves incubating pineal glands from rats in culture medium with radioactive serotonin for 24 hours. During this incubation period, the pineal glands take up the radioactive serotonin and metabolize it. The metabolites are released into the culture medium. After 24 hours, the pineal glands are removed and an aliquot of the culture medium is analysed for its content of radioactive serotonin metabolites by using TLC and scintillometry.

The culture technique used for the enzyme studies again had to be modified to meet the conditions required for this study. The volume of culture medium (BGJb) was reduced to 200 ul per culture vessel and 5 pineal glands were incubated per vessel. It was decided to use 5 pineals because this number was found to give the desired c.p.m.. The stainless steel gauze was no longer necessary because with this volume of culture medium, the pineal glands were not totally submerged in the medium thus achieving the same effect as with the gauze. The volume of medium used contained more than sufficient nutrients for the pineals during the 24 hour culture period. The volume of the culture medium was reduced to 200 ul in these studies so that less radioactive material was required to achieve the desired concentration.

Rats were sacrificed and five pineal glands from rats in the same test group were incubated per culture vessel in 200 µl of BGJb medium. Following this, 2 µCi of (side,chain-2-^{14}C)

serotonin creatinine sulphate (specific activity 57 mCi / m mol) was added to the culture vessels. The culture vessels were placed in sterile glass bottles (medical flats) with a humid atmosphere as described earlier, and the bottles were gassed with carbogen, the lids were screwed on tightly and the bottles were placed in an incubator at 37°C for 24 hours. Only one culture vessel was placed per bottle.

After 24 hours, the reaction was stopped by removal of the pineal glands from the culture vessels. A 20 µl aliquot of the culture medium was spotted on TLC plates in duplicate. The TLC plates used were 20 x 20cm glass plates coated with 0,25 mm Kieselgel (Merck). Following this, standards of all the pineal serotonin metabolites measured, were spotted on top of the culture medium spot. A total of 20 µl of the solution containing 4 µg/20µl of each standard was spotted. The solution containing the standards was made up as follows :- 1mg of each of the 8 standards used was dissolved together in a test tube containing 2,5 ml of 95% ethanol. To this, 2,5 ml of a solution of 1% ascorbic acid in 0,1 N HCL was added. The solution was stored in the dark at -20°C. In both cases while spotting the plates, a gentle stream of nitrogen was used to dry the spots, which were not larger than 0,5cm. The nitrogen was used to prevent atmospheric oxidation of the metabolites.

The standards used are listed in Fig. 21. The plates were spotted about two metres from a Phillips R5 photographic bulb in a dark room. After spotting the plates, they were

placed in TLC tanks containing solvent A which consisted of chloroform-methanol-glacial acetic acid (93:7:1). The solvent was allowed to run up to 17cm. After the solvent had reached 17 cm ($\pm$ 1 hr), the plates were taken out of the TLC tanks, dried under nitrogen and once again placed back into the same solvent and run in the same direction up to 17cm. Following this, the plates were dried under nitrogen, turned ninety degrees and then run in this second direction up to a height of 10cm using ethyl acetate (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 the plates with nitrogen and the plates were sprayed with Van Urks reagent (1g of paradimethylbenzaldehyde dissolved in 50 ml of 25% HCL followed by the addition of 50 ml of 95% ethanol). The plates were then placed in an oven at 60^{o}C for 20 minutes to allow development of the spots. In preliminary studies, individual standards were chromatographed in this manner to identify the location of all the various metabolites.

After the development, the various spots were scraped off the TLC plate and placed into scintillation vials. Following this, 1 ml of 95% ethanol was added to the scrapings in the vials and then 7 ml of scintillation cocktail (Instagel $^{(R)}$) was added. The vials were placed in duplicate in a Beckman LS 3150 T scintillation counter and each vial was counted for a total of 20 minutes. The c.p.m. were converted to d.p.m. using the ESCR method of quench correction.

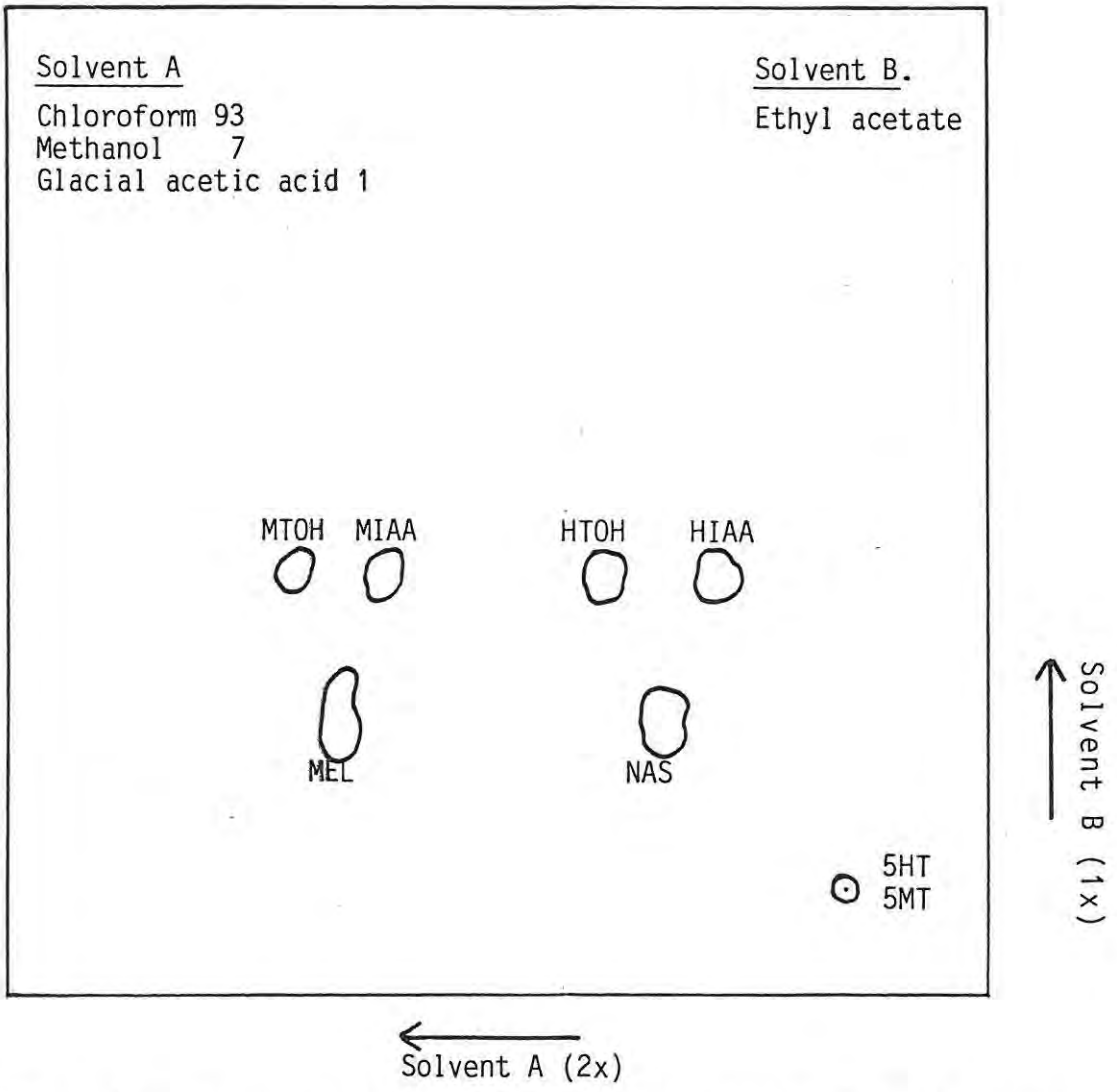

Fig. 21. Tracing of chromatographic separation of pineal indole compounds.

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

The results were taken the mean of the duplicates ± S.E.M. and finally expressed as d.p.m. of product formed / 20 µl of medium / pineal gland ± S.E.M. Each plate was spotted with 280 854 ± 2235 d.p.m. Blanks were run in exactly the same way except that no tissue was included.

The blank values, which were very low, were subtracted from the results before expressing the results. Tissue blanks using other tissues were not used because monoamine oxidase (MAO) which is present in most tissues, can oxidize serotonin and hence give incorrect blank readings.

3.2.6.1.3 Results

Good separation and high counts of the metabolites were obtained. Serotonin and methoxytryptamine did not however migrate from the origin. The results expressed for the origin are therefore those of methoxytryptamine plus serotonin.

3.2.6.2 Variation in (^{14}C) - serotonin metabolism by pineal glands from rats in different stages of the estrus cycle.

3.2.6.2.1 Materials and Methods

Intact female rats with a mass of 200 - 220 g were used.

The estrus cycles of these rats were monitored daily at 09h00 and only those rats showing at least 2 regular consecutive cycles were used. Five rats in the same stage of the estrus cycle were sacrificed at 09h00 during proestrus, estrus, metestrus and diestrus, the pineal glands were removed under aseptic conditions and placed into culture with radioactive serotonin as described earlier. The medium was analysed after 24 hours incubation as previously described.

3.2.6.2.2 Results (See Table 11)

3.2.6.2.3 Discussion

A tremendous variation in metabolism was observed at the various stages.

It is strange that the level of NAS varied during the different stages of the estrus cycle since SNAT activity is known not to vary during the estrus cycle (Cardinali and Vacas, 1978; Shivers and Yochim, 1979). Even though NAS is a substrate for H1OMT for the synthesis of MEL, there was no distinct correllation between NAS levels and MEL levels indicating that NAS levels are not the same in all the stages due to different levels of synthesis of NAS. Bearing in mind that SNAT activity is not a function of the estrus cycle, it is possible that the high estradiol levels expected to be present in the pineals obtained from proestrus rats induces an increase in H1OMT activity during the 24 hour incubation period, resulting in the high MEL production by pineals from proestrus rats.

METABOLITE	PROESTRUS	ESTRUS	METESTRUS	DIESTRUS	SIGNIFICANCE
NAS	477,3 ± 22	339,2 ± 13	232,2 ± 24 *	185,5 ± 10 *	P<0,05
MEL	320,2 ± 23 *	223,5 ± 3 **	175,9 ± 18 **	269,3 ± 10 *	P<0,05
H1AA	20455 ± 74	18932 ± 250	21161 ± 350	27087 ± 480	P<0,02
M1AA	461,1 ± 7	355,4 ± 17 *	341,1 ± 2 *	606,4 ± 15	P<0,02
HTOH	4034 ± 467 *	7001 ± 86	6463 ± 130	5181 ± 356 *	P<0,05
MTOH	740,4 ± 14 *	537,0 ± 16	778,1 ± 21 *	1016,0 ± 92	P<0,05
ORIGIN	129866 ± 4638	109082 ± 3159 *	113342 ± 706 *	77605 ± 694	P<0,05

* = not significantly different.

** = not significantly different.

TABLE 11. Variation in $[^{14}C]$-Serotonin metabolism by rat pineal organ cultures over 24 hrs by pineals taken from rats in different stages of the estrus cycle. 5 pineals were incubated per flask.
Results are expressed as dpm/20 µl Medium/pineal ± S.E.M.

It is possible that there are different H1OMT enzymes in the pineal gland since the pineals from diestrus rats produced the highest levels of MIAA and MTOH, both of which are synthesized by H1OMT, in contrast to MEL synthesis which was highest by pineals from proestrus and diestrus rats.

A correlation between HTOH and MTOH levels reveals that the variation in MTOH during the different stages is probably due to a variation of H1OMT activity and is not due to the availability of HTOH as a substrate. A correlation between HIAA and MIAA levles also reveals that the variation in MIAA levels is probably due to a variation in H1OMT levels and not due to the availability of HIAA as a substrate. It is likely that HIAA has some important role to play other than to be a substrate for MIAA production since it is produced in vast quantities compared to the other products. The other interesting observation is that MEL, from these studies, does not seem to be the most important product produced by the pineal gland. The other methoxyindoles appear to be produced in similar quantities. Whether this is so in pineals obtained from rats in the dark phase remains to be seen.

3.2.6.3. Effect of estradiol administration on (^{14}C)-serotonin metabolism by pineal glands in organ culture.

3.2.6.3.1. Materials and Methods

Female rats with a mass of 200 - 220 g and which had been

ovariectomized two weeks previously were used. Five rats were injected with estradiol benzoate at a dose of 1 µg / day for 3 days. Five control rats were injected with the vehicle at a dose of 0,1 ml olive oil / day for 3 days. All rats were dosed at 09h00. At 09h00 on the 4th day, the rats were sacrificed by neck fracture and the pineals placed into culture aseptically. Radioactive serotonin was added as described earlier. After the 24 hour incubation period, 20 µl aliquots of the media were chromatographed in duplicate and the levels of the metabolites were measured as previously described.

3.2.6.3.2 Results

See table 12.

3.2.6.3.3 Discussion

The reduction in NAS formation is probably not due to a decreased production of NAS but rather due to an increased production of MEL from NAS as observed in the estradiol treated group. The MEL production then causes a fall in the NAS level. Once again, this study confirms the stimulatory effect of estradiol in low concentrations on MEL production, probably by stimulating H1OMT activity (observed in Chapter 2).

Estradiol, at the dose used, suppresses MIAA production indicating that a different H1OMT enzyme might be responsible for MIAA production.

Assuming that the vehicle has no effect, it does appear that ovariectomy reduces the ability of pineals to synthesize HTOH compared to levels found at different stages of the

Surgery	OVX	OVX	—
Dose of estradiol benzoate (µg/day for 3 days)	0	1	Significance
Metabolite			
NAS	459,1 ± 17	187,6 ± 11	P<0,01
MEL	183,3 ± 7	310,7 ± 4	P<0,01
H1AA	25605 ± 155	25013 ± 167	N.S.
M1AA	704,2 ± 19	429,4 ± 68	P<0,05
HTOH	3583 ± 214	5979 ± 107	P<0,01
MTOH	1244,9 ± 198	679,4 ± 42	N.S.
Origin	97199 ± 1741	105499 ± 2511	N.S.

TABLE 12. Effect of estradiol benzoate administration to OVX rats on $[^{14}C]$-Serotonin metabolism in organ culture for 24 hrs. Rats were injected with either estradiol benzoate or 0,1 ml olive oil for 3 days at 9 a.m. and sacrificed at 9 a.m. on the 4th day. Pineal glands, were removed and placed into culture, 5 per flask with 2µCi of $[^{14}C]$-Serotonin for 24 hrs. Results are expressed as dpm/20µl Medium/pineal ± S.E.M.

estrus cycle. The HTOH production capacity is restored in pineals of the estradiol treated group, indicating that estradiol could possibly increase alcohol dehydrogenase activity.

3.2.6.4 Effect of 10nM estradiol in the culture medium on (^{14}C)-serotonin metabolism by rat pineal organ cultures.

3.2.6.4.1 Introduction

This experiment was performed to observe the direct effects of estradiol on pineal serotonin metabolism and to compare these results to those observed in the previous experiment where estradiol was administered *in vivo*.

3.2.6.4.2 Materials and Methods

A total of 10 rats which had been ovariectomized two weeks previously and with a mass of 200 - 220 g were used. The rats were sacrificed at 09h00 and the pineals were placed into culture immediately. One group of five pineals was incubated with 10nM 17-β-estradiol. Another group of five pineals was incubated in the presence of $4X10^{-4}$M ethanol which served as the vehicle for the estradiol, thus the latter group acted as the control. Both groups of pineals were cultured with radioactive serotonin as described earlier. After 24 hours of incubation, 20 µl aliquots of the media were chromatographed in duplicate and the levels of the metabolites measured as described earlier.

3.2.6.4.3 Results

See table 13.

SURGERY	OVX	OVX	-
Concentration of Estradiol (nM)	0	10	-
Metabolite	-	-	Significance
NAS	249,7 ± 0,4	215,8 ± 3	P<0,01
MEL	167,2 ± 2	319,5 ± 6	P<0,005
H1AA	29504 ± 241	22365 ± 376	P<0,005
M1AA	715,2 ± 13	390,1 ± 20	P<0,01
HTOH	5034 ± 648	5274 ± 135	N.S.
MTOH	1205,3 ± 51	551,9 ± 37	P<0,01
Origin	84362 ± 1171	114512 ± 2234	P<0,01

TABLE 13. Effect of 10nM estradiol on [^{14}C]-Serotonin metabolism by pineals from ovariectomised rats in organ culture for 24 hrs. Pineals from OVX rats were cultured* for 24 hrs in the presence and absence of 10nM estradiol, with [^{14}C]-Serotonin. Results are expressed as dpm / 20 µl medium / pineal. ± S.E.M.

* (5 pineals per flask).

3.2.6.4.4 Discussion

Again, the decrease in NAS production by pineals incubated with estradiol was probably not due to decreased synthesis of NAS but due to increased MEL production by a stimulation of H1OMT activity brought about by estradiol. The stimulatory effect of a physiological concentration of estradiol on MEL production was confirmed yet again. Estradiol suppressed the synthesis of MIAA and MTOH as in the previous experiment, supporting the hypothesis of the presence of different H1OMT enzymes in the pineal. It appears that estradiol, in the concentrations used, stimulates MEL synthesis and suppresses MIAA and MTOH synthesis.

The results of this experiment are very similar to those of the previous experiment, indicating that estradiol acts directly on the pineal to modulate pineal metabolism.

3.2.6.5. Effect of progesterone administration on (^{14}C)-serotonin metabolism by pineal organ cultures.

3.2.6.5.1 Materials and Methods

Female rats which had been ovariectomized two weeks previously and with a mass of 200 - 220 g were used. Five of the rats were dosed with 100 µg progesterone subcutaneously daily for 3 days at 09h00and another five control rats were dosed with 0,1 ml olive oil daily for 3 days at 09h00. The rats were sacrificed at 09h00 on the 4th day and pineals from the two groups were placed into culture, five per culture vessel as described earlier, with radioactive serotonin.

After the 24 hour incubation period, 20 µl. aliquots of the media were chromatographed in duplicate and the levels of the metabolites measured as described earlier.

3.2.6.5.2 Results

See table 14.

3.2.6.5.3 Discussion

The reduction in NAS synthesis was again not due to an actual reduction in NAS synthesis in the progesterone treated group, but due to increased conversion of NAS to MEL. The stimulatory effect of acute progesterone administration (at the dose used) on MEL production is further support for the stimulatory effect of this dose of progesterone on pineal H1OMT activity observed in the progesterone experiments in Chapter 2. A stimulatory effect of MIAA production was also noted in the progesterone treated group. This observation is in contrast to the results of the estradiol treated group in the previous experiment, where estradiol decreased MIAA synthesis. It is possible therefore that progesterone and estradiol increase H1OMT activity for MEL production at the doses used and that estradiol inhibits and progesterone stimulates H1OMT responsible for MIAA production. However, the higher levels of MIAA production observed in the progesterone treated group could be due to the higher levels of HIAA production in this group, making more substrate available for conversion to MIAA, although this does not seem feasible as the HIAA which acts as a substrate for H1OMT appears to be present in excess.

Surgery	OVX	OVX	Significance
Dose of progesterone (µg/day for 3 days)	0	100	Significance
Metabolite			
NAS	459,1 ± 17	203,0 ± 30	P<0,01
MEL	183,3 ± 7	268,8 ± 4	P<0,01
H1AA	25605 ± 155	34260 ± 159	P<0,001
M1AA	704,2 ± 19	793,4 ± 5	P<0,025
HTOH	3583 ± 214	5465 ± 85	P<0,01
MTOH	1244,9 ± 198	885,1 ± 12	N.S.
Origin	97199 ± 1741	79899 ± 2161	P<0,02.

TABLE 14. Effect of progesterone administration to OVX rats on [^{14}C]-Serotonin metabolism in organ cultures for 24 hrs. Rats were injected with either progesterone or 0,1 ml olive oil (vehicle) for 3 days at 9 a.m. and sacrificed at 9 a.m. on the 4th day. Pineal glands were removed and placed into culture, 5 per flask with 2µCi of [^{14}C]-Serotonin for 24 hrs. Results are expressed as dpm/20 µl medium/pineal±S.E.M.

It is possible that progesterone affects HIAA production by stimulating the enzyme, aldehyde dehydrogenase which is responsible for the synthesis of HIAA.

It appears therefore that progesterone does modulate the metabolism of serotonin by the pineal gland.

3.2.6.6 Effect of castration and testosterone administration on the metabolism of (^{14}C)-serotonin by pineals in organ culture.

3.2.6.6.1 Materials and Methods

Sham-operated and castrated male rats with a mass of 180 - 200 g were used. Surgery was performed about two weeks prior to use of the animals. Three groups of rats were used :- five sham-operated rats, five castrated rats and five castrated rats treated with 100 µg testosterone proprionate in olive oil per day for 3 days. The other two groups were injected with 0,1 ml olive oil per day for 3 days. All injections were administered at 09h00. The rats were sacrificed at 09h00 on the 4th day and the pineal glands were placed into culture with radioactive serotonin as described earlier. After the 24 hour incubation period 20 µl aliquots of the media were chromatographed in duplicate and the levels of the metabolites measured as described earlier.

3.2.6.6.2 Results

See table 15.

	1	2		3	
SURGERY	SHAM OPERATED	CAST	—	CAST	—
Dose of Testosterone proprionate µg/day for 3 days	0	0	—	100	—
Metabolite	—	—	Significance	—	Significance compared to 2
NAS	156,6 ± 15	349,1 ± 51	P<0,05	375,3 ± 6	N.S.
MEL	441,2 ± 18	432,2 ± 17	N.S.	1346,0 ± 28	P<0,005
H1AA	13631 ± 725	21827 ± 215	P<0,005	14572 ± 685	P<0,005
M1AA	434,0 ± 37	437,3 ± 24	N.S.	573,5 ± 2	P<0,05
HTOH	5477 ± 224	7351 ± 598	P<0,05	9256 ± 558	P<0,05
MTOH	1069,7 ± 39	980,6 ± 28	N.S.	783,6 ± 14	P<0,02
Origin	139493 ± 2343	72282 ± 3262	P<0,005	101869 ± 2399	P<0,01

TABLE 15 Effect of castration and testosterone administration to castrated rats on $[^{14}C]$-Serotonin metabolism by rat pineal organ cultures for 24 hrs. Rats were injected with either testosterone proprionate or 0,1 ml of olive oil (vehicle) for 3 days at 9 a.m. and sacrificed at 9 a.m. on the 4th day. Pineal glands were removed and placed into culture, 5 per flask with 2µCi of $[^{14}C]$-Serotonin for 24 hrs. Results are expressed as dpm/20µl Medium/pineal ± S.E.M.

3.2.6.6.3 Discussion

No significant difference in MEL production by pineals from sham-operated and castrated controls could be detected. This indicates that the higher NAS production by pineals from the castrated control group was due to increased SNAT activity. The increase in SNAT activity was probably due to the elevation of the basal levels of this enzyme which is observed after castration (Chapter 2). However, an expected reduction in MEL production by pineals from the castrated control group was not observed, whereas pineals from the testosterone treated group produced approximately three times more MEL than that produced by pineals from the sham-operated and castrated control groups. This supports the results of the enzyme studies in Chapter 2 which show that testosterone at the dose used, increases HIOMT activity and this results in higher MEL production. Testosterone at the dose used, also appears to increase HIOMT activity for the production of MTOH. Castration appears to elevate HIAA production markedly whereas testosterone treatment suppresses this elevation to levels close to those of the sham-operated controls. It is possible therefore, that testosterone affects the enzyme, aldehyde dehydrogenase which is responsible for the synthesis of HIAA in the pineal gland.

The HTOH production was elevated by castration and further elevated by testosterone treatment. It is possible that other factors such as gonadotropin levels affect the synthesis of this product since variations in HTOH levels in the various

groups was not accompanied by similar variations in MTOH production.

From the results it appears that testosterone affects pineal indole metabolism, particularly MEL production.

3.2.6.7 Effect of 10nM testosterone and 10nM estradiol in the culture medium on the metabolism of (^{14}C)-serotonin by organ cultures of pineals from castrated male rats.

3.2.6.7.1 Materials and Methods

A total of fifteen rats which had been castrated two weeks prior to the experiment and with a mass of 200 - 220 g were used. The rats were divided into three groups consisting of five rats per group. The rats were sacrificed and the pineal glands were removed aseptically and placed into culture with radioactive serotonin. One group of pineals was cultured in the presence of 4 x 10^{-4}M ethanol which acted as vehicle and these were used as the controls. The second group of pineals were cultured in the presence of 10nM testosterone; this is the physiological level of testosterone in the adult male rat. The third group of pineals were incubated in the presence of 10nM estradiol. After the 24 hour incubation period, 20 µl aliquots of the media were chromatographed in duplicate and the levels of the metabolites were measured as described earlier.

3.2.6.7.2 Results

See table 16.

	1	2		3	
Surgery	CAST	CAST	—	CAST	—
Concentration of Testosterone (nM)	0	10	—	0	—
Concentration of Estradiol (nM)	0	0	—	10	—
Metabolite	—	—	Significance	—	Significance compared to 1
NAS	181,7 ± 5	225,1 ± 11	P<0,05	221,8 ± 34	N.S.
MEL	286,7 ± 8	454,2 ± 12	P<0.005	205,7 ± 25	N.S.
H1AA	17012 ± 202	24813 ± 1614	P<0.025	25377 ± 73	P<0,001
M1AA	484,6 ± 6	545,7 ± 13	P<0.05	653,4 ± 4	P<0,001
HTOH	2909 ± 198	3830 ± 192	P<0,05	6021 ± 770	P<0,05
MTOH	721,7 ± 12	981,4 ± 35	P<0,02	840,1 ± 46	N.S.
Origin	122955 ± 2393	103007 ± 1998	P<0,02	120143 ± 36	N.S.

TABLE 16 .Effect of 10nM estradiol and 10nM Testosterone on $[^{14}C]$-Serotonin metabolism in rat pineal organ cultures for 24 hrs. Pineal glands from castrated rats were incubated in the presence of ethanol (vehicle) 4 x 10^{-4}M, 10nM estradiol and 10nM testosterone respectively per flask with 2µCi $[^{14}C]$-Serotonin per flask for 24 hrs. (5 pineals per flask). Results are expressed as dpm/20µl Medium/pineal ± S.E.M.

3.2.6.7.3 Discussion

A correllation between NAS and MEL levels in the testosterone-treated and control groups clearly indicates that testosterone increased NAS synthesis. This was probably due to restoration of pineal cyclic AMP content to normal levels (Karasek et al, 1978). Estradiol, at the concentration used does not seem to affect NAS levels in this experiment supporting the generally held view and the results of the enzyme studies in Chapter 2, that estradiol does not affect SNAT activity. Pineals from the testosterone-treated group produced more melatonin giving further support that testosterone stimulates melatonin synthesis. However, estradiol at the concentration used, did not increase melatonin synthesis indicating that the stimulatory effect of testosterone on melatonin production is probably not due to the conversion of testosterone to estradiol in the pineal gland. Testosterone and estradiol have stimulatory effects on HIAA, MIAA and HTOH production. It is possible that these sex steriods exert their effects independently or that testosterone requires prior conversion to estradiol to exert these stimulatory effects or possibly by a combination of these possibilities. It is interesting to note that the same concentration of estradiol suppresses the synthesis of MIAA by pineals from ovariectomized rats. Testosterone increases MTOH production whereas estradiol does not affect it.

In contrast to the effects of estradiol on the synthesis of

methoxyindoles in pineals from ovariectomized rats, testosterone treatment of pineals from castrated rats increases the synthesis of all three methoxyindoles measured.

The results of this experiment show that estradiol and testosterone can influence the metabolism of some or most of the pineal indoles. As discussed, the possibility that testosterone exerts some of its effects by prior conversion to estradiol cannot be excluded.

CHAPTER 4

4.1 CONCLUSION

4.1.1 H1OMT Activity Studies

The pineal H1OMT activity varies as a function of the estrus cycle, being highest during the estrus phase and lowest during the proestrus phase of the estrus cycle. This is possibly due to fluctuations in the levels of the circulating sex hormones. The organ culture study involving the time-related effect of a physiological concentration of estradiol (10nM) on pineal H1OMT activity enables the formulation of a hypothesis to explain the high level of H1OMT activity found during the estrus phase of the estrus cycle. Since 10nM estradiol increases H1OMT activity in the cultures of pineals from ovariectomized rats from about 7 hours to three times the starting level at 24 hours in culture, it is possible that in the intact rat, the high levels of plasma estradiol prevailing during proestrus (Hori et al, 1968) result in the increase in H1OMT activity to the high levels found in estrus (which is about 24 hours after proestrus).

Ovariectomy results in a significant reduction in pineal H1OMT activity compared to that of sham-operated rats in the estrus phase of the estrus cycle. The administration of low doses of estradiol (0,01 - 1 µg/day for 3 days) to ovariectomised rats restores this reduction of H1OMT activity to levels approximating and above those of the controls. A higher dose of estradiol (10 µg/day for 3 days) administered to ovariectomised rats results in a significant reduction in pineal H1OMT activity. Thus, the lower doses of

estradiol stimulate H1OMT activity while higher doses inhibit it.

Progesterone administration to ovariectomized rats in doses ranging from 10 to 1 000 µg/day for 3 days significantly elevates pineal H1OMT activity. Chronic administration of progesterone in doses of 10 and 100 µg/day for 30 days to ovariectomized rats results in a very significant elevation (10 µg group) and depression (100 µg group) of H1OMT activity compared to controls.

Castration also results in a reduction of pineal H1OMT activity. Pineals of castrated rats have H1OMT activities which are approximately half those of the sham-operated controls. The administration of testosterone to castrated rats at a dose of 0,01 to 1 000 µg/day for 3 days results in an elevation of pineal H1OMT activity to levels approximating those of the controls. However, a dose of testosterone at 10 000 µg/day for 3 days administered to these rats results in a depression of H1OMT activity to levels approximating those of the controls.

In organ cultures of rat pineals obtained from ovariectomized rats, a dose-response relationship of estradiol concentration versus H1OMT activity was observed. Estradiol at concentrations ranging from 0,1 to 100 nM increases H1OMT activity with maximal stimulation at 10nM estradiol. A concentration of estradiol of 1 000 nM suppresses H1OMT activity to levels below that found in the controls. A biphasic dose-response relationship was therefore observed.

All three of these sex steroids investigated have been found to have biphasic dose-dependent effects on pineal H1OMT activity in these studies.

4.1.2 SNAT activity studies.

In all the SNAT activity studies, a diurnal variation in enzyme activity was observed. The nocturnal levels of this enzyme were found to be more than fifty times higher than the basal levels, confirming the reports of many other researchers in this field.

Ovariectomy does not affect the basal and nocturnal levels of this enzyme nor the diurnal rhythm of this enzyme. The administration of estradiol to ovariectomized rats in doses ranging from 1 to 100 µg/day for 3 days does not affect the basal and nocturnal nor the diurnal rhythm of this enzyme under the experimental conditions in which these experiments were performed. This indicates that estradiol probably does not play a role in the regulation of melatonin synthesis at the level of SNAT.

The administration of progesterone to ovariectomized rats in doses ranging from 1 to 100 µg/day for 3 days does not affect the levels nor the rhythm of this enzyme either under these experimental conditions. Progesterone therefore probably does not play a role in the regulation of pineal melatonin synthesis at the level of SNAT either.

Castration results in a very significant reduction in the nocturnal rise of SNAT activity and a significant elevation

of the basal level of activity of this enzyme. The administration of testosterone to these castrated rats at a dose of 100 µg/day for 3 days results in a restoration of the nocturnal rise of this enzyme to levels approximating those of the controls and a depression of the basal levels of this enzyme.

Testosterone is the only gonadal sex steroid which affects SNAT activity in these studies. This steroid can therefore modulate pineal melatonin synthesis at the level of both SNAT and H1OMT.

4.1.3 ^{14}C-serotonin metabolism studies using organ culture

Pineal glands obtained from rats in different stages of the estrus cycle and placed into organ culture have definite differences in their ability to metabolize ^{14}C-serotonin. This indicates that pineal indole metabolism varies as a function of the stage of the estrus cycle.

When pineal glands from estradiol-treated ovariectomized rats are cultured with ^{14}C-serotonin, they produce significantly more radiolabeled MEL from the radiolabeled serotonin. However, the formation of radiolabeled MIAA and MTOH is significantly reduced compared to controls. These results are very similar to those where pineals from ovariectomized rats were cultured in the presence of 10nM estradiol. It can therefore be concluded that at the doses of estradiol used in these studies, estradiol has a stimulatory effect on MEL production and an inhibitory effect on MIAA and MTOH production.

Castration does not produce any change in the ability of pineals from these castrated rats to form radiolabeled MEL from ^{14}C-serotonin but elevates the production of radiolabeled HIAA, and NAS. However, the administration of testosterone to castrated rats prior to sacrifice, increases the ability of these rat pineals to form radiolabeled MEL, MIAA and HTOH from the ^{14}C-serotonin markedly and reduces the elevated production of HIAA to those of control values.

Pineal glands from untreated castrated rats cultured in the presence of 10nM testosterone markedly form more radiolabeled NAS, MEL, HIAA, MIAA and HTOH from ^{14}C-serotonin. From results of these studies it can be concluded that testosterone enhances synthesis of NAS, MEL, MIAA and HTOH.

Estradiol (10nM) in the culture medium containing pineals from untreated castrated rats, does not influence pineal indole metabolism to a great extent. It has a stimulatory effect on MIAA, HIAA and HTOH production. Due to the lack of a stimulatory effect of estradiol on MEL, NAS and HTOH synthesis in these pineals, it can be concluded that testosterone in the pineal glands of male rats probably does not rely on prior conversion to estradiol for all of its activity to influence pineal indole metabolism.

From these metabolism studies it can be seen that MEL might not be the most important methoxyindole produced by the pineal

gland, since all the methoxyindoles measured were found to be produced in similar amounts. Whether this is so in pineals obtained from rats in the dark phase remains to be seen. It is likely that only MEL is produced in far larger amounts in the dark phase since SNAT activity is high during the dark phase. The biological effects of these methoxyindoles and the hydroxyindoles produced by the pineal need further investigation to gain a better understanding of pineal function.

CHAPTER 5

SUMMARY

CHAPTER 1 :

A general review involving pineal location, anatomy, function and the modulatory effects of other organs of the body and environmental illumination on pineal function has been presented.

CHAPTER 2 :

The effects of the gonadal sex steroids viz. estradiol, progesterone and testosterone on the level of rat pineal H1OMT and SNAT activity were investigated.

The level of pineal H1OMT activity was found to vary as a function of the estrus cycle, with highest levels during the estrus phase and lowest levels during the proestrus phase of the estrus cycle of the rat.

Ovariectomy and castration were found to significantly lower pineal H1OMT activity. The administration of estradiol to ovariectomized rats and testosterone to castrated rats was found to have dose-dependent effects on rat pineal H1OMT activity. Lower doses were stimulatory while the higher doses were found to by inhibitory. Progesterone administration to ovariectomized rats elevated pineal H1OMT activity but only had dose-dependent effects such as of estradiol and testosterone when it was administered chronically.

Neither ovariectomy nor estradiol and progesterone administration to ovariectomized rats affected the basal and

nocturnal levels of pineal SNAT activity. However, castration of adult male rats significantly lowered the nocturnal and raised the basal levels of the activity of this enzyme. Testosterone administration to these castrated rats restored the nocturnal rise of this enzyme to control levels but depressed the basal levels.

CHAPTER 3 :

A technique for growing monolayer cell cultures using rat pineal explants is described. This technique, although successful, was not used for any further studies as the required microscope was not available. Instead, an organ culture technique using whole rat pineal glands was developed. A time related study involving H1OMT activity as a function of time, in the presence of a physiological concentration of estradiol in the culture medium was performed. It was found that pineals cultured in the presence of estradiol showed an increase in H1OMT activity from about 7 hours to 24 hours in culture. Following this, a dose-response study of H1OMT activity versus estradiol concentration using organ culture for 24 hours was performed. A biphasic dose-response relationship was observed, with lower concentrations of estradiol being stimulatory while higher concentrations were inhibitory.

In further studies, rat pineal organ cultures were used to investigate the influence of sex steroids on ^{14}C-serotonin metabolism by these pineal glands. For these studies, the organ culture system had to be modified further to suit the experimental conditions.

A tremendous variation in ^{14}C-serotonin metabolism by pineals obtained from rats in different stages of the estrus cycle was observed. Estradiol administration to ovariectomized rats or a physiological concentration of estradiol in the culture medium containing pineals from untreated ovariectomized rats both result in increased synthesis of radiolabeled MEL from the ^{14}C-serotonin, in organ cultures. The synthesis of radiolabeled MIAA and MTOH by these pineals is however suppressed.

Pineals from castrated rats did not show any significant difference in radiolabeled MEL production compared to pineals from sham-operated controls. Pineals from castrated rats which were treated with testosterone prior to sacrifice formed about three times more radiolabeled MEL from ^{14}C-serotonin than the controls, using organ cultures. A significant elevation in radiolabeled MTOH production by pineals from the testosterone treated group was also observed.

Organ cultures of pineal glands from untreated castrated rats placed into culture with a physiological concentration of testosterone formed significantly more radiolabeled NAS, MEL, MIAA, HIAA and HTOH from ^{14}C-serotonin compared to controls. The addition of estradiol to organ cultures of pineals from untreated castrated rats results in the formation of significantly more radiolabeled MIAA, HIAA and HTOH from ^{14}C-serotonin.

CHAPTER 4 (Conclusion) :

The gonadal sex steroids have biphasic dose-dependent effects on the pineal H1OMT activity responsible for the synthesis of melatonin. The only one of these sex steroids which affects SNAT activity is testosterone. These sex steroids, by affecting the enzymes responsible for the synthesis of melatonin can possibly have modulatory effects on melatonin synthesis.

The technique of organ culture proved to be an invaluable tool for the determination of the effects of sex steroids on the pineal enzymes and metabolism, eliminating the complexities of organ interaction. The results of the pineal metabolism studies indicate a definite modulatory effect of the gonadal sex steroids on pineal indole metabolism, particularly the elevation in melatonin synthesis. These studies also revealed that melatonin might not be the most important pineal indole produced, since other indoles such as HIAA and HTOH were produced in very high quantities. It does not seem logical that the pineal would produce such high quantities of these indoles for conversion to methoxyindoles only, which were found to be produced in very low quantities. The biological effects of these hydroxyindoles and the other methoxyindoles should be evaluated further for a better understanding of pineal function.

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