ADRENO-ACTIVE SUBSTANCES AND THE PINEAL GLAND

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by

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ABSTRACT

The pineal gland, a biochemically very active neuroendocrine transducer which is innervated by the sympathetic nervous system, was used *in vivo* to evaluate the effect of different β-adrenoceptor agonists and antagonists on pineal enzyme levels.

Hydroxyindole-O-methyltransferase (HIOMT), an enzyme with a circadian activity and unknown control was not significantly affected by these drugs. The activity of serotonin N-acetyltransferase, another pineal enzyme with a greater amplitude of circadian rhythmicity and which is under noradrenergic neural control, was greatly enhanced by β-adrenoceptor agonists. This effect could be blocked by β-adrenoceptor blocking agents, the degree of blockade depending on the selectivity and affinity of the agent used.

An attempt was also made to alter the oestrous cycle of the rat by dosing with β-active substances. Only propranolol had any effect on the oestrous cycle. It was not possible to establish an absolute link between the alteration in pineal enzyme activity and an influence on the oestrous cycle. It was concluded that the pineal enzyme studies are useful pharmacological means for evaluating β-active substances.

CHAPTER 1

LITERATURE REVIEW

1.1 PINEAL ANATOMY

1.1.1 Pineal Innervation and Blood Supply

The pineal gland of the rat is situated between the two cerebral hemispheres of the brain, just forward of the cerebellum; viewed laterally, it is superficially located just below the skull (fig. 1). It derives its name from the pine cone which it resembles in shape. In the rat, the pineal gland has a mass of about 1 mg. It is covered by the confluence of the superior sagittal sinus and the transverse sinus. The gland is supported by strands of connective tissue which attach it to the tela chorioidea rostrally and to the inferior surface of the transverse sinus caudally (fig. 2). In the rat, relay of sensory information to the pineal takes place via nerve fibres; the majority of these fibres are sympathetic and originate bilaterally in the superior cervical ganglion (Kappers, 1960). These penetrate the pineal as the nervi conarii in two bilateral symmetrical nerve tracts. Some nerves enter the pineal along small pial vessels (fig. 2). After leaving the tentorium cerebelli, the nerves enter the pineal symmetrically at the dorso-caudal surface of the organ just beneath the floor of the confluens sinuum. At this point, two symmetrical branches of the posterior cerebral arteries usually enter the organ. These are the main source of blood supply to the pineal.

A small number of nerves travelling up the pineal stalk carry direct information from the brain (fig. 1), but these are generally considered to be merely aberrant neurons arising from the habenular commissure and posterior commissure with no functional significance (Clark, 1940). However, Gardner (1953) has suggested that these nerves which are

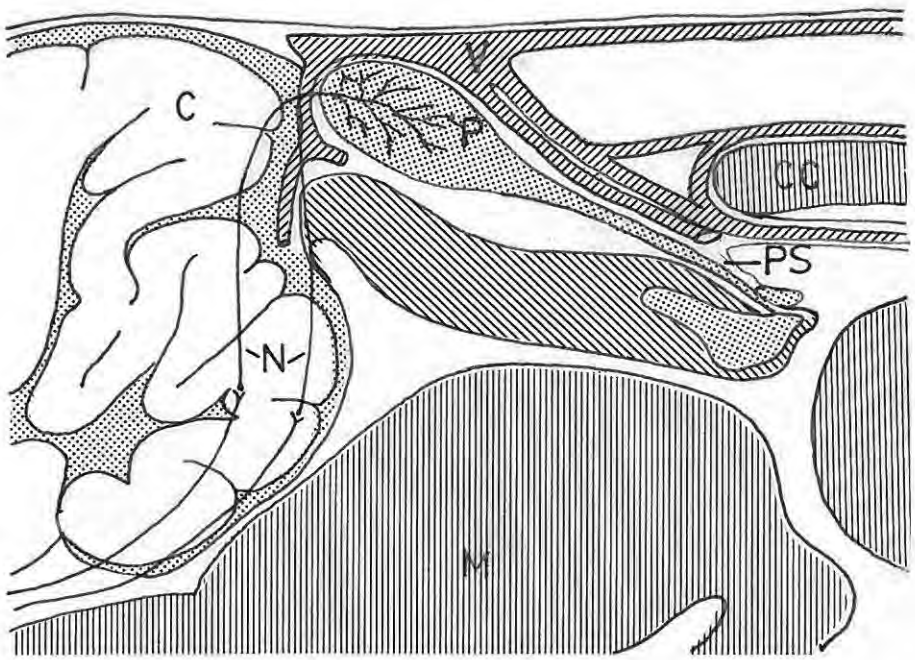


Fig. 1. Lateral view through the midplane of the adult rat brain indicating location of the pineal gland and sympathetic nervous innervation.

C, cerebellum; P, pineal gland; CC, corpus callosum; V, veins; PS, pineal stalk; M, medulla; N, nerves from superior cervical ganglia (Wurtman and Axelrod, 1965).

derived from the commissures may have some functional significance. The so-called Fibrae pineales, ventrales or posteriores reach the organ from the caudal or posterior commissure. Fibrae prepineales, pineales superiores, anteriores or dorsales arise from the habenular commissure. It is now generally accepted that these fibres from the commissures have their cells of origin in the brain (Kappers, 1965). Owing to difficulty in tracing these nerve pathways, it has been referred to as the "habenulo-caudo-commissural epiphysial system" or the "epithalamico-epiphyseal tract" (fig. 2).

1.1.2 The Pineal Stalk

The pineal stalk develops by growing out of the most proximal part of the rostro-dorsal and caudo-ventral area of the pineal. The stalk consists of pinealocytes which show a follicular arrangement in some places, as well as pinealoblasts and fibrocytes. The stalk can be divided into three parts, a proximal-, a mid- and a distal part. At the midpart the stalk is often very thin (Kappers, 1960). Some nerve fibres from the commissures form loops and pass back down the stalk. Some however may penetrate deep into the pineal and some may pass on to neighbouring tissues (Kappers, 1965). There do not seem to be any afferent nerves originating in the pineal and going to the brain (fig. 3).

Dafny (1977) found electrophysiological evidence of reciprocal central connections between the pineal and the ventromedial hypothalamus as well as the existence of pineal inputs from acoustic, olfactory, photic and amygdaloid pathways. These pathways are thought to originate in the habenular commissure.

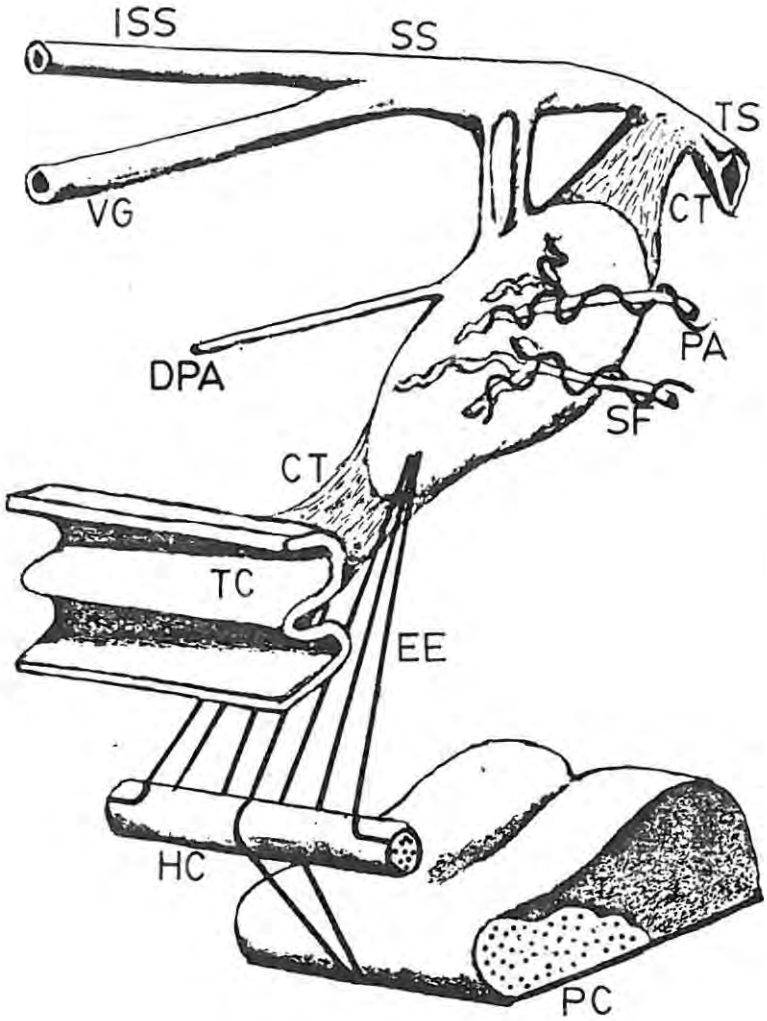


Fig. 2. Diagrammatic representation of the blood and nerve supply of the rat pineal gland.

ISS, inferior sagittal sinus; VG, great vein of Galen; SS, straight sinus; DPA, dorsal pineal artery; TS, transverse sinus; PA, pial arteries to the pineal; CT, connective tissue strands supporting the pineal; TC, tela chorioidea; EE, epithalamico-epiphyseal nerve fibres; SF, sympathetic nerve fibres; HC, habenular commissure; PC, posterior commissure (Gardner, 1953).

Matsushima et al. (1977) showed that large bundles of unmyelinated nerve fibres penetrate the pineal gland through relatively wide intercellular spaces. The nerve fibres and endings are situated mainly within these intercellular or pericapillary spaces. These nerve endings contain small granulated and non-granulated vesicles of about 50 nm in diameter as well as a few larger granulated ones of about 100 nm in diameter. The presence of these vesicles indicates that the nerve fibres here are probably sympathetic adrenergic postganglionic in nature, with their cell bodies located in the superior cervical ganglia (Pellegrino de Iraldi et al., 1965). Axonal dilations have also been observed in the pineal gland. These contain inclusions such as mitochondria and vesicles. The swellings are found mainly within the pineal parenchyma, surrounded by parenchymal cells and less often in pericapillary spaces (fig. 4a and 4b).

Bulbous nerve terminals lying adjacent to pineal capillaries were also described by Milofsky (1957). These terminals contain many small vesicles most of which enclose a dense granule. Pellegrino de Iraldi et al. (1961), using electron microscopy, observed pinealocytes with club-shaped perivesicular expansions connected to the cell body by thin pedicles. These expansions were seen to be rather large pericapillary spaces. It was assumed that these expansions may contain biogenic amines. Reserpine causes heterogeneous granular vesicles to disappear almost entirely, suggesting therefore that they are storage sites for biogenic amines. These club-shaped terminals are now considered to be autonomic nerve endings.

Wolfe et al. (1962) has shown that noradrenaline is stored or associated in pineal autonomic nerves and endings. The neurotransmitter, most probably noradrenaline, released from the neuronal varicosities, stimulates

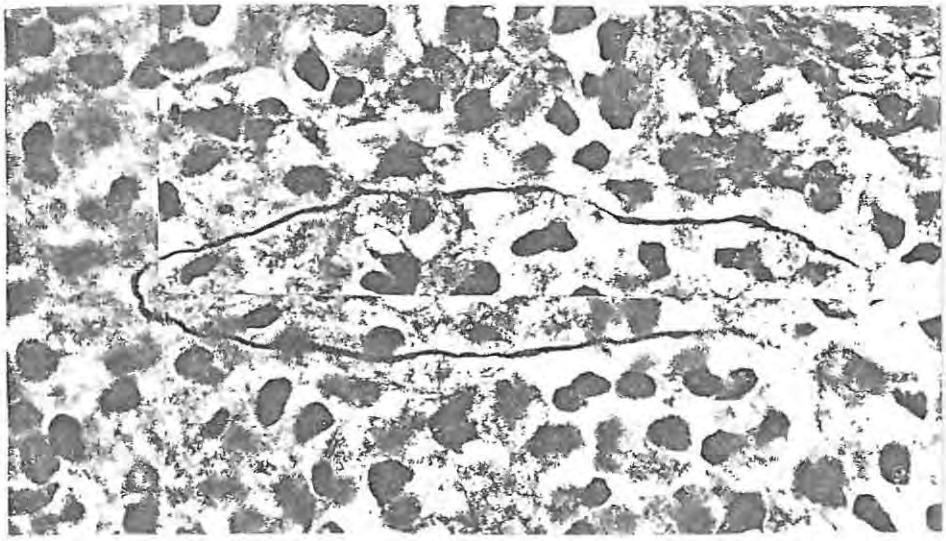


Fig. 3. Aberrant commissural-epiphyseal fibre making a hair-pin loop in the most rostral part of the rat pineal body, X800 (Kappers, 1965).

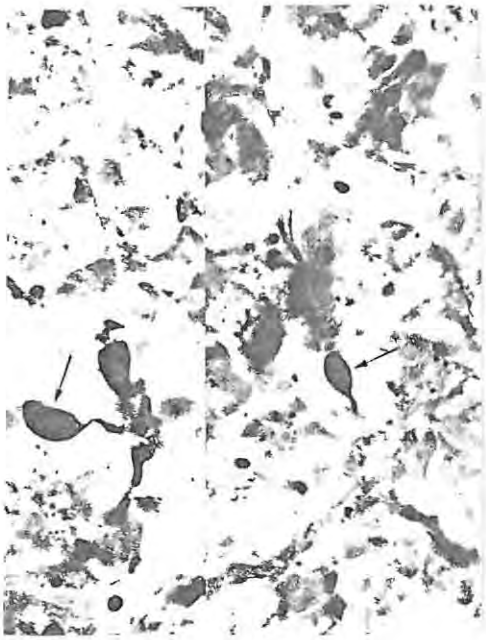


Fig. 4a. Probable autonomic nerve terminals in pineal parenchyma X800 (Kappers, 1965).

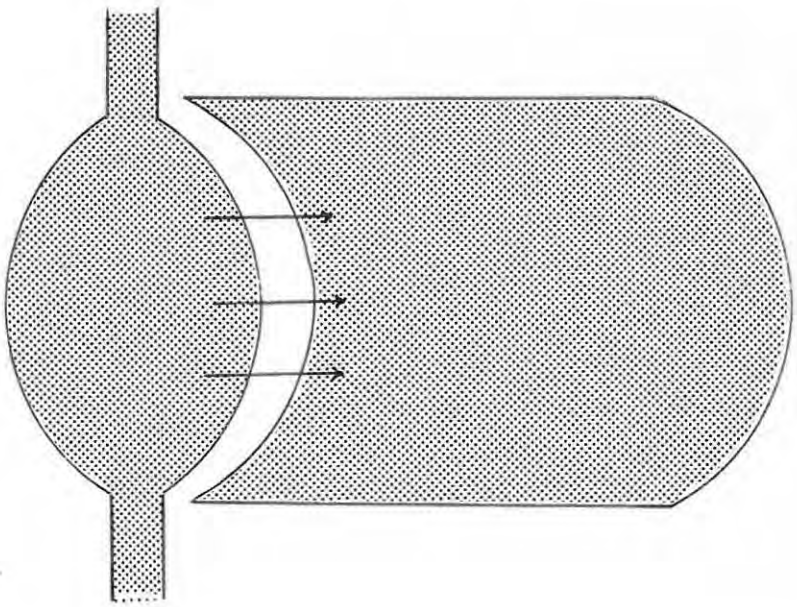


Fig. 4b. Representation of an axonal dilation considered to be an autonomic nerve ending, adjacent to a pineal cell.

the parenchyma cells or pinealocytes by diffusing through the basement membrane lining the pineal parenchyma. Intraparenchymal sympathetic fibres end in close contact with processes of pinealocytes and their terminal buds, but never on the bodies of these cells.

1.1.3 The Pineal Cell

The mammalian pinealocyte is a secretory cell which produces, stores and secretes different pineal substances. Storage, and possibly also part of the production of secretory substances, occurs in the terminal buds of the pinealocyte processes. These buds usually terminate either on the basement membrane of the pineal parenchyma or on intercellular spaces which communicate with the pericapillary spaces.

It seems that pinealocytes secrete their products into the blood circulating in the capillaries via the basement membrane of the pineal parenchyma, the pericapillary space, the basement membrane which covers the capillary endothelium and the endothelial wall. Secretion also occurs from the parenchyma into intercellular spaces which are in open communication with the pericapillary spaces. Sympathetic nerve endings are by no means limited to areas around a capillary, but have also been observed either within the pineal parenchyma or adjacent to the terminal clubs indicating that the function is not merely the regulation of bloodflow through the pineal.

Although synapse-like structures have not been observed between nerve endings and pinealocytes, there is a close relationship between them. It is possible that a nerve impulse may spread from an innervated terminal club to adjacent terminal clubs by means of low-resistance pathways.

Indoleamines such as serotonin have been detected in pinealocytes by fluorescence microscopy (Owman, 1964). However, the ultrastructural location of melatonin and serotonin remains to be clarified.

1.1.3.1 Structure of Pineal Cells

The main type of cell in the rat pineal accounting for almost 90% of the cell population is the epiphyseal cell. It has also been called "parenchymal", "glandular" and "fundamental". The other element constituting the remainder of the pineal is a distinctive population of interstitial cells. They are so named because this describes their disposition in pineal tissue. The number and situation of these cells suggests that they correspond to most of the "neuroglial cells" (usually astrocytes) traditionally described in the pineal (Wolfe, 1965).

Pineal cell clusters tend to be haphazard (fig. 5). Cytoplasmic prolongations or polar processes arise from the perikaryon of a pineal cell and taper to a neck-like or collicular segment. A polar process is joined to adjacent pineal cells by a particular type of intercellular junction called zonula or fascia adhaerens (Farquhar and Palade, 1963). The fine structure of polar processes suggests that they are homologous to the club-shaped prolongations of pineal cells in the human pineal body.

Pineal cells in a cluster are tightly grouped, a gap of about 15 to 25 nm separating most cells. Sometimes these clefts widen abruptly, forming an interfacial lake (fig. 5).

1.1.3.2 The Epiphyseal Nucleus

The epiphyseal nucleus has the fine structure of interphase nuclei of

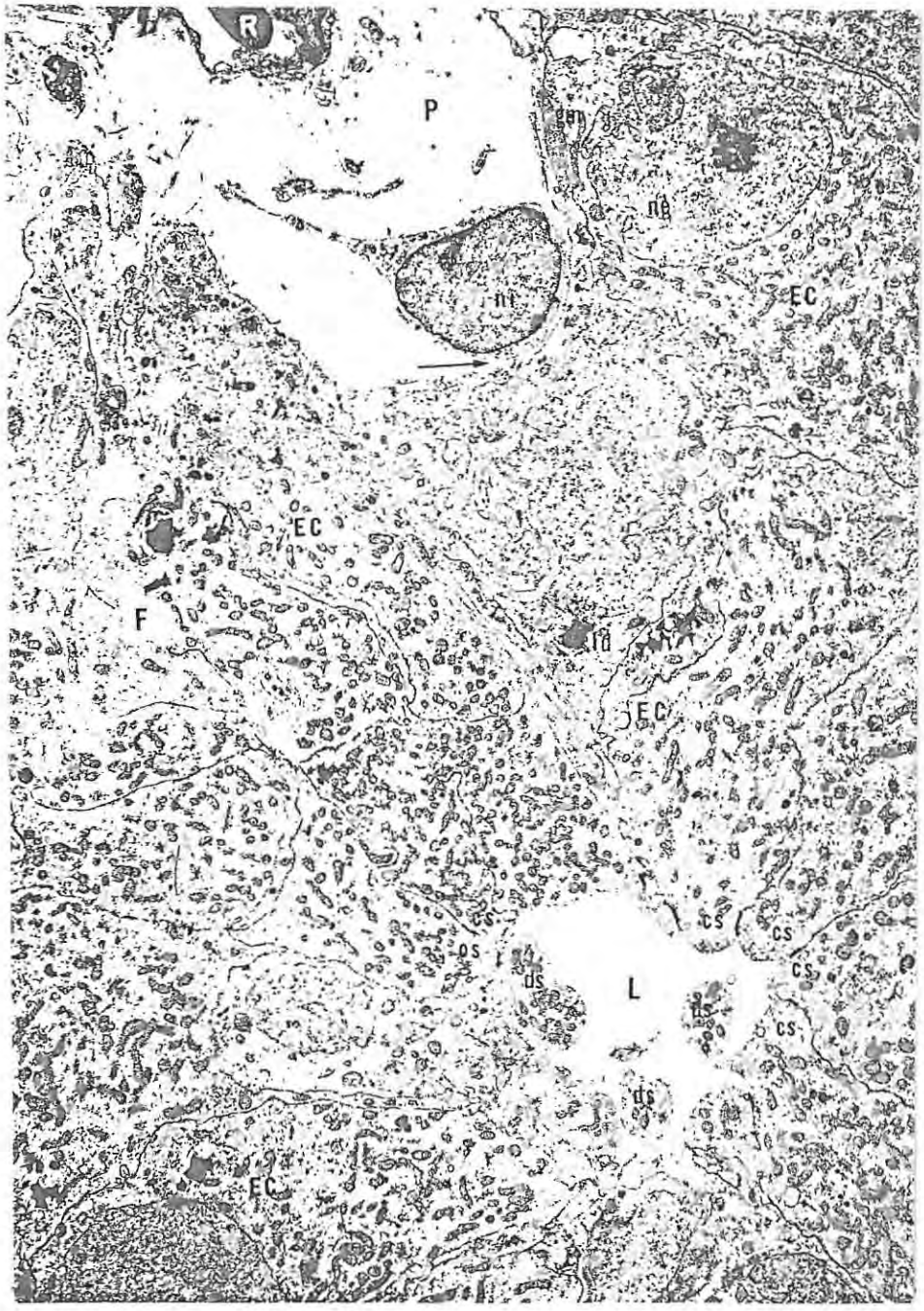


Fig. 5. Typical epiphyseal cluster showing compact cell arrangement.

P, perivascular space; L, circumluminal array; F, interfacial lake; EC, epiphyseal cells; ld, lipid droplets; R, erythrocyte; cs, collicular segments of polar processes; ds, distal segments of polar processes; gm, giant mitochondria; ne, pineal nuclei; ni, interstitial cell nuclei X5200 (Wolfe, 1965).

many tissues (Bernhard and Granboulon, 1963). The nucleoplasm is highly electron-lucent. Chromatin is localized in an uneven marginal zone sporadically confluent with areas deeper in the nucleus. Chromatin areas are filled with areas of low electron density which are arranged in granular and variegated masses and thin helical strands. Nucleoli probably also exist in all pineal cell nuclei, their marked electron-density contrasting with the relatively uniform appearances of the remaining nucleoplasm and cytoplasm (fig. 6).

1.1.3.3 Pinealocyte Cytoplasm

Single ribosomes and clusters of 4 to 12 ribosomes are sprinkled throughout the cytoplasm and are not associated with membranes. A small number of ribosomes are associated with paired cytoplasmic membranes and with the outer layer of the nuclear envelope to form the rough surfaced or granular endoplasmic reticulum of the pineal cell. Cytoplasmic membranes are rarely observed in their full complexity due to the disruptive action of preparative techniques for electron microscopy (Wolfe, 1965).

Pineal cells contain a large number of lipid inclusions which vary widely in shape. Close to the lipid droplets, material of similar electron density is frequently present within cisternae of the endoplasmic reticulum.

Pineal cells have a fairly abundant number of mitochondria varying markedly in size (fig. 5). Zig-zag arrays of cristae are sparse in the mitochondria (Revel et al., 1963).

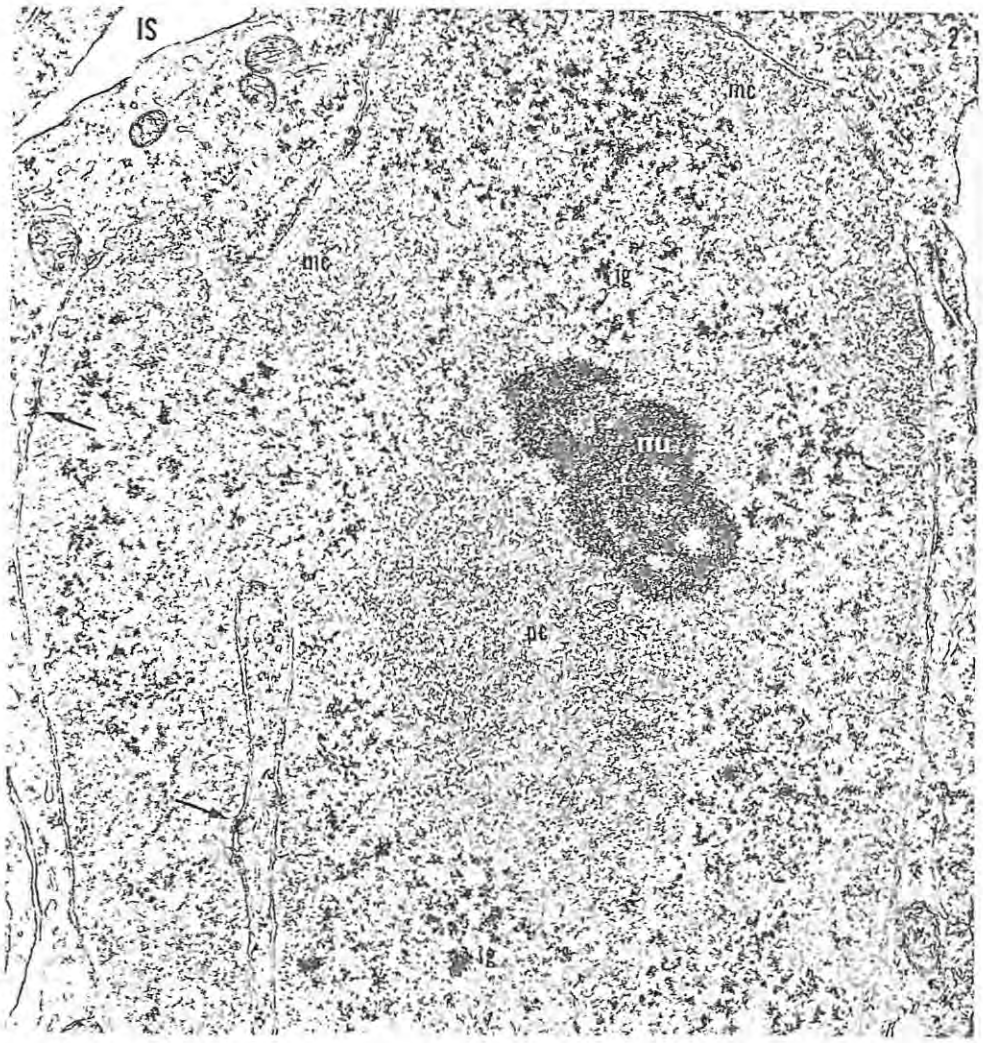


Fig. 6. Pineal cell nucleus showing pattern of chromatin in the marginal (mc) and perinucleolar chromatin areas.

nu, nucleolonema; ig, interchromatin granules; IS, interstitial space. Nuclear pores are arrowed. (Wolfe, 1965).

1.1.3.4 Vernate Bodies in the Pinealocyte

These are irregular convolutions of lamellae surrounding a central zone of ill-defined granular material in which lipid droplets, glycogen particles, mitochondria and vesicles are present. The whorled lamellae may be part of pineal cell cytoplasm.

Filaments about 8 nm thick are common in autonomic axons and interstitial cells but are rare in epiphyseal cells.

The presence of a microtubular arrangement is the most distinctive cytoplasmic component of the pineal cell. Membranes of the Golgi apparatus occur randomly in epiphyseal cells. "Grumose bodies", common in pineal cells usually contain finely-textured granular material distributed evenly in a dense amorphous matrix. These bodies usually populate the polar terminals (Wolfe, 1965).

1.1.4 Blood Flow

By using an isotope indicator method, Goldman and Wurtman (1964) clarified the nature of blood flow in the pineal. The rate of flow was found to be at least 2 to 4 ml/min/g. This rate of flow through the pineal is higher than in most other endocrine organs; it is equalled by that of the neurohypophysis and surpassed only by the rate of flow through the kidney.

1.2 PINEAL BIOCHEMISTRY

1.2.1 Melatonin Biosynthetic Pathway

For an organ of such small size, the rat pineal gland is of extreme

biochemical significance. The initial stimulus to study its physiology and biochemistry was provided by Lerner (1958) when he isolated and characterized melatonin. Melatonin is regarded as the primary pineal indoleamine. It is synthesized in the organ as shown in fig. 7. These biosynthetic conversions are under the sympathetic neural control of the nerves arising from the superior cervical ganglia (Volkman and Heller, 1971; Wurtman et al., 1971; Zatz et al., 1976). It has been shown that the sympathetic nerve endings contain noradrenaline, serotonin and histamine (Giarman and Day, 1959). Noradrenaline is released to act on β-receptors in the pineal gland (Axelrod et al., 1969) and thus influences the metabolic processes in the gland. The precursor in the biosynthesis of melatonin (fig. 7) is tryptophan, an amino acid. The uptake of tryptophan by the pineal parenchymal cells is enhanced by nervous stimulation which results in a greater release of noradrenaline (Wurtman et al., 1969). Tryptophan is then hydroxylated by the enzyme tryptophan hydroxylase to form 5-hydroxytryptophan (Lovenberg et al., 1967). Another enzyme, aromatic-L-amino acid decarboxylase, catalyses the formation of 5-hydroxytryptamine or serotonin (Snyder and Axelrod, 1964a) This enzyme has a concentration in the pineal greater than in any other tissues examined (Snyder and Axelrod, 1964b). Another enzyme serotonin-N-acetyl transferase (SNAT) transfers an acetyl group from acetyl coenzyme A to form N-acetylserotonin (Weissbach et al., 1960). The final step of melatonin synthesis, i.e. O-methylation in the 5 position, takes place in the presence of the enzyme hydroxyindole-O-methyltransferase (HIOMT)(Axelrod and Weissbach, 1960; 1961).

1.2.2 Secondary Indole Pathways

There are also a number of secondary indole metabolic pathways in the

MELATONIN BIOSYNTHETIC PATHWAY

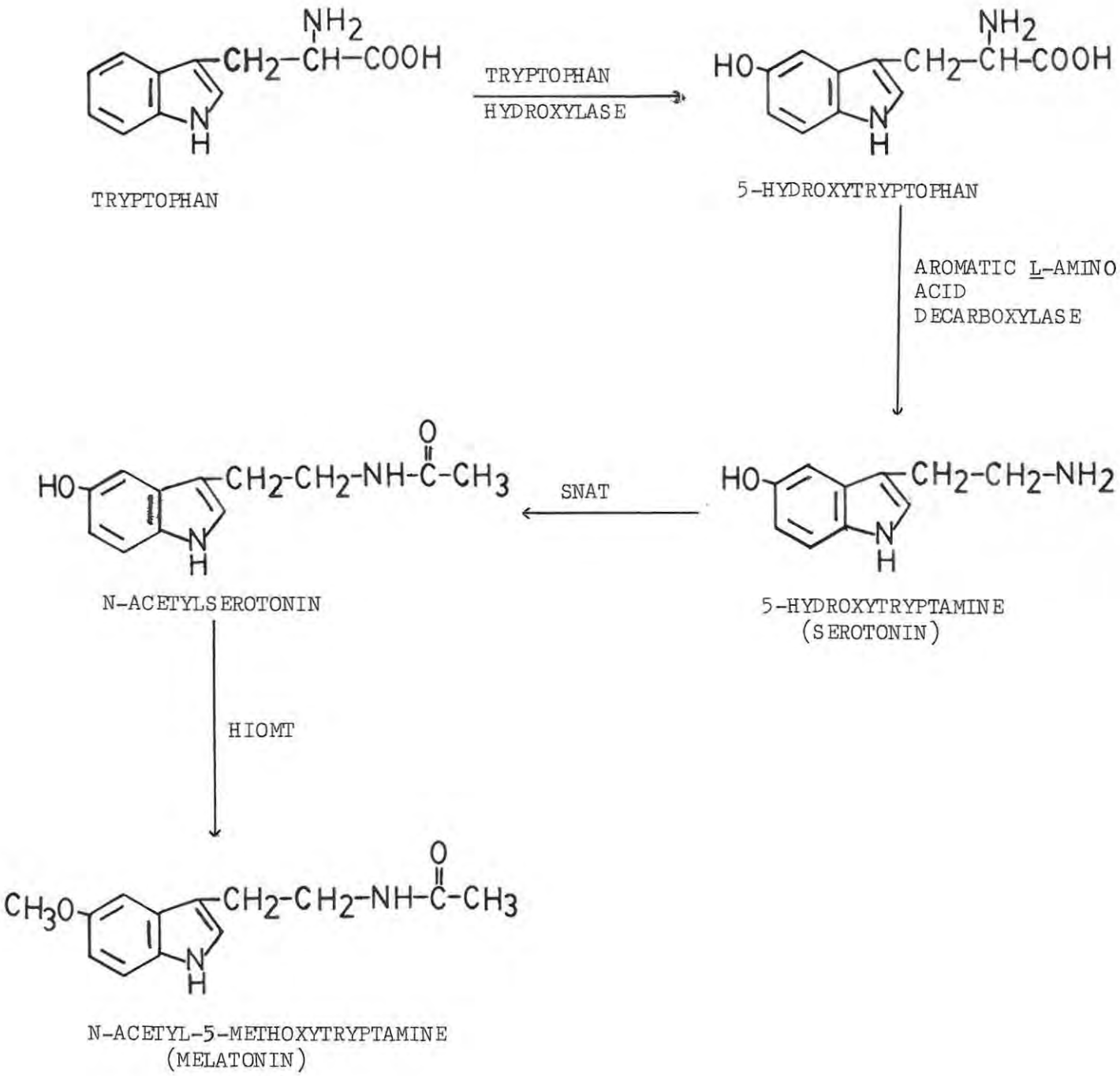


Fig. 7.

pineal gland (fig. 8). Serotonin can be oxidized to 5-hydroxyindole-acetaldehyde by the enzyme monoamine oxidase (Axelrod et al., 1969). A further oxidation leads to the formation of 5-hydroxyindoleacetic acid (Wurtman and Larin, 1968). This can then be O-methylated in the 5 position (as was the case with N-acetylserotonin) by HIOMT to form 5-methoxyindoleacetic acid (Wurtman and Axelrod, 1967). 5-Hydroxy-indoleacetic acid formed from serotonin can alternatively be reduced to 5-hydroxytryptophol and then converted to 5-methoxytryptophol by HIOMT (Wurtman and Axelrod, 1967; Wurtman et al., 1967).

1.2.3 The Receptors

1.2.3.1 The β-Adrenergic Receptor

Once it had been established by Kappers (1960) that the pineal is heavily innervated by the sympathetic nervous system and that the pineal body contains high concentrations of noradrenaline (Wolfe et al., 1962) as well as serotonin and histamine (Giarman and Day, 1959), the question arose whether the noradrenergic receptors in the pineal were alpha (α) or beta (β) or both. To find out, organ culture systems were developed by Wurtman and Larin (1968); Klein et al., (1970b). Radioactive DL-[3-^{14}C]-tryptophan was added to a pineal organ culture and the formation of radioactive serotonin, melatonin and 5-hydroxyindoleacetic acid monitored. Addition of the catecholamine, L-noradrenaline greatly enhances the synthesis of these tryptophan derivatives. Propranolol, a β-adrenergic blocking agent, prevents this increased synthesis, (Wurtman et al., 1971) whereas phenoxybenzamine, an α-adrenergic blocking agent, does not. This observation indicated that the mechanism of stimulation involves 'classic' β-receptors. Other sympathomimetic amines such as tyramine, dopamine and octapamine also stimulate the formation of melatonin from

PINEAL SECONDARY INDOLE BIOSYNTHETIC PATHWAY

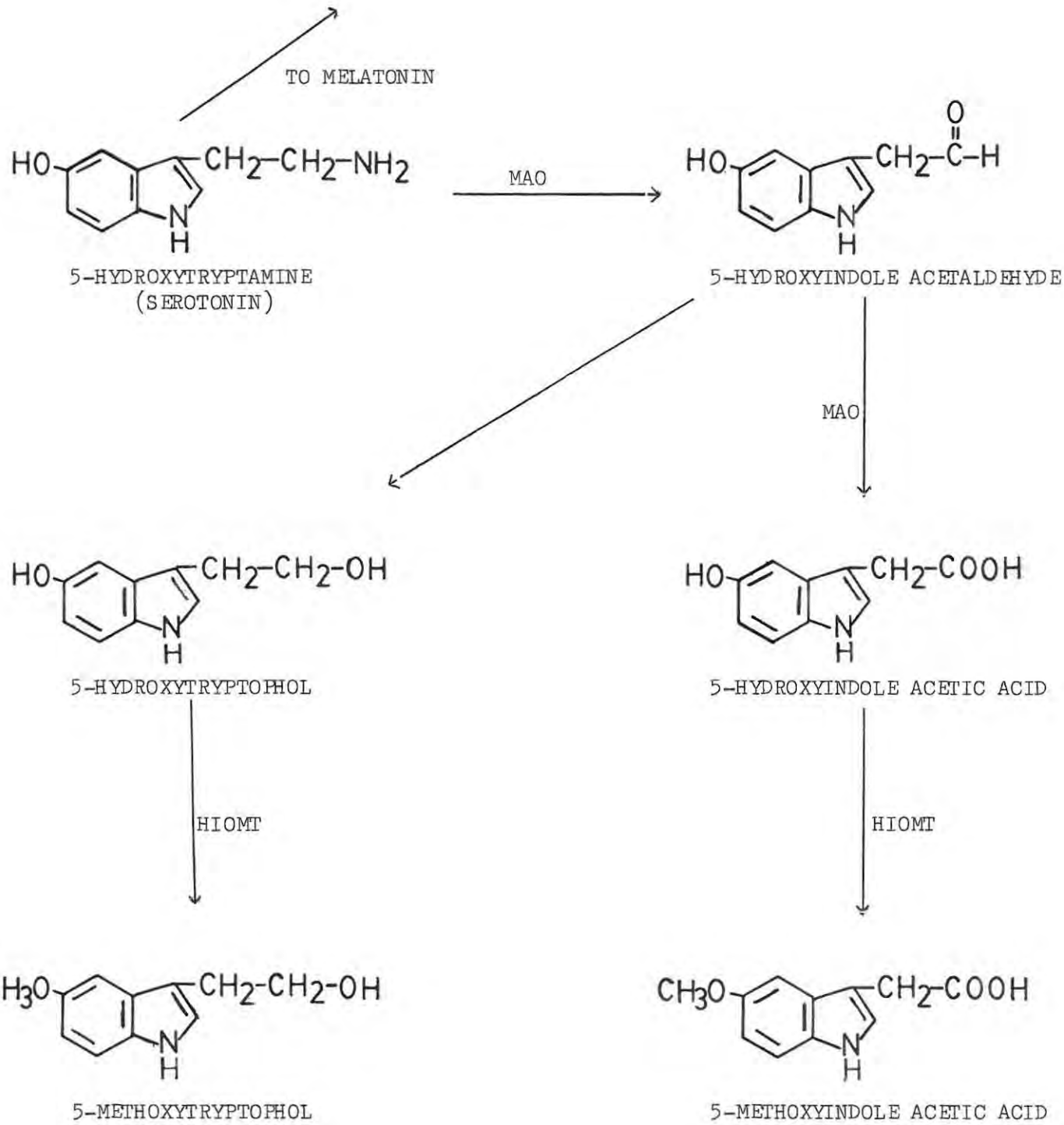


Fig. 8.

tryptophan, but to a lesser extent (Axelrod et al., 1969). The assumption that β-adrenergic receptor stimulation effects the synthesis of melatonin by acting on the cyclic adenosine-3',5'-monophosphate (cyclic AMP or cAMP) "second messenger" system was verified by adding dibutyryl cyclic AMP to a pineal organ culture. This compound causes an increase in melatonin production (Klein et al., 1970b; Wurtman et al., 1971). Stimulation of the β-receptor activates the enzyme adenyl cyclase (Breckenridge, 1964) which in turn, catalyses the conversion of adenosine-triphosphate (ATP) to cAMP (Robison et al., 1968a). Cyclic AMP activates the enzyme serotonin N-acetyl transferase (SNAT) which acetylates serotonin to form N-acetylserotonin (Deguchi and Axelrod, 1972b; Deguchi, 1972; Klein et al., 1970b).

1.2.3.2 Variation in Sensitivity of the β-Adrenergic Receptor: Supersenstivity and Subsensitivity

Deguchi and Axelrod (1973) found that the response of a pineal β-adrenergic receptor (or β-adrenoceptor) to nervous stimulation depends on the previous exposure of the receptor to the neurotransmitter noradrenaline. When noradrenaline, liberated from the nerve ending, is removed by denervation or reduced by reserpine administration, decentralisation, or continuous lighting (which reduces sympathetic nervous stimulation as will be discussed later), the response of cyclic AMP or serotonin N-acetyltransferase to stimulation of the β-receptor is greatly enhanced. Conversely, if the number of catecholamine molecules reacting with the β-adrenoceptor is increased for a period of time, the sensitivity of the receptor to its agonist is markedly reduced. (Larger amounts of catecholamine are then needed to elicit the same degree of increase in adenyl cyclase and SNAT activity compared with that exerted on β-receptors that have not been previously exposed to catecholamine.

The overall sensitivity of the β-adrenoceptor varies diurnally as the amount of noradrenaline released by the sympathetic nerves varies during the course of a day (Romero and Axelrod, 1974). Romero and Axelrod (1975) showed that although β-adrenoceptor supersensitivity increased the overall resultant activity of SNAT, there was a lag period prior to the induction of SNAT. Conversely, subsensitivity results in reduced SNAT activity, but the induction of the enzyme (SNAT) is far more rapid in onset. This phenomenon suggests the possibility that a precursor is necessary before final production of enzyme protein, and that β-receptor stimulation is necessary to produce this precursor.

Kebabian et al. (1975) showed that β-receptor stimulation causes a rapid decrease in the number of specific sites which will bind l-[^{3}H]-alprenolol, thus decreasing the sensitivity of adenyl cyclase to β-adrenergic stimulation. Romero et al. (1975a) found that the number of [^{3}H]-alprenolol binding sites varies with a circadian periodicity inversely related to the rhythm of neurotransmitter release. Inhibition of this β-receptor stimulation (by exposure to light for example) causes a rapid increase in the number of specific binding sites. This phenomenon can be interpreted as being related to an increase or decrease in the number of β-adrenergic receptors available when the pineal gland is subjected to nervous inhibition or stimulation respectively. This would explain the mechanism of supersensitivity and subsensitivity which occurs during the course of a day (fig. 9).

1.2.4 Cyclic AMP and Its Influence on SNAT

Cyclic adenosine-3',5-monophosphate is now well established as the intracellular mediator of the actions of a variety of substances that affect

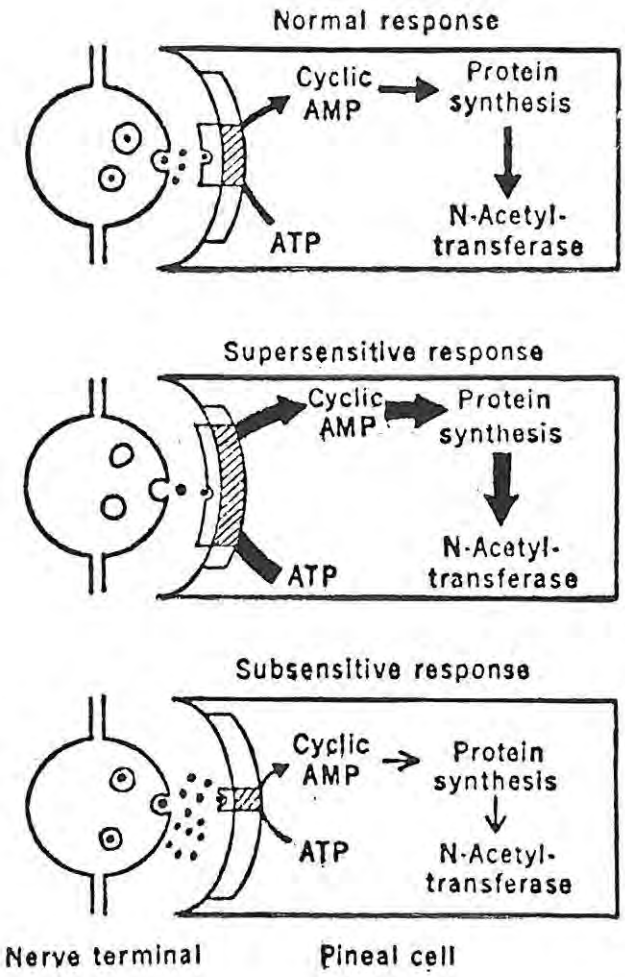


Fig. 9. Possible mechanism for the development of supersensitivity and subsensitivity which leads to an increase or decrease in cAMP and SNAT levels. (Axelrod, 1974).

cell function following the interaction of a neurotransmitter with extracellular receptors (Robison et al., 1968b). In the pineal gland, release of noradrenaline at the nerve endings stimulates adenyl cyclase activity (Weiss and Costa, 1967) which converts ATP to cAMP (Shein and Wurtman, 1969; Klein et al., 1970a; 1970b). Cyclic AMP, the "second messenger", (Robison et al., 1968a) increases SNAT activity (Deguchi, 1972) resulting in an increased formation of N-acetylserotonin and subsequent formation of melatonin (fig. 9). This can also take place in organ culture (Klein et al., 1970b) or in vivo (Deguchi and Axelrod, 1972a) and can be caused not only by catecholamines but by their precursor, L-Dopa as well. It seems that the control of melatonin synthesis is at the serotonin N-acetyltransferase level.

It has been shown that theophylline raises the intracellular concentration of cyclic AMP (Kakiuchi and Rall, 1968) by inhibiting the cyclic nucleotide phosphodiesterase (Butcher and Sutherland, 1962; Cheung, 1967), which converts cAMP to adenosine monophosphate (AMP). Any exogenous cAMP added to a pineal organ culture is degraded by the phosphodiesterase. To overcome such degradation, dibutyryl cyclic AMP ($N^6O^{2'}$-dibutyryl adenosine-3',5'-monophosphate), the diacylated derivative of cAMP, has been used instead as it is not a substrate for the enzyme. The addition of either noradrenaline (Axelrod et al., 1969) or dibutyryl cAMP (Shein and Wurtman, 1969; Klein et al., 1970a) to organ cultures, stimulates the conversion of radiolabelled tryptophan and serotonin to melatonin. These compounds do so by mimicking the action of cAMP and stimulating SNAT activity (Klein et al., 1970b), which, as stated before, converts serotonin to N-acetylserotonin (Weissbach and Axelrod, 1960; Berg and Klein, 1971). Accelerated enzymic formation of melatonin by simple

mass action (Klein and Rowe, 1970) and a net eflux of N-acetylserotonin and melatonin from the pineal cell results. When theophylline and dibutyryl cAMP are added to pineals that have already been stimulated by noradrenaline, no further stimulation of melatonin production is observed (Weiss and Costa, 1967; Kakiuchi and Rall, 1968) which suggests that these three compounds are acting at the same site, and that this is already maximally stimulated. Lynch et al. (1977) showed that pineal SNAT activity is not only controlled by sympathetic nervous innervation controlling cAMP levels, but also by an adrenal effect; catecholamines released from the adrenals stimulate SNAT activity.

Berg and Klein (1971) showed also that actinomycin D and cycloheximide block the dibutyryl cAMP stimulation of [^{3}H]-melatonin production from [^{3}H]-tryptophan, a result which indicates that synthesis of new protein is needed. This was further substantiated by Deguchi (1972). When he injected rats i.v. with isoprenaline, cAMP level increased immediately and fell to its initial level after 15 minutes. SNAT rose one hour after injection and returned to its initial level after 5 h. However, prior treatment with propranolol blocks an increase in SNAT activity. If propranolol is injected when SNAT activity is at a maximum, the level of activity drops rapidly showing that maintenance of high SNAT levels requires continuous stimulation of the β-adrenergic receptor of the pineal cell. When he gave cycloheximide before or after cAMP had dropped back to its normal level, there was no increase in SNAT activity. After SNAT activity had already increased, no rapid effect by cycloheximide was observed as new protein would by then already have been synthesized. Cyclic AMP seems to act on the translation process in the new enzyme molecules as actinomycin-D has no immediate effect on

the induction of SNAT (Deguchi and Axelrod, 1972a). The lag period before SNAT increases after exposure to light and the β-stimulation appears to be due to a lack of messenger RNA which first has to be synthesized during this lag period (Zatz et al., 1967). Cycloheximide actually causes a slow fall in high SNAT activity. These findings indicate that there are two types of changes in SNAT activity: a rapid change mediated by the β-receptor-second messenger system and a slow one which represents enzyme turnover and is dependent on enzyme protein synthesis (Deguchi and Axelrod, 1972b).

1.2.5 Other Enzymes in the Pineal Indole Biosynthetic Pathway

1.2.5.1 Tryptophan Hydroxylase

This is the enzyme which hydroxylates tryptophan in the 5-position. It has highest activity in the rat pineal gland (Lovenberg et al., 1967). It has generally been accepted that hydroxylation of tryptophan by this enzyme is the rate-limiting step in serotonin (5-hydroxytryptamine) biosynthesis.

1.2.5.2 Aromatic L-Amino Acid Decarboxylase (5-Hydroxytryptophan) Decarboxylase

This is a non-specific enzyme and is responsible for the conversion of 5-hydroxytryptophan to 5-hydroxytryptamine (serotonin). The pineal gland has the highest concentration of this enzyme of all mammalian tissues so far examined (Snyder and Axelrod, 1964b). The activity of pineal aromatic L-amino acid decarboxylase is under sympathetic neural and photic control (Snyder et al., 1964b; Snyder et al., 1965a). The enzyme can be assayed by a method described by Snyder and Axelrod (1964a).

1.2.5.3 Oxidative Enzymes, Non-Specific Esterases and Other Enzymes

Many enzymes occur in pineal parenchymal cells (Tapp et al., 1973), including glucose-6-phosphate dehydrogenase (Hess et al., 1958), succinate dehydrogenase (Nachlas et al., 1957a), lactate dehydrogenase (Hess et al., 1958), NAD diaphorase (Scarpelli et al., 1956), cytochrome oxidase (Burnstone, 1961), monoamine oxidase (Glenner et al., 1957), non-specific esterase (Gomori, 1950), leucine amino peptidase (Nachlas et al., 1957b), acid phosphatase (Grogg and Pearse, 1952), and alkaline phosphatase (Gomori, 1951). Most of these, with the exception of alkaline phosphatase, were found to be present in moderate to high levels. The presence of high levels of oxidative enzymes suggests a high overall degree of metabolic activity in the pineal gland.

1.2.5.4 Hydroxyindole-O-methyltransferase: the Terminal Enzyme in the Melatonin Biosynthetic Pathway

Hydroxyindole-O-methyltransferase (HIOMT) catalyses the conversion of N-acetylserotonin to melatonin (N-acetyl-5-methoxytryptamine) by transferring a methyl group from S-adenosyl-L-methionine to the hydroxy group in position 5 on N-acetylserotonin (Axelrod and Weissbach, 1960). It has been shown that HIOMT from different animals differs in electrophoretic mobility on a starch block (Axelrod and Vesell, 1970). The enzymes differ also in heat stability, Km values and substrate specificity. Jackson and Lovenberg (1971) established that the molecular mass of HIOMT is about 76000 to 78000 and that is also exists in higher molecular mass aggregates. It exists as two differently-charged subunits which can be separated. They also showed that in the pineal, HIOMT may be regarded as a major protein constituting about 4% of the soluble protein in the

gland. Studies by Cardinali and Wurtman (1972) showed that HIOMT is present in the rat retina and harderian gland. From their Km values and stability in storage, these authors conclude that pineal and retinal HIOMT is very similar but that harderian HIOMT is a different enzyme and that its physiological role may involve substrates other than indoles. Deguchi and Barchas (1971) showed that S-adenosylhomocysteine, to which S-adenosylmethionine is converted as a result of the transfer of a methyl group, is a potent inhibitor of the transmethylation process. This occurs because HIOMT exhibits a much greater affinity for S-adenosylhomocysteine than for S-adenosylmethionine. They also found that the supernatant fraction of brain homogenate contains an enzyme which stimulates transmethylation. Yang and Neff (1976) established that the diurnal variation in pineal HIOMT activity is due to an alteration of enzyme molecule numbers rather than a change in enzyme kinetics.

1.2.6 Serotonin

Serotonin, or 5-hydroxytryptamine, is present in the pineal gland and its sympathetic nerve endings in large quantities (Quay, 1963). The content of serotonin in the pineal follows a 24-hour cycle. Serotonin levels appear to be under the control of sympathetic nerves (Snyder et al., 1964b; 1965b). Serotonin is synthesized according to the indole biosynthetic pathway (fig. 7). Tryptophan hydroxylase appears to be the rate-limiting enzyme in the biosynthesis of serotonin (Lovenberg et al., 1967). Brownstein et al. (1973b) and Klein et al. (1973) demonstrated that serotonin levels in the pineal appear to be regulated by the β-adrenergic receptor stimulation which controls serotonin N-acetyltransferase activity. Serotonin in the pineal is metabolized to melatonin and 5-methoxyindole acetic acid.

1.2.7 Melatonin

Melatonin is an indoleamine synthesized primarily in the pineal gland. As far back as 1917, it was shown that this substance (although not yet identified as melatonin) could cause blanching of the skin of reptiles and amphibians (McCord and Allen, 1917). Today it is regarded as "the pineal hormone". Melatonin and 5-methoxyindole-3-acetic acid were isolated first from bovine pineal glands (Lerner et al., 1960). This finding demonstrated that hydroxyindoles could be O-methylated in the same way as catecholamines. Kopin et al. (1961) elucidated the metabolic pathway of melatonin in mice. They showed that labelled melatonin is rapidly metabolized after injection; the major pathway is by way of 6-hydroxylation, followed by conjugation, mainly with sulphate (70%) and to a small extent with glucuronic acid (6%). A small portion remains tissue bound. Circulating melatonin is rapidly taken up by all tissues, including the brain. Anton-Tay and Wurtman (1969) showed that tritiated melatonin is concentrated in the brain after both intravenous and intraventricular injection, although with the latter route, the brain concentration is almost a hundred times higher.

By using an organ culture technique, Axelrod et al., (1969) showed that noradrenaline liberated from the sympathetic nerves, stimulates melatonin formation.

1.2.8 Glycoproteins

Lott et al. (1972) on incubating [^{3}H]-fucose with rat pineal glands, found that it was taken up, synthesized into [^{3}H]-fucose-containing glycoproteins and released back into the medium. It has been suggested that these

glycoproteins could act as carrier proteins responsible for the transport of a hormonal substance like melatonin from the pineal parenchymal cells into the interstitial spaces prior to its release.

1.2.9 Melatonin and Enterochromaffin Cells

Raikhlin et al. (1975) found melatonin in enterochromaffin cells of the human gastro-intestinal tract. They later showed that active biosynthesis of melatonin was actually taking place in these cells (Raikhlin and Kvetnoy, 1976). They proposed that as the enterochromaffin cells and pinealocytes both produce melatonin, they could be grouped together as one functionally active neuroendocrine system. These two sources of melatonin, although anatomically distinct may well be fulfilling the same role.

1.3 PINEAL PHYSIOLOGY

1.3.1 Influence of Light on the Pineal

The pineal gland exhibits a number of biosynthetic rhythms of varying duration. No other endocrine organ exhibits as many rhythms as the pineal. Some of these rhythms have periods of 24 hours, other of 7 days and others as long as one year (Reiter, 1976). Control of metabolic function in the pineal gland is mediated via the sympathetic nervous system. An increase in sympathetic stimulation increases the production of melatonin. When the sympathetic nerves to the pineal gland are cut, light no longer has an effect on melatonin synthesis (Wurtman et al., 1964c). Brownstein and Heller (1968) reported that stimulation of the preganglionic fibres to the superior cervical ganglion decreases HIOMT activity. This is similar to the effect of light, suggesting that the photic inhibiting effect is mediated by autonomic innervation to

the pineal. In rats less than 12 days old (Zweig et al., 1966) there is an extra-retinal pathway for light which influences the pineal gland. It is assumed that while rats are still young, light can penetrate the skull and control physiological processes before visual function can be demonstrated.

Exposure to light reduces the ability of the pineal gland to synthesize melatonin (Wurtman et al., 1964c) and also decreases the mass of the gland (Fiske et al., 1960). Moore et al. (1967) showed that the central visual pathway essential for mediation of this response is the inferior accessory optic tract (fig. 10). It was also shown that the effect of light on melatonin synthesis was probably due to the marked change in HIOMT activity (Wurtman et al., 1963b) when rats are placed in continuous darkness for 6 days, there is a large increase in the level of this enzyme. Exposure to constant light has the opposite effect, HIOMT activity being greatly reduced. These findings suggest that there is a naturally occuring diurnal rhythm in HIOMT activity and that this fluctuation caused by light is probably due to a change in the rate of protein synthesis (Axelrod et al., 1965). Taylor and Wilson (1970) provided electrophysiological evidence for the action of light on the pineal by demonstrating that the spontaneous tonic level of pineal electrical activity is markedly reduced by photic stimulation. In an apparent contradiction of these findings, it was shown that light increases the concentration of cAMP in the pineal gland (Ebadi et al., 1970). It was suggested that this was due to an increased synthesis of the cyclic nucleotide or that the endocrine system may be involved. Klein and Weller (1972) on the other hand found that light acting via its established route, viz. retina, inferior accessory optic tract, median

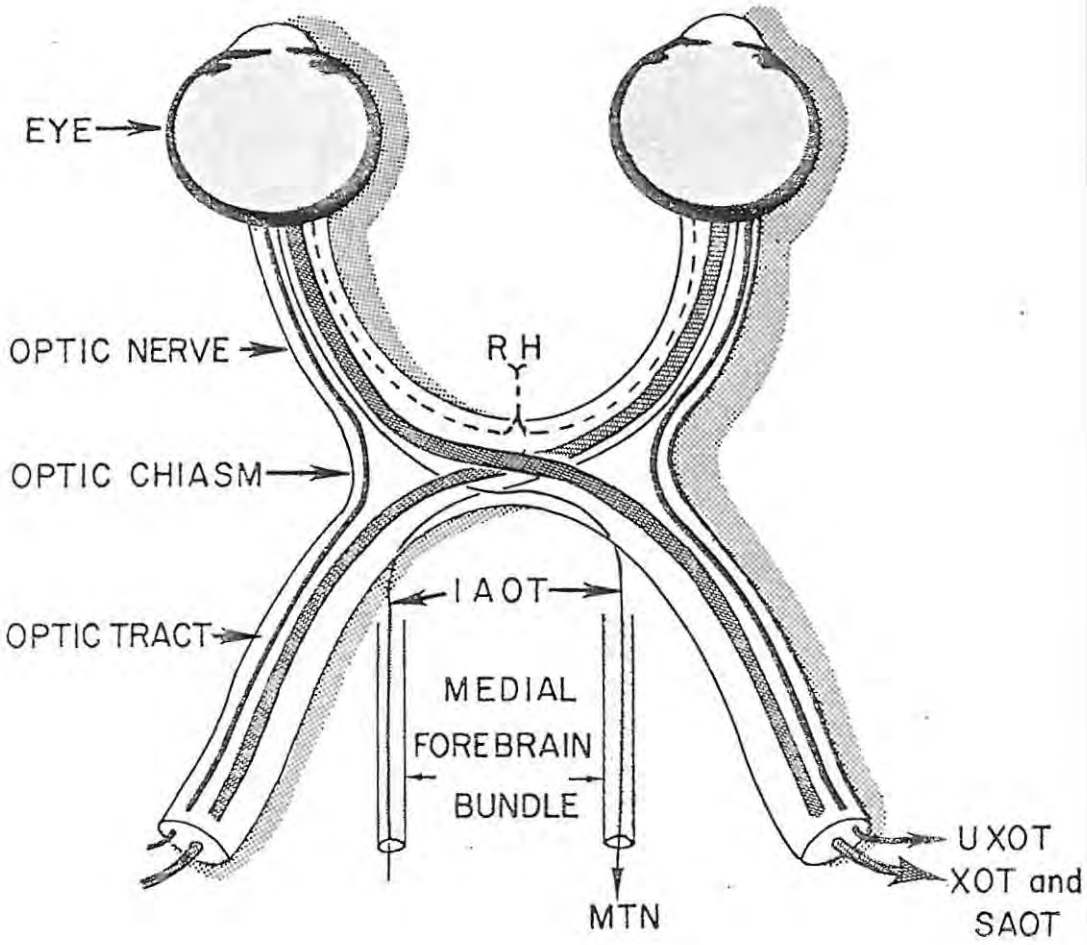


Fig. 10. Central visual projections in the rat.

XOT, crossed component of primary optic tract; UXOT, uncrossed component of primary optic tract; SAOT, superior accessory optic tract; RH, retino-hypothalamic fibres; IAOT, inferior accessory optic tract; MTN, medial terminal nucleus (Moore et al., 1968).

forebrain bundle, superior cervical ganglion and nervi conarii causes the dark-induced activity of serotonin N-acetyltransferase to decrease very rapidly (Heller and Moore, 1965; Axelrod and Snyder, 1966). This phenomenon could involve simple inhibition of adrenergic neuronal stimulation. Alternative hypotheses could include: (i) the non-adrenergic neural transmission of a visual signal (ii) the release of a second transmitter from nerve endings in response to a net uptake of noradrenaline, which would occur at the termination of the dark-induced noradrenaline release, (iii) electrochemical changes in the pinealocyte processes which could be associated with termination of neural stimulation. The overall effect of light on the rat pineal therefore is one of metabolic inhibition. The circadian fluctuation in this inhibitory input is largely responsible for the presence of various biorhythms in the pineal gland.

1.3.1.1 Rhythmicity in Pineal Noradrenaline Turnover

Brownstein and Axelrod (1974) demonstrated the existence of a 24-hour rhythm in the turnover of noradrenaline in the sympathetic nerves innervating the rat pineal gland. By measuring the rate of disappearance of [^{3}H]-noradrenaline from the pineal over the course of a day, they found that the turnover of this catecholamine is more than twice as rapid during the night than during the day. They established also that the rhythm is endogenous as it persists in blinded animals. In normal animals, exposure to light during the night causes a marked reduction in the rate of noradrenaline turnover. The authors conclude that the 24-hour rhythm in noradrenaline turnover probably reflects diurnal variations in the release of the neurotransmitter. By extension, it was also concluded that the daily variation in pineal β-adrenergic receptor stimulation is probably responsible for the circadian rhythms in the metabolism of

pineal indoleamines (serotonin, N-acetylserotonin etc.). The fluctuation in noradrenaline is most certainly responsible for a daily rhythm in adenyl cyclase activity, cyclic AMP concentration and, consequently, serotonin N-acetyltransferase (SNAT) activity. Klein et al. (1971) found that superior cervical sympathectomy abolished the rhythm in SNAT activity, strongly suggesting that sympathetic innervation and noradrenaline release control SNAT levels. It could also be assumed that there is no sympathetic or photic control over tryptophan hydroxylase, as it exhibits no circadian rhythm (Deguchi and Barchas, 1972).

1.3.1.2 Serotonin and Associated Enzyme Rhythms

Pineal serotonin content has been shown to exhibit a circadian rhythm (Quay, 1963) under sympathetic nervous control (Fiske, 1964). This rhythm ranges from about 10 ng/pineal at the nocturnal minimum to over 90 ng/pineal at the midday maximum. There is a rapid decrease in serotonin content at the onset of darkness, the level falling at a rate of about 25 ng/hour. From midnight to midday, there is a gradual increase of about 6 ng/hour.

Snyder et al. (1965b) demonstrated that constant light abolishes circadian changes in pineal serotonin content whereas the rhythm persists in rats kept in constant darkness. Disruption of the sympathetic nerve supply abolishes the rhythm, but blinding does not. These facts suggest that the pineal circadian serotonin rhythm is endogenous, but is cued or regulated via the sympathetic nerve supply by an external synchronizer. (This could be feeding or any other sympathetically controlled event.) The serotonin rhythm appears to depend on alterations in the rate at which serotonin is released from intracellular binding sites (Snyder and

Axelrod, 1965). During the day, the amine is bound (within parenchymal cells or sympathetic nerve endings) in such a manner as to make it unavailable for conversion to melatonin or destruction by monoamine oxidase. The onset of darkness seems to trigger the release of bound serotonin.

Serotonin rhythmicity can be ascribed to two main factors, both influenced by light: (1) Snyder et al. (1964a) showed that exposure of rats to constant light causes a marked increase in aromatic-L-amino acid decarboxylase activity, the serotonin-forming enzyme (Snyder and Axelrod, 1964a) when compared with its levels in rats kept in constant darkness. This enzyme thus exhibits a circadian rhythm which is under photic influence, peaking in the day and declining at night in parallel with the serotonin rhythm. Snyder et al. (1965a) showed also that aromatic-L-amino acid decarboxylase activity is controlled by the sympathetic nervous system. When rats are subjected to superior cervical ganglionectomy, the light-induced rise in enzyme activity is prevented. (2) Brownstein et al. (1973a) found that the enzyme SNAT shows a diurnal rhythm as well, which is 180° out of phase with the rhythms of serotonin and aromatic-L-amino acid decarboxylase. SNAT activity is more than 15 times higher at night than during the day. This circadian rhythm persists in complete darkness or in blinded animals and is suppressed in constant lighting (Klein and Weller, 1970a).

The circadian rhythm in SNAT activity appears 4 days after birth in rats and develops most rapidly over a 7-day period one week after birth. A concomitant development in sympathetic nervous innervation and in the adenyl cyclase system probably accounts in part for the development of this rhythm (Ellison et al., 1972).

When rats are exposed to light during the dark phase, SNAT activity, which peaks at night, declines rapidly. This is probably due to the inhibitory effect of light on sympathetic nervous stimulation which is responsible for inducing SNAT activity in the first place. The degree of pineal SNAT suppression by light depends on its intensity. As little as 0,5 mcW/cm^2 causes more than 50% suppression of the enzyme and maximum inhibition has been observed with .15 mcW/cm^2 light intensity (Minneman et al., 1974). It is the rhythmic fluctuations of the serotonin-forming enzyme, aromatic-L-amino acid decarboxylase, and of the enzyme which converts serotonin to N-acetylserotonin, i.e. SNAT, which are responsible for the circadian rhythm shown by serotonin in the pineal gland.

As a consequence of the SNAT rhythm, the product of N-acetylation, i.e. N-acetylserotonin, shows a diurnal rhythm in phase with that of SNAT. It exhibits a nocturnal peak and a diurnal trough. If rats are exposed to light during the night, the level of N-acetylserotonin falls rapidly due to the light-induced decline of SNAT (Brownstein et al., 1973a).

1.3.1.3 Hydroxyindole-O-methyltransferase Rhythm

HIOMT activity undergoes a 24-hour cyclic fluctuation peaking at night and dropping during the day (Axelrod et al., 1965). This rhythm is also controlled by light (Wurtman et al., 1963b) which suppresses the activity of the enzyme. Cardinali et al. (1972) investigated the relationship between various light source spectra and HIOMT activity. When rats are exposed to green, blue or yellow light after 7 days of darkness, time-related decreases in pineal HIOMT activity are observed. Green light has the most pronounced effect in decreasing enzyme activity. Red light has no effect. Cool white fluorescent tubes have been shown

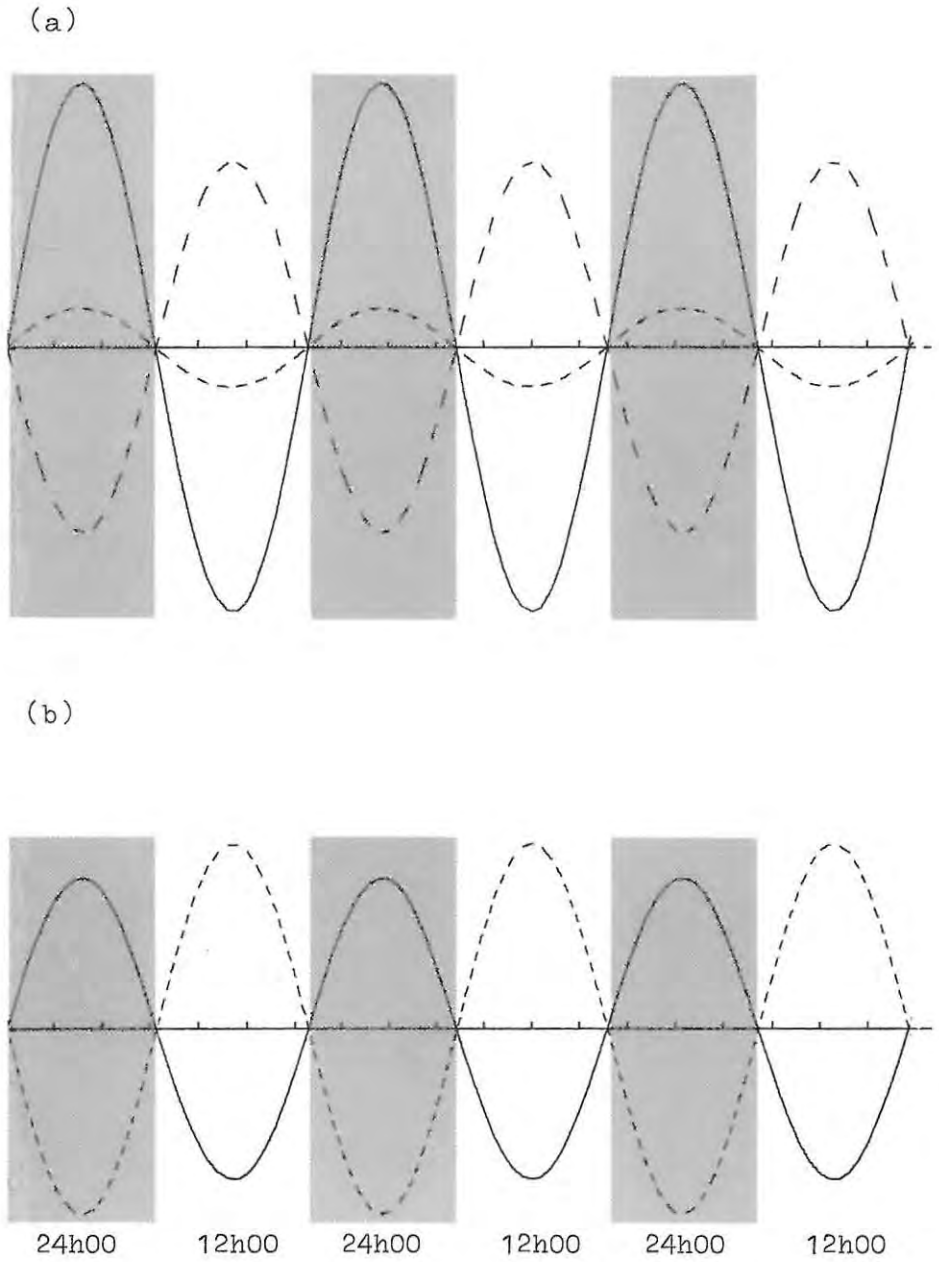


Fig. 11a & 11b. Schematic representation of pineal circadian rhythms showing phase relationships.

(a) ——— SNAT
— — — aromatic L-amino acid decarboxylase
------- HIOMT

(b) ——— melatonin
-------- serotonin

to be more effective than "vita-lite" sunlight-simulating sources in decreasing HIOMT activity. (Fig. 11a).

Wallen and Yochim (1974a) found that the level of enzyme activity in female rats also depends on the stage of the rat oestrous cycle. The enzyme activity is highest during metoestrous and dioestrous and lowest during oestrous. The irregular fluctuation of HIOMT was ascribed to the existence of two component rhythms of different frequencies. The interaction of these rhythms creates a "beat frequency" oscillation.

If rats are placed in continuous dark, HIOMT activity rises and remains at a high level (Wurtman and Axelrod, 1965), but an "oestrous cycle" pattern of HIOMT activity still remains (Yochim and Wallen, 1974a). Continuous light completely abolishes HIOMT rhythm. Although HIOMT rhythm appears to be externally regulated and almost entirely dependent on shifts in environmental lighting (therefore suggesting that this enzyme rhythm may be more important in carrying information about light than other circadian rhythms that do not depend on light for their existence), the presence of a rhythm in the dark varying with oestrous cycle demonstrates an endogenous aspect of the rhythm. Three important factors have thus emerged from the studies (Yochim and Wallen, 1974a) of constant conditions on HIOMT rhythms: (1) an endogenous pattern of HIOMT activity exists in constant darkness with smaller amplitudes and a decreased rate of activity; (2) both theoretical rhythm components of the HIOMT pattern are endogenous ; (3) constant light gradually abolishes rhythmicity and causes the activity of HIOMT to decrease with time. In terms of Wallen and Yochim's mixed-wave hypothesis (1974a), all effects can be accounted for simply by changes in the numerical values

of the non-periodic terms of the mixed-wave equation. It can therefore be assumed that HIOMT activity varies seasonally and, as a result, can modify basal release of gonadotrophins. This is due to the anti-gonadotrophic action of melatonin which will be discussed.

1.3.1.4 Melatonin Rhythm

As HIOMT, which has a circadian rhythm, is responsible for converting N-acetylserotonin to melatonin, it can be assumed that melatonin undergoes a diurnal fluctuation as well, which is in phase with the HIOMT rhythm. This happens to be the case. It undergoes a 24-hour rhythm, attaining a maximum peak at night and dropping to a low level during the day. Lynch et al.(1975) collected melatonin from human volunteers and concentrated it on an Amberlite XAD-2 resin column. By using a bioassay technique, they showed that the amount of melatonin from urine samples collected between 23h00 and 07h00 was several times higher than that of samples collected between 07h00 and 15h00 or 15h00 and 23h00. This not only confirms that melatonin is released in a rhythmic fashion, but also that at least some melatonin in human beings finds its way chemically unchanged into the urine. Hedlund et al. (1977), using a radioimmunoassay technique, showed that the level of melatonin in the cerebrospinal fluid of calves increases 17-fold at night in comparison with daytime levels. Plasma concentrations increased approximately 6-fold. (Fig. 11b).

1.3.2 Other Effects of Light

1.3.2.1 Pineal Gonadal Function

Besides the direct effects of light on the pineal gland (in inhibiting

HIOMT activity for example) studies have shown secondary manifestations of light effects. Light may affect other organs indirectly owing to an effect on the pineal gland, or due to a direct effect. In the latter case, it is possible that some retinal target-organ neuronal pathway exists which allows light to have some modulating influence on that particular organ, or light may impinge directly on some area of the brain. Lisk and Kannwischer (1964) for example found that light apparently has a direct effect on the suprachiasmatic nuclei of rats. They allowed light to impinge on this area by way of glass rods through the skull and thus demonstrated a significant increase in the incidence of vaginal oestrous. Fiske (1939) also has found that rat pituitary gonadotrophin levels vary according to a lighting schedule. This variation is most likely due to a pineal-mediated effect, but it is conceivable that the changes may be directly mediated. Wurtman et al. (1964b) found that [^{3}H]-melatonin was highly concentrated by the pineal, iris-choroid and ovary as well as other endocrine organs and peripheral and sympathetic nerves. Constant exposure to light was shown to diminish the uptake of melatonin by the ovary. This may be another way in which light affects the oestrous cycle. Constant light enhances the incidence of vaginal oestrous in mature rats, whereas darkness has the opposite effect (Wurtman et al., 1964a). Interruption of the photic pathway by blinding or by superior cervical ganglionectomy blocks these light effects and prevents light-induced hypertrophy of the ovaries. Hoffmann (1967) found that rats deprived of light by blinding or by being kept in constant darkness at the age of 21 days continued their oestrous cycles regularly for 8 months, while animals deprived of light at the age of about 90 days began to show prolonged cycles and in some cases ceased after 3 months. It appears that if cycles begin in the absence of light, they can continue fairly

normally, but if they commence in the presence of light, their continuance is dependent on light, and deprivation of light in this case has profound effects on most rats within 3 months. Light therefore appears to be necessary for the normal functioning of the pituitary-ovarian axis.

In another report Hoffmann (1973) discusses the possible effects of early lighting on oestrous cycles in rats. Most rats which had been born and raised in constant light, if then blinded or exposed to light/dark of 12h/12h, had predominantly 4-day oestrous cycle lengths. If the light/dark ratio was changed to 16h/8h, these rats tended to show a 5-day cycle. Rats born and raised under a diurnal light/dark regimen of 12h/12h also showed 4-day cycles after blinding, but if kept in a 12h/12h or 16h/8h photoperiod, they showed mainly 5-day cycles. It seems that both environmental and genetic factors play a part in responses which the female rat reproductive system manifests to light in adulthood. Early constant lighting also seems to render a female rat less sensitive to dark. However, genetic differences among rats seem to determine inherent length of cycle and to affect reproductive responses to light deprivation.

Lawton and Schwartz (1967) studied the effects of constant light on gonadal-adenohypophyseal-hypothalamic function in rats. They found constant light led to a desynchronization of 24-hour rhythms. A state of persistent oestrous eventually developed which was characterised by a failure to release a surge of LH necessary for ovulation. A steady state of ovarian steroid release also developed. It was possible to divide persistent oestrous into two stages, which depended on the number of days the rats had been exposed to constant light before persistent

oestrous developed. The first group, which had been in constant light for 60 days or less, showed prolonged vaginal cornification which was interrupted occasionally by ovulation. Pituitary LH level was high. The second group, which had been in constant light for more than 60 days, had prolonged vaginal cornification, no breakthrough ovulation and low pituitary LH content. It seems likely that reproductive cyclicity depends on the synchronization of various rhythms within the hypothalamo-hypophyseal-ovarian axis, which in turn is dependent on an alternating light-dark rhythm. It has been noted however (Brown-Grant et al., 1973), that ovulation in rats exposed to constant light may be induced by mating, penile intromission being responsible for its occurrence.

Wallen and Yochim (1974c) exposed female rats to a 20-hour day in an attempt to modify their oestrous cycles predictably. Their results indicate that the function of the pineal gland appears to be related primarily to regulating the duration of the degree of activity of the hypothalamic-pituitary-ovarian axis, which is functionally essential to the oestrous cycle. In another report, they proposed that HIOMT activity, and in turn vaginal oestrous cyclicity, are governed by the interaction of two rhythms, one of which although endogenous, is cued by photoperiod (Yochim and Wallen, 1974c). They found that under phase conditions in which maximal amplitudes of HIOMT activity were generated, the incidence of vaginal cornification and persistent oestrous, which are indicators of oestrogen secretion, was high and vice versa. These results offer strong support for the concept that a photoperiodic rhythm affects both pineal HIOMT and aspects of reproductive function identically. (Fig. 12).

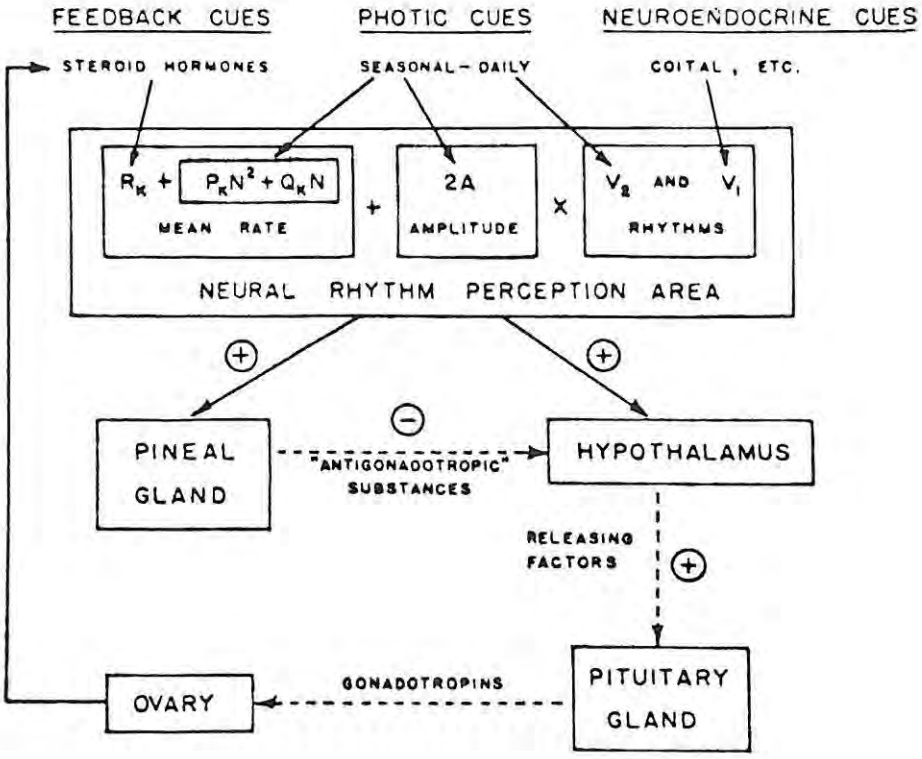


Fig. 12. Relationship between pineal function and reproductive neuroendocrinology in the rat. Solid and dashed arrows connect a framework of the basic neuroendocrine unit involved in reproductive function (Yochim and Wallen, 1974c).

1.3.2.2 Drinking

Continuous illumination suppresses water intake of male and female rats (Zucker, 1971). Bilateral transection of the inferior accessory optic tracts completely abolishes this effect, suggesting that these tracts form part of the principal visual pathway by which excessive light exposure depresses drinking (Stephan and Zucker, 1972). Constant light exposure was found also to cause hypertrophy in neurosecretory cells of the supra-optic nucleus, indicating increased antidiuretic hormone (ADH) synthesis and release. This response appears to be a direct effect of light rather than being a consequence of reduced water intake. The effect of light on drinking seems to be independent of pineal and gonadal changes as the suppression of water intake was seen to exist in both pinealectomised males and ovariectomised females. It is possible that direct retino-hypothalamic innervation of neurosecretory cells which are involved in this suppressive effect of light, may exist.

1.3.2.3 Core Temperature

Spencer et al. (1976) established the existence of a 24-hour bimodal rhythm in female rat core temperature. By shifting the phase of the photoperiod, or altering the proportion of light per day, they showed that the rhythm is entrained by light but that the two component peaks are controlled by different aspects of the lighting regimen. The primary rhythm seems to be endogenous, entrained only by a circadian photoperiod, whereas the secondary rhythm is exogenous, requiring a circadian light-dark rhythm. The relationship between mean core temperature and the proportion of daily light was drastically altered by pinealectomy, but no effect on rhythmicity was observed. This

suggests that the pineal regulates a light-dark dependent set point about which oscillations in core temperature may be generated.

1.3.2.4 Growth

Relkin (1972) reported that constant darkness reduced the production and release of growth hormone (GH) by the pituitary with consequent growth retardation. This effect is probably exerted by increased pineal secretion which inhibits growth hormone releasing factor (GHRF) release from the hypothalamus.

1.4 INFLUENCE OF MELATONIN AND OTHER PINEAL ANTIGONADOTROPHINS ON ENDOCRINE ORGANS

1.4.1 The Hypothalamic-Hypophyseal Axis

1.4.1.1 LH and FSH

The antigonadotrophic action of the pineal gland is now an established fact. However, because pinealectomy does not necessarily have a pronounced effect on gonadotrophic function in most species, it may be assumed that the role of the pineal here is as a modulating influence on overall reproductive function.

The first conclusive evidence of pineal inhibition of gonadotrophic function was obtained by pinealectomising seasonal breeders such as the hamster (Hoffmann and Reiter, 1965). This operation resulted in marked alteration in gonadal mass. Wurtman et al. (1963a) have demonstrated that melatonin administered to female rats causes a significant reduction in the number of positive oestrous vaginal smears. They gave immature

female rats intraperitoneal (i.p.) injections of melatonin, which also resulted in delayed vaginal opening and a large reduction in ovarian mass. In addition, they found that tritiated melatonin was taken up largely by the ovary and pineal gland, suggesting a direct effect on the ovary. Constant lighting, which normally has an inhibitory effect on pineal function, was found to reduce this effect.

McIsaac et al. (1964) showed however that 5-methoxytryptophol, another methoxyindole, had an even more pronounced effect in reducing the frequency of positive oestrous smears. In addition, ovarian masses in the 5-methoxytryptophol-treated rats were significantly less than control or melatonin-treated groups. Although its physiological effects seem similar to those of melatonin, in several instances it has been shown to be significantly more potent than melatonin in inhibiting the growth of the sexual organs. It is capable of blocking the rapid rise in plasma FSH after castration in adult male rats (Talbot and Reiter, 1973-1974). It has been concluded that this indole specifically inhibits FSH (Fraschini and Martini, 1970).

Chu et al. (1964) confirmed the inhibitory effect of melatonin on the oestrous phase of the rat oestrous cycle and showed that this effect was not exhibited by the precursors of melatonin, i.e. serotonin or N-acetylserotonin, or the major metabolite of melatonin, 6-hydroxy-melatonin. Soffer et al. (1965) found that defatted bovine pineal extracts exhibited a gonadotrophin-inhibiting activity when administered to prepuberal HCG-treated female mice. On the other hand Schnitman and Debeljuk (1971), using an aqueous defatted extract of human pineal glands, were unable to detect any inhibition of the HCG-induced increase of

uterine mass of young Wistar rats. This failure could well have been because accepted antigonadotrophins such as melatonin are fat soluble and therefore would not have been present in this extract. It is also possible that degradation of pineal principles took place post mortem.

Hoffman and Reiter (1965) have shown that pinealectomy stimulates gonadotrophin secretion. The question arose whether these anti-gonadotrophic effects occurred as a result of direct pineal action on the reproductive organs, or whether the hypothalamo-hypophyseal complex was implicated. Fraschini et al. (1968a; 1968b) found that when various pineal indole compounds, e.g. 5-hydroxytryptophol, melatonin and 5-methoxytryptophol were implanted in the median eminence or midbrain of the rat, pituitary stores of the gonadotrophin, LH, were significantly reduced. This result not only suggests that the pineal gland may exert its antigonadotrophic influence via the pituitary gland, but also that the brain has indole-sensitive receptors which may be implicated in the synthesis and release of LH from the pituitary gland. By what route the indoles reach these areas of the brain remains still to be conclusively established. Fraschini and Martini (1970) also showed that by removing the pineal gland from young adult male rats, a conspicuous and highly significant enhancement of pituitary LH content resulted. Adams et al. (1965) have shown however that melatonin administration increased pituitary LH content, which could mean that LH release was inhibited.

Hipkin (1970) reaffirmed the antigonadotrophic potential of the pineal gland after injecting pineal extracts into mice. These extracts blocked the human chorionic gonadotrophin (HCG) and pregnant mares serum gonadotrophin (PMSG) - induced increases in uterine mass. He pointed

out that this effect could, however, also have been due to toxicity of the injected material. Cheesman and Forsham (1974) have suggested that the effects in this case may be due to a direct breakdown of HCG or PMSG by pineal indoles. Systemically administered melatonin has been shown to affect the adrenals, ovaries, uterus and pituitary. However, de Prospo and Hurley (1971) were unable to detect any effects on these organs when melatonin was administered intraventricularly.

Kamberi et al. (1970), using an anterior pituitary perfusion technique, found that LH release by indoleamines such as melatonin and serotonin was not caused by a direct action on the anterior pituitary. They infused melatonin (2 μg/min for 30 min) into the stalk portal vessel, which drains into the sinusoids of the anterior pituitary. No direct effect on peripheral serum LH concentration was noticed. However, melatonin injected intraventricularly caused a marked decrease in serum LH concentration. This seems to indicate that melatonin acts indirectly via the hypothalamic-hypophyseal complex.

Several investigators have reported that melatonin has an inhibitory effect on reproduction. Collu et al. (1971), by implanting a cannula into a lateral ventricle, showed that melatonin could indeed inhibit ovulation in the rat when administered by this route. Subcutaneous administration of melatonin had no effect. They proposed that this finding was possibly due to inhibition of LH secretion. It has also been suggested that as melatonin has a central hypnotic effect, it may block ovulation in a manner similar to that of the barbiturates. Other possibilities are inhibition of ACTH release or an increase in brain serotonin levels.

Moszkowska et al. (1973) showed that serotonin inhibits the synthesis and release of hypothalamic releasing factors i.e. FSH-releasing factor (FSH-RF) in vitro. This effect was noted after in vivo administration of serotonin into the third ventricle as well. This finding reinforces the suggested mode of action proposed by Kamberi et al. (1970). It has been found that chronic administration of serotonin can cause ovarian atrophy (Kamberi et al., 1971). This is probably due to inhibition of the release of FSH-RF and LH-releasing factor (LH-RF). Melatonin however, seems only to modify the synthesis of FSH-RF in synergy with serotonin or by an increase in cerebral serotonin levels.

Other evidence implicating the pineal gland in the control of FSH was suggested by Debeljuk et al. (1970) who found that daily injections of 300 µg of melatonin from day 21 to day 51 of age caused a substantial decrease in the pituitary FSH content of male rats. Testicular retardation was also observed. Fraschini and Martini (1970) have maintained that 5-methoxytryptophol and serotonin are responsible for the antagonistic action of the pineal on accumulation of FSH within the pituitary. When the pineal is removed, there is a three-fold rise in pituitary FSH content. They regard these two indoles as being exclusive to FSH control and melatonin and 5-hydroxytroptophol as being the indoles concerned with the regulation of LH (Fraschini, 1969). Kamberi et al. (1970) however demonstrated that melatonin seems to control FSH secretion by an effect on a hypothalamic releasing factor.

Martin and Klein (1976), demonstrated that physiologic concentrations of melatonin could directly inhibit the LH-RF-induced release of LH from the cultured pituitary gland of the rat. Other related indoleamines,

i.e. N-acetylserotonin and 5-methoxytryptamine, had no effect. Although Kamberi et al. (1970) were unable to demonstrate a direct effect by melatonin on the pituitary, it was concluded that melatonin acts both directly on the pituitary and indirectly on the hypothalamus to suppress the release of LH into the systemic circulation.

Using pituitary glands in an organ culture, Martin et al. (1977) blocked LH-RF-induced release of LH with melatonin. They also found that serotonin and 5-methoxytryptamine were able to suppress LH release from the pituitary. It seems possible that melatonin, serotonin and 5-methoxytryptamine react with a specific receptor on the pituitary to elicit their effect. The inability of Kamberi et al. (1970) to demonstrate a direct inhibitory effect on the pituitary may be ascribed to their use of adult rats while Martin et al. used neonatal ones. Another important factor which may account for this difference in experimental results is that Kamberi measured basal LH levels, whereas Martin et al., measured LH-RF-induced levels.

Moszkowska et al. (1971), using chromatographic separation techniques, established that two different types of pineal factors seemed to account for the antigonadotrophic activity of the pineal gland. A purified fraction (F_3) with a molecular mass of about 700 was assumed to act directly on the pituitary to antagonize the release of gonadotrophic hormones particularly FSH, in vitro. This fraction was considered to be free of melatonin and 5-methoxytryptophol. The other fraction of indoleaminergic material (F_2), acts at hypothalamic level to regulate LH secretion. The exact role of the pineal gland in controlling gonadotrophin secretion was further clouded by results indicating

that subcutaneous implants of melatonin reversed the antigonadotrophic effects of smell and sight deprivation (which cause a decrease in pituitary LH and prolactin)(Reiter et al., 1975). In other words, it exhibited a counter-antigonadotrophic effect. The effect of melatonin here may be due to an inhibitory effect on the synthesis or release of primary pineal antigonadotrophins. It is also possible that melatonin renders the neuroendocrine axis insensitive to inhibition by the pineal hormones, directly stimulates the peripheral end organs, e.g. gonads, or increases the sensitivity of the end organs to circulating gonadotrophins. Melatonin may even stimulate the release of a progonadotrophic substance from the pineal gland or another organ. It is also possible that the effects of melatonin may depend on the time of day when it is administered. In this connection Fiske and Huppert (1968) reported that a melatonin injection during hour 8 of the light period suppressed the normal rhythm in pineal serotonin, but when given after onset of darkness, no such effect was seen.

In pursuance of the possibility that the function of the pineal gland may not necessarily be an antigonadotrophic one, Kao and Weisz (1977) incubated medial-basal hypothalami of rats and showed that melatonin could release a gonadotrophin-releasing hormone (Gn-RH). Other putative neurotransmitters had no effect. These facts seem to conflict with the view that melatonin has an inhibitory effect on gonadotrophin release, be it through a direct or indirect mechanism.

The findings of White et al. (1974) reinforced this current line of pineal progonadotrophic research. They found unusually large quantities of Gn-RH, which is normally liberated from the hypothalamus, and

thyrotrophin-releasing hormone (TRH) present in the pineal gland of the rat. In fact, the pineal glands contained 4 to 10 times as much Gn-RH as the hypothalami of the same species. Furthermore, these pineal extracts were shown to be capable of inducing ovulation. TRH content was found to be of the same order as that of the hypothalami of the same species. It seems as though the pineal gland may be a supplementary source of these two releasing factors. They may exert a maintenance or tonic effect on the pituitary, or alternatively act on extra-pituitary areas in the brain.

1.4.1.2 Growth Hormone (GH)

Smythe and Lazarus (1973b) proposed that melatonin and serotonin play an important role in the regulation of growth hormone (GH) release from the pituitary gland. Previous researchers had shown that pineal inhibition, by a tumour for example, encouraged growth (Kitay and Altschule, 1954) whereas pineal stimulation caused by darkness led to the opposite effect (Wurtman et al., 1968; Sorrentino et al., 1972). It seems therefore that conditions favouring high melatonin production result in retarded growth. It had already been shown that serotonin enhances GH release (Collu et al., 1972), and Smythe and Lazarus (1973b) demonstrated that both melatonin and serotonin have antagonistic effects on GH release. They did so by administering melatonin and the serotonin precursor, 5-hydroxytryptophan to rats and assaying blood samples for GH content. Melatonin greatly reduced the GH levels, whereas 5-hydroxy-tryptophan increased it, suggesting that melatonin can block the GH response to serotonin. It seems that, in this case, melatonin can act as a competitive blocker of serotonin receptors at the hypothalamic level.

On further investigation, Smythe and Lazarus (1974) found that, contrary to expectations, oral administration of melatonin to human male subjects elevated serum GH. They postulated that this phenomenon could be due to an interaction with hypothalamic receptor sites mediating the release of GH from the pituitary. The fact that other work by these two investigators has shown an inhibitory effect by melatonin on GH release could be a dose related effect due to melatonin inhibition of GH release at a low dose and stimulation at a higher dose (Smythe and Lazarus, 1973a; 1973b).

1.4.1.3 Prolactin

Not many studies have been conducted on pineal-prolactin interrelationships. It has been shown however that blind rats are deficient in milk and prolactin production, phenomena which could be due to an activated pineal gland (Reiter, 1972). Nir et al. (1968) found that pinealectomy resulted in a 13% decline in milk yield during the second half of the nursing period. Kamberi et al. (1971) reported a correlation between pineal activity and prolactin release. After injecting melatonin, either into the cerebral ventricles or a cannulated portal vessel, prolactin levels were measured. Following intraventricular administration of melatonin, it was found that the indoleamine suppressed the discharge of prolactin inhibiting factor, thereby increasing prolactin levels in the blood. Lu and Meites (1973) found that i.v. administration of 5-hydroxytryptophan significantly increased serum prolactin levels in rats, whereas serotonin given intravenously had no significant effect. Melatonin also increased serum prolactin significantly. Serotonin has been shown not to stimulate pituitary release of prolactin (Talwalker et al., 1963), but it is possible that 5-hydroxytryptophan or melatonin may do so.

It is also possible that 5-hydroxytryptophan and melatonin may stimulate the release of "prolactin releasing factor" from the hypothalamus into the systemic circulation.

Although the pineal gland is obviously of importance in prolactin release and milk production, more research is needed to elucidate its exact involvement.

1.4.2 The Adrenal Glands

Since Farrell (1959) described a pineal substance, glomerulotrophin, which specifically stimulates aldosterone production by the adrenals, studies have been conducted in an attempt to clarify the possible relationship between the pineal gland and adrenals. Farrell also postulated the existence of a second pineal factor, possibly melatonin, capable of inhibiting adrenal steroidogenesis (Farrell, 1960). Ogle and Kitay (1977) injected melatonin (50 μg i.p.) daily into ovariectomised and hypophysectomised rats and found that it stimulates adrenal 5 α-reductase activity. It was therefore suggested that melatonin could be a potential contributor to the physiologic regulation of adrenal steroidogenesis. Farrell (1964) later indicated that the supposed glomerulotrophin was present in insignificant amounts. Wurtman et al. (1959) found that pinealectomy increased adrenal mass in female rats, whereas a protein-free pineal extract retarded adrenal growth. Lommer (1966) reported that a pineal extract inhibits adrenal β-hydroxylase activity, which blocks the synthesis of aldosterone, cortisol and cortisone. Jouan and Samperez (1964) postulated that serotonin, a hormone widely present in the central nervous system, and especially in pineal extracts, is responsible for aldosterone-releasing activity.

Gromova et al. (1967) demonstrated that melatonin inhibits aldosterone production in vitro. Hypophysectomy abolishes the inhibitory effect of melatonin on aldosterone production. Narang et al. (1967) in agreement with Gromova, found that melatonin increases adrenal mass in female rats.

There are insufficient data at present to define a specific role of the pineal gland in modifying overall adrenal gland function, glucocorticoid and mineralocorticoid activity.

1.4.3 The Thyroid Gland

Not much is known at present about the relationship between the pineal gland and thyroid function. Scepovic (1963) has shown that pinealectomy causes thyroid hypertrophy and an increase in ^{131}I uptake. It seems also to increase thyroid hormone secretions (Ishibashi et al., 1966), but does not modify the thyroid hypertrophy induced by propylthiouracil administration (Yamada, 1961). Melatonin given subcutaneously to the rat has been shown to reduce thyroid mass as well as ^{131}I uptake (Baschieri et al., 1963). It was assumed that this could be due to an inhibitory effect of melatonin on TSH or on the action of this hormone on the thyroid gland. Thiéblot et al. (1966) found that, in contrast, melatonin given to prepuberal rats produced marked hypertrophy of the thyroid gland. Mess (1969), however, was unable to show any effect of pinealectomy on the gland.

Panda and Turner (1968) demonstrated a goitrogenic effect of melatonin. It caused plasma thyroid stimulating hormone (TSH) level as well as thyroid size to increase. A similar effect would have been obtained by thyroidectomy. This finding seems to substantiate the view that

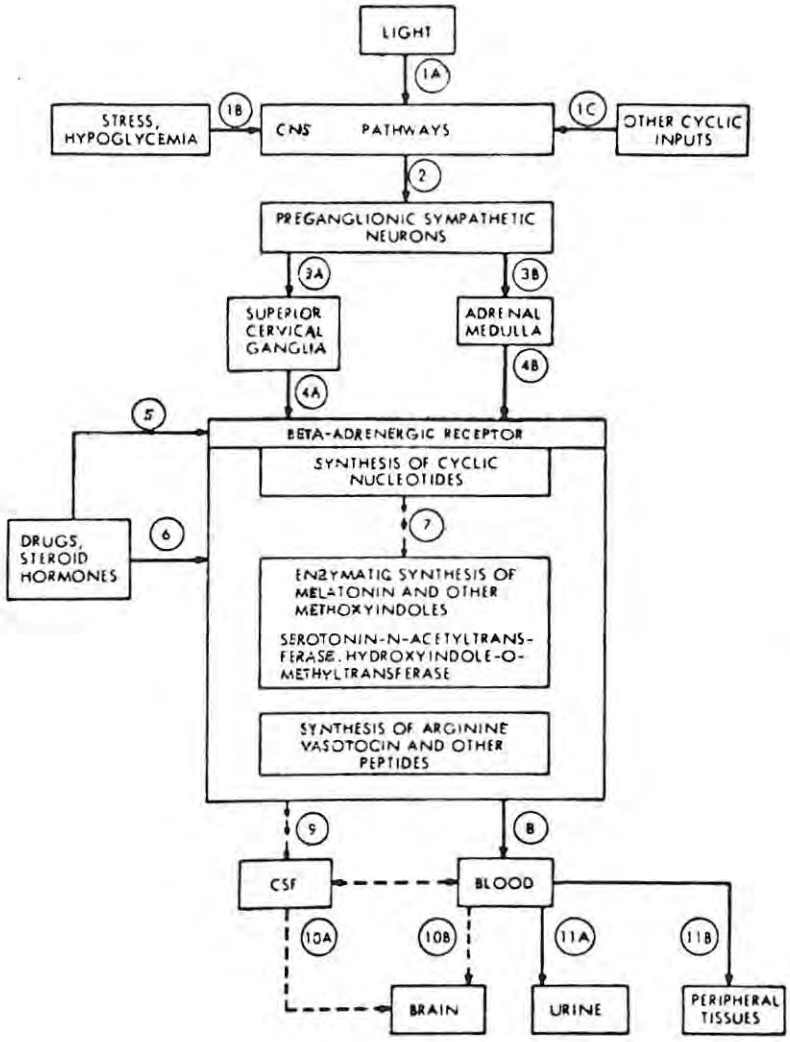


Fig. 13. Schematic diagram showing the factors that control pineal hormone secretion (7) and the delivery of pineal hormones to target organs. Noradrenaline is released from postganglionic sympathetic nerves (3A and 4A). The rate of release is suppressed by light (1A). Noradrenaline acts via β-adrenoceptors in the pineal gland (4A and 4B). (Wurtman and Moskowitz, 1977).

melatonin has a direct action on the thyroid gland. The effect on TSH would be due to a decrease in plasma thyroid hormone owing to inhibition of its synthesis or release in the thyroid.

The pineal gland could thus influence the thyroid gland by: (1) a central action on neural centres controlling TSH or on the pituitary gland, or (2) by direct action on the thyroid.

1.4.4 The Pineal Gland

Freire and Cardinali (1975) found that melatonin administered subcutaneously to rats or exposing them to constant darkness for 14 days induces changes in pineal ultrastructure characteristic of increased pineal activity. Ribosomes, procentrioles, microtubules, nucleoli and golgi apparatus are all increased in number. The activities of SNAT and HIOMT are also increased. Microtubule protein content increases by 85%. Pineal noradrenaline turnover is unaffected. The pineal gland is possibly a target organ for exogenously administered melatonin.

1.5 DIVERSE EFFECTS OF MELATONIN

Barchas et al. (1967) showed that melatonin administered to mice (25 mg/kg i.p.) increased the hexobarbitone (100 mg/kg i.p.) sleeping time by 50%. Marczynski et al. (1964) found that melatonin implanted into the hypothalamus of the cat induced sleeping behaviour. A dose of 2,5 mg/kg i.v. also caused 4-day old chicks to assume the roosting position (Barchas et al., 1967). This sleeping effect could be due to an increase in brain serotonin concentration caused by melatonin administration (Anton-Tay et al., 1968). Melatonin has no detectable

effects on vertebrate blood pressure and does not alter the vasoactive effects of other substances such as noradrenaline and tyramine. Electrocardiograms are also unaltered. Kastin et al. (1973) have found that melatonin injections (200 µg/180 g male rat) have no significant effect on their locomotor activity. It is possible that the dose was too small.

It has been shown that melatonin is capable of inhibiting rat liver N-acetyltransferase (Howd et al., 1976). This can be used as a basis for a melatonin assay where other methods are unsuitable.

Anton-Tay et al. (1968), as already mentioned (see page 53) found that i.p. administration of melatonin to rats increased brain serotonin content by an unknown mechanism. They have however, shown that melatonin 2,8 mg/kg i.p. does alter rat brain noradrenaline concentrations. Fiske and Huppert (1968) have found that subcutaneous injections of melatonin in young adult female rats shift or block the usual rhythm, depending on the time of day the melatonin was administered.

Cotzias et al. (1971) have noted that an i.p. injection of melatonin (400 mg/kg) does not result in any significant alterations in brain dopamine content. However, they were able to demonstrate an increase in brain serotonin levels after melatonin administration. Piezzi and Wurtman (1970) found that daily i.p. injections of melatonin (1 mg/kg) to male rats causes a 44% increase in the serotonin content of the pars intermedia of the pineal gland. Intra-arterial and intracisternal injections of melatonin cause an increase in rat brain dopamine and noradrenaline content (Wendel et al., 1974). One hour after an intra-arterial injection of 250 µg/kg melatonin, the dopamine content nearly

doubled and the noradrenaline content significantly increased. An intracisternal injection had the same effect. Injection of 6-hydroxy-melatonin, the main metabolite of melatonin, had no effect.

Heldmaier and Hoffmann (1974) demonstrated that melatonin administered to hamsters increased brown adipose tissue growth. This tissue is necessary for non-shivering heat production. Although they did not ascertain the mechanism by which this growth occurs, they concluded that it was unlikely to be due to an effect on the thyroid gland, but rather a direct effect. Narang et al. (1967) reported that melatonin injections of 50 µg depressed food intake in rats by about 10%.

Melatonin also causes bronchodilation in the dog (Rahamimoff et al., 1965) and inhibits the spontaneous contractions of isolated rat uterus (Hertz-Eschel and Rahamimoff, 1965). Melatonin apparently competitively inhibits smooth muscle contraction induced by serotonin.

1.6 POSSIBLE ANTIGONADOTROPHIC SUBSTANCES

1.6.1 Serotonin

Some investigators have described serotonin as a pineal antigonadotrophic agent. It has been shown that serotonin implants into the brain depress pituitary levels of bioassayable FSH, suggesting that serotonin may play a role in the control of gonodotrophin production and release (Fraschini and Martini, 1970).

1.6.2 N-Acetylserotonin

Not much work has been done to determine the hormonal activity of this

indole compound and some reports about its activity seem contradictory. According to Porter et al. (1971-72), intraventricular administration of N-acetylserotonin of rats gives rise to elevated levels of LH in the plasma. Another publication from the same laboratory however, states that N-acetylserotonin inhibits LH release from the pituitary (Kamberi et al., 1971).

1.6.3 5-Hydroxytryptophol

This substance does not seem to alter ovarian growth or inhibit the oestrous cycle in the rat (McIsaac et al., 1964) but does inhibit compensatory ovarian hypertrophy in unilaterally ovariectomised mice (Vaughan et al., 1972). It also reduces the percentage of immature rats which ovulate after subcutaneous administration of pregnant mares serum (PMS) (Pomerantz and Reiter, 1974). It is possible that it acts by restricting the synthesis of LH by the anterior pituitary of the rat (Fraschini and Martini, 1970).

1.6.4 Polypeptides: Arginine Vasotocin

Arginine vasotocin (AVT), a biologically active octapeptide, was identified in pineal extracts by Milcu et al. in 1963. It was found to be a potent inhibitor of reproduction in immature rodents pretreated with gonadotrophins, its effect on endocrine tissues being about a million times greater than that of melatonin. Pavel and Petrescu (1966) found that AVT inhibited PMS-induced hypertrophy of ovaries in immature mice while Moszkowska et al. (1968) and Vaughan et al. (1975) found that it inhibited the HCG-induced hypertrophy of ovaries and uterus in immature mice. These latter workers have also shown that daily injections of AVT prolonged

oestrous cycles and caused abortion in mice (Vaughan et al., 1976). In addition, it is known to be capable of blocking compensatory ovarian hypertrophy after unilateral ovariectomy and reducing the size of the reproductive organs when injected into young male and female mice. This retarding effect seems to be specific as no other organs were affected.

Pavel (1973) showed that an injection of melatonin could induce AVT release from the pineal gland, the only known source of it in mammals. It has therefore been suggested that melatonin is possibly only an intermediate in neuroendocrine transduction i.e. a releasing factor for AVT, which is the final effector. This would then adequately account for the antigonadotrophic properties of both substances. However, it fails to account for the action of melatonin on pinealectomised animals.

Other structurally unidentified compounds of a peptidic nature have been isolated from mammalian pineal glands. These also possess antigonadotrophic activity.

1.6.5 GABA

Mata et al. (1976) investigated the possible role of GABA in the pineal gland. They were unable to establish its exact function, but concluded that GABA content is not influenced by light, is not affected in any way by the adrenergic nervous system and it in turn has no modifying effect on the adrenergic nervous system.

1.7 ENDOCRINE EFFECTS ON THE PINEAL GLAND (REVERSE EFFECTS)

1.7.1 Pineal Biochemistry (See also 1.8)

Although much work has been done on the effects of the pineal on other organs, very little research has been done on the reciprocal effects of gonadal and other hormones on the pineal. The pineal has the capacity to respond to a variety of hormonal signals in the circulation. These include gonadal steroids (Cardinali et al., 1974a; Nagle et al., 1975) and catecholamines (Lynch et al., 1973a).

Wurtman et al. (1965) reported that pineal HIOMT activity in female rats is two-fold higher during dioestrous than during oestrous. Although castration does not affect pineal activity, oestradiol benzoate has been shown in inhibit pineal HIOMT activity (Wurtman et al., 1965); Alexander et al., 1970). However, Houssay and Barcelo (1972) have reported that daily injections of 20 μg of oestradiol benzoate into ovariectomised female rats has a stimulatory effect on pineal HIOMT activity and that progesterone decreases HIOMT activity. It appears that low doses of oestradiol benzoate have a stimulatory effect on HIOMT and that higher doses are inhibitory. Oestrogens, apparently, have little effect on SNAT activity (Preslock, 1977). Weiss and Crayton (1970) have demonstrated that progesterone is ineffective in reversing the noradrenaline-induced increase in pineal adenyl cyclase activity, but Houssay and Barcelo (1972) showed that it can decrease HIOMT activity.

Nagle et al. (1974) have shown that castration decreases HIOMT activity in pineals of male rats and that testosterone propionate injections restore the activity. Higher doses of this substance however, decrease pineal HIOMT activity.

It has also been shown that the low dose oestradiol-induced increase in pineal HIOMT activity can be antagonized by simultaneous administration of noradrenaline. Cardinali et al. (1974a; 1974b) have shown that oestradiol or testosterone enhances melatonin synthesis and this suggests the possibility of a negative feedback system between the gonads and the pineal gland. Sympathetic innervation is essential for the steroid to exert such an effect (Nagle et al., 1975) and the change in pineal synthetic activity seems to be brought about by an increased eflux of noradrenaline from pineal nerve terminals (Cardinali et al., 1975a).

Although the effects of gonadal steroids on the pineal have received much attention, not much information is available on the influence of gonadotrophins on this organ. Cardinali et al. (1975a) have demonstrated that gonadotrophin (LH and FSH) and prolactin injections could induce HIOMT activity. Pineal denervation prevents this effect which seems to be a direct one. Experiments indicate that the stimulatory effect of the pituitary on HIOMT may decrease with age of the animal (Alexander et al., 1970). It is possible that the effect of pituitary hormones on the pineal may be effected through secretions from specific target organs. For example, any suggestions of gonadotrophin effects on the pineal must take into account the effects of secretion of gonadal steroids because gonadotrophins such as LH are known to influence the secretion of gonadal steroids both from the ovary and the testis.

Gonadectomy causes an increase in gonadotrophin levels (Gay and Midgley, 1964). Smith et al. (1975) investigated this phenomenon in a histochemical and biochemical study and concluded that there seems to be no

effect of increased gonadotrophin levels on the pineal serotonin level.

1.7.2 Pregnancy

Huang and Everitt (1965) investigated effects of pregnancy on the pineal gland. They found that pineal mass in pregnant rats decreases as the pregnancy progresses and that pineal mass is inversely proportional to the number of foetuses carried. Induction of pseudo-pregnancy was found to result in a significant change in pineal HIOMT rhythm from that measured during the oestrous cycle (Yochim and Wallen, 1974a). Yochim and Wallen therefore suggested that the pineal gland may have a dual role in reproduction: (1) as a transducer of photo-periodic information for seasonal breeding activity and (2) as a regulator of gonadotrophin secretion during the reproductive cycle, both phenomena being demonstrable in the rat.

1.8 DRUG EFFECTS ON THE PINEAL GLAND

1.8.1 β-Receptors, cAMP and Adenyl Cyclase Activity

The catecholamine, l-noradrenaline has been shown by Weiss and Costa (1967) to activate adenyl cyclase in pineal homogenates. This led to further investigation of the possible effect of various catecholamines on adenyl cyclase and cyclic AMP (Weiss and Costa, 1968). l-Noradrenaline, l-adrenaline and l-isoprenaline all increase adenyl cyclase activity. Substances such as serotonin, histamine, d-noradrenaline, dl-normetanephrine, tyramine, dopamine, dl-dihydroxymandelic acid, d-amphetamine or l-phenylephrine have no effect on the enzyme activity in pineal homogenates. Guanethidine,

desmethylimipramine and cocaine, all of which alter sympathetic nerve impulses into the pineal, also have no effect. β-Blockers have been shown to antagonize the noradrenaline-induced stimulation of adenyl cyclase activity, whereas the α-blockers phenoxybenzamine and phentolamine have negligible activity. These results suggest that pineal adenyl cyclase responds selectively to specific adrenergic and adrenolytic drugs in a manner similar to that of a postjunctional β-receptor.

Weiss and Costa (1968) studied the effects of gonadal hormones in regulating pineal adenyl cyclase activity. They found adenyl cyclase in female rats to be less responsive to the stimulatory effects of noradrenaline than that of male rats. Testosterone propionate failed to alter adenyl cyclase activity in either male or female rats. Noradrenaline was found to activate adenyl cyclase in female rats at oestrous, metoestrous or dioestrous. Adenyl cyclase of rats at prooestrous failed to respond. This finding suggests that female sex hormones may inhibit the enzyme since oestrogen, progestin and gonadotrophin levels rise during prooestrous. Further investigation showed that progesterone alone has no significant effect on adenyl cyclase when administered to ovariectomised rats. Oestradiol plus progesterone completely abolishes the noradrenaline stimulatory effect on adenyl cyclase and markedly reduces the response of the enzyme to sodium fluoride. Oestrogen alone also inhibits the noradrenaline-induced activity without affecting the basal level of adenyl cyclase. Ovarian hormones seem, therefore, to affect the pineal gland and this suggests the possible presence of a hormonal (as well as neuronal) control of pineal adenyl cyclase activity and cyclic AMP levels. This suppressant effect of oestrogens may be a

direct one on pinealocytes or an indirect one modulating neuronal input. Furthermore Cardinali et al. (1975a) showed that treatment of rats for 3 days with testosterone or oestradiol increases noradrenaline turnover without affecting the overall transmitter levels in the gland. These steroidal effects seem to tie in with Weiss and Costa's observations on adenyl cyclase activity at prooestrous and suggest strongly the presence of a feedback mechanism regulating gonadal function by altering the rate of pineal antigonadotrophin synthesis and release. In theory, gonadal hormones could accelerate noradrenaline turnover by acting (a) at any point of the multisynaptic neural pathway between the retina and pineal gland, (b) at the level of pineal nerve endings, or (c) through a hypothetical feedback loop involving the pinealocytes, which are target cells for sex steroids. The sensitivity of these target cells to steroids may possibly fluctuate with changing noradrenaline levels.

1.8.2 Aromatic L-Amino Acid Decarboxylase Activity

Drugs interfering with peripheral sympathetic nerve impulses to the pineal gland, e.g. guanethidine and bretylium, were administered by Snyder et al. (1965a) to rats (20 mg/kg for both drugs) to determine their effect on the response of the enzyme to constant light. Bretylium blocks the rise of the enzyme level but guanethidine has no effect. Both drugs were found to block the constant light-induced reduction in pineal mass. These effects are probably due to the neuronal blocking ability of the two drugs. Why guanethidine has no effect on enzyme activity in this case is unclear. Trentini et al. (1973) showed also that bretylium and guanethidine block the increase of pineal mass associated with hypergonadotrophinaemia, thus

suggesting that experimental hypergonadotrophinaemia affects the pineal by way of the sympathetic nervous system and not the blood-stream.

1.8.3 Serotonin Production

The monoamine oxidase inhibitor (MAOI) β-phenylisopropylhydrazine was administered to rats to see if this could block the nocturnal drop in serotonin concentration in the pineal (Snyder and Axelrod, 1965). It did so, suggesting that serotonin is released from a bound form at night, making it accessible to degradation by monoamine oxidase. The MAOI pargyline, however, has been shown to have no significant effect on serotonin levels after prior treatment with l-isoprenaline (Brownstein *et al.*, 1973b).

Serotonin rhythm, which appears to be controlled by sympathetic nerves, is abolished by reserpine but guanethidine and bretylium have no effect. The latter two drugs thus do not seem to alter nervous impulses to the pineal gland in the same way as they affect those of other peripheral sympathetic nerves.

Hyyppä *et al.* (1971) found that intraperitoneal dosing of rats with L-dopa plus a peripheral decarboxylase inhibitor resulted in a drop in pineal serotonin content. This could be due to conversion of some of the L-dopa in the CNS to noradrenaline, which, when acting on pineal β-receptors, causes an increase in SNAT activity and therefore a drop in pineal serotonin level.

Isoprenaline was shown *in vivo* to cause a fall of serotonin levels

during the day. Propranolol, a β-adrenergic blocker, reverses this fall. Phentolamine, an α-adrenergic blocker, is without effect. L-Propranolol, conversely, causes a rapid increase in serotonin concentration at night when its levels are normally low (Brownstein et al., 1973b).

1.8.4 Serotonin N-Acetyltransferase Activity

Rat pineal SNAT activity is markedly and rapidly elevated in vivo after subcutaneous injection of drugs such as L-dopa, noradrenaline, adrenaline, isoprenaline, monoamine oxidase inhibitors or theophylline (a phosphodiesterase inhibitor)(Deguchi and Axelrod, 1972a). The effect of MAOI's is apparently not due to stimulation by serotonin, the oxidation of which is prevented by the inhibition of MAO, but rather due to inhibition of noradrenaline catabolism (by MAO), which leads to a greater stimulation of SNAT activity (Klein and Weller, 1970b). β-Blockers and protein synthesis inhibitors completely block the effect of these drugs, suggesting that their effects are mediated via the β-adrenoceptor and require the synthesis of new enzyme molecules. It is assumed that L-dopa acts by being converted to a catecholamine, possibly noradrenaline. Phenoxybenzamine, (an α-adrenoceptor blocker), is unable to block the stimulatory effects of these drugs on SNAT activity (Deguchi and Axelrod, 1972a). Monoamine oxidase inhibitors probably act by raising catecholamine concentrations. On the assumption that pargyline, an MAOI, can increase noradrenaline concentrations in the brain by inhibiting the breakdown of this catecholamine, Illnerová (1974) administered the drug to rats to determine its effect on pineal SNAT activity. When it was given during the day, a transient decrease, then an

increase in SNAT activity occurred. Pargyline however, did not block a decrease in SNAT activity at night after exposure to light. It seems that noradrenaline levels decrease not because of an action by MAO but because of nervous reuptake and/or action of catechol-O-methyltransferase (COMT). The stimulatory effect of theophylline suggests that the effect of the aforementioned drugs is mediated *via* cyclic AMP.

The diurnal rhythm of SNAT, which increases in activity at night and drops off during the day, is abolished by reserpine (which depletes noradrenaline stores in nerve endings), further emphasizing that the activity of the enzyme is controlled by release of noradrenaline from nerve terminals (Deguchi and Axelrod, 1972b).

Other factors such as stress induced by physical immobilization, or insulin-induced hypoglycaemia, have been shown by Lynch *et al.* (1973b) to cause an increase in pineal SNAT activity, which can be blocked by propranolol suggesting that these responses are also mediated by a catecholamine acting on the β-receptor. This again could either be due to noradrenaline being released from the nerve terminals or adrenaline being released from the adrenal medulla. The latter mechanism is a strong possibility as sympathetic denervation of the pineal actually potentiates the stress induced increase in SNAT activity.

Bäckström (1977) used an organ culture to investigate and categorize the β-adrenergic receptor of the pineal gland. The non-selective β-receptor antagonist, *dl*-propranolol and the selective β_1-receptor

antagonist, practolol, both decrease the amount of [^{14}C]-N-acetylserotonin formed by the pineal when it is stimulated by the directly-acting β-adrenergic agonist terbutaline. To cause a 50% decrease in [^{14}C] -N-acetylserotonin, compared with that formed by terbutaline alone, a 1000-times greater concentration of practolol than propranolol is needed. Two selective β_2-receptor antagonists, butoxamine and H35/25, have no effect or a minimal effect respectively on this system. These results indicate that pineal adrenoreceptors in an organ culture behave as β_1-receptors.

Holz et al. (1974) also used an organ culture to investigate the stimulatory effects of certain drugs on SNAT induction in intact and denervated pineal glands. They found that cocaine, procaine, pheniprazine, veratridine and the absence of potassium stimulates SNAT in innervated but not in denervated pineals. The stimulatory effects increase enzyme activity 100 to 2000-fold and this can be blocked by propranolol. It was concluded that these drugs and lack of potassium act on nerve terminals to cause an accumulation of extra-neuronal noradrenaline, which stimulates, a β-receptor to induce production of SNAT. In the case of veratridine, it was suggested that it works not only by inhibiting noradrenaline uptake, but also by stimulating its release.

Although the oestrogen, oestradiol has been shown by Weiss and Crayton (1970) to suppress adenyl cyclase-cyclic AMP function in vitro, Illnerová (1975) was unable to demonstrate any effect of oestradiol in vivo on SNAT which is assumed to be controlled by the "second messenger". This finding suggests that SNAT activity is not in fact under control of the "second messenger" or that the

activation of adenyl cyclase by noradrenaline is not suppressed to the same extent *in vivo* as *in vitro*.

1.8.5 HIOMT Activity, Melatonin Production and Pineal Protein Synthesis

Using an organ culture system, Axelrod *et al*. (1969) showed that, besides noradrenaline, structurally related compounds such as adrenaline, tyramine, tryptamine and octopamine also stimulate the formation of [^{14}C]-melatonin. Serotonin, melatonin and 5-hydroxyindole-3-acetic acid have no effect. This stimulation was assumed to be due partly to adrenergic stimulation and partly to monoamine oxidase inhibition. Klein and Rowe (1970) also investigated the effect of harmine, a MAOI, on the production of melatonin in an organ culture system. They found that pineal glands treated with harmine produce up to 5 times as much melatonin as untreated glands and that N-acetylserotonin levels are increased as well. Serotonin breakdown products like 5-hydroxyindole-3-acetic acid, caused by the action of monoamine oxidase, were found to be present at reduced levels. They concluded that, by inhibiting serotonin oxidation, an enhancement of N-acetylation results. This yields N-acetylserotonin and, hence, more melatonin can be produced by simple mass action. Houssay and Barcelo (1972) found that oestrogen administration to rats produces a significant decrease in pineal mass and a marked increase in HIOMT activity. Progesterone however, while not affecting pineal mass, significantly lowers HIOMT activity. Although they did not offer an explanation, it seems likely that these observations indicate the presence of a steroidal feedback system controlling HIOMT activity. This

phenomenon may be part of the same system mentioned by Cardinali et al. (1975a).

Hartley et al. (1972) reported that the neuroleptic drugs haloperidol and fluphenazine inhibit pineal HIOMT activity in vitro. They also found that psychotomimetic drugs such as dimethyltryptamine stimulate HIOMT activity in vitro by almost 30% (Hartley and Smith, 1973a). Other substances such as methoxybufotenin, mescaline and lysergide tartrate cause about a 10% increase in activity. Seeing that haloperidol and fluphenazine, which are used to treat schizophrenia, inhibit HIOMT activity and therefore melatonin production in vitro, and that psychotomimetic drugs produce psychoses similar to those observed in schizophrenia, they suggest that melatonin production may be implicated in schizophrenic states. Altschule (1957), Eldred et al. (1961) and Bigelow (1974) have found that aqueous pineal extracts exert a beneficial effect in some schizophrenics. The mechanism by which this occurs is not known.

Hartley and Smith (1973b), continuing in this field, found that HIOMT catalyses the formation of N-acetyl-3,4-dimethoxyphenethylamine (NADMPEA) from N-acetyl-3-hydroxy-4-methoxyphenethylamine and N-acetyl-4-hydroxy-3-methoxyphenethylamine in vitro. This conversion was found also to be inhibited by haloperidol, which is concentrated in the pineal gland more than in any other part of the brain (Naylor and Olley, 1969). It seems that in schizophrenia, HIOMT may act on abnormal substrates such as isomeric methyl esters of dopamine to produce the dimethylated metabolites which have been implicated in the disease. This blockade of the formation of a psychotogenic amine would then offer a plausible alternative mechanism of action of haloperidol.

Nir et al. (1976) found that the vitamin pyridoxal-5'-phosphate inhibits HIOMT activity and thus reduces the conversion of N-acetylserotonin to melatonin. Noradrenaline blocks this effect by forming a complex with the vitamin. Pyridoxal-5'-phosphate may therefore play a role in regulation of the activity of HIOMT and perhaps of other methyltransferases in the brain.

Besides demonstrating that the pineal gland is a target for ovarian steroids, Cardinali et al. (1974a) have also investigated the effects of physiological doses of oestradiol on HIOMT activity (and therefore melatonin production) which was found to be enhanced by the steroids. Furthermore, they found that oestradiol benzoate significantly increases [^{3}H]-leucine incorporation into pineal protein in vivo. If the apparent increase in protein synthesis reflects an increased production of pineal antigonadal peptides as well, this, together with the increased HIOMT activity, would provide the basis for an ovarian-pineal negative feedback system. With so much evidence suggesting the presence of such a system, it seems most likely that it does exist.

Wurtman et al. (1969) used an organ culture to show that l-noradrenaline can profoundly increase the incorporation of [^{14}C]-tryptophan into [^{14}C]-protein. Dopamine and l-adrenaline have similar effects, but dopamine is more potent, and l-adrenaline less potent than l-noradrenaline. Indoles such as serotonin have no effect. The mechanism by which noradrenaline exerts this effect is probably by enhancing the rate at which the amino acid enters pineal parenchymal cells. It may also increase intracellular specific activity. The amount of [^{14}C]-tryptophan incorporated into the

indoleamine biosynthetic pathway is however at least 40 times that incorporated into protein.

1.9 EFFECTS OF SURGERY ON PINEAL AND RELATED FUNCTIONS

1.9.1 Denervation

The sympathetic nervous system which innervates the pineal gland in mammals has its origins in the superior cervical ganglia in the neck (Kappers, 1960). Bilateral ganglionectomy involves surgical removal of both ganglia; decentralisation entails removal of a section of the preganglionic fibres of the ganglia (Moore and Rapport, 1971). Although it is generally accepted that the pineal stalk is of little importance with respect to pineal innervation, it has been demonstrated that disruption of the pineal stalk leads to a reduction in pineal size (Quay, 1971).

Weiss and Costa (1967) investigated the possible effects of bilateral superior cervical ganglionectomy (S.C.G.) on adenyl cyclase activity in the pineal gland. They found that there was little or no effect, although this procedure does sensitize the adenyl cyclase system to stimulation by noradrenaline. Wurtman et al. (1964c) had already shown that bilateral S.C.G. abolishes HIOMT rhythm by removing photic control of this enzyme. This applies also to pineal serotonin rhythm (Fiske, 1964) which fluctuates diurnally in relation to the photoperiod (Quay, 1963). Chu et al. (1964) found that melatonin had an inhibitory effect on the oestrous cycle whereas pinealectomy increases the incidence of oestrous. However, Brown-Grant and Östberg (1964) found that pineal denervation had no significantly different effect from sham-operated animals regarding effect on

the oestrous cycle and ovulation.

Moore and Rapport (1971) further investigated the effects of S.C.G. and decentralisation on HIOMT activity. They confirm that HIOMT response to light is abolished after these surgical procedures. It was found also that HIOMT activity levels are significantly less after decentralisation than after ganglionectomy, and these are in turn significantly less than constant-light control levels. This confirms that intact sympathetic innervation is necessary not only to enable it to respond to light, but also for normal rhythmic enzyme activity. Furthermore they found that denervation has a negligible effect on the oestrous cycle in the female rat. These findings agree with those of Brown-Grant and Östberg (1974). Snyder et al. (1965b) suggest that, although serotonin rhythm is abolished by S.C.G., the rhythm is essentially intrinsic to the pineal gland but requires intact sympathetic nerves for expression. They thought initially that some controlling mechanism for the rhythm could be present in the ganglia, but as severing the preganglionic nerves has the same effect on the serotonin rhythm, this is obviously not so.

1.9.2 Bilateral Orbital Enucleation (Blinding)

This procedure has no effect on pineal serotonin rhythm, a finding which further confirms the endogenous character of the rhythm (Snyder et al., 1965b). Removal of other endocrine glands such as adrenals, thyroid, ovaries and pituitary also has no effect on the serotonin rhythm.

1.9.3 Pinealectomy

In 1956, Holmes reported that pinealectomy in the rat has no apparent anatomical effect on the pituitary gland as seen under the light microscope. (It also has no effect on the renin content of the kidneys which is increased by hypophysectomy (Bruinvels *et al.*, 1964)). These findings suggest that pinealectomy either is of minimal physiological significance or that its potential effects are obscure or slow in appearing. Blake (1976), by pinealectomising or sham-pinealectomising and, in addition, subjecting rats to ovariectomy, showed that the pineal gland apparently has no major effect on the timing or magnitude of LH, FSH and prolactin release at prooestrous. The length of the oestrous cycle is also unaffected. Other workers have also investigated this field in an attempt to arrive at more definite conclusions about the effects of pinealectomy. Piacsek *et al.* (1969) reported that pinealectomy causes an increase in ovarian size in hypophysectomised rats with pituitary transplants. However, in this case, relatively low ovarian and uterine masses indicate that oestrogen and gonadotrophin levels are far below normal so that the entire hypothalamic-pituitary-gonadal feedback system is probably altered. As a result, very small changes in pineal secretion could be magnified by acting on a very steep portion of a hypothetical dose-response curve. Reiter (1973) showed that pinealectomy is ineffective in preventing persistent oestrous in female rats placed in conditions of constant light. However, pinealectomised rats tend to show fewer four-day oestrous cycles than intact rats. The pineal also seems to have an influence in controlling cycle length during shorter periods of light (Hoffmann and Cullin, 1975).

Moszkowska et al. (1971) have reported that pinealectomy results in lowered hypothalamic serotonin levels. Cardinali (1975b) found that the hypothalamic synaptosomes of rats which had been pinealectomised or pinealectomised plus superior cervical ganglionectomised show a reduced uptake of serotonin, whereas noradrenaline, dopamine and glutamate uptake are unaffected. The findings suggest a close relationship between pineal gland and serotoninergic systems in the brain.

It is possible that pinealectomy does not have gross after-effects because pineal substances such as melatonin are still present in the body. Ozaki and Lynch (1976) found that pinealectomised rats still exhibit melatonin in their plasma and urine, although the level is only about 20% of that normally present. It was assumed that this melatonin comes from undiscovered extrapineal sources. These sources could be the enterochromaffin cells of the harderian gland.

1.9.4 Hypophysectomy and Gonadectomy

Zweens (1963) noted that ovariectomy in young rats causes a sharp rise in pineal phospholipid content. He assumed that since the phospholipid content varies with the oestrous cycle, this is a gonadal effect which is reinforced by the effect of ovariectomy. This surgical procedure apparently alters the accumulation of phospholipids due either to deprivation of ovarian hormones or overproduction of gonadotrophins.

Ito and Matsushima (1968) found that hypophysectomy causes degenerative

cellular atrophy and nuclear changes in the pineal gland. Lupulescu (1968), in addition, reported that the numbers of ribosomes are reduced, secretory granules are depleted, the endoplasmic reticulum atrophies and the mitochondria are altered. Alexander et al. (1970) reported that hypophysectomy of 53-day old rats reduces pineal mass and HIOMT activity, whereas ovariectomy has no effect on pineal mass in 28-day old rats but HIOMT activity is increased. Urry et al. (1972) found that hypophysectomy of young, immature male rats causes a decrease in pineal mass and HIOMT activity. In mature rats, only pineal mass is decreased. This suggests that the pituitary may release a substance necessary for growth and development of the pineal and of HIOMT activity.

In an attempt to define a more precise relationship between HIOMT activity and reproductive cyclicity, Wallen and Yochim (1974b) tested the pattern of HIOMT enzyme activity after ovariectomy and oestrogen replacement in the rat. After surgery, mean enzyme activity is significantly reduced, but rhythmicity is maintained. Oestrogen replacement restores the overall level of activity. It was concluded that the ovaries are not necessary for maintenance of pineal rhythmicity, but a feedback relationship obviously exists between ovarian function and the mean level of pineal HIOMT activity. (See also 1.7).

1.9.5 Combined Blinding and Anosmia

If female rats are rendered blind and anosmic, this significantly increases their locomotor activity (Sackman and Reiter, 1977). Their frequency of defaecation is also increased. By pinealectomising

these animals, or by giving them melatonin implants, this increased frequency of locomotor activity is completely reversed. However, the frequency of defaecation is only partly reversed. It has so far not been possible to define the exact role of the pineal in these phenomena. Blinding also abolishes HIOMT rhythm, which is dependent on light for its diurnal fluctuation.

1.9.6 Lateral Hypothalamic Lesioning

Heller and Moore (1965) found that lateral hypothalamic lesions which section the medial forebrain bundle can produce decreased noradrenaline and serotonin levels in the brain and also abolish their rhythms in the pineal gland. These levels depend on the integrity of central fibre systems relevant to each particular amine. Axelrod and Snyder (1966) placed bilateral lesions stereotaxically which transected the medial forebrain bundle in the lateral hypothalamus of young male rats. This procedure was shown to abolish the light-induced rhythm in HIOMT activity. It seems that this part of the brain is involved in transmission of impulses from the retina to the pineal gland.

From this review, it can be seen that the pineal has become a focus of much attention. Although much work has been done, there is plenty of scope for further research. It was decided, therefore, to conduct an investigation into the effects of β-adreno-active substances on pineal metabolism and see if they caused any secondary gonadal changes.

CHAPTER 2

2.1 MATERIALS AND METHODS

2.1.1 Animals

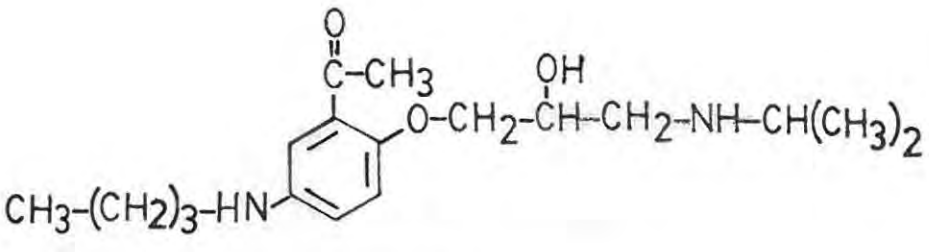
Albino female rats of the Wistar strain were used. Animals were not more than 6 months old and the mass of each rat was approximately 180-200 g. Each rat, individually housed in a plastic cage, had free access to food and water. The animal room was lit by white fluorescent tubes to give a light intensity of 300 $\mu W/cm^2$. Use of a timer enabled the amount of light and dark to be varied according to requirements. The rats were maintained under a 12h/12h light/dark regimen. The room was well ventilated and the temperature controlled at 20 ^{o}C.

2.1.2 Administration of Drugs

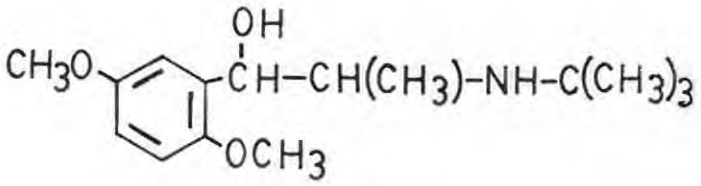
Drugs were administered orally or intraperitoneally to rats on a mg/kg or mmol/kg basis. All drugs were dissolved in normal saline prior to administration. Pindolol and hexoprenaline sulphate were dissolved in very dilute acetic acid. A hypodermic needle of which the point had been rounded with epoxy resin, was used to dose the rats orally.

2.1.3 Surgery

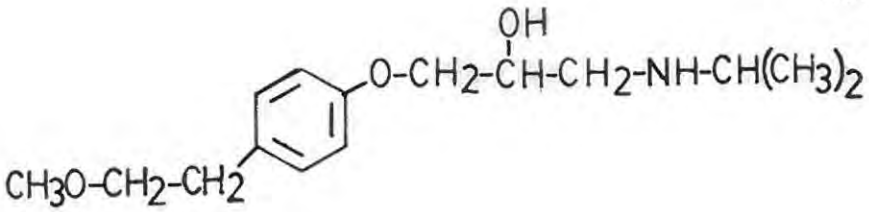
Initial surgical work entailed dissection of the brain and localization of the pineal gland. One atlas (The Stoelting Co.) was found to be inaccurate in its indication of the pineal location. By trial and error the gland was eventually located and mapped.



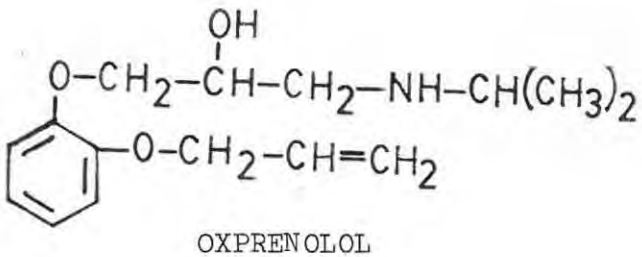
ACEBUTOLOL



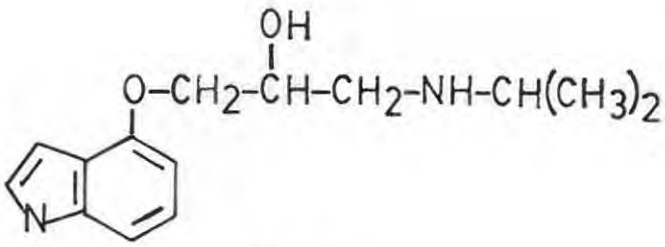
BUTOXAMINE



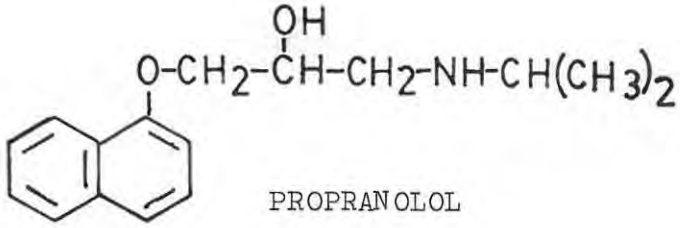
METOPROLOL



OXPRENOLOL

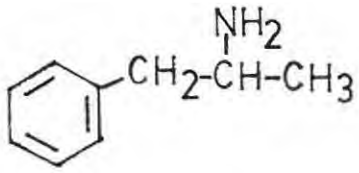


PINDOLOL

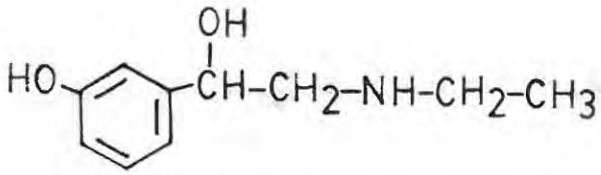


PROPRANOLOL

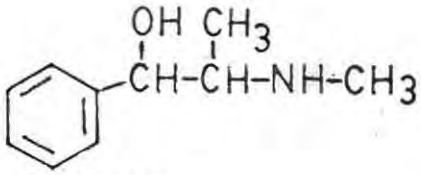
Fig. 14. β-Adrenergic blocking agents used.



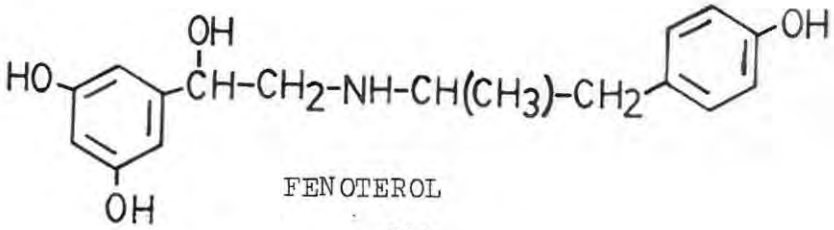
AMPHETAMINE



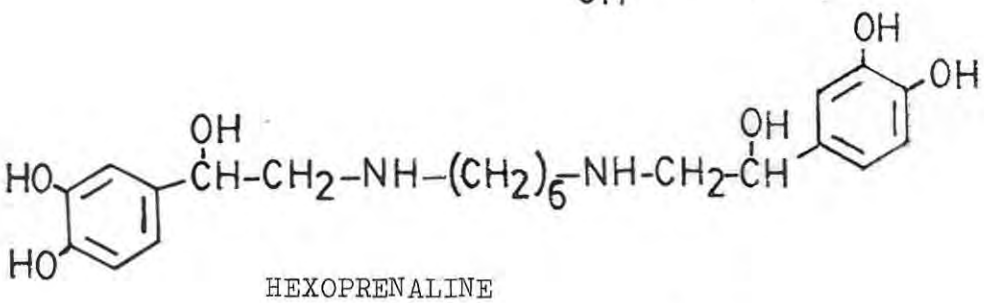
ETHYLNORPHENYLEPHRINE



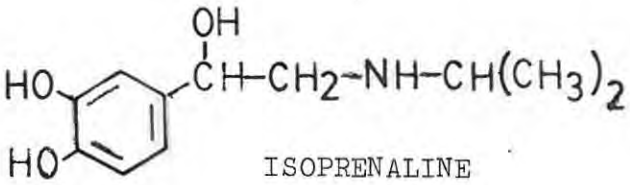
EPHEDRINE



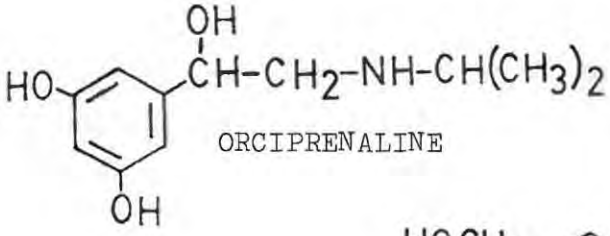
FENOTEROL



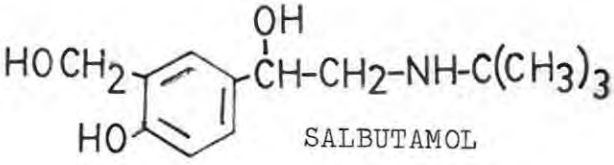
HEXOPRENALINE



ISOPRENALINE



ORCIPRENALINE



SALBUTAMOL

Fig. 15. β-Adrenergic stimulating agents used.

This location agrees with that given in another atlas (Skinner, 1971).

For the purpose of hardening the brain for dissection, freezing with liquid air proved to be impractical and therefore brains were removed from the skull and fixed in 10-20% formalin solution prior to dissection.

2.1.3.1 Pinealectomy

Surgery was undertaken to establish the precise location of the pineal gland and to become familiar with the technique of pinealectomy.

Several different methods were tried. The rat was anaesthetized with pentobarbitone (40-60 mg/kg i.p.) and placed in a stereotaxic apparatus. The scalp of the rat was then incised and the skull scraped clear of tissue between the bregma and the lambda to a width of 3 mm on either side. With the aid of a dental drill, a plate of bone approximately 4 mm x 7 mm was removed directly over the pineal gland where the lambda and sagittal sutures intersect. Alternatively, bone just anterior to the lambda suture and lateral to the sagittal suture was removed, leaving the intact dura mater exposed (fig. 16). Because of the presence of large venous sinuses above the pineal, it was found to be extremely difficult to pinealectomise the animal without causing severe bleeding. A pair of fine iridectomy forceps was inserted through an incision in the dura mater and the pineal gland grasped and removed as rapidly as possible. A dissecting microscope was used to facilitate this procedure. The bone chip was replaced and the skin sutured back into place. The rat was allowed to recover in a warm place.

Female rats were found to be far more difficult to pinealectomise than male rats. They tended to bleed more and were also more vulnerable to the effect of the anaesthetic. This resulted in high mortality.

Other anaesthetics were tried in an attempt to overcome this problem. Alfathesin(R) has virtually no anaesthetic effect at all, even in large doses. Combination anaesthesia using ether or halothane plus pentobarbitone was found to be unpredictable in effect and unreliable.

2.1.4 Electrocardiogram

As it was envisaged that rats would in due course be dosed with β-adrenergic sympathomimetics and sympatholytics, it was decided to monitor the heartbeat of the rat as an approximate parameter for determining the amount of drug to be administered. An ECG machine was linked up to a rat under anaesthesia. Hypodermic needles soldered to leads were embedded under the skin of the rat. The standard 3-lead system (lead III) was used. The chart speed was 25 mm/s. A very clear ECG was obtained in this way and it was possible to get a reasonable assessment of the effect of the drug on chronotropic activity. However, the anaesthetics used, viz. pentobarbitone (± 40 mg/kg), ether and halothane, also had an effect on the heart rate. Moreover, it was found that many of the β-adrenergic blocking drugs had some intrinsic stimulatory activity, thus making it difficult to adjust dose levels on the basis of heartbeat alone. The system was therefore used to give an approximate indication of β-receptor blockade. (Fig. 17).

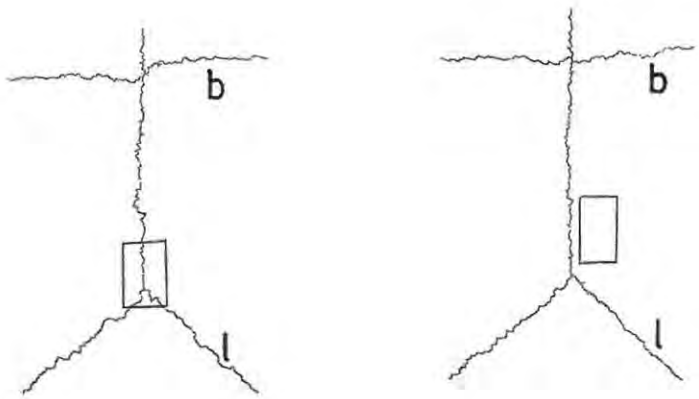


Fig. 16. Dorsal view of rat skull showing where bone was removed for pinealectomy.

b, bregma suture; l, lambda suture.

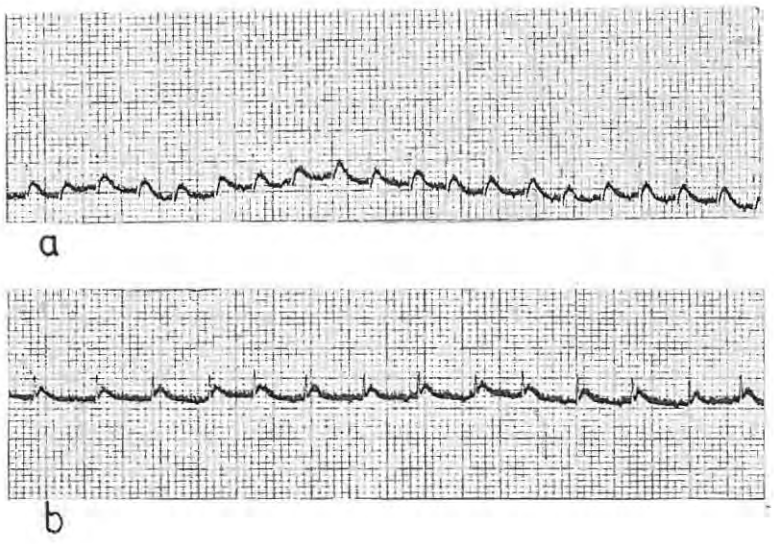


Fig. 17. Example of a rat ECG (lead III).

a. normal ECG; b. after administration of propranolol 20 mg/kg.

2.1.5 Vaginal Smears

A cotton bud, dampened with a small amount of water, was inserted into the vagina to obtain vaginal material for the assessment of the stage of oestrous of the rat. The smear was placed on a glass slide with a drop of water and examined under a magnification of 100. Cell cornification indicates the state of oestrous.

Vaginal smears were taken between 09h00 and 10h00 every day. Only rats which exhibited regular cycles were used for experimental work.

2.1.6 Thin-Layer Chromatography (TLC)

TLC was undertaken to develop solvent systems for the separation or identification of pineal indole compounds. Several systems were tried (tables 1; 2). Solvent system 4 was found to give the best results.

2.1.7 Assay of Pineal Enzymes by Liquid Scintillation Counting

The pineal gland is heavily innervated by the sympathetic nervous system (Kappers, 1960), and it is apparently not protected by the blood-brain barrier. Only β-receptors have been found in the pineal and therefore it seemed likely that drugs acting on the β-adrenoceptor would affect pineal metabolic function. In fact, the effect of β-active drugs on pineal gland enzymes, especially on SNAT, which is under control of adenyl cyclase-cyclic AMP second messenger system (Klein *et al.*, 1970b), has been demonstrated by several researchers (Weiss and Costa, 1968; Deguchi and Axelrod, 1972a; 1972b; Romero and Axelrod, 1975). It was decided, therefore, to investigate

TABLE 1

SOLVENT SYSTEMS TRIED FOR INDOLE COMPOUNDS

NO.	SYSTEM	REMARKS
1	methyl acetate:isopropanol:ammonium hydroxide (45:35:20)	satisfactory
2	methyl acetate:isopropanol:ammonium hydroxide (50:30:20)	good
3	ethyl acetate:glacial acetic acid:water (30:30:20)	poor
4	ethyl acetate:isopropanol:ammonium hydroxide (45:30:10)	excellent
5	methyl acetate:isopropanol:ammonium hydroxide (45:30:10)	good
6	n-butanol:pyridine:glacial acetic acid:water (30:4:10)	moderate, slow
7	ethanol:ethyl acetate:ammonium hydroxide:water (10:10:1:4)	discrete spots; too closely space
8	chloroform:glacial acetic acid (95:5)	virtually ineffective
9	n-butanol:glacial acetic acid:water (15:3:5)	poor
10	ethyl acetate:glacial acetic acid:water (15:15:10)	good, but causes tailing
11	sodium chloride 8% in water	very slow; high Rf value with poor separation
12	n-butanol:ethyl acetate:pyridine:glacial acetic acid: water (20:10:7:7:5)	very good; comparable with 4 and 5

TABLE 2

EXAMPLE OF Rf VALUES OBTAINED USING SILICA GEL G

SUBSTANCE		SYSTEM				
		1	2	4	9	10
tryptophan	Rf	4,9	5	1,5	8,5	4,5
tryptamine		7,7	8,1	8,5	8,3	6,5
5-hydroxytryptamine		5,9	7	6,5	8,2	5,8
5-hydroxyindole-3-acetic acid		5,6	5,8	4,2	8,4	8,0
indole		5,6	6	3,3	-	-

TABLE 3

VISUALIZERS USED FOR TLC

NO.	VISUALIZER	REMARKS
1	Iodine Vapour	good, general purpose visualizer
2	Ehrlichs Reagent	good
3	Ninhydrin/ethanol/glacial acetic acid	pale spots, not very sensitive
4	Ferric chloride/potassium ferricyanide	good
5	Phosphomolybdic acid/ethanol/hydrochloric acid	good
6	Ultraviolet light	short and long wave not active

further the possible effects of β-active substances on pineal gland SNAT and HIOMT activities, especially the latter as, although its activity was known to be modified by light (Wurtman et al., 1963b; Axelrod et al., 1965), very little was known about the possible direct sympathetic influence on this enzyme. It had also not been conclusively established whether adenyl cyclase was responsible for controlling the activity of HIOMT. An attempt was made to correlate the effects of these drugs on pineal enzymes with the effects observed on the oestrous cycle of the rat.

2.1.7.1 Determination of HIOMT Activity

The assay of HIOMT used is a modification of that developed by Axelrod and Weissbach (1961). 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). HIOMT is responsible for the formation of melatonin from N-acetylserotonin. The amount of radioactive melatonin formed can be detected by liquid scintillometry and this can be used as a parameter for enzyme activity.

Rats were killed by a blow on the neck followed by decapitation. The pineal gland was rapidly removed by cutting off the top of the skull with a large pair of scissors. Groups of 3 pineal glands were pooled and after all extraneous tissue had been removed, their mass was determined on a Sartorius analytical balance accurate to 0,1 mg. All three glands were then homogenized in 1,5 ml ice-cold 0,05 M phosphate buffer, pH 7,9, in a 1 ml glass homogenizer with a teflon pestle. As the quantity of tissue is very small, it was found that

homogenization by hand was quite adequate. The pestle was taken through 8 excursions of the homogenizer tube. This provided adequate mixing and homogenization.

S-Adenosyl-L-[methyl-^{14}C] methionine was supplied by The Radiochemical Centre, Amersham in glass bottles of 5 µCi each. The radioactive material was diluted to a volume containing 1 nCi/µl and quantities of 500 nCi were stored in glass vials at -20 °C.

Glass scintillation vials (Beckman "Extra" vials) were used for incubation purposes. Six vials were used for each group of 3 pineals. Prior to incubation, 200 µl aliquots of the pineal homogenate were placed in each vial containing 50 µg N-acetylserotonin in 50 µl phosphate buffer and 20 nCi S-adenosyl-L-[methyl-^{14}C]-methionine (specific activity 0,4 - 0,5 mCi/mmol) in 20 µl H_2SO_4, pH 3. A tissue blank in which brain cortex was substituted for pineal tissue and a reagent blank in which tissue was omitted were incubated along with every batch of vials. The average calculated disintegrations per minute (d.p.m.) for these two vials was subtracted from the d.p.m. obtained with the other vials before conversion to picomoles of melatonin formed/90 min/mg pineal tissue. A BASIC computer programme was used for this calculation. The total incubation volume in each vial was 270 µl. Initially, 50 nCi S-adenosyl-L-[methyl-^{14}C]methionine was used, but the quantity was reduced as it is far in excess of that needed for transmethylation purposes and because there is a chance that S-adenosylhomocysteine, the resultant product after the removal of the [^{14}C]-methyl group, may be present. S-Adenosylhomocysteine

has been shown by Deguchi and Barchas (1971) to be a potent inhibitor of HIOMT activity. The vials were incubated at 37 °C for 90 minutes in a shaking reaction incubator, the vials being agitated very gently at 80 cycles per minute. The reaction was stopped by the addition of 1 ml 0,2 M borate buffer, pH 10, to each vial. This was followed by the addition of 8 ml chloroform to each vial by means of pipette with a pipette-sucker. Thereafter the vials were shaken for 10 minutes on a mechanical shaker to transfer the radioactive melatonin formed to the organic phase. The aqueous phase was removed by aspiration with a pasteur pipette attached to a suction venturi on a water tap, another 1 ml borate buffer was added and the organic phase washed by shaking for a further 5 minutes. After removal of the aqueous buffer phase, 4 ml aliquots of the chloroform layer were pipetted into clean scintillation vials and evaporated to dryness in a water bath at 75 °C. (Chloroform is a powerful quenching agent in liquid scintillation counting and therefore had to be removed.) The residue was taken up in 1 ml ethanol, 10 ml Istagel(R) (Packard Instrument Company) added to each vial which was then counted in a Beckman LS3150T liquid scintillation counter. A quench-correction curve was drawn up using a set of Beckman quenched standards. This curve, in conjunction with an external standard channels ratio (ESCR), obtained from the scintillation counter printout, was used to convert c.p.m. to d.p.m. Each sample was counted three times (20 minutes for each count) and the mean c.p.m. value calculated. To facilitate processing of results, a Wang mini-computer model 2200 was used. Programmes in BASIC were designed to calculate mean ESCR values and convert c.p.m. to d.p.m. The d.p.m. values were then converted to picomoles of substance formed over a period of 90 minutes and expressed as picomoles/period of time/pineal gland or picomoles/period of time/

mg pineal tissue. Statistical programmes were also used. The Students t-test was used to evaluate significance.

As a precautionary measure, both S-adenosyl-L-[methyl-^{14}C] methionine and N-acetylserotonin were checked chromatographically for impurities by spotting onto TLC plates (Gelman ITLC type SA). For the methyl donor, two solvent systems consisting of (a) n-butanol:glacial acetic acid:water (15:3:1) and (b) sodium chloride 8% in water were used. With the first system, one discrete spot (Rf $\simeq$ 0,2) was seen. The second solvent system gave a single spot with an Rf value of 0,68, showing the substance to be chromatographically pure. Two solvent systems consisting of (a) benzene:methanol:glacial acetic acid (94:5:1) and (b) chloroform:methanol:glacial acetic acid (94:5:1), were used to check the purity of N-acetylserotonin. Both systems gave single spots, the Rf values being 0,18 and 0,32 respectively.

2.1.7.2 Determination of SNAT Activity

The method of assay used is a modification of that developed by Deguchi and Axelrod (1972c). The principle of the assay involves acetylation of tryptamine with [1-^{14}C] acetyl-coenzyme A. SNAT is the enzyme responsible for the transfer of a radioactive acetyl group to tryptamine to form [^{14}C]-acetyltryptamine. This product can then be extracted into a non-polar solvent system i.e. toluene-isoamyl alcohol (97:3) and measured quantitatively by liquid scintillation counting. For each assay, four rats were used. The rats were killed by a blow on the neck and the pineal glands removed by cutting off the top of the skull, thus exposing the gland. After extraneous tissue had been removed from each pineal, the mass of the glands was

determined as described previously and homogenized in ice-cold phosphate buffer (0,05 M), pH 6,5, containing tryptamine hydrochloride (3×10^{-3} M) and [1-^{14}C]-acetyl-coenzyme A (15 nmol; specific activity 4,99 nCi/nmol, The Radiochemical Centre, Amersham) with a 2 ml glass homogenizer and hand-held teflon pestle. The pestle was taken through 8 excursions of the tube to ensure adequate homogenization of the tissue.

The homogenizer tubes were incubated at 37 °C for 15 minutes in a shaking reaction incubator. The tubes were agitated gently at 80 cycles per minute. The reaction was stopped by the addition of 0,2 M borate buffer, pH 10. A total of 2 ml of the buffer was used in small quantities to rinse out each tube thoroughly. A pasteur pipette was used to transfer the contents of the tubes to a 6 ml glass-stoppered test-tube which contained 3 ml of toluene-isoamyl alcohol (97:3). After shaking on a vortex mixer for 30 seconds, the tube was centrifuged at 2000 r.p.m. for 5 minutes to separate organic and aqueous phases. A 2 ml aliquot of the organic layer was pipetted into a scintillation vial, 10 ml Instagel$^{(R)}$ scintillation cocktail added and the radioactivity measured in a Beckman LS3150T liquid scintillation counter. Each vial was counted three times for a total of 60 minutes. The ESCR method of quench correction was used to convert c.p.m. to d.p.m. (see HIOMT assay) and the amount of [^{14}C]-acetyltryptamine formed in 15 minutes calculated. Each assay was performed in duplicate and a blank incubation, in which brain tissue was substituted for pineal tissue, was performed as well to determine background c.p.m.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 THE RAT OESTROUS CYCLE

The pineal gland exerts an antigonadotrophic influence in rats (Wurtman et al., 1963a). Because the pineal is dependent on sympathetic nervous stimulation for indoleamine production (Axelrod et al., 1969), it was decided to investigate the possible effect of β-adrenoceptor blockade on the oestrous cycle of the rat.

3.1.1 Effect of Propranolol Hydrochloride

3.1.1.1 Dosage during Dark

A group of 20 individually-housed female rats, each with a mass of 180 g - 220 g, were selected for regularity of oestrous cycle. An attempt was made to block sympathetic activity during the 12-hour dark phase. Propranolol hydrochloride was chosen initially for its non-selective β-blocking properties and also because of its lipophilicity even though it is thought that the pineal gland is not protected by the blood-brain barrier.

A dosage of 20 mg/kg of the drug was administered orally twice daily, once 30 minutes before the onset of darkness and again 6 hours later. To ensure that the dose of propranolol was sufficient to suppress β-sympathetic activity over the whole 12-hour dark period, ECGs were taken while the rat was under light ether anaesthesia during the hour preceding the second dose and again during the hour preceding the light period.

Depression of chronotropic activity by 15% or more was taken as an indication of sustained β-blockade. Doses of 10 mg/kg and 5 mg/kg proved insufficient to produce β-blockade of sufficient duration. The stage of the oestrous cycle was determined by taking vaginal smears and noting the cell morphology and degree of cornification. The oestrous cycle of each rat was monitored daily at 09h00 and the number of positive oestrous smears taken over a 28-day period preceding drug dosage compared with the number of positive oestrous smears taken during 28 days of dosing. A control group of rats received normal saline.

After 4 weeks, the data were analysed by using the Wilcoxon matched-pairs signed-ranks test for significance. As no assumption had been made as to how the drug treatment would affect the oestrous cycles, two-tailed probabilities are quoted.

From the data obtained it was found that the 10 rats dosed with propranolol gave a significantly greater number of positive oestrous smears, the oestrous cycle apparently being shortened (table 4). This shortening may be explained on the basis of pineal β-adrenoceptor blockade. Blockade of these receptors before the onset of darkness should prevent the surge in the activities of the pineal enzymes SNAT and HIOMT, resulting in reduced melatonin production. Melatonin injections have been shown to reduce the incidence of oestrous (Chu et al., 1964). Conversely, inhibition of production of this anti-gonadotrophic substance should increase the incidence of oestrous as was found to be the case in these experiments.

3.1.1.2 Dosage during Light

The above experiment was repeated under similar conditions but the propranolol was administered at 6-hour intervals during the light period. To determine the effect of different dose levels of propranolol on the oestrous cycle, 40 rats were used. They were divided into 4 groups of 10 rats each. The first 3 groups received propranolol hydrochloride 20 mg/kg, 10 mg/kg, and 5 mg/kg respectively. The fourth group received normal saline.

In this instance the oestrous cycle was lengthened. These results were again analysed for significance using the Wilcoxon matched-pairs signed-ranks test (table 4). The first two groups, taken together showed a significant lengthening of the cycle ($P < 0,05$, two-tailed). The latter two groups, taken together, showed no significant difference.

Blockade of β-adrenoceptors during the day, when sympathetic activity (and consequently pineal enzyme activity) is low, would not be expected to affect the oestrous cycle to any great extent. However, contrary to expectations, the results showed that the incidence of oestrous had been significantly diminished. The reason for this observation is not clear. It could however be due to an increase in super-sensitivity of the β-receptors which then respond to rising sympathetic activity during the dark phase by causing greater formation of melatonin. The antigonadotrophic effect of melatonin would then be responsible for prolonging the cycle.

3.1.2 Effect of Acebutolol Hydrochloride, Metoprolol Tartrate and Oxprenolol Hydrochloride

Five groups of 10 rats each were dosed orally during the day for

TABLE 4

EFFECT OF β-ADENERGIC BLOCKERS ON RAT CORNIFIED OESTROUS SMEARS

DRUG USED	DOSE mg/kg (oral)	NO. OF RATS	TIME OF DOSING	DURATION OF DOSING/ DAYS	TOTAL NO. OF CORNI-FIED SMEARS BEFORE DOSING	TOTAL NO. OF CORNI-FIED SMEARS DURING DOSING	SIGNIFICANCE
PROPRANOLOL	20	10	dark	28	65	79	$P < 0,05$
PROPRANOLOL	5	10	light	28	62	63	N.S.
PROPRANOLOL	10	10	light	28	65	55	$P < 0,05$
PROPRANOLOL	20	10	light	28	68	48	$P < 0,05$
ACEBUTOLOL	10	10	light	28	70	65	N.S.
ACEBUTOLOL	20	10	light	28	69	64	N.S.
METOPROLOL	10	10	light	28	70	63	N.S.
METOPROLOL	20	10	light	28	69	70	N.S.
OXPRENOLOL	10	10	light	28	65	67	N.S.
OXPRENOLOL	20	10	light	28	64	67	N.S.
NORMAL SALINE	-	10	dark	28	68	70	N.S.
NORMAL SALINE	-	10	light	28	70	67	N.S.

Each rat served as its own control.

three weeks with acebutolol hydrochloride or metoprolol tartrate in the same manner as described in the previous experiment, in order to establish whether these specific β_1-adrenoceptor antagonists would be able to exert any effect on the oestrous cycle. The cycles of the rats were monitored for 28 days prior to dosing, two of the groups then received acebutolol hydrochloride or metoprolol tartrate in a dose of 10 mg/kg. Two of the remaining groups received the same drugs orally, in a dose of 20 mg/kg, and a control group received normal saline. Rats were placed under light ether or halothane anaesthesia and electrocardiographs were taken to give an indication of dosage levels to be used. However, interfering factors such as the effect of the anaesthetic and possible intrinsic sympathomimetic activity of the drugs precluded the use of the ECG for accurate or effective dose determination.

The data were again analysed for significance using the Wilcoxon matched-pairs signed-ranks test. No significant alteration in the oestrous cycle was observed. The experiment was repeated with oxprenolol hydrochloride in a dose of 10 or 20 mg/kg. Again there was no significant effect on the oestrous cycle. (Table 4).

The reason for this apparent inconsistency in the effect of propranolol on the oestrous cycle when compared with the other three β-blockers used is not known. It is possible that the greater lipophilicity, greater binding constant or lack of β_1 or β_2-adrenoceptor selectivity of propranolol enables it to interact with β-adrenoceptors in the pineal gland or other parts of the brain such as the pituitary gland and thereby influences the oestrous cycle by altering gonadotrophin secretion. Too little acebutolol,

metoprolol and oxprenolol may reach the brain to elicit any effect. Because of the inconsistency of these results and the fact that the experimental procedure is extremely time-consuming, it was decided to carry out more specific and selective experiments on the effects of the various β-blockers directly on the pineal enzymes.

3.2 HIOMT ACTIVITY

In preliminary studies, counts proved to be low owing to the very low specific activity of the radioactive methyl donor (0,5 mCi/mmol) and the presence of very small amounts of the enzyme being assayed. In an attempt to improve the sensitivity of the assay and increase the number of counts, the duration of incubation was varied (fig. 18). The quantity of [^{14}C]-melatonin formed increased for a period up to approximately 100 minutes before levelling off. This reduction in the rate of [^{14}C]-melatonin production could be due to the formation of S-adenosylhomocysteine which exerts an inhibitory effect on HIOMT activity. The quantities of N-acetylserotonin and S-adenosyl-L-[methyl ^{14}C] methionine used were also varied, but this modification had little effect as the compounds were still both present in concentrations far in excess of that capable of being utilized by the enzyme. Although equimolar quantities of N-acetylserotonin and S-adenosyl-L-[methyl ^{14}C]methionine react to form an equimolar amount of [^{14}C]-melatonin, the quantity of radioactive methyl donor used was reduced to approximately half of that of N-acetylserotonin as no reduction in efficacy of the assay was noted and it also constituted a considerable saving on an expensive radiochemical.

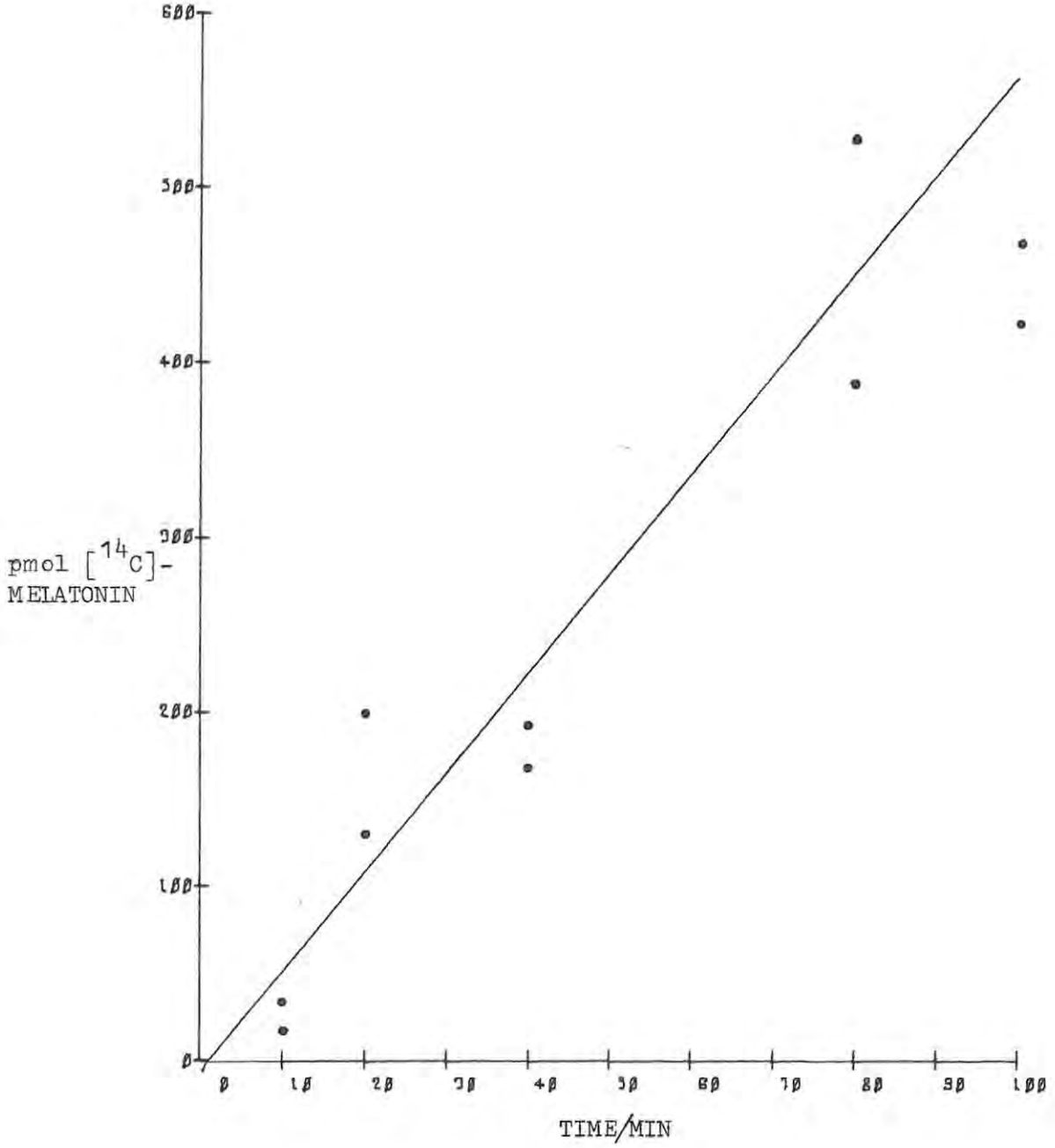


Fig. 18. HIOMT Activity (pmol [^{14}C]-melatonin formed) vs. time. There is a linear increase with time which falls off after 100 minutes.

3.2.1 Effect of β-Adrenoceptor Blocking Agents

An attempt was made to block the nocturnal rise in HIOMT activity by dosing female rats with propranolol 4×10^{-2} mmol/kg 30 minutes before the onset of darkness. Control animals were dosed with normal saline. The rats were housed under a light/dark schedule of 12h/12h with the lights on from 22h00 to 10h00. Three rats in metoestrous were used in both the test group and the control group. HIOMT activity fluctuates during the oestrous cycle and is at its highest at metoestrous (Yochim and Wallen, 1974a). The rats were killed in the dark five hours after being dosed, the pineals from each group pooled, and their combined mass and mean mass determined prior to being assayed for HIOMT activity.

No significant difference was noticed in HIOMT levels between the two groups when the data were analysed by the Students t-test. Propranolol failed to block the nocturnal surge in HIOMT activity. This finding (a) may be due to an insufficient quantity of propranolol reaching the pineal gland to exert a measurable blocking effect or (b) it may be an indication that HIOMT activity is not under sympathetic β-adrenergic control.

Experiments with acebutolol hydrochloride and metoprolol tartrate confirmed the lack of activity of β-blockers on HIOMT activity (fig. 19; table 5).

3.2.2 Effect of β-Adrenoceptor Stimulating Agents

None of the β-sympathomimetics, whether non-selective, selective or indirect-acting, seem to exert any appreciable effect on HIOMT

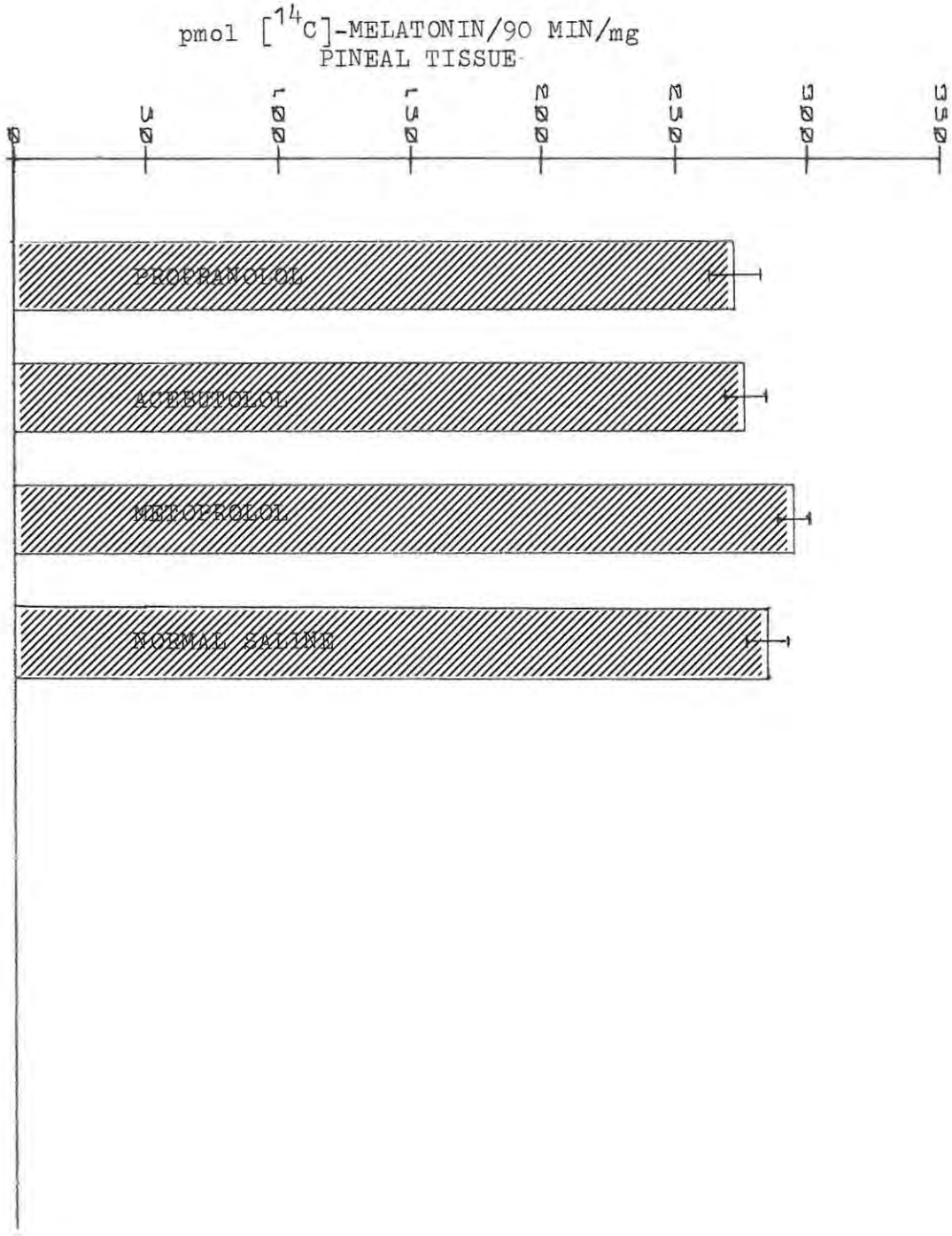


Fig. 19. Effect of β-adrenoceptor blocking agents (4×10^{-2} mmol/kg) HIOMT activity (see table 5).

TABLE 5

EFFECT OF β-ADRENERGIC BLOCKING AGENTS ON HIOMT ACTIVITY

DRUG	DOSE (mmol/kg)	ROUTE OF DOSING	HIOMT ACTIVITY (pmol [^{14}C]-MELATONIN/90 MIN/mg PINEAL TISSUE ± S.E.M.	HIOMT ACTIVITY %	SIGNIFICANCE
ACEBUTOLOL	$4x10^{-2}$	p.o.	277 ± 8	96	not significant
METOPROLOL	$4x10^{-2}$	p.o.	295 ± 6	103	not significant
PROPRANOLOL	$4x10^{-2}$	p.o.	273 ± 8	95	not significant
NORMAL SALINE		p.o.	287 ± 5	100	-

activity (fig. 20; table 6). Had HIOMT activity been under adenyl cyclase-cyclic AMP control, an effect should have been observed. It is possible that the availability of N-acetylserotonin or other factors may be of much greater importance than sympathetic stimulation. Oral absorption of the drug was probably adequate so dosage by the intraperitoneal route was no more effective in influencing HIOMT activity.

HIOMT activity has a relatively small amplitude of diurnal fluctuation (fig. 11a), thus alterations of levels are difficult to detect at the best of times. Any stimulatory or inhibitory effect on HIOMT activity by β-active substances, unless β-sympathomimetics or the sympathetic nervous system was strongly implicated in the control of HIOMT activity, would thus be of a very low order, and could possibly go unnoticed.

The only significant alteration in HIOMT activity noted was exerted by ephedrine hydrochloride which caused an apparent inhibition of enzymic activity (fig. 20). This could have been due to effects on higher centres in the brain as ephedrine has the ability to cross the blood-brain barrier. Amphetamine however did not cause a similar decrease in activity thus mitigating against this explanation.

3.3 SNAT ACTIVITY

Various β-active substances were administered intraperitoneally to female rats in an attempt to alter SNAT activity. It had been shown that β-adrenoceptor agonists such as isoprenaline could markedly increase SNAT activity. β-Adrenergic blocking agents such as propranolol had the opposite effect. This work was undertaken in

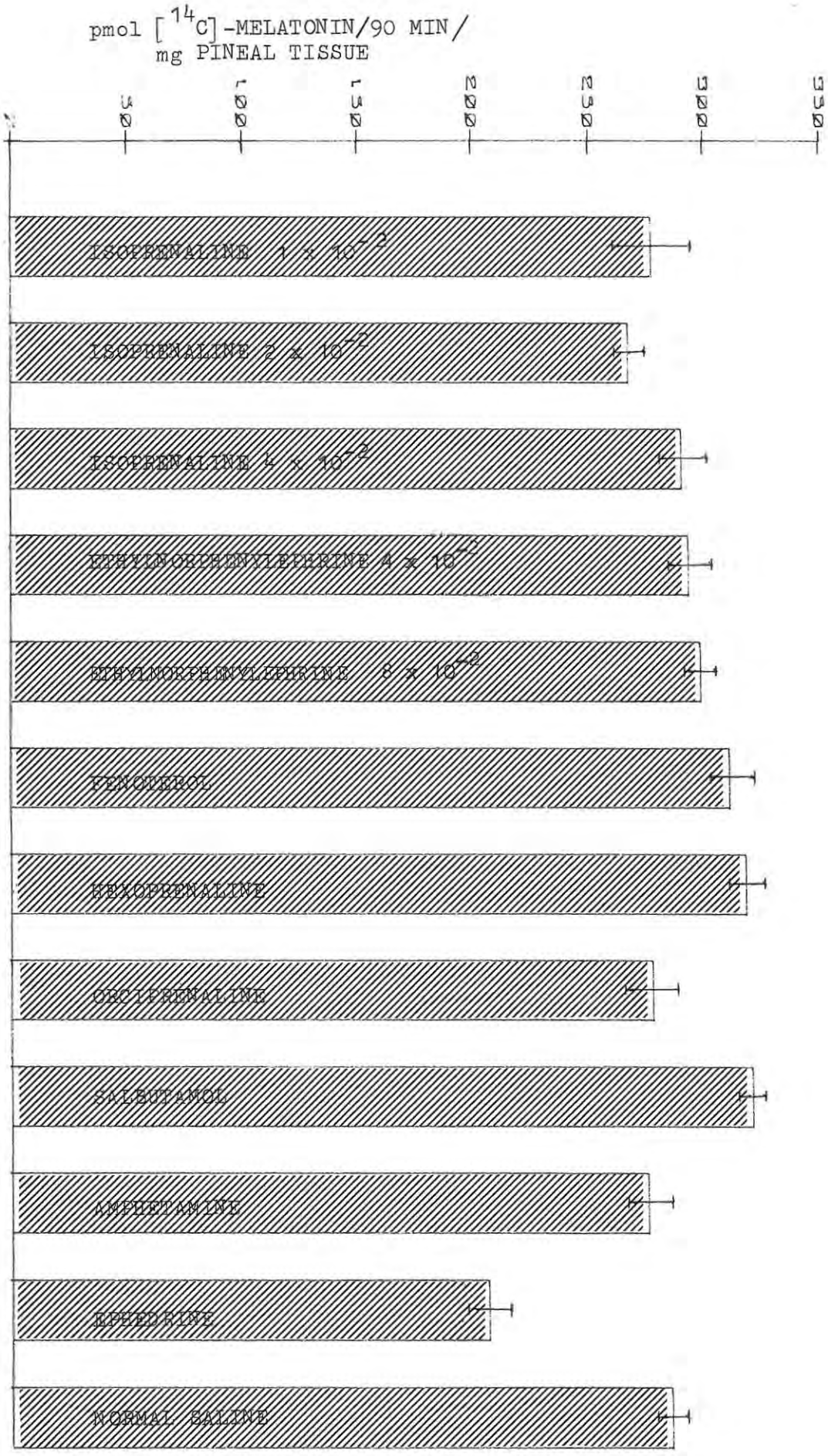


Fig. 20. Effect of β-adrenoceptor stimulating agents (mmol/kg) on HIOMT activity (see table 6).

TABLE 6

EFFECT OF β-ADRENERGIC SYMPATHOMIMETICS ON HIOMT ACTIVITY

DRUG	DOSE (mmol/kg)	ROUTE OF DOSING	HIOMT ACTIVITY (pmol [^{14}C]-MELATONIN/90 MIN/mg PINEAL TISSUE) ± S.E.M.	HIOMT ACTIVITY %	SIGNIFICANCE
ISOPRENALINE	$1x10^{-2}$	p.o.	287 ± 16	97	not significant
ISOPRENALINE	$2x10^{-2}$	p.o.	268 ± 6	93	not significant
ISOPRENALINE	$4x10^{-2}$	p.o.	291 ± 10	101	not significant
ETHYLNORPHENYL-EPHRINE	$4x10^{-2}$	p.o.	294 ± 9	102	not significant
ETHYLNORPHENYL-EPHRINE	$8x10^{-2}$	p.o.	299 ± 6	104	not significant
FENOTEROL	$4x10^{-2}$	p.o.	312 ± 9	109	P< 0,01
HEXOPRENALINE	$4x10^{-2}$	p.o.	319 ± 7	111	P< 0,02
ORCIPRENALINE	$4x10^{-2}$	p.o.	279 ± 12	97	not significant
SALBUTAMOL	$4x10^{-2}$	p.o.	322 ± 4	112	P< 0,02
AMPHETAMINE	$1,4x10^{-2}$	p.o.	277 ± 8	96	not significant
EPHEDRINE	$4x10^{-2}$	p.o.	108 ± 7	73	P< 0,001
NORMAL SALINE		p.o.	2,5 ± 0,5	100	

order to investigate further the effects of β-sympathomimetic drugs and also, if possible, to determine the type of the β-receptor involved in the stimulation of SNAT activity. The rats were again housed under conditions of 12 hours light and 12 hours dark with light on at 06h00 and off at 18h00. β-Sympathomimetic drugs, when injected soon after the onset of the light phase, elicit a rapid rise in SNAT activity which soon declines to base levels again; the activity is not greatly increased. If the drug is administered later on in the day, induction of enzyme activity is slower but the activity also declines more slowly. The increase in activity however is much greater (Romero and Axelrod, 1975). Dosing rats at midday and killing them 3 hours later gave optimal results. The time of dosing, and the period between dosing and killing enabled any alteration in enzyme activity to be easily detected.

In cell cultures the pineal cells maintain the ability to produce enzymes which respond to sympathomimetics. Thus pre-incubation of the pineal homogenate containing the enzyme for two hours with the drug being tested was also tried to see if any activation of the enzyme *in vitro* would result. No effect on enzyme activity was noted.

3.3.1 Effect of β-Adrenoceptor Blocking Agents

Female rats of mass 150 - 180 g were used. Under a lighting schedule of light from 06h00 to 18h00 and dark from 18h00 to 06h00, groups of 4 rats were dosed i.p. at 11h30 with the blocking agent (4×10^{-2} mmol/kg). A control group of 4 rats was dosed i.p. with normal saline. At 12h00, both groups of rats were dosed i.p. with

isoprenaline sulphate 2 x 10^{-2} mmol/kg. At 15h00, the rats were killed and their pineal glands assayed for SNAT activity.

3.3.2 Effect of β-Adrenoceptor Stimulating Agents

The assay of SNAT was performed under exactly the same conditions as with the blocking agents. The rats were dosed at 12h00 with equimolar quantities of different β-sympathomimetics, killed at 15h00 and the pineals assayed for SNAT activity.

Irrespective of their selectivity, all the β-adrenoceptor blocking agents used were active in significantly reducing the amount of [^{14}C]-acetyltryptamine formed by SNAT (fig. 21; table 7). The ability of isoprenaline to stimulate SNAT activity is ascribed to its activation of adenyl cyclase which converts adenosine triphosphate (ATP) to cyclic AMP which in turn controls the synthesis of SNAT enzyme protein (Klein *et al.*, 1970b). β-Blockers prevent the formation of cAMP by blocking the receptor which activates the adenyl cyclase. After induction with isoprenaline on an equimolar basis, pindolol showed the greatest blocking activity while acebutolol showed the least. Of the sympathomimetics used, isoprenaline exerted the greatest stimulatory effect on SNAT and amphetamine elicited the least stimulatory activity (fig. 22; table 8).

From the results, no absolute conclusion can be drawn about the nature of the β-adrenoceptor of the pineal gland. The β_2-selective stimulants i.e. fenoterol, orciprenaline, hexoprenaline and salbutamol exert much less stimulatory activity on SNAT than isoprenaline, suggesting that the receptors involved are probably of a β_1 nature. However,

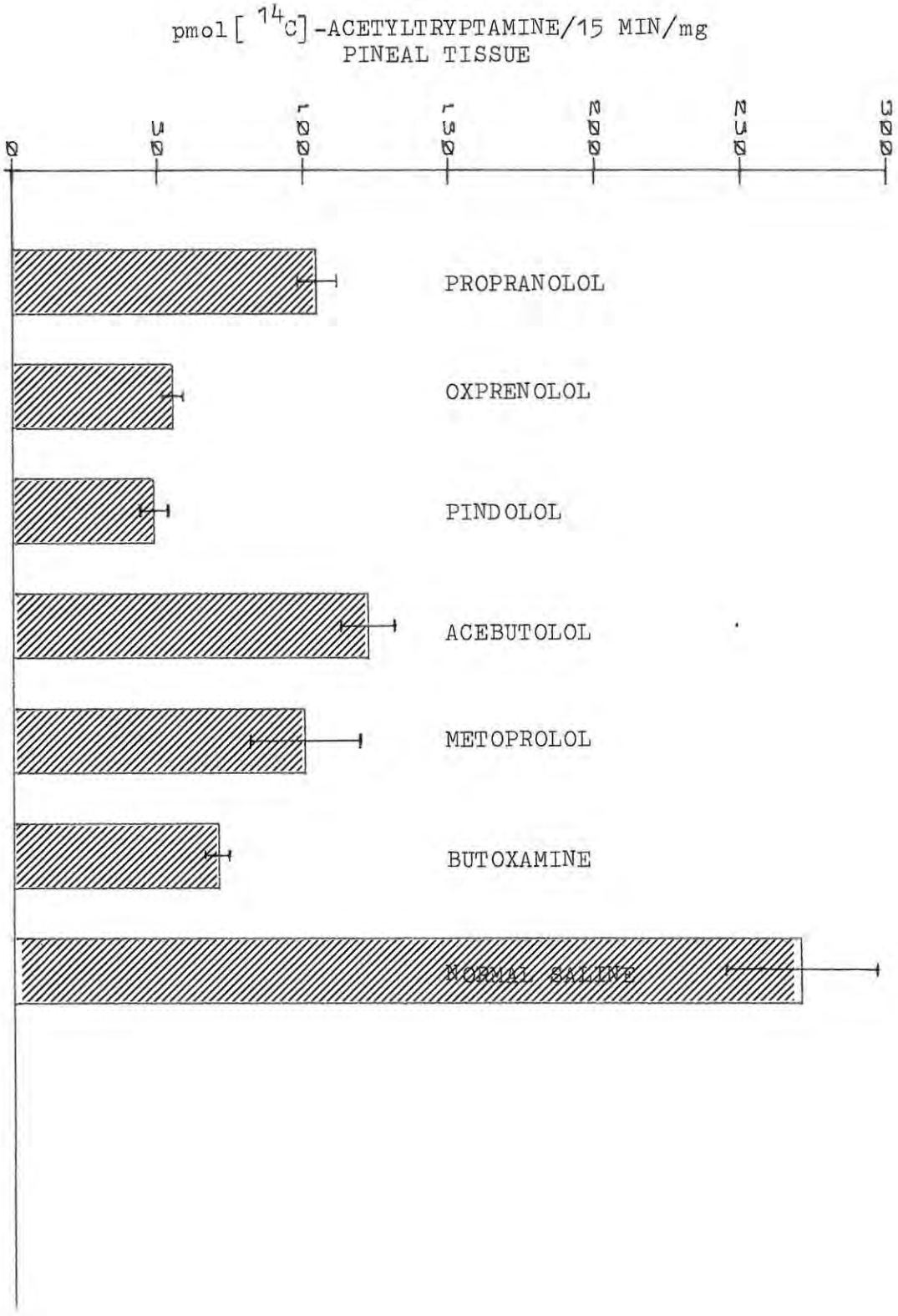


Fig. 21. Effect of β-adrenoceptor blocking agents (4×10^{-2} mmol/kg) on isoprenoline-induced (2×10^{-2} mmol/kg) SNAT activity (see table 7).

TABLE 7

EFFECT OF β-ADRENERGIC BLOCKING AGENTS ON ISOPRENALINE-INDUCED SNAT ACTIVITY

SUBSTANCE	DOSE (mmol/kg)	ROUTE OF DOSING	SNAT ACTIVITY (pmol [^{14}C]-ACETYLTRYPTAMINE FORMED/15 MIN/mg PINEAL TISSUE) ± S.E.M.	PERCENTAGE REDUCTION BELOW NORMAL SALINE (RELATIVE POTENCY)	SIGNIFICANCE
PROPRANOLOL	4×10^{-2}	i.p.	103,7 ± 7	38,5	$P < 0,0005$
OXPRENOLOL	"	i.p.	54,3 ± 3	20	$P < 0,0005$
PINDOLOL	"	i.p.	47,6 ± 5	17,8	$P < 0,0005$
ACEBUTOLOL	"	i.p.	121,2 ± 10	44,8	$P < 0,0005$
METOPROLOL	"	i.p.	99,4 ± 19	36,7	$P < 0,0005$
BUTOXAMINE	"	i.p.	69,8 ± 2	25,9	$P < 0,0005$
NORMAL SALINE		i.p.	269,5 ± 26	100	$P < 0,0005$

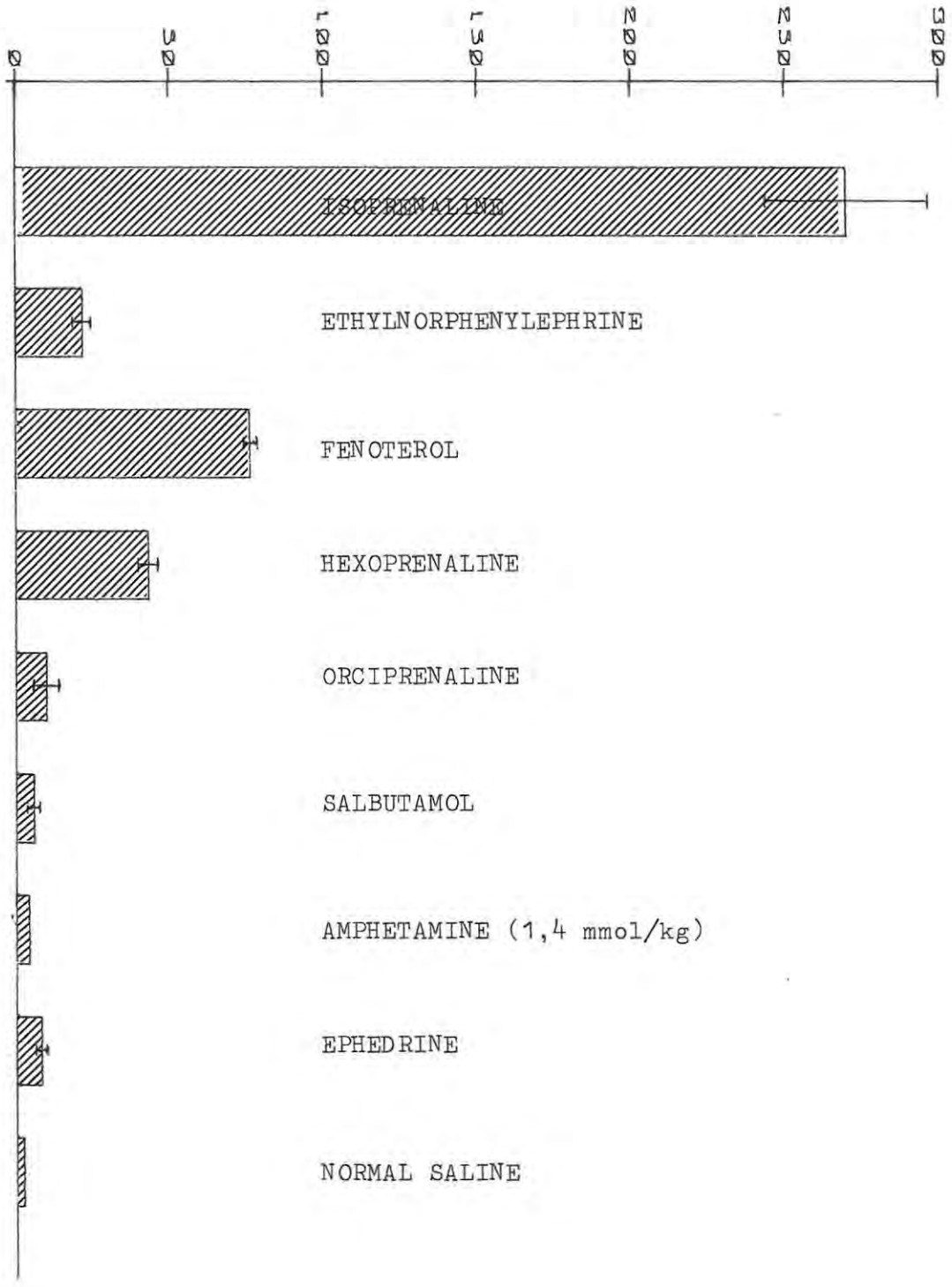


Fig. 22. Effect of β-adrenoceptor stimulating agents (2 x 10^{-2} mmol/kg) on SNAT activity (see table 8).

TABLE 8

EFFECT OF β-ADRENERGIC SYMPATHOMIMETICS ON SNAT ACTIVITY

SUBSTANCE	DOSE (mmol/kg)	ROUTE OF DOSING	SNAT ACTIVITY (pmol [^{14}C]-ACETYLTRYPTAMINE FORMED/15 MIN/mg PINEAL TISSUE) ± S.E.M.	PERCENTAGE BOOST ABOVE NORMAL SALINE (RELATIVE POTENCY)	SIGNIFICANCE
ISOPRENALINE	2×10^{-2}	i.p.	269,5 ± 26	107,8	$P < 0,0005$
ETHYLNORPHENYL-EPHRINE	"	i.p.	21,5 ± 2	8,6	$P < 0,0025$
FENOTEROL	"	i.p.	75,8 ± 2	3,03	$P < 0,0005$
HEXOPRENALINE	"	i.p.	42,9 ± 2	1,72	$P < 0,05$
ORCIPRENALINE	"	i.p.	9,8 ± 3	3,92	$P < 0,005$
SALBUTAMOL	"	i.p.	5,5 ± 2	2,4	$P < 0,0125$
AMPHETAMINE	"	i.p.	4,1 ± 0,5	1,64	$P < 0,025$
EPHEDRINE	"	i.p.	8,0 ± 1,5	3,2	$P < 0,01$
NORMAL SALINE		i.p.	2,5 ± 0,5	1	not significant

ethylnorphenylephrine, which is reputedly a β_1-selective sympathomimetic, also exerts only a small stimulatory effect on SNAT activity. It is possible that the pineal β-receptors are predominantly β_1 in nature but that ethylnorphenylephrine does not reach the pineal in sufficient amounts or its affinity for pineal β-receptors is too low to elicit a pronounced effect on SNAT levels (fig. 22).

The indirectly acting sympathomimetics, amphetamine and ephedrine, have the least effect on SNAT activity (fig. 22). This could be due to their inability to release noradrenaline, the levels of which are low during the day, from nerve terminals. The slightly greater effect of ephedrine may be due to a partial direct action which is exerted by para-hydroxylated ephedrine which is formed in the liver.

It is difficult to classify the pineal β-receptor on the basis of the degree of β-receptor blockade. β_1-Adrenoceptor blocking agents such as metoprolol or acebutolol reduced isoprenaline-induced SNAT activity significantly as did non-selective blocking agents such as isoprenaline and oxprenolol. Butoxamine, a β_2-selective blocking agent also caused a significant reduction in SNAT activity (fig. 21), strongly suggesting that both β_1 and β_2-type receptors are present. This finding differs from that of Bäckström (1977) who used an *in vitro* system to classify pineal β-receptors as predominantly of the β_1 type.

The β-sympatholytics used are compared with propranolol in their ability to reduce enzyme activity. The β-sympathomimetics are compared with isoprenaline in their ability to stimulate the activity of this enzyme. The degree of inhibition or stimulation of SNAT

activity may depend on many other factors such as lipophilicity, intrinsic activity, affinity and metabolism of the substances used as well as biological variation among the rats used.

CHAPTER 4

4.1 DISCUSSION

An attempt was made to categorize the effects of adreno-active substances on the pineal gland by monitoring enzymic changes and to establish whether a link exists between these changes and possible gonadal effects of these substances. In other words, two main lines of attack were planned: (1) to determine whether any of the pineal enzymes were affected by β-adreno-active substances and (2) whether this had any effect on the oestrous cycle. The substances used were selected as representing the major β-adrenergic blocking and β-stimulating substances used in South Africa.

4.1.1 SNAT Sensitivity

In the various experiments performed, SNAT levels proved to be very sensitive to stimulation or inhibition by the substances used. Isoprenaline in particular, a non-selective β-sympathomimetic agent, stimulated enzyme activity the most effectively. The overall degree of enzyme sensitivity depends not only on the time of day, but also on the time of year, enzyme activity fluctuating seasonally as well (Reiter, 1976). As the experiments extended over a period of months, enzyme activity would probably have fluctuated slightly as a result of the seasonal influence. This fluctuation is of no consequence in evaluating the degree of stimulation or inhibition of the enzyme system as results were compared with concurrently-run controls. SNAT appeared to be susceptible to the greatest degree of stimulation during the period preceding the onset of the dark phase, the activity of the stimulated enzyme increasing as the day progresses. This

progressive increase in enzyme sensitivity is accompanied by a concomitant increase in the lag period between dosing and onset of enzyme induction. The lag period is apparently due to messenger RNA being required prior to SNAT activity being increased and this mRNA must first be synthesized (Romero et al., 1975b). Maintenance of high SNAT levels requires continuous occupation of the β-receptor by an agonist. The β-blockers, which compete with β-adrenoceptor stimulants for the receptor sites, exert their effect by displacing the agonist and thereby causing reduction in enzyme activity.

Agents such as fenoterol or salbutamol, which are β_2-selective adrenergic stimulants, were less effective than isoprenaline in stimulating enzyme activity. Ethylnorphenylephrine, a β_1-selective sympathomimetic, showed very little enzyme stimulatory activity. It is quite possible therefore that the pineal β-adrenoceptor is of such a nature that an agonist or antagonist possessing non-specific characteristics is required to elicit a response on this receptor. The results obtained seem to substantiate this point of view. On the other hand, there may be a mixed population of β_1 and β_2 receptors in the pineal. Occupation of neither group alone would exert maximal stimulant or inhibitory effects.

Pindolol, a non-selective β-adrenergic blocking agent, proved to be most effective in reducing isoprenaline-induced SNAT activity. Butoxamine, a β_2-selective adrenergic blocker, was also found to be a moderately proficient blocking agent. According to the results obtained, the more β_1-selective agents were less effective.

The receptors of the pineal thus seem to be of mixed nature. This difference from the results of Bäckström (1977) who classified pineal β-receptors as mainly β_1 could be as a result of using an *in vivo* system instead of an *in vitro* one. In the latter system which was used by Bäckström, the substances being tested would have had greater accessibility to the receptors than is the case with the *in vivo* model. Liver degradation and first-pass clearance, formation of active metabolites and other effects may also be implicated in the difference in results.

Of the agents used, the β-adrenoceptor antagonists, in addition to their receptor-blocking activity, also possess other properties on cardiac tissue such as intrinsic sympathomimetic activity (i.s.a.), membrane stabilizing activity (m.s.a.) and cardioselectivity. Although one or more of these effects may be implicated in the overall effect of the drug on the pineal, they do not necessarily contribute to such an effect. The effect of pindolol and oxprenolol, which possess i.s.a., on SNAT activity may however be a combination of blocking activity plus this partial agonist effect.

The age of the animals used was also found to influence the effect of adreno-active substances on SNAT activity. Enzyme activity was less susceptible to stimulation as the age of the rats increased. This is probably due to a decreased sensitivity of the β-receptors as discussed by Greenberg and Weiss (1978). A decrease in the number of biochemically active pinealocytes due to calcification with increasing age may also be of importance. Other factors such as hepatic blood flow, cardiac output and drug-metabolizing enzyme activity may also influence the degree of effect of the agents used on SNAT activity.

Possible variation in the metabolism and distribution of β-active substances or their metabolites by individual animals may also contribute to slight differences in the effect of these drugs. These variations among the rats used were however limited by using rats from one population with approximately the same mass and age.

4.1.2 HIOMT Activity and the Oestrous Cycle

Although no apparent effect on HIOMT activity was noted, it is possible that β-active substances may have exerted an effect on this enzyme which could not be detected as such an effect was very small and the technique used was relatively insensitive. In view of the fact that the diurnal rhythm of HIOMT has a relatively small fluctuation in amplitude, stimulation or inhibition of the production of enzyme protein (by variation in light intensity for example) may already be occurring at limits approaching the maximum. A β-active substance would thus elicit a negligible effect assuming of course that HIOMT activity is controlled by stimulation of the pineal β-adrenoceptor. One should not rule out the possibility that HIOMT levels are controlled by another physiological mechanism such as the dopaminergic system or negative feedback by hormones — especially oestrogen. The intermediate second messenger system may not be cAMP but cyclic guanosine monophosphate (cGMP). If this is the case, it would account for the lack of effect by the agents used on HIOMT activity.

It could be argued that the significant effect of ephedrine on HIOMT activity is due to an influence on other centres in the brain. If this is the case, one would have expected a similar result with amphetamine. As no such effect was found when amphetamine was used, some other unknown factors may be present which caused ephedrine

to act in an inhibitory fashion on the enzyme.

The pineal exerts a demonstrated antigonadotrophic effect in the rat. With the knowledge that β-active substances modify pineal metabolic activity, it seems logical to assume that these substances would consequently exert some sort of influence on reproductive physiology. The oestrous cycle may be regarded as a fairly useful parameter for monitoring any such gonadal effects. Although, with the exception of propranolol, β-active substances did not influence the oestrous cycle to any marked degree, a more intensive study of effects on the oestrous cycle may well have produced results from which more absolute conclusions would have been drawn. For example, significant change in dosage levels, route of administration, time and frequency of dosing and proportion of light/dark may well have produced more significant results. Unfortunately, this would have required great numbers of rats and would have been extremely laborious and time-consuming, thus mitigating against extending that line of investigation. Propranolol, which did exert some effect on the oestrous cycle, may well have caused this effect by a direct action on the hypothalamus or pituitary or the effect may have been due to a combined action on these two areas as well as on the pineal. However, as the effect of propranolol differed during dark and light, this effect on the oestrous cycle seems most likely to be pineal-mediated. Propranolol was also a very effective blocker of pineal SNAT, thus reinforcing the concept of pineal involvement.

Aside from any central or pineal-mediated influence of β-sympatholytics or sympathomimetics on the oestrous cycle, these substances may well influence the oestrous cycle directly by acting on uterine β-receptors. The effect here depends not only on the

species of the animal, but also on the stage of the sexual cycle, the stage of gestation if conception has taken place and the dose given. In vitro, sympathomimetics have been shown to contract strips of pregnant or non-pregnant uterus. These effects differ in situ however, making precise predictions of overall effects of β-active substances on uterine muscle difficult. Just before, and at parturition, β_2-selective sympathomimetics have been shown to inhibit uterine tone and contractions in vivo. Presumably β-adrenoceptor blocking agents would reverse this effect. More selective β_2-receptor stimulants such as fenoterol or salbutamol have been used successfully to delay premature labour thus adequately demonstrating the effectiveness of β-active agents in inhibiting uterine tone (Liggins and Vaughan, 1973). Propranolol may well have affected the oestrous cycle by altering uterine tone.

If one considers the involvement of the pineal in gonadal function, it is apparent that from a clinical point of view, administration of β-active substances, which are currently widely used in medicine, may have far-reaching secondary effects not yet discovered. This applies especially to patients taking medication on a long-term basis. Examples of such are hypertensive patients taking β-adrenergic blocking agents or asthmatic patients on β_2-adrenergic stimulating agents. It is possible that fertility may be affected. The pineal, which has many rhythms of its own, may also control other biorhythms and these may in turn be affected by adreno-active substances. Interference with these rhythms (by blocking them or causing rhythmic phase changes for example) may in the long run seriously impair physiological processes or behaviours which are of a rhythmic nature. Rhythmic functions such

as sodium excretion, insulin release and ACTH release, which are endogenous in nature, may be significantly altered, thereby affecting mood and drive for example. With light and dark having an influence on the pineal as well, these potential effects may vary at different times of the day thus complicating the issue, this would again lead to increased attention to chronopharmacology as opposed to factors such as bioavailability. It must also be realised that any such effects of adreno-active substances are not necessarily mediated only by the pineal. They may involve other areas of the brain alone or in combination with the pineal or they may work at a more direct level on peripheral organs such as the uterus or ovaries.

In addition to possible gonadal effects, the apparent existence of a pineal-adrenal axis and a pineal-thyroid axis as discussed in Chapter 1 gives rise to speculation on the potential effects of adreno-active substances on adrenal and thyroid function and on overall metabolic function. Rhythms such as that of corticosterone as well as production of glucocorticoids, mineralocorticoids and thyroid hormones may be markedly influenced. Melatonin for example, is known to influence TSH (thyroid stimulating hormone) production. β-active substances, which can alter melatonin biosynthesis, are thus most likely to exert an effect on TSH. All these potential pineal effects may be either direct or <u>via</u> the hypothalamus or pituitary. Overall adrenal and thyroid metabolic effects thus suggested may in turn indirectly influence fertility as it is known that the thyroid gland for example is implicated in reproductive function. Viewed as a whole, pineal involvement in reproductive physiology is quite likely to be multi-factorial, a combination of

effects on other endocrine systems contributing to an overall effect of the pineal.

4.2 CONCLUSION

The rat pineal gland is a useful model for studying substances which act on the β-adrenoceptor. A parameter such as effect on SNAT activity may be used to classify such substances in order of potency. The β-receptors appear to be both β_1 and β_2 in nature. HIOMT does not appear to respond to β-active substances. One can speculate on the likelihood of the sympathetic nervous system not exerting any control over this enzyme. Alternatively, any alteration in HIOMT levels is of such a low order that it is not possible to detect it using the existing method of enzyme assay.

There does not appear to be a conclusive link between the alteration in pineal enzyme activity caused by β-active substances and an influence on the oestrous cycle. As further research in this field is needed, it would be interesting to investigate the possible pineal-mediated effect of β-active substances on adrenal and thyroid function and see if any such effects can influence overall body metabolism and gonadal function. Instead of the oestrous cycle, one could possibly also investigate the effect of β-active substances on other gonadal processes such as ovulation or number of foetuses produced. Any research in this direction would be well worth the effort as much remains to be learnt about the pineal gland which is indeed a unique organ. Chrono-pharmacology is now taking its place as a relevant and important field of study and it is bound to become even more so in the near future.

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