THE PINEAL GLAND
AS A MODEL TO ELUCIDATE
THE PRIMARY MODE OF ACTION OF SYMPATHOACTIVE AGENTS

THESIS
Submitted in Fulfilment of the
Requirements for the Degree of
MASTER OF SCIENCE
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by
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"Then said a teacher, Speak to us of Teaching.

And he said:

No man can reveal to you aught but that which already lies half asleep in the dawning of your knowledge.

The teacher who walks in the shadow of the temple, among his followers, gives not of his wisdom but rather of his faith and his lovingness.

If he is indeed wise he does not bid you enter the house of his wisdom, but rather leads you to the threshold of your own mind."

Kahlil Gibran (1883 - 1931)

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List of Abbreviations Used

aHT	N-Acetylserotonin (5-Hydroxy-N-Acetyltryptamine)
aMSH	Alpha-Melanocyte-Stimulating Hormone
aMT	Melatonin (5-Methoxy-N-Acetyltryptamine)
BP	Blood Pressure
CAT	Choline Acetyltransferase
cAMP	Adenosine 3'5'-Monophosphate (cyclic AMP)
cGMP	Guanosine 3'5'-Monophosphate (cyclic GMP)
CNS	Central Nervous System
COMT	Catechol-O-Methyltransferase
cpm	Counts Per Minute
DBH	Dopamine-beta-hydroxylase
DDC	Dopa Decarboxylase
DG	Diacylglycerol
DNA	Deoxyribonucleic Acid
dpm	Disintegrations Per Minute
DSIP	Delta-Sleep Inducing Peptide
FA	Fatty Acids
GABA	Gamma-Aminobutyric Acid
GER	Granular Endoplasmic Reticulum
GFA	Glial Fibrillary Acidic (Protein)
GV	Granular Vesicles
h	Hours
HIOMT	Hydroxyindole-O-Methyltransferase
HNMT	Histamine-N-Methyltransferase
5-HT	Serotonin (5-Hydroxytryptamine)
5-HIAA	5-Hydroxyindoleacetic Acid
HPLC	High Performance Liquid Chromatography
5-HTOH	5-Hydroxytryptophol
i.p.	Intraperitoneal
L-Dopa	L-Dihydroxyphenylalanine
LH-RH	Luteinizing Hormone-Releasing Hormone
min	Minutes
MAO	Monoamine Oxidase
MDA	Alpha-Methyldopamine
MeDopa	Alpha-Methyldopa

ME	Alpha-Methylepinephrine
MEM	Minimal Essential Medium
5-MIAA	5-Methoxyindoleacetic Acid
MNE	Alpha-Methylnorepinephrine
MRIH	MSH-Release Inhibiting Factor
5-MTOH	5-Methoxytryptophol
NADP	Nicotinamide Adenine Dinucleotide Phosphate
NAT	Serotonin-N-Acetyltransferase
NE	Norepinephrine
NGF	Nerve Growth Factor
NTS	Nucleus Tractus Solitarius
PBS	Phosphate Buffered Saline
RNA	Ribonucleic Acid
s	Seconds
S.E.M.	Standard Error of Mean
TLC	Thin Layer Chromatography
t_r	Retention Time
TRH	Thyrotropin Releasing Hormone
UV	Ultra-Violet
VCR	Vesicle Crowned Rodlets

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Abstract

An attempt was made to use the pineal gland as a model for the study of the primary mode of action of sympathoactive agents. Two drugs were investigated, viz. alpha-methyldopa and ephedrine whose mode of action is not entirely clear. Organ cultures of pineal glands from rats treated chronically with alpha-methyldopa showed enhanced conversion of radioactive serotonin to melatonin (aMT), as well as its precursor N-acetylserotonin (aHT). This treatment was also found to raise N-acetyltransferase (NAT) activity. These increases associated with alpha-methyldopa treatment were further enhanced by the beta-adrenergic agonist, isoproterenol, suggesting a supersensitivity-type effect occurring at the level of the beta-receptor. A subsequent binding study, however, showed a decrease in beta-receptor binding with exposure to alpha-methyldopa, providing mitigating evidence against the occurrence of a supersensitivity phenomenon. It is possible that a metabolite of alpha-methyldopa acts as an alpha 1 and beta-adrenergic agonist, resulting in greater melatonin (aMT) and N-acetylserotonin (aHT) synthesis than by a beta-adrenergic agonist, isoproterenol. Combined treatment of pineals with alpha-methyldopa and an alpha-receptor blocker, phentolamine, resulted in melatonin (aMT), N-acetylserotonin (aHT), and N-acetyltransferase (NAT) activity levels which were lower than those obtained with alpha-methyldopa treatment alone, thus confirming the alpha-adrenergic activity of the metabolite of alpha-methyldopa. Additional pineal metabolites were isolated and measured simultaneously in the organ culture experiments. Organ cultures of rat pineal glands treated with ephedrine showed raised levels of melatonin (aMT) and N-acetylserotonin (aHT). Treatment with ephedrine also produced raised N-acetyltransferase activity. A further enhancement of these parameters was induced by norepinephrine, suggesting a supersensitivity-type effect occurring at the level of the beta-adrenergic receptor. Rats were treated with reserpine (a norepinephrine depleter) and the pineals exposed to ephedrine. Endogenous norepinephrine normally released by the action of ephedrine was thus absent, and under these conditions, levels of melatonin (aMT) and N-acetylserotonin (aHT) were reduced. N-acetyltransferase (NAT) activity was also reduced, but maintained levels pointing to substantial adrenergic activity of ephedrine as well as norepinephrine

released by virtue of the drug's action. A subsequent binding study showed a decrease in beta-adrenergic receptor binding with exposure to ephedrine and a further decrease in ephedrine treated pineals from reserpine treated rats, thus ruling out the occurrence of a super-sensitivity phenomenon. It is possible that both ephedrine and released norepinephrine have alpha- and beta-receptor activity. Additional pineal metabolites were isolated and measured in the organ culture experiments. A 16-hour time profile of the production of melatonin (aMT) and N-acetylserotonin (aHT) with norepinephrine and ephedrine treatment provided useful information regarding the course of action of the two agents. A pineal cell-culture system was developed and exposed to ephedrine and norepinephrine. N-acetyltransferase (NAT) activity levels measured after exposure to these agents were raised, confirming the adrenergic activity of both in the model. Finally, an HPLC system coupled to a UV detector was used in an attempt to measure melatonin (aMT) extracted from pineal organ culture media. The results showed that melatonin could be measured by this method, however, a more sensitive detection system was recommended for future work.

CHAPTER 1
LITERATURE REVIEW

1.1 Introduction

Located near the centre of the mammalian brain, is a diminutive white structure, which assumes the shape of a pine-cone, the pineal gland. Described by René Descartes as being the seat of the rational soul, the organ has become an object of mystery, attracting the attention of a variety of disciplines, keen to unravel the secrets locked within. Studies showed it to be susceptible to periodic nervous activity generated by light, converting this input into hormonal information, namely melatonin, a ubiquitous biochemical envoy, charged with numerous effects on normal metabolism.

The advent of neuroendocrinology provided a useful conceptual framework within which the functions of the pineal gland could begin to be described. The cyclic nature of the gland's activity earned it the title of a "biological clock", a switching mechanism, regulating the activity of the sex glands in response to the changing seasons and exposure to light and dark. Perhaps appropriately labelled as "the last great frontier" in the study of physiology, increased emphasis has been placed on this "neuroendocrine transducer" and its chemical association with the functions of other organs.

The fundamentally unique nature of the pineal gland, and its discreet association with the sympathetic nervous system has inspired novel avenues of research. The original idea that the pineal might respond when challenged with certain agents lent a new tool to the armoury of biochemists and pharmacologists involved with this type of study.

Over the last three decades extensive investigation into the effects of administered substances on the pineal gland has been performed. These studies have afforded a substantial measure of understanding of the biochemistry of the gland, as well as providing important information on the pharmacology of various sympathomimetic drugs. The rat pineal is useful for this purpose for a variety of reasons. In the first instance, it is situated outside the blood-brain barrier, and is thus accessible to drugs administered peripherally. It has the

ability to maintain its metabolic functions in organ culture, which enables investigations of the effects of various drugs on the gland. The pineal gland is innervated exclusively by sympathetic nerves, from the superior cervical ganglia, thus making it an inviting prospect for the study of sympathoactive agents and their antagonists. A well-characterized biochemical pathway linked via a second-messenger system to a rich supply of beta-adrenergic receptors, as well as alpha-adrenergic receptors provides a useful model for the study of such drugs. Pineal metabolites, in particular, melatonin and its precursor N-acetyl-serotonin, as well as associated enzymes can be conveniently assayed. Important information, therefore, on the effects of agents on the internal metabolism of the pinealocyte, including the status of the beta-adrenergic receptor, can be derived.

This study served to explore the use of the rat pineal gland as a model for investigation into the modes of action of two sympathoactive agents viz. alpha-methyldopa, and ephedrine, whose mechanisms of action have not been entirely elucidated. In an effort to expand the experimental boundaries of the study, and to thereby improve the suitability of the pineal gland as a model for such an investigation, a variety of techniques were employed. This document, therefore, will hopefully serve as a useful experimental base from which further endeavours of this nature may be planned.

1.2 History of Pineal Discovery and Research

Three separate periods of pineal research can be described.

1.2.1 The First Period

Herophilos (325 - 280 B.C.), a renowned anatomist at the University of Alexandria in Egypt, was probably first in discovering the pineal gland in man. He understood the pineal to be a tap, regulating the stream of the "pneuma" or spirit from the third to the fourth ventricle.

Galen (± 130 - 300 A.D.) did not support the tap function concept, as his observations placed the pineal outside of the brain ventricular system. Galen considered the pineal to have an exclusively glandular function, occupying the space between the internal cerebral veins at

the point where they both drain into the great vein of Galen. Galen coined the anatomical term "soma konoeides" or "konareion" to describe the pine cone-shaped gland.

The Renaissance heralded a more personal approach to the study of the pineal. Berengario da Carpi (1470 - 1530) was first in performing a more careful examination of the gland, while Andreas Vesalius (1514 - 1564) in the famous "De Humani Corporis Fabrica, Libri Septem", provided a more modern illustration of the organ. Vesalius (1555) described the topography and consistency of the pineal gland but offered little in terms of a suggested function for the gland.

René Descartes (1596 - 1650) is widely cited as stating that the pineal gland is the seat of the soul. Localization of the soul was to the minds of philosophers and scientists at that time, a fundamental area of research. Descartes in point of fact specifically states that in principle, the soul cannot be localized in any precise region of the body, because it is related with all of its parts, according to the doctrine of Aristotle. He considered, however, that the soul would be more expressed in the pineal, the only unpaired part of the brain. According to Descartes, an optical image can pass from the retina to the pineal (or "gland H," as he refers to it). The pineal gland is thereby induced to transmit its spirits, by moving in different directions, to different motor nerves, thus "stimulating" them. Paired with this mechanism, an "âme raisonable" would operate, influencing the activity of the pineal gland.

Ancient theories on the soul and spiritus haunted the province of neuroscience research for more than 20 centuries, before new concepts cleared the path for more useful research.

1.2.2 The Second Period

A renewed and more systematic interest in pineal comparative anatomy, histology and embryology typified the commencement of the second era in pineal research.

Ahlborn (1884) and Rabl-Rückhardt (1886) pointed out the analogy between the vesicular non-mammalian pineal organ and the primary optic

vesicles. According to De Graaff (1886a, b) and Korschelt and Spencer (cit. Studnicka, 1905), the amphibian and reptilian epiphysis is a photosensory organ and homologous to the mammalian pineal gland. The pineal thus exhibits phylogenetic regression. Studnicka (1905) states that, phylogenetically, the pineal develops from a photoreceptor organ into a complicated gland possessing an enigmatical function.

Subsequent to the discovery of the endocrine organs by Claude Bernard (1813 - 1878) and Brown-Sequard (1817 - 1894), the interest of physiologists became more vivid. By the close of the 19th century, the mammalian pineal gland was being considered as having an endocrine function. Heubner (1898) was first to describe a boy suffering from a pinealoma and presenting signs of precocious puberty. According to Marburg, the human pineal is an endocrine gland which, in youth, would exert an inhibitory effect on hypothalamic function and, via the hypothalamus, on the development of the reproductive organs. Berblinger (1920) and Engel (1936) were convinced that the pineal gland exercises an inhibitory effect on gonadal development.

These views were however criticised when the structure and function of the hypothalamus became clearer. It was felt that the pressure exercised by the pinealoma on the hypothalamic centres associated with regulation of sexual functions were responsible for the abnormalities in sexual development, rather than as a result of any effects exerted on the pineal itself (Bustamente et al., 1942; Bustamente, 1943; Driggs and Spatz, 1939; Spatz, 1942, 1955).

A valuable contribution to the literature on pineal research is the literature survey of Bargmann (1943). In his work, the author provides details of the microscopic anatomy and histology of the pineal organ in all vertebrates, also devoting attention to the dependency of the microscopic structure of the mammalian pineal on internal and external conditions. The author emphasizes the necessity of investigations on the influence of light on the pineal gland. Bargmann also provides an extensive revue of all endocrinological work on the vertebrate pineal performed to date. He is of the opinion that the organ would possibly modulate hypothalamic function, an idea based upon the earlier work of Roussy and Mosinger (1938a, b) who suggested

the existence of neural pathways between the pineal and the hypothalamus.

Following Bargmann, comparative anatomical data confirm the theory that phylogenetically, the organ changes from a photoreceptor into a secretary organ, the photosensory cells losing their receptor organelles and developing into mammalian pinealocytes.

It is apparent that, in the first half of the 20th century, the main line of phylogenetic evolution was known, while also the idea of an antigonadotropic pineal influence and the functional cooperation between the pineal, the hypothalamus and the pituitary was a strong supposition.

1.2.3 The Third Period

The third period corresponds to history of pineal research as well as present and future trends for research. In this era, pineology in vertebrates has developed into a generally recognized field of research, becoming multi-disciplinary in nature. The number of special investigations, and consequently of articles published has increased dramatically during this period. Important developments have occurred in the areas of pineal biochemistry, pineal cytology, pineal histochemistry, pineal pharmacology, and pineal endocrinology.

1.3 Pineal Anatomy

1.3.1 Location of the Rat Pineal Gland

The pineal gland, or ephiphysis cerebri, is situated in the rat brain, between the two cerebral hemispheres, just forward of the cerebellum. Viewed laterally, it is superficially located just below the skull plate (Fig. 1.1). The rat pineal is covered by the confluence of the superior sagittal sinus and the transverse sinus.

1.3.2 The Pineal Stalk

In the rat, the pineal body is connected to the commisural region by a thin pineal stalk. The stalk is formed by fusing and growing out of the anterior and posterior epiphyseal peduncles originating from the most proximal parts of the rostro-dorsal and caudo-ventral walls of the original epiphyseal evagination.

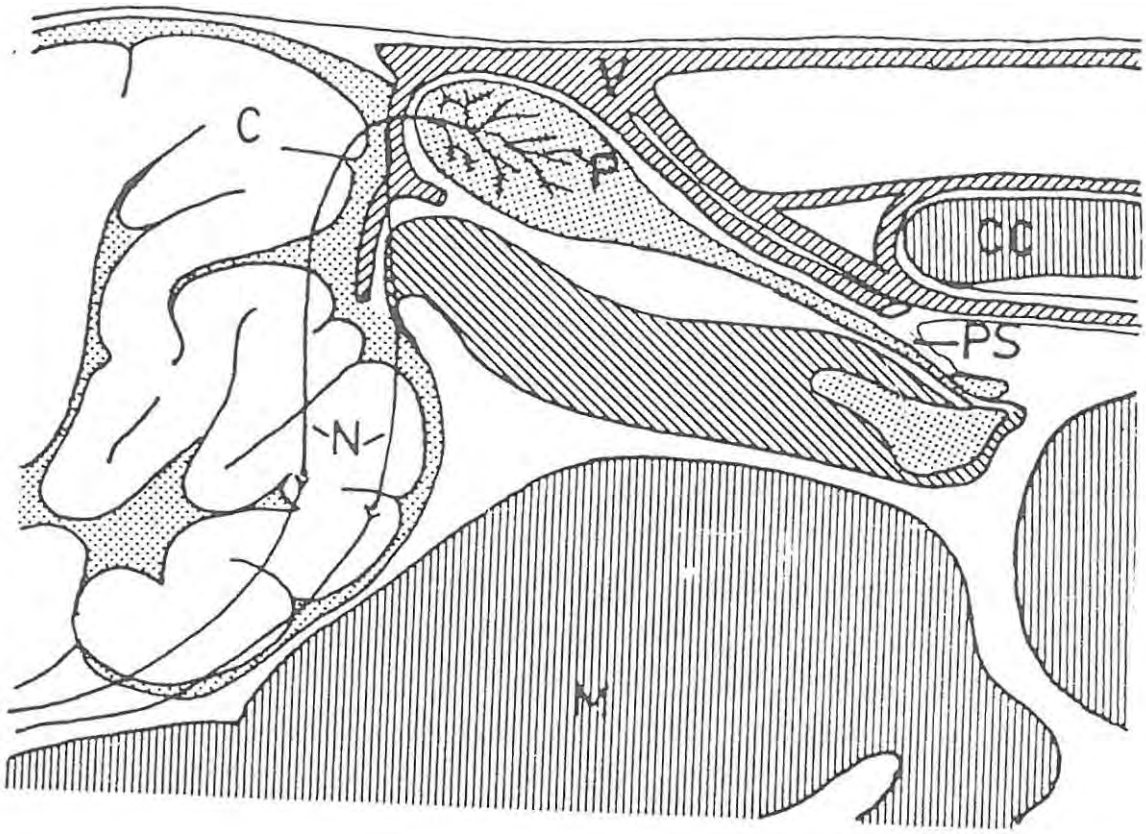

Fig 1.1 Lateral view through the midplane of the adult rat brain indicating location of the pineal gland and sympathetic nervous innervation. C = cerebellum; P = pineal gland; CC = corpus callosum; V = veins; PS = pineal stalk; M = medulla; N = nerve from superior cervical ganglia.

The stalk consists of three distinct parts, viz: a proximal part, a midpart and a distal part. The proximal part is situated between the habenular and caudal commissures. The midpart is a very thin structure. The distal part is the largest of the three components of the stalk and is located between both the rostral and caudal collicles.

1.3.3 Vascularization of the Rat Pineal

The rat pineal is a densely and uniformly vascularized organ with a separate arterial supply, consisting of up to four branches of the posterior cerebral artery. The capillary network has a similar density as in the cerebral cortex, and the venous drainage consists of 12 - 16 short veins, all draining in the great cerebral vein and thereafter via the confluens sinuum immediately in the systematic venous circulation (Hodde, 1979).

1.3.4 Pineal Innervation

Two types of pineal innervation can be identified viz: a pinealofugal (afferent) and a pinealopetal (efferent) one. As pinealofugal innervation is related to the transport of the influence of photic stimuli, transduced by photosensory cells, to brain centres, it is exclusively observed in non-mammalian pineals containing functional photoreceptor cells. Pinealofugal innervation is achieved by pineal sensory neurones amongst which several types have been identified viz: multipolar, bipolar and pseudounipolar elements.

The mammalian pineal gland is extensively innervated by pinealopetal orthosympathetic fibres which originate in the superior cervical ganglia and reach the organ via nervi canarii and along pineal vessels (Kappers, 1960). Subsequently, it has been shown that sympathetic pineal innervation is of cardinal importance for the pineal secretory process in mammals. Wolfe *et al.*, 1962 showed that the neurotransmitter involved is norepinephrine.

In certain mammals, the existence of a parasympathetic pinealopetal innervation alongside a sympathetic innervation was demonstrated (Kenny, 1961; Romijn, 1973a, b, 1975). The terminals of neither the postganglionic noradrenergic sympathetic nor the postganglionic

acetylcholinergic parasympathetic fibres form true synaptic contacts with the pinealocytes, ending either in the parenchyma or in pericapillary spaces.

"Hairpin loops" are often formed by aberrant commissural fibres of the habenular and posterior commissure, in the proximal part of the mammalian pineal stalk, or in the deep part of the pineal, if present (Kappers, 1960). Electrophysiological investigations (Dafny et al., 1975; McClung and Dafny, 1975; Dafny, 1977; Ueck, 1979) demonstrate that stimuli originating in parts of the limbic system of the brain travel to the pineal by way of the medullary striae, the habenular nuclei and the pineal stalk. It may be observed, thus, that pineal function may not exclusively depend on stimuli of various physiological origin reaching the gland via its sympathetic innervation.

There exists indirect histophysiological, histochemical and biochemical evidence for the possible presence of extrahypothalamic neurosecretory fibres in the pineal stalk of cattle and sheep (Lukaszyk and Reiter, 1975), rat (Pévet et al., 1978, 1979) and monkey (Lukaszyk and Reiter, 1974). The function of such pineal neurosecretory fibres is unclear, but with regard to peptidergic compounds demonstrated in the organ their presence should be taken into consideration.

The mammalian pineal gland is referred to as a "neuroendocrine transducer organ" by virtue of its ability to synthesize and release melatonin in response to a neural input. An external light stimulus is converted to photic signals in the retina. These signals pass via the suprachiasmatic nucleus, to the tubural hypothalamus, over the medial forebrain bundle, reticular formation, and upper thoracic intermediolateral cell column, to the superior cervical ganglion, whose postganglionic sympathetic fibres, travelling along the tentorium cerebelli, enter the pineal gland via the conarian nerve (Ebadi et al., 1986).

Neural innervation of the pineal is illustrated in Fig. 1.2.

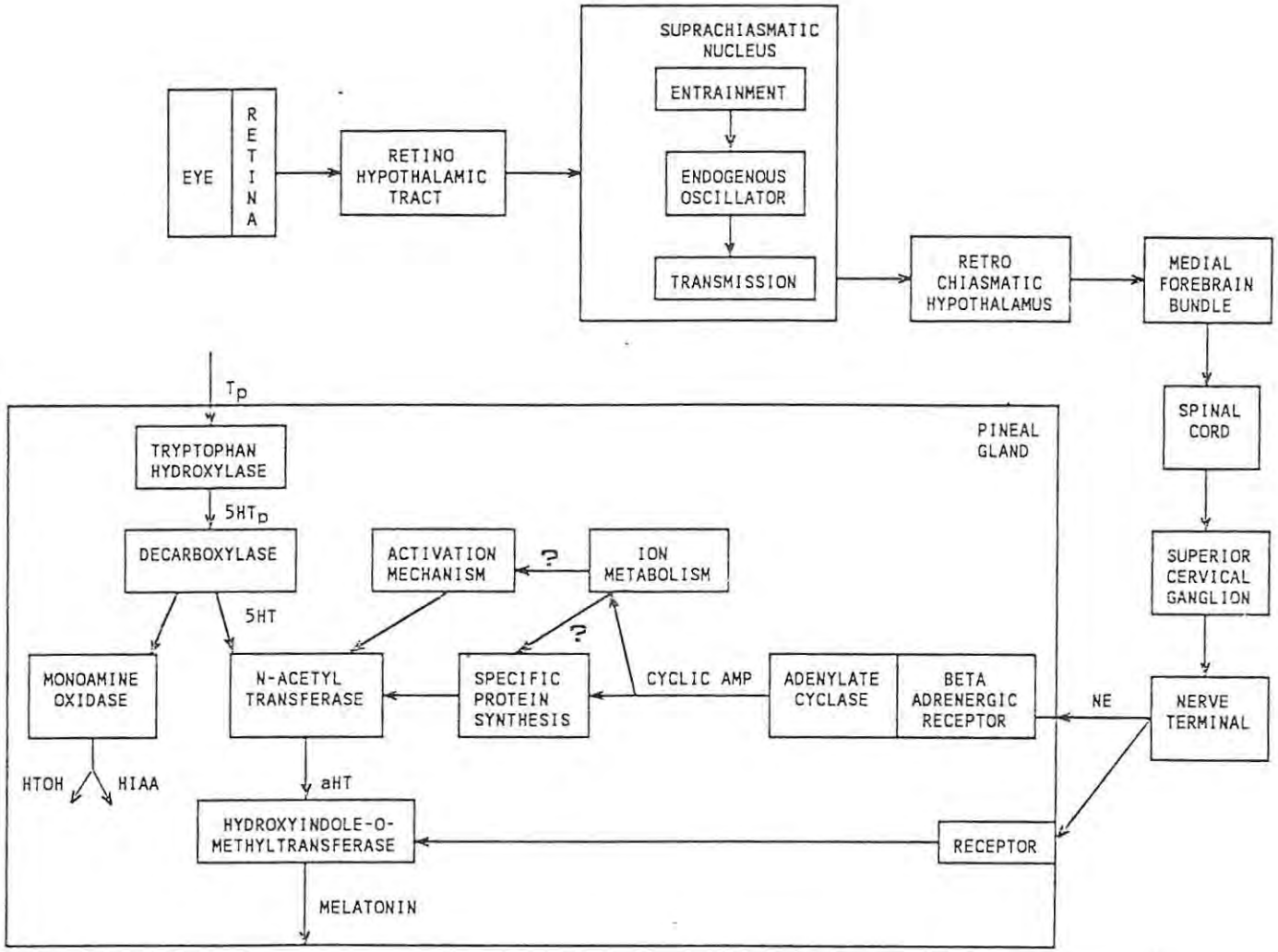

Fig 1.2 A schematic representation of the neural control of the pineal gland. NE, norepinephrine; cyclic aMP, adenosine 3′5′-cyclic monophosphate; T_p, tryptophan; 5HTp, 5-hydroxytryptophan; 5HT, 5-hydroxytryptamine, serotonin; HIAA, 5-hydroxyindole acetic acid; HTOH, 5-hydroxytryptophol; aHT, N-acetylserotonin. The question marks indicate unproven hypotheses.

1.4 Pineal Cytology

1.4.1 First category of cells

In mammalian pineals, one category of cells is predominant viz: the first category. These cells are referred to in the literature as pinealocytes, pinealocytes of population I, light pinealocytes, parenchymal cells, clear pinealocytes and light or dark chief cells. These cells are virtually all characterized by the presence of granular vesicles (GV). It has been shown by Pévet et al., (1977) that phylogenetically, these cells are derived from the neurosensory photoreceptor cells present in the pineal organ of submammalian vertebrates, and that they are members of the sensory cell line as established by Collin (1971). As a consequence thereof, this category of cells may be termed true pinealocytes.

1.4.2 Second Category of Cells

The second category of pineal cells, typified by their location close to the perivascular space and by the absence of GV, is extremely complex in composition. Cells of this category most often represent the so-called interstitial or glial cells, but in certain mammals another group of cells different from both glial cells and true pinealocytes, is present. These cells have been termed pinealocytes of population II in noctule bats and mole-rats, pigment-containing cells in chinchilla and pocket gophers, and dark pinealocytes in rabbit and guinea pigs.

1.4.3 Pineal Cells of the Rat

Only one single population of true pinealocytes has been described in the rat, with the exception of a description by Arstla, (1967).

1.4.4 Ultrastructure of the Pinealocyte

1.4.4.1 Cytoplasm

Ribosomes are spread throughout the cytoplasm, singly, and in clusters of 4 - 12 ribosomes. The ribosomes do not form any association with membranes except in the case where a few are associated with paired cytoplasmic membranes and with the exterior layer of the nuclear envelope to form the rough endoplasmic reticulum.

1.4.4.2 Nucleus

The nuclear envelope comprises perinuclear cisterna which are joined by nuclear pores. Chromatin material is situated in an irregular peripheral area which in certain places penetrates further into the nucleus. The chromatin material is arranged in a combination of helices, granular masses , and mesh structures. The nucleoli appear as amorphous areas, surrounded by a dense nucleolonema. Ribonucleoprotein granules are also present.

1.4.4.3 Secretory Processes in the Pinealocyte

At least two secretory processes involved in the synthesis of pineal peptidergic compounds are present in the mammalian pinealocyte. One involves the formation of granular vesicles by the Golgi apparatus and the other, the production of material by the cisternae of the granular endoplasmic reticulum. The latter process has been termed an ependymal-like secretory process (Pévet, 1979).

1.4.4.4 Granular Vesicles

Granular vesicles (GV) have been observed in the pinealocytes of many mammalian species (Pévet, 1979). GV originate from the Golgi saccules and are found in the perikarya, including the processes of the pinealocytes. The idea that GV represent a major secretory product in a "packaged" form is generally accepted. Certain authors maintain that the GV may contain an antigonadotrophic compound (Benson and Krasovich, 1977; Upson and Benson, 1977).

1.4.4.5 The Ependymal-Like Secretory Process

In many mammals, including the rat (Karasek and Marek, 1978), a peculiar activity of the granular endoplasmic reticulum (GER) leading to either an accumulation of proteinaceous material in its cisterns, or to the formation by the cisterns of vacuoles containing flocculant material has been described. It would appear that the ependymal-like secretory process plays an important role in pineal physiology. The formation of these vacuoles has been linked to antigonadotrophic pineal activity in the rat (Karasek et al., 1976), as well as other mammals. Pineal antigonadotrophic compounds, however, have not yet been demonstrated to be produced by this type of secretory process.

1.4.4.6 Concentric Lamellae

Concentric lamellae or membrane-whorls are observed in the pinealocytes of certain mammalian species. Similar circularly organized reticular formations have been described in numerous cell types, particularly in endocrine glands. Although the function of these structures in the endocrine cells is unclear, some authors infer that their formation is in some manner associated with secretory activity (Pévet, 1979).

1.4.4.7 Glycogen, Vacuolar Systems, and Calcareous Concentrations

Glycogen granules are observed in pinealocytes, and are especially abundant in the pineals of species which are exposed to low environmental lighting. The precise function of glycogen metabolism and its relationship with other pinealocyte biochemical activities is unclear.

A different vacuolar system has been described in some mammals. This is characterized by the formation of a single very large vacuole which occupies virtually the complete cell. The vacuolar system appears to originate from cisterns of the GER (Pévet, 1979).

Calcareous concretions, known also as corpora arenacea, acervuli, psammoma bodies and brain sand, are found almost without exception in the adult human pineal gland. The prevalence of these concretions of calcified material in the pineal gland progressively increases with age (Allen et al., 1981). The concretions are found in a number of mammalian species, including the rat. Further investigations are necessary in order to elucidate the formation and function of these calcarious concretions.

1.4.4.8 Mitochondria and Lipid Droplets

Mammalian pinealocytes contain a large number of mitochondria in the cytoplasm, and are responsible for the transduction of energy from food into the high energy bond of ATP, as in all eukaryotic cells.

Lipid droplets have been observed in the pinealocytes of all mammalian species studies (Pévet, 1979). At the ultrastructural level, observations indicate that the lipid droplets could be either directly

or indirectly involved in the process of synthesis and/or secretion of the pinealocytes (Karasek and Marek, 1978; Karasek et al., 1976). A report by Pévet and Kuyper (1978) that lipid droplets are morphologically involved in the formation of a vacuolar system supports this opinion.

1.4.4.9 Annulate Lamellae

Several structures of unknown function in the pinealocytes have been termed "annulate lamellae", and are found in only a few mammalian species, including the rat. McNeil (1977) suggests that these organelles could originate from an elaborate modification of the granular endoplasmic reticulum.

1.4.4.10 Vesicle-Crowned Rodlets

Vesicle-crowned rodlets (VCR) (or synaptic ribbons) comprise an electron-dense rodlet surrounded by a single layer of small clear vesicles lying singly or in groups in the pinealocyte cytoplasm, often in close proximity to the cell membrane.

Collin (1971) suggested that VCR could by "phylogenetic relics", however the majority of authors are of the opinion that VCR have a definite function, but of which is unclear.

The number, size and location in the cell of VCR appear to be dependent upon environmental illumination. A circadian rhythm of this nature has been observed in the rat (Kurumado and Waturu, 1977). A number of other factors appear to affect the number of VCR. For example a considerable increase in number of VCR in rat pinealocytes was observed after orchidectomy as well as in rat pinealocytes cultured in vitro (Karasek, 1976).

1.4.4.11 Subsurface Cisterns

Subsurface cisterns are found in the pinealocytes of numerous mammalian species. They are situated close to the cell membrane and are generally found in the perikarya. It appears that the number of these organelles is relatively large only in animals in which the pineal is very active (Pévet, 1979). The precise function of these cisterns is unclear, but they are suggestive of creating a pathway for

the exchange of metabolites on ions between neighbouring cells.

1.5 Pineal Biochemistry

1.5.1 Circadian Rhythms and the Pineal Gland

The function and biochemistry of the pineal involve circadian rhythms. Circadian rhythms have been implicated in three areas of pineal function viz: control of locomotor activity, circadian time measurement in photoperiodism and colour changes. Metabolic pathways of the pineal gland are also subject to circadian rhythms. Circadian rhythm is defined as a periodic event that recurs approximately, but not exactly, every 24 hours.

1.5.2 Melatonin Synthesis by the Pineal Gland

The pathway for the conversion of serotonin to melatonin is illustrated in Fig. 1.3 and appears to be common to pineal glands in general. The serotonin substrate is thought to derive from nerve endings or by pineal synthesis from tryptophan.

1.5.3 Daily Rhythms in Pineal Serotonin Metabolism

Fiske (1964) observed a large daily change in serotonin in rat pineal glands, with light-time serotonin 13 times as high as dark-time serotonin. N-acetylserotonin transferase (NAT), hydroxyindole-O-methyltransferase (HIOMT) activity as well as melatonin, all exhibit peak dark-time levels (Axelrod et al., 1965; Klein and Weller 1970; Klein 1972).

1.5.4 HIOMT Activity with respect to Season and Photoperiod

There exists a daily change in HIOMT activity (up to three-fold in the rat) (Axelrod et al., 1965). This remains, however, a controversial issue. Originally, this change was viewed to be responsible for the daily rhythm in melatonin, but it is more probable that the daily melatonin change is due primarily to NAT.

Large seasonal changes in HIOMT have also been reported. In addition, large HIOMT changes can to a degree be reproduced with constant light or constant dark eg: dark/light, 5.6 fold in rats (Wurtman et al., 1968).

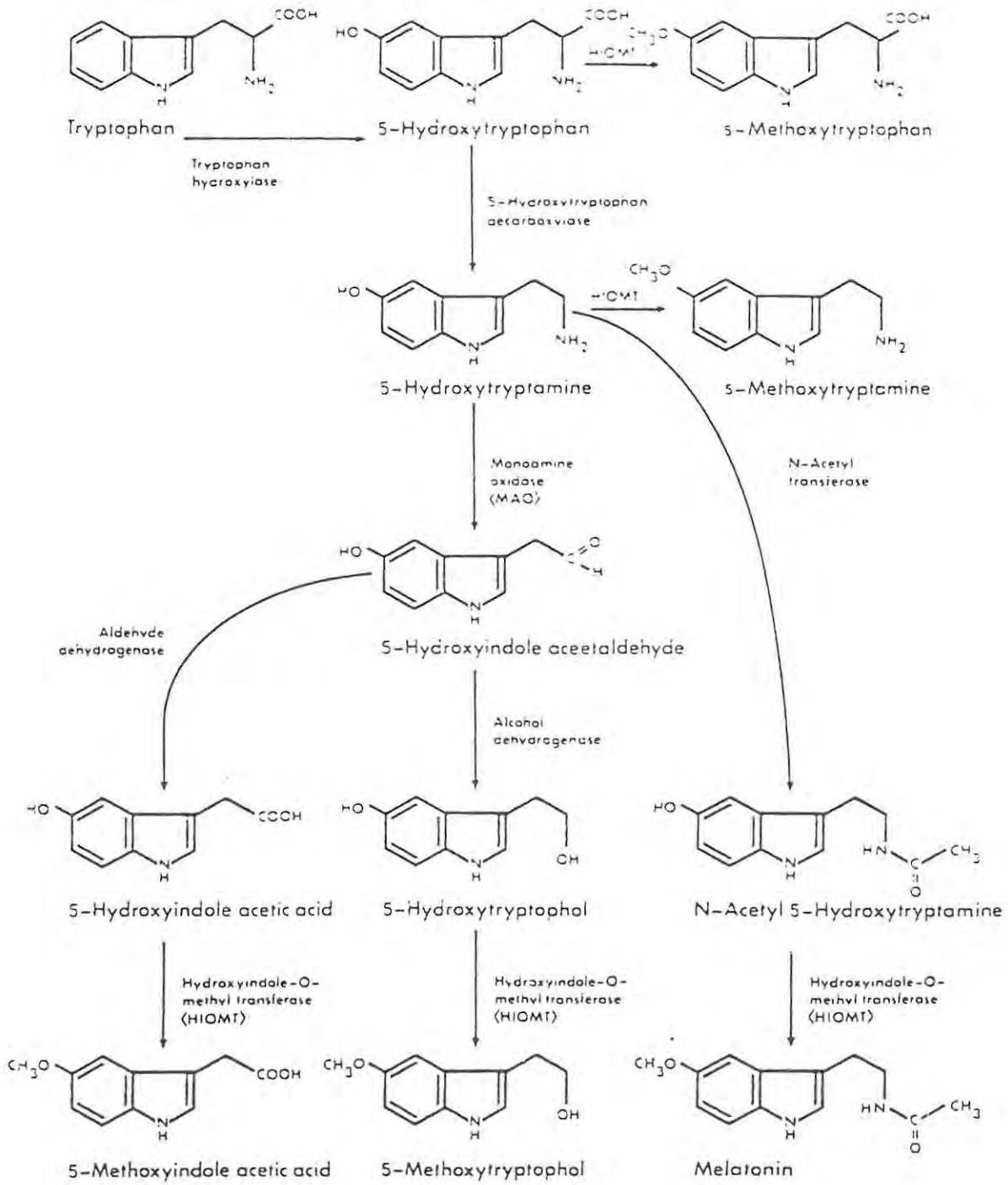

Fig. 1.3 The indole metabolism in the pineal gland.

1.5.5 Effect of Constant Darkness and Constant Light on Pineal Rhythms

NAT and melatonin rhythms have been demonstrated to persist in constant darkness in rats for up to a fortnight (Ralph et al, 1971). The persistence is construed as evidence that the NAT rhythm is a true circadian rhythm with a period of approximately 24 hours. NAT and melatonin rhythms damp in constant light (Klein and Weller, 1970).

1.5.6 Lighting Transitions

Unexpected "dusk" (dark) transitions imposed on rats result in a refractory period, which occurs approximately coincident with the light-time during which darkness will not initiate a rise in NAT or melatonin (Binkley et al., 1973). Transitions from constant light to dark or to light of different intensities results in a rise in NAT activity in the rat. It has been shown that the light intensity affects the degree of the increase in NAT (Minneman et al., 1976). Unexpected "dawn" (light) transitions imposed on rats during the dark-time results in a rapid decrease in NAT and melatonin (Klein and Weller, 1972).

Light, therefore, can at any time "switch off" NAT once it has been initiated and dark will only initiate NAT during a sensitive time.

1.5.7 Inorganic Constituents of the Pineal Gland

The main calcareous structural material of human pineal corpora arenacea is hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$ or $Ca_5(PO_4)_3OH$. Carbonite apatite, $Ca_{10}(PO_4, CO_3, OH)_6(OH)_6$ has been shown to be present as well (Earle, 1964). Iron is present in the pineal, particularly in the more superficial lamellae of the corpora arenacea (Earle, 1964). Pineal concretions and similar types of deposits to those seen in humans have been reported in some rodent species and laboratory rat strains (Diehl, 1978).

Pineal uptake and release of K^+ shows major changes in relation to time of day and the daily light-dark cycle (Piechowiak and Schnizer, 1976).

The pineal gland contains high concentrations of copper, manganese,

and zinc in relation to other organs (Wang and Fritze, 1969). It is probable that these inorganic constituents are mainly associated with specific proteins and enzymes, as in many other organs.

Calcium has been associated with a number of molecular control mechanisms in the pineal viz: closure of pineal canaliculi (Quay, 1974), sensitivity of pineal beta-receptors (Wilkinson et al., 1976), and the activation of enzymes involved with pineal synthesis of catechol- and indoleamines. These enzymes include adenylate cyclase, phosphodiesterase (Cantor and Weiss, 1978), tyrosine hydroxylase (Morgenroth et al., 1975) and N-acetyltransferase (Zatz and Romero, 1978).

1.5.8 Pineal Lipids and Carbohydrates

Pineal lipids in man and other animals differ in quantity and localization relative to species, age, and a wide variety of circumstances and experimental manipulations. Generally, from 3% to over 10% of wet weight of the organ is lipid. More than half of this fraction is phospholipid, with most of the remainder consisting of free cholesterol and glycerides. Pineal phospholipids consist mainly of phosphatidyl choline (45 to 47%), phosphatidyl ethanolamine (22 to 27%) and sphingomyelin (11 to 14%). Pineal phospholipid metabolism can be altered by certain neurotransmitters and related drugs. Norepinephrine, for example, can stimulate incorporation of inorganic phosphate into phospholipids (Muraki, 1972). Pharmacological studies by Hauser and Nijjar (1977) demonstrated that pineal phospholipid metabolism is mediated by alpha-receptors.

1.5.9 Pineal Amino Acids

In human, bovine, and laboratory rat pineals, taurine, glutamic acid, glycine, glutamine, and alanine constitute the largest proportions of the total free amino acids. Aromatic amino acids are of interest in the pineal gland as they include the precursors to chemical mediators of the catechol-, imidazol-, and indoleamine series.

1.5.10 Pineal Nucleotides and Nucleic Acids

Adenine nucleotides and their derivatives possess a wide range of important roles in energy metabolism as well as biosynthetic control

mechanisms within the pineal gland. Adenosine, a nucleoside, has been shown by Quay (1958) to have vasodilatory effects in the pineal. Wakade and Wakade (1978) demonstrated that adenosine has an inhibitory effect on the release of NE.

An important nucleotide in the pineal, from a research aspect, is adenosine 3',5'-monophosphate (cyclic-AMP, cAMP). cAMP functions in the pineal, as well as in many other target cells of the body, as a "second messenger", responding to certain hormonal or neurotransmitter molecules ("first messengers") via receptor-bound adenylate cyclase. Levels of cAMP in the pineal have been shown to be higher than those of pituitary gland and brain regions (Ebadi et al., 1971). Synthesis of cAMP from ATP in the pineal is dependent upon adenylate cyclase, the activity of which can be raised by L-NE.

Pineal DNA remains virtually constant from 1 - 365 days of age in male rats. Rat pineal RNA undergoes more variation through development of the animal. There exists a circadian rhythm in pineal RNA, which remains in animals raised in constant light, but is greatly reduced or absent in those raised from birth in constant darkness (Nir et al., 1969).

1.5.11 Pineal Enzymes

Five major categories of pineal enzymes may be defined by virtue of their catalytic effects viz: oxidoreductases, transferases, hydrolases, lyases, and isomerases.

1.5.11.1 Oxidoreductases

Three groups of oxidoreductases are of importance in pineal research. Oxidoreductases or dehydrogenases play major roles in energy metabolism in the pineal. Examples of these are: lactate dehydrogenase (EC 1.1.1.27, LDH), isocitrate dehydrogenase (EC 1.1.1.41), glucose-6-phosphate dehydrogenase (EC 1.1.1.49), glycerolphosphate dehydrogenase (EC 1.1.2.1), and reduced NADP and NAD dehydrogenases (EC 1.6.99.1 and EC 1.6.99.3). High levels of these enzymes have been recorded in the pineal gland (Yang et al., 1972).

Monoamine oxidases (EC 1.4.3.4., MAO) have been detected in pineal

tissue. Two types of MAO have been defined in the pineal. Type A monoamine oxidase occurs in sympathetic nerve fibres, acts on tyramine and 5-HT, and comprises approximately 15% of pineal MAO. Type B MAO includes that found within pinealocytes, and does not act on 5-HT (Yang et al., 1972). These enzymes are important in certain steps in the metabolism of biogenic amines in the pineal.

Specific hydroxylating enzymes which convert certain amino acids to biologically active amines or their precursors, occur in the pineal as well. Phenylalanine, for example, is hydroxylated by phenylalanine 4-hydroxylase (EC 1.14.3.1) (Bagchi and Zarycki, 1971), tyrosine by tyrosine 3-hydroxylase, (EC 1.14.3.a) (Vogel et al., 1969), and tryptophan by tryptophan 5-hydroxylase (EC 1.14.3.b) (Lovenberg et al., 1967).

1.5.11.2 Transferases

A wide range of transferases have been discovered in pineal tissue. The methyltransferases include hydroxyindole-O-methyltransferase (EC 2.1.1.4, HIOMT, 5-adenosylmethionine:N-acetyl-serotonin-O-methyltransferase); catechol-O-methyltransferase (EC 2.1.1.6, COMT, 5-adenosylmethionine:catechol-O-methyltransferase); histamine-N-methyltransferase (EC 2.1.1.8, HNMT, 5-adenosylmethionine:histamine-N-methyltransferase); and methanol-forming enzyme (EC 2.1.1.b, 5-adenosylmethionine:H_2O-methyltransferase), which is of lesser physiological importance in the pineal.

HIOMT is responsible for the conversion of N-acetylserotonin (aHT) to melatonin (aMT) in the pinealocyte. Much of the literature regarding changes in HIOMT is obscure, and it is therefore difficult to relate these differences to circadian changes.

COMT is found in erythrocytes, and various organs including the pineal. Although NE, a factor in the circadian mechanism, is inactivated by COMT, current opinion does not support the possibility that COMT contributes to adrenergic mechanisms driving circadian and hormone-induced changes in pineal synthetic activities.

Two of the acetyltransferases have received close attention.

Serotonin-N-acetyltransferase (EC 2.3.1.5, NAT, acetyl-CoA:arylamine-N-acetyltransferase). NAT is responsible for the conversion of serotonin (5-HT) to N-acetylserotonin (aHT), which is regarded as a rate-limiting step in the conversion of serotonin to melatonin and related methoxyindoles in the pineal. Choline acetyltransferase (EC 2.3.1.6, CAT, acetyl-CoA:choline-O-acetyltransferase or choline acetylase) is responsible for the synthesis of the neurotransmitter acetylcholine.

The pineal gland is an abundant source of cyclic AMP-dependant protein kinase (EC 2.7.1.37, cyclic-AMP:protein phosphotransferase) which incorporates a regulatory protein (cyclic-AMP binding), and a catalytic protein (protein kinase). This enzyme system phosphorylates histones, following activation by cAMP. This enzyme can induce certain pineal enzymes, and in particular, NAT (Winters et al., 1977). Rat pineal glands exposed in vivo to 24 hours continuous light become supersensitive to beta-adrenergic stimulation. Simultaneously, there occurs a 50% increase in protein kinase activity, either with or without added cAMP (Zatz and O'Dea, 1976).

1.5.11.3 Hydrolases

Two physiologically significant pineal hydrolases are phospho-diesterase, and adenylate cyclase.

Pineal cyclic nucleotides phosphodiesterase (EC 3.1.4-) transforms cAMP to 5'-AMP and is the only known enzymatic mechanism for the physiological termination of the action of cAMP. Various forms of this enzyme have been identified in pineal tissue, and one or more of these have been shown to be affected by beta-adrenergic-linked mechanisms (Cantor and Weiss, 1978).

Adenylate cyclase (EC 3.6.1.-) induces the cleavage of ATP to form cAMP and inorganic pyrophosphate. Large amounts of this enzyme occur in the pineal, their levels being affected by environmental light and other factors via hormonal and adrenergic mechanisms (Weiss, 1969). It is generally accepted that adenylate cyclase is located in cell-membranes, and closely associated with a receptor system.

1.5.11.4 Lyases

The most commonly studied lyases in pineal tissue are DOPA decarboxylase (EC 4.1.1.26, 3,4-dihydroxy-L-phenylalanine carboxy-lyase) and 5-hydroxytryptophan decarboxylase (EC 4.1.1.28, 5-hydroxy-L-tryptophan carboxy-lyase), which are involved in the synthesis of catecholamines and indoleamines respectively. There appears to exist a common substrate specificity between the two enzymes in certain instances. Coupled with the fact that these enzymes do not exercise a major rate-limiting role in the pineal, research in this area is limited. Carbonic anhydrase (EC 4.2.1.1., carbonate hydro-lyase) exists in relatively low levels in rat and human pineal glands.

1.5.11.5 Isomerases

Glucose phosphate isomerase (EC 5.3.1.9, D-glucose-6-phosphate ketol-isomerase) have been found in high levels in lamb pineals. Information on the characteristics of this enzyme is limited.

1.5.12 Pineal Proteins

Various endogenous and exogenous protein fractions have been identified in the pineal. In general, it has been shown that gonadal hormones, NE, and time of day interact in the stimulation and modulation of pineal protein synthesis in the rat.

Pineal uptake *in vivo* of exogenous radioactively labelled peptides has been reported for thyrotropin-releasing hormone (TRH) (Dupont *et al.*, 1972), luteinizing hormone-releasing hormone (LH-RH) (Dupont *et al.*, 1974), alpha-melanocyte-stimulating hormone (alpha-MSH) (Dupont *et al*, 1975, MSH-release inhibiting factor (MRIH) (Redding *et al.*, 1973), somatostatin (Nair and Colwell, 1975), and methionine-enkephalin (Kastin *et al.*, 1976). Other peptides that have been measured in mammalian pineal glands include arginine vasotocin (Cheesman, 1970), oxytocin (Legros *et al.*, 1975), substance P (Duffy *et al.*, 1975), neurophysins (Reinharz and Vallotton, 1977), lysine vasotocin (Pavel, 1965), nerve growth factor (NGF) (Perez-Polo *et al.*, 1978), delta-sleep inducing peptide (DSIP), Kastin *et al.*, (1978), and S-100 protein and glial fibrillary acidic (GFA) protein (Møller *et al*, 1978).

1.5.13 Pineal Indoleamine Metabolism

Pineal metabolism of indoleamines may be classified into two principal groups viz: the synthesis of serotonin from tryptophan, and the conversion of serotonin to melatonin and other deaminated products. Pineal indole metabolism is represented in Fig. 1.3. Neural control of the pineal gland is represented schematically in Fig. 1.2.

1.5.13.1 Enzymes Involved in Serotonin Biosynthesis

The synthesis of serotonin from tryptophan involves two enzymes, tryptophan hydroxylase and aromatic amino acid decarboxylase (Fig. 1.3). Both enzymes are found in pinealocytes (Lovenberg et al, 1968). The hydroxylation of tryptophan, which is regarded as the rate-limiting step in the synthesis of serotonin, occurs in the mitochondria where tryptophan hydroxylase is located (Lovenberg et al., 1968). Measurements of the biological half-life of tryptophan hydroxylase suggest that the enzyme is continually being synthesized (Sitaram and Lees, 1978). Conflicting evidence regarding the regulation of tryptophan hydroxylase exists. Deguchi (1977) demonstrated no physiological circadian change in enzyme activity, however Shibuya et al. (1978) contradicts this statement, reporting a 1.5-fold increase in enzyme activity at the middle of the dark period. This increase in activity was also shown to be blocked by propanolol, suggesting that norepinephrine which is known to be released at night (in the dark) is the neurotransmitter responsible for these effects. Organ culture studies, however, contradict these results. Wurtman et al. (1971) suggested that the rate at which serotonin is produced from radio-labelled tryptophan is raised by norepinephrine, acting via a cyclic AMP mechanism. Other studies, however, were unable to show an adrenergic-cyclic AMP induced increment in tryptophan hydroxylation (Bensinger et al., 1974).

Aromatic-L-amino acid decarboxylase is present in the pineal gland at relatively high concentrations and is confined mainly to the cytosol fraction of pinealocytes (Snyder and Axelrod, 1964). The enzyme converts 5-hydroxytryptophan to serotonin, utilizing pyridoxal phosphate as a cofactor. The activity of aromatic-L-amino acid decarboxylase has been demonstrated to be influenced by environmental lighting acting via sympathetic nerves (Snyder et al., 1965). These

effects on the activity of the enzyme only become evident after several days of exposure to constant light, indicating that alterations in the activity of aromatic-L-amino acid decarboxylase cannot be implicated in daily indoleamine metabolic rhythms.

1.5.13.2 Conversion of Serotonin to Deaminated and N-Acetylated Products

Two major pathways of pineal serotonin metabolism exist (Fig. 1.3). In the first instance, serotonin (5-HT) is deaminated by MAO, resulting in the formation of 5-hydroxyindoleacetaldehyde, which is subsequently oxidized by aldehyde dehydrogenase to 5-hydroxy-indoleacetic acid (5-HIAA), or reduced by alcohol dehydrogenase to 5-hydroxytryptophol (5-HTOH). Secondly, serotonin may be N-acetylated by serotonin N-acetyltransferase (NAT) to form N-acetylserotonin (aHT) which is thereafter O-methylated by hydroxyindole-O-methyltransferase (HIOMT) to form melatonin (aMT). It has been suggested that this pathway is only characteristic of mammalian pineals. Evidence exists pointing to the formation of melatonin from serotonin in other tissues, such as the retina and the Harderian gland (Pang et al., 1977).

1.5.13.3 Enzymes Involved in Serotonin Metabolism

Monoamine oxidase activity has been reported to be high in the pineal (Snyder et al., 1965), the enzyme being mainly confined to the mitochondria. The principal function of the enzymes is to counter-balance amine production and to maintain a constant level of amine.

Alcohol dehydrogenase requires a reduced pyridine cofactor in order to convert aldehydes into alcohols (Feldstein and Williamson, 1968).

Aldehyde dehydrogenase, which transforms aldehydes into acids, requires an oxidized pyridine cofactor (Feldstein and Williamson, 1968).

Serotonin N-acetyltransferase (NAT) undergoes large daily changes (Axelrod, 1974). Rat NAT activity is 30- to 70-fold higher during the night than during the day. Pineal NAT is found entirely in the cytoplasm, and acts to transfer an acetyl group from the co-factor

acetyl CoA to acceptor amines (Weissbach et al., 1961). The main acetyl group acceptor is considered to be serotonin, although 5-methoxytryptamine can also be N-acetylated. It has been speculated that a physiological disulphide in the pinealocyte is involved in NAT regulation. Pineal NAT activity is regulated by an adrenergic-cyclic AMP mechanism (Klein et al., 1978). Inhibition of cAMP destruction by drugs or cAMP analogs results in an increase in NAT activity (Klein et al., 1970). Cycloheximide and actinomycin D block these effects, indicating that the increase in NAT requires the synthesis of new RNA and protein. It has also been suggested that cAMP-induced hyperpolarization is necessary for NAT stimulation. An additional important piece of evidence concerning the role of cAMP, is that propanolol treatment reduces the amount of cAMP in the cell, as well as NAT (Klein et al., 1978).

The amount of cAMP in the cell is a function of its rate of production, which is under adrenergic control. Pineal NAT activity is hence regulated by the second-messenger, cAMP, the production of which is controlled by a beta-adrenergic receptor associated with adenylate cyclase in the membranes of the pinealocyte.

Norepinephrine is the physiological catecholamine involved in the transsynaptic regulation of NAT. NE interacts with adrenergic receptors after being released from sympathetic nerves in the pineal gland, thus any drug which induces an increase in norepinephrine at the synapse is able to stimulate N-acetyltransferase activity eg. cocaine, desmethylimipramine, and MAO inhibitors (Holz et al., 1974).

Environmental factors known to regulate pineal NAT activity are drugs, light, and stress. An analog of norepinephrine, isoproterenol, is a drug commonly used in vivo to induce an increase in NAT activity. A strong beta-receptor blocking agent, l-propanolol is a drug frequently used experimentally to reduce elevated pineal NAT activity. Environmental lighting exercises a marked effect on pineal NAT activity. During the day, when NAT activity is diminished, exposure to darkness does not induce an increase in NAT activity supposedly because the biological clock is not synchronized to stimulate the pineal gland at that time (Binkley et al., 1974). After

the 12 hours of normal light period, the pineal gland can be stimulated, and exposure to darkness at this time will result in the increase in NAT activity (Binkley et al., 1974). Stress has been shown to induce a small increase in NAT activity (Lynch et al., 1973). It is important, thus, for these factors to be strictly controlled during experimental procedures which directly or indirectly involve NAT.

In the pineal gland, hydroxyindole-O-methyltransferase (HIOMT) catalyses the conversion of N-acetylserotonin to melatonin and to a lesser degree converts 5-hydroxyindole acetic acid to methoxyindole acetic acid, hydroxytryptamine to methoxytryptamine, and hydroxytryptophol to methoxytryptophol (Axelrod and Weissbach, 1961). The enzyme is located almost entirely in the cytosol of the pinealocyte.

HIOMT is regulated by a neural mechanism modulated by light, and circulating sex steroids.

Pineal HIOMT activity alters slowly over a period of days in response to changes in enviromental lighting conditions (Axelrod and Wurtman, 1978). During exposure to constant darkness there is a gradual increase in HIOMT activity, and in constant light there is a gradual decrease in HIOMT activity. It is generally accepted that signals generated endogenously on a circadian basis in the suprachiasmatic nucleus stimulate the release of transmitter in the pineal gland, resulting in an increase in HIOMT activity. Light appears to block transmission of signals to the pineal gland. Intracellular mechanisms involved in HIOMT regulation have not been entirely elucidated. No direct evidence implicating norepinephrine release and cAMP stimulation exists. Attempts to stimulate HIOMT activity in organ culture with norepinephrine or cAMP have been unsuccessful (Berg and Klein, 1971). The increment in HIOMT activity observed in constant darkness is due to an increase in the number of HIOMT molecules (Yang and Neff, 1975).

Evidence exists to support the idea of a gonadal-pineal axis. It has been reported that at low concentrations, testosterone can increase

HIOMT activity, whereas at high concentrations, HIOMT is inhibited (Preslock, 1977).

N-acetylserotonin appears to occupy the role of a "fulcrum" in terms of melatonin production. At night, when NAT levels are high, and when saturating amounts of N-acetylserotonin are produced, it would appear that HIOMT would function as the rate-limiting enzyme. During the day, however, when N-acetylserotonin levels are low, NAT would most probably assume dominance in this role.

1.5.13.4 Pineal Indole Compounds

1.5.13.4.1 Tryptophan

Tryptophan levels in the pineal gland appear to be modulated by the concentration of tryptophan in circulation. Injections of tryptophan can lead to elevated pineal levels of tryptophan (Deguchi and Barchas, 1972).

1.5.13.4.2 Serotonin

Serotonin in the pineal gland occurs mainly in pinealocytes (Neff et al., 1969). The concentration of serotonin in the pineal is regulated by three factors viz: synthesis, the rate at which serotonin is converted to oxidation products by MAO, and the rate at which serotonin is converted to N-Acetylserotonin by NAT. The neural circuit regulating NAT (Fig. 1.5) regulates the amount of serotonin in the pineal gland. Drugs which affect NAT accordingly will also indirectly affect the concentrations of serotonin in the pineal gland.

1.5.13.4.3 N-Acetylserotonin

N-acetylserotonin in the pineal gland is regulated mainly by the activity of NAT (Klein and Weller, 1972). At night, when NAT activity is elevated, the concentration of N-acetylserotonin is also raised. Drugs which stimulate NAT also raise N-acetylserotonin levels, and likewise, those drugs which block the adrenergically induced increase in NAT also block an increase in N-acetylserotonin normally seen due to adrenergic stimulation (Klein and Weller, 1972).

1.5.13.4.4. Melatonin

Melatonin exists in the cytosol of the pinealocyte, as a soluble

molecule. The existence of melatonin in this form might provide an explanation why rapid and large changes in melatonin levels are seen in the gland, and why melatonin does not appear to be stored in substantial amounts in the pineal gland. Melatonin in the pineal gland is regulated in a manner similar to N-acetylserotonin viz: by the neural-cAMP mechanism. In all instances, melatonin levels are raised in the pineal gland during darkness.

1.6 Pharmacology of the Pineal Gland

1.6.1 Receptors and Pharmacology

The mammalian pineal gland contains a variety of receptors eg: beta-adrenergic, alpha-adrenergic (post-synaptic), alpha 2-adrenergic (pre-synaptic), GABAergic, benzodiazepinergic, glutamatergic, serotoninergic and cholinergic.

Catecholamines capable of eliciting a response at the receptor site are referred to as adrenergic agonists. Agents which block specifically the response elicited by an adrenergic agonist are referred to as adrenergic antagonists or blockers. For the purposes of this study the author is primarily concerned with beta- and alpha-adrenergic receptors.

1.6.2 Classification of beta- and alpha- Receptors

In view of the complexity of the type of responses mediated by alpha- and beta-receptors, the basis for classification is the chemistry of the stimulating agonist and of the blocking antagonist (Levitzki, 1976).

1.6.2.1 The Beta-Receptor

A beta-receptor is one which mediates a response pharmacologically characterized by: (1) a relative potency series: isoproterenol > epinephrine > norepinephrine > phenylephrine, and (2) a susceptibility to specific blockade by either propanolol or pronethalol at relatively low concentrations (Levitzki, 1976).

1.6.2.2 The Alpha-Receptor

An alpha-receptor is one which mediates a response pharmacologically characterized by: (1) a relative potency series in which

norepinephrine > epinephrine > phenylephrine >> isoproterenol, and (2) a susceptibility to specific blockade by phentolamine, dibenamine or phenoxybenzamine at low concentrations (Levitzki, 1976).

1.6.3 Probing of Catecholamine Receptors

Two principal approaches are used, both involving binding studies with specific ligands. Firstly, ligand binding can be measured using radioactive ligands or fluorescent ligands in order to measure both the quantity of receptors and their affinity towards the ligand. In the second instance, irreversible affinity labels based on either agonist or antagonist can be used (Levitzki, 1976).

1.6.4 The Phenomenon of Super- and Sub-Sensitivity

The responsiveness of a pinealocyte depends upon its prior exposure of the beta-adrenergic receptors to norepinephrine. Abolition of noradrenergic activity by denervation, or reduction by reserpine or continuous lighting, results in a great enhancement in beta-adrenergic receptor activity with respect to the response by cAMP or NAT. If the number of catecholamine molecules reacting with beta-adrenergic receptors are increased for a period of time, the pineal beta-receptors become less responsive to the agonist (Deguchi and Axelrod, 1973). These phenomena are referred to as "super- and sub-sensitivity". Investigations performed by Kebabian et al., (1975), show that the phenomena of super- and sub-sensitivity can be ascribed to an increase or decrease in the number of beta-adrenergic receptors available.

1.6.5 Suitability of the Pineal Gland as a Pharmacological Model

The pineal gland provides an attractive model system for investigating molecular mechanism at cellular and subcellular levels. Two characteristics of the pineal gland lend themselves to this cause viz: the large amplitude circadian rhythms in biochemical functions, and innervation exclusively by sympathetic nerves, involving to a large degree, beta-adrenergic receptors. Lying outside of the blood-brain barrier, the gland is accessible to peripherally administered drugs. in addition its well characterized biochemical pathway can be conveniently assayed.

1.6.6 Effect of Sympathomimetics and Related Agents on the Pineal Gland

Extensive research on the effects of sympathomimetic agents on the rat pineal gland has been performed. The most commonly used agents are norepinephrine, the physiological neurotransmitter, and isoproterenol, a specific beta-adrenergic agonist. Stimulation of the pineal with sympathoactive agents produces the same physiological events that are associated with the gland at night. In terms of beta-adrenergic stimulation, the sympathomimetic binds to the beta-adrenergic receptor of the pineal (Zatz et al., 1976), activating adenylate cyclase (Weiss and Costa, 1968) resulting in the elevation of cAMP (Deguchi, 1973). Cyclic AMP-dependent protein kinase is activated (Zatz and O'Dea, 1976) and endogenous proteins are phosphorylated with the induction of NAT activity (Winters et al., 1977). Serotonin levels are subsequently reduced and N-acetylserotonin levels become raised (Brownstein et al., 1969). A reduction of sensitivity of the pineal to subsequent stimulation has been shown by measurement of various parameters (Deguchi and Axelrod, 1973). Examples of mimetics which stimulate the pineal beta-adrenergic receptor are L-DOPA, (norepinephrine precursor), pargyline (MAO inhibitor), theophylline (phosphodiesterase inhibitor), dibutyryl cAMP (a cAMP-analog) (Deguchi and Axelrod, 1972), tyramine (induces neurotransmitter release) and cocaine (an uptake blocker) (Holz et al., 1974), and the two frequently used sympathomimetics, norepinephrine and isoproterenol (Klein et al., 1970). The abovementioned agents increase the concentration of norepinephrine at the receptor sites by a variety of mechanisms, and can mimic or potentiate the actions of norepinephrine.

Certain agents can block the beta-adrenergic receptors. These agents have been demonstrated to diminish the stimulation of indoleamine metabolism (by norepinephrine). Examples of such agents are reserpine which depletes the sympathetic nerve endings of physiological catecholamines, and propanolol, which actively blocks the pineal beta-adrenergic receptors (Deguchi and Axelrod, 1972). These agents are useful tools in elucidating pharmacological induced events, as well as comparing these mechanisms with physiological ones.

Other agents which inhibit events distal to cAMP have also been

utilized. Examples are actinomycin D, which inhibits RNA transcription in the pinealocyte (Romero et al., 1975), and cycloheximide, which inhibits translation to protein (Klein et al., 1970).

1.6.7 Effect of Cations on the Pineal Gland

High concentrations of potassium have been shown to significantly inhibit the induction of NAT activity by norepinephrine (Parfitt et al., 1975). A step in the induction distal to the accumulation of cAMP appears to be affected. The precise nature of the mechanism by which potassium induces its effects is unclear at this time.

The induction of NAT activity by isoproterenol or dibutyryl cAMP is inhibited when calcium is absent (Zatz and Romero, 1978). High concentrations of calcium were shown to enhance the induction. Calcium appears to have a general effect on the rate of protein synthesis, which is reduced in the absence of the ion. It would appear that the protein synthesis necessary for enzyme induction was inhibited, reducing the induction of NAT.

Zinc appears to exert the opposite effect to calcium, inhibiting an extensive number of calcium-dependent biochemical events such as calcium-mediated activation of phosphodiesterase (Ca^{2+} ATPase, phosphorylase kinase, and adenylate cyclase (Ebadi, 1984).

1.6.8 Effect of Agents on Pineal cGMP Levels

In the presence of extracellular calcium, exposure of rat pineal glands to norepinephrine or to depolarizing concentrations of potassium results in raised cGMP levels (O'Dea and Zatz, 1976). Isobutylmethylxanthine, a phosphodiesterase inhibitor, potentiates the effects of stimulation (Zatz and O'Dea, 1977). The changes in cGMP appear to occur in the presynaptic nerve terminals. Isoproterenol, an exclusive beta-agonist, is less effective than norepinephrine in stimulating cGMP. The responses by cGMP to norepinephrine can be blocked by alpha-adrenoreceptor antagonists (Vanacek et al., 1985).

NE regulates pinealocyte cAMP and cGMP by an unusual synergistic mechanism involving alpha- and beta-adrenoreceptors (Vanacek et al.,

1985).
In both instances, beta-adrenergic stimulation is an absolute requirement, and alone induces an 8-fold increase in cAMP and a 3-fold increase in cGMP. No detectable alteration in either cyclic nucleotide is produced by alpha 1-adrenergic activation alone, however, potentiation of beta-adrenergic stimulation of cAMP occurs (10-fold) apparently by a Ca^{2+}-phospholipid-dependent protein kinase (Sugden et al., 1985), and of cGMP (100-fold) by an unidentified mechanism.

The adrenergic stimulation of pineal cAMP and cGMP is an unusual feature because stimulus deprivation enhances the cAMP response and virtually abolishes the cGMP response, even though the number of alpha- and beta-receptors in pinealocytes increase under these conditions. The phenomenon is known as see-saw signal processing and is probably governed by intracellular mechanisms (Vanacek et al., 1986).

Vanacek et al. (1986) firmly support the hypothesis that alpha 1-adrenergic stimulation causes Ca^{2+} influx. Under the lighting conditions LD 14:12, this influx of Ca^{2+} could activate phospholipid hydrolysis to diacylglycerol (DG) and fatty acids (FA). This would potentiate beta-adrenergic stimulation of cAMP via a DG-sensitive mechanism and that of cGMP via a FA-dependent mechanism.

1.7 Review of Sympathetic Agents Investigated

1.7.1 Alpha-Methyldopa: Introduction

Alpha-methyldihydroxy-phenylalanine (alpha-methyldopa or MeDOPA) was synthesized by Stein et al. (1955). Sourkes (1954) reported that MeDOPA is a patent inhibitor of decarboxylase. Oates et al. (1960) were the first to administer the drug to hypertensive patients, after stringent toxicological studies on animals. They found that daily oral administration of 1 - 2 g MeDOPA was followed by an average reduction in the recipient's blood pressure of 10 - 30 mm Hg. Subsequently, MeDOPA has been extensively used as a hypotensive drug. The hypotheses regarding its mechanism of action have, however, changed several times.

1.7.1.1 Metabolism and Site of Action of Alpha-Methyldopa

Goldberg et al. (1960) were the first to publish information on the pharmacology of MeDOPA, in which it was reported that difficulty was encountered in showing interference with peripheral sympathetic nerve-mediated responses in the dog. Weissbach et al. (1960) demonstrated that MeDOPA inhibited dopa-decarboxylase (DDC). Porter et al. (1961) reported that alpha-methyldopamine (MDA), which is formed initially from MeDOPA by a decarboxylation reaction, lowered tissue norepinephrine. Carlsson and Lindquist (1962) demonstrated that MDA and its derivative, alpha-methylnorepinephrine (MNE), formed by beta-hydroxylation, were present in the brain after the administration of MeDOPA to mice, suggesting that MDA and MNA could possibly usurp the functions of the physiological catecholamines.

It was demonstrated by Day and Rand (1963) that MDA and MNE are similar to norepinephrine in terms of their pharmacological activity. MNE was shown to be taken up and stored in sympathetic nerve endings (Stone, 1971). It was hypothesized that MNE displaces norepinephrine from the storage vesicles in the nerve endings and acts as an inadequate "false transmitter" (Kopin, 1968).

In 1968, Henning and Van Zwieten studied the hypotensive activity of MeDOPA when administered into the vertebral artery of the cat. This route of administration was shown to be more effective in eliciting a hypotensive response than the intravenous route, Heise and Kroneberg (1973) demonstrated a reduction in the anti-hypertensive effect of MeDOPA with prior administration of dopamine-beta-hydroxylase DBH inhibitors thereby presenting a centrally-acting mechanism for MeDOPA.

Subsequently, it has been shown that the hypotensive action of MeDOPA is mediated through the CNS (Freed et al., 1977).

It has thus been established that MeDOPA is converted by DDC into MDA, following which MDA is converted by DBH into MNE. MeDOPA, therefore, acts as an "imposter", utilizing enzymes required for the conversion of DOPA to NE in its transformation to MNE (Fig. 1.4). More recent studies have shown that MNE is converted to alpha-methylepinephrine (ME) (Beart et al., 1983).

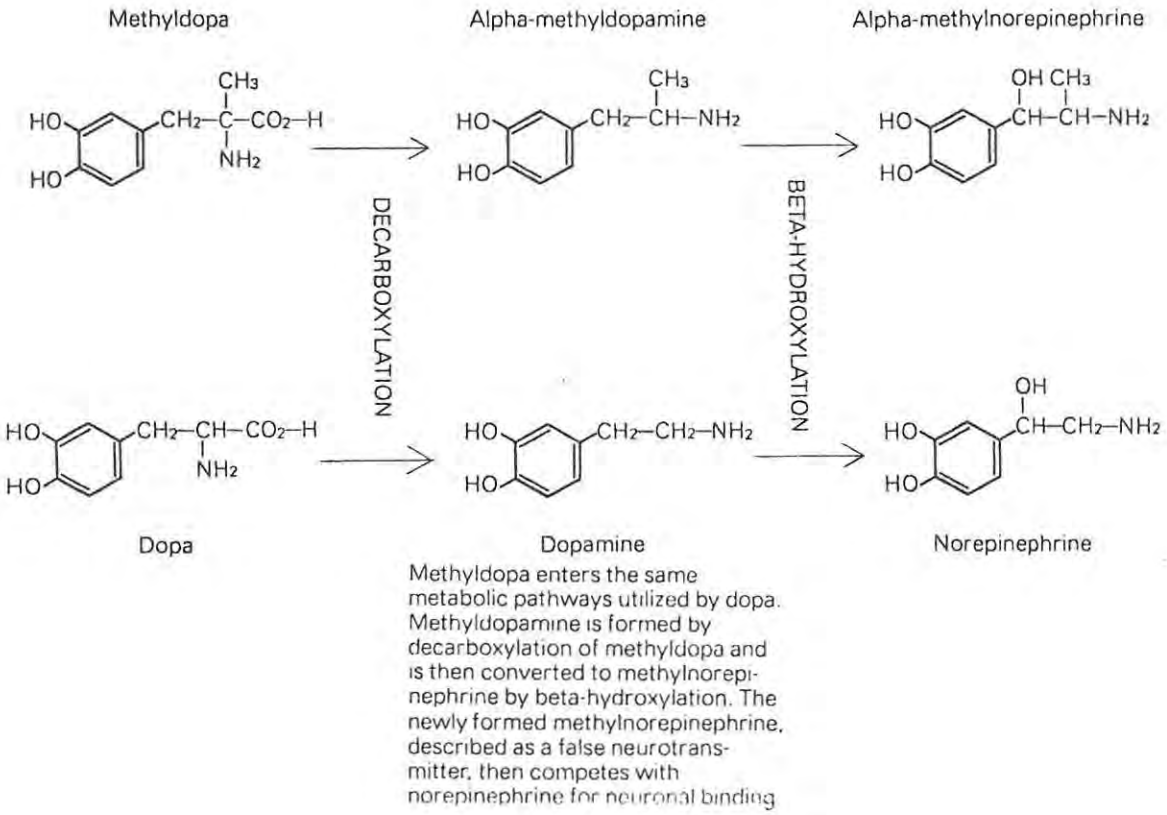

Fig. 1.4 The metabolic pathways of dopa and alpha-methyldopa.

1.7.1.2 Association of Alpha-Methyldopa and its Metabolites with Alpha-Receptors

With regard to blood pressure (BP) regulation by the alpha-receptor in the CNS, investigations using other centrally acting antihypertensive drugs, such as clonidine, indicated that hypotension could be induced by the inhibition of peripheral sympathetic nerve activity via stimulation of the alpha-receptors (Scholtysik, 1980). The alpha-receptor associated with regulation of BP was originally held to be of the alpha 2-subtype (Timmermans et al., 1981). Huchet et al., in 1982, however, showed that blockade of the central alpha 1-receptor acceleration of vagus nerve activity and inhibition of sympathetic tone, lowered BP. Thus stimulation of the central alpha 1-receptor, or blockade of the alpha 2-receptor (or blockade of both the alpha 1- and alpha 2-receptors) can induce a hypotensive effect by suppression of sympathetic activity and stimulation of vagus nerve activity, respectively.

MeDOPA does not have any alpha-adrenergic activity, however its physiological metabolites are considered to have hypotensive activity (Goldberg et al., 1981). In a comparative study involving competitive experiments using [^{3}H]-prazosin, [^{3}H]-clonidine, and [^{3}H]-dihydroxyprenol, the potency of action of MDA on alpha 1- alpha 2- and beta-receptors was shown to be relatively weak in comparison with that of MNE and ME. It was thus deduced that the hypotensive role of MDA was weak (Goldberg et al., 1982). Inhibition of DBH in the CNS causes a decrease in hypotensive activity (Henning and van Zwieten, 1968). This information suggests that the hypotensive role played by MNE or ME is greater than that played by MDA. In an investigation of alpha-receptors, Louis et al. (1982) observed that MNE possessed a 7-fold strength of action on the alpha 2-receptor and a 70-fold affinity towards the alpha 2-receptor in comparison with norepinephrine.

It is possible that a large amount of MNE is formed in central noradrenergic neurons instead of norepinephrine, after the administration of MeDOPA. The MNE produced may then be released from the nerve endings, stimulating the alpha 2-receptor. It was reported by de Jong et al. (1976) that trace amounts of MNE injected into the nucleus tractus solitarius (NTS), an important regulating site, induce

a significant decrease in BP. This is suggestive of an important role for MNE in the hypotensive mechanism of MeDOPA.

With regard to ME, Goldberg et al. (1981) demonstrated that synthesized ME is an alpha 2-adrenergic agent with less potency than MNE. Robertson et al. (1984) reported on the beta-adrenergic activity of ME. In terms of competitive binding capacity towards the beta-receptor using [^{3}H]-dihydro-alprenol, ME was found to be the strongest agent, followed by MNE, epinephrine and norepinephrine in order of decreasing strength. In addition, timolol, a beta-antagonist, suppressed BP after the micro-infusion of ME into the NTS, as occurs with yohimbine, an alpha 2-blocking agent. Simultaneous administration of yohimbine and timolol caused a total inhibition of the hypotensive activity of ME. This is suggestive of a mechanism in which ME acted upon alpha 2- and beta-receptors in the NTS, resulting in the reduction of BP (Goldberg and Robertson, 1983; Robertson et al., 1984; Tung et al., 1983b). Himene et al. (1985) investigated the intracerebral production of ME. The amounts of ME produced in the hypothalamus and the brainstem, in the C_1 - C_2 region, corresponded to 0.14 and 0.08% of the amount of MNE, respectively. Further studies are thus necessary in order to confirm whether ME plays a more important role than MNE in the hypotensive mechanism of MeDOPA.

Alterations in the status of alpha 1- and alpha 2-receptors have been shown to occur in the CNS after MeDOPA treatment. The number of binding sites in alpha 1-receptors have been found to increase and those in alpha 2-receptors to decrease (Goldberg et al., 1983). It has been observed that the alpha 2-affecting MNE suppresses the release of catecholamines through the presynaptic alpha 2-receptor, which induces the supersensitivity of the alpha 1-receptor postsynaptically (Freed et al., 1984).

The mode of action of MeDOPA has not been clarified. A more complex neural circuit probably serves in the regulation of BP, involving possibly many more neurotransmitters than has previously been considered. It is thus a difficult task to describe the mechanism of the hypotensive action of MeDOPA in terms of a single metabolite.

1.7.2 Ephedrine Hydrochloride

Ephedrine Hydrochloride ((-)-(1R,2S)-N-(1-Hydroxy-1-phenylprop-2-yl)-N-methylammonium chloride) is a sympathomimetic amine with direct and indirect effects on adrenoreceptors. Ephedrine is obtained from certain species of Ephedra, particularly E. sinica Stapf and E. equisetina Bunge, indigenous to China, and E. gerardiania Wall, indigenous to India (The Pharmaceutical Codex 1979). Ephedrine Hydrochloride is the form in which ephedrine is usually given orally or by injection.

Ephedrine stimulates both alpha- and beta-receptors and has clinical uses related to both modes of action. Part of the drug's peripheral action is achieved by inducing release of the neurotransmitter, norepinephrine from its stores at the sympathetic nerve endings (Ariëns, 1979). Ephedrine also has direct effects on receptors, and exhibits marked effects in reserpine-treated animals.

Tachyphylaxis develops to its peripheral actions, and subsequent rapidly repeated doses lose their efficacy (Weiner, 1985).

Ephedrine is used clinically in the prevention of bronchial spasm in asthma, in the treatment of nasal congestion and allergic disorders, to combat a fall in BP during spinal anaesthesia, and occasionally for narcolepsy (Martindale, 1982).

CHAPTER 2

2.1 The Effect of Alpha-Methyldopa and Ephedrine on Rat Pineal Production of Serotonin Metabolites in Organ Culture, NAT Activity and Beta-Adrenergic Receptor Binding

2.1.1 Materials and Methods

2.1.2 Animals

Albino male rats of the Wistar strain were used and monitored under an automatically regulated lighting cycle of LD 12:12 in well-ventilated plastic cages. The temperature was controlled at 20°C and the rats enjoyed free access to food and water.

2.1.3 Administration of Drugs to Animals

For all the experiments undertaken, the animals were divided into groups of five animals each, with one group serving as the control. In the instances where alpha-methyldopa was injected, it was given i.p. at a concentration of 50 mg/kg (Barnes and Etherington, 1973) over a 1½ day period (1000 h, 1700 h, and 1000 h). Alpha-methyldopa was dissolved in deionized, distilled water, and fully solubilized with 0.05% HCl. Animals in the control group received the vehicle for alpha-methyldopa only.

In the instances where isoproterenol (1-[3',4'-dihydroxyphenyl]-2-isopropylaminoethanol; isopropylarterenol hydrochloride) was given in vivo, the drug was administered i.p. at a concentration of 5 mg/kg (Barnes and Etherington, 1973), three hours prior to sacrifice. The injectable solution was prepared by dissolving isoproterenol in deionized, distilled water shortly prior to dosing.

Reserpine (methyl 11,17 alpha-dimethoxy-18beta-(3,4,5-trimethoxy-benzoyloxy)-3beta, 20alpha-yohimbine-16beta-carboxylate; methyl 18-O-(3,4,5-trimethoxybenzoyl) reserpate) was administered i.p. at a concentration of 5 mg/kg (Barnes and Etherington, 1973), allowing at least 18 hours for the drug to deplete norepinephrine from their storage vesicles. The injectable solution was prepared by dissolving reserpine in benzyl alcohol at a concentration of 10 mg/ml. Citric acid was added to serve as an antioxidant, and caution was taken to prevent the solution from being exposed to light. Control groups

received the identical vehicle for reserpine.

2.1.4 Dissection

In all the experiments, the rats were killed swiftly by neck fracture and decapitated by guillotine. A pair of scissors was used to make an incision through the bone on either side of the head, from the foramen magnum to near the orbit. The top of the skull was removed with forceps and the pineal gland taken out rapidly. Care was taken to remove the pineal stalk from the pineal gland and to remove any visible traces of blood. All pineal excision procedures were begun at 14h30. In the instances where pineal glands were bisected and half-pineals used in a particular assay, the corresponding half-pineals remaining, were frozen at -80°C for later use in various analyses.

2.1.5 Investigation of Rat Pineal Serotonin Metabolism by Organ Culture

Organ culture experiments and separation of [^{14}C]-serotonin metabolites (Klein and Notides, 1969) were performed in order to determine the effect of alpha-methyldopa on the pineal metabolites aHT and aMT. Additional serotonin metabolites were assayed as well.

The experimental technique involves incubating pineal glands from rats for 24 hours in culture medium with radioactive ^{14}C-serotonin. During the incubation period, the pineal glands take up the [^{14}C]-serotonin and metabolize it. The metabolites are subsequently released into the culture medium. After the 24 hour incubation period, a fraction of the culture medium is analysed for levels of radioactive serotonin metabolites using thin-layer chromatography (TLC) and liquid scintillometry.

2.1.5.1 Chemicals and Materials

Alpha-methyldopa, ± isoproterenol and phentolamine were obtained from Sigma Chemical Co. (St. Louis, Mo). BGJb culture medium (Fitton-Jackson modification) was obtained from Gibco (Europe). Chromatography plates (Kieselgel 60F_{254}) were obtained from Merck (West Germany). The 5-hydroxy-[side chain-2-^{14}C]tryptamine creatinine sulphate (specific activity = 57 mCi/mmol) was obtained from Amersham (England). Beckman Ready-Solv™ MP multi-purpose pre-mixed liquid scintillation solution was obtained from Beckman RIIC, Ltd (Scotland).

2.1.5.2 Organ Culture

Individual pineal glands were collected and cultured in BGJb culture medium containing 2 μCi 5-hydroxy-[side chain-2-^{14}C] tryptamine creatinine sulphate (specific activity 57 mi Ci/mmol) in organ culture tubes at 37°C for 24 hours (total volume of 60 μl). A 5% CO_2:95% O_2 environment was obtained by gassing the tubes with carbagen prior to incubation. The BGJ6 culture medium is a "Fitton-Jackson modification", to which was added 0.05 mg of the antibiotics benzyl penicillin, streptomycin sulphate, and amphotericin B per millilitre of the culture medium.

In experiments involving drug addition to the culture medium, 10 μl of the drug was added to 52 μl of the culture medium (containing the pineal gland) prior to addition of the [^{14}C]-serotonin. Where basal pineal [^{14}C]-serotonin metabolite levels had to be determined, 10 μl of deionized and distilled water was substituted for the drug. After 24 hours, the reaction was stopped by removal of the pineal glands from the culture media.

2.1.5.3 Thin-Layer Chromatography

A 10 μl aliquot of the culture medium was spotted on 10 cm x 10 cm TLC plates. The TLC plates used were aluminium foil based and coated with kieselgel, and 0.2 mm thick. Standards of all the pineal serotonin metabolites measured were subsequently spotted on top of the culture medium spot. A mixture (10 μl) of these standards was used. The solution containing the standards was prepared by dissolving approximately 1 mg of each of the standards used together in 2.5 ml 95% ethanol. A few grains of ascorbic acid were added to the solution as an antioxidant. The solution was stored in the dark at -20°C. During the spotting process, a gentle stream of nitrogen was used to dry the spots and prevent atmospheric oxidation of the metabolites.

Prior to performing separation of the organ culture [^{14}C]-serotonin metabolites by TLC, the standards were separated by TLC in order to identify the location of the metabolites, and to evaluate the quality of the separation. A two-dimensional chromatographic separation of the metabolites was performed. The first solvent used (solvent A) was composed of chloroform:methanol:glacial acetic acid 93:7:1 and was

Solvent A		Solvent B
Chloroform	93	Ethyl acetate
Methanol	7	
Glacial acetic acid	1	

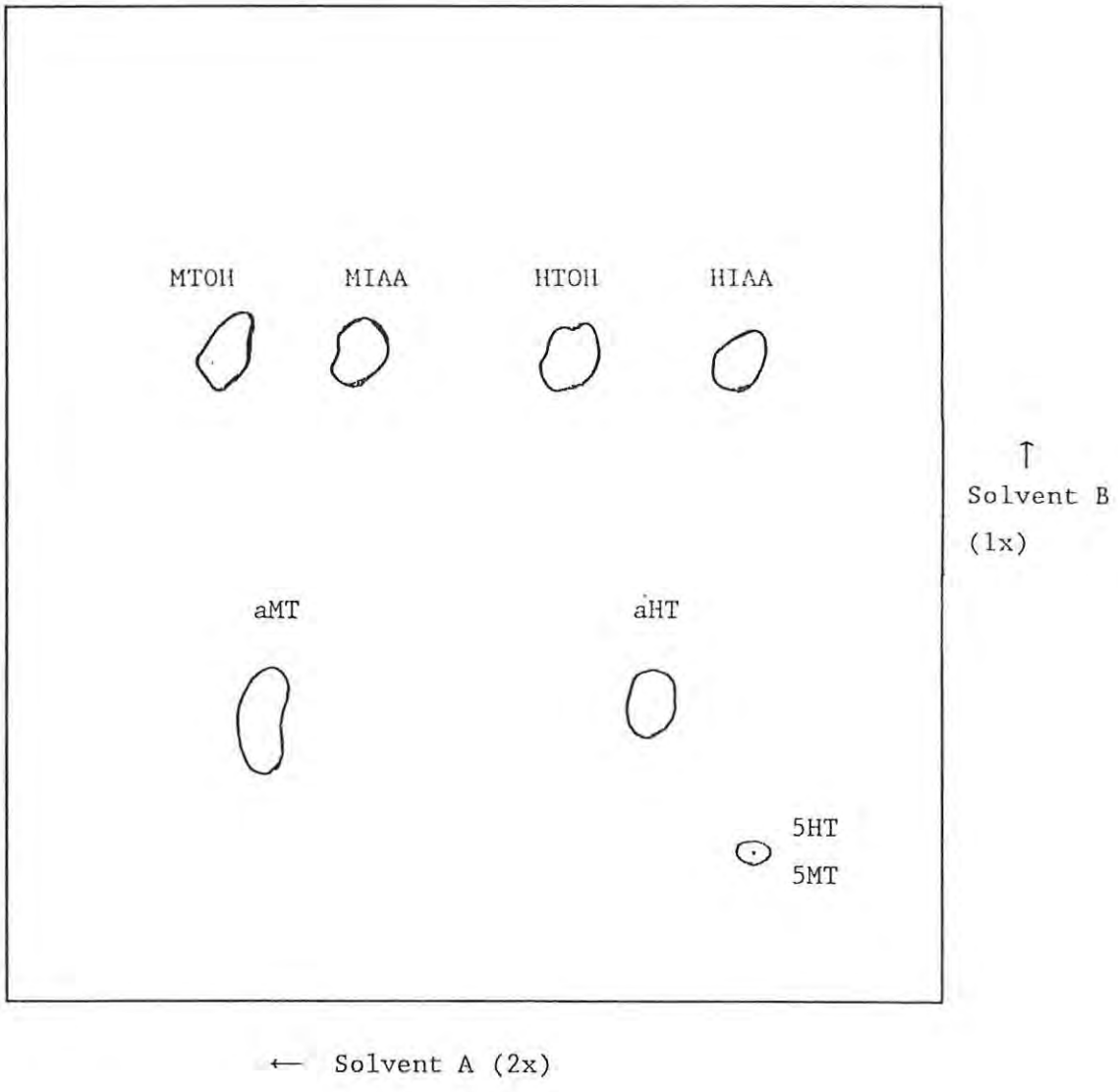

Fig. 2.1 Illustration of chromatographic separation of pineal indole compounds.

allowed to run up to approximately 9 cm (± 40 min). The plates were then removed from the tanks, dried under nitrogen, and then re-run in the same solvent up to the same point as in the previous run. The plates were removed, dried under nitrogen, and run in the second direction up to a height of approximately 5 cm using ethyl acetate (solvent B) (± 10 min). A stream of nitrogen was used to evaporate the ethyl acetate off the plates. The plates were then sprayed with Van Urk's reagent (1 g paradimethylbenzaldehyde dissolved in 50 ml 25% HCl followed by the addition of 50 ml 95% ethanol). Development of the spots was performed by placing the plates in an oven at 60°C for 20 minutes, and the position of each spot was marked with a pencil. The relative positions of these spots is illustrated in Fig. 2.1.

2.1.5.4 Liquid Scintillometry

Following development, sections corresponding to each [^{14}C]-serotonin metabolite were cut out and placed into scintillation vials. Caution was exercised in containing the spread of radioactivity during this procedure. To each of the vials was added 1 ml absolute ethanol and 3 ml scintillation cocktail (Beckman HP-B), and shaken on a mechanical shaker for 20 min.

Scintillation readings were obtained using a Beckman LS2800 scintillation counter. The CPM were converted to DPM using a pre-programmed quench correction curve, and the results expressed as DPM "pineal metabolite"/10 μl medium/pineal gland ± S.E.M. Blank samples were run in an identical fashion with the exception that the pineal glands were excluded. The blank valves obtained were subtracted from the pineal metabolite DPM readings. Tissue blanks using other tissues were not used as monoamine oxidase (MAO), which is present in most tissues, has the ability to oxidise serotonin.

2.1.5.5 The Effect of Alpha-Methyldopa on Pineal Serotonin Metabolites in Organ Culture

The following organ culture experiments were performed:

(i) The determination of pineal aHT and aMT levels after administration of alpha-methyldopa (50 mg/kg i.p.) to rats (n=5). Distilled water (10 μl) was also added to each culture medium tube

containing a pineal gland prior to incubation with [^{14}C]-serotonin. Comparative basal pineal metabolite levels were determined for untreated rats as well (n=5).

(ii) The determination of pineal aHT and aMT levels after in vitro exposure of the glands to 10 μM alpha-methyldopa (n=5). Alpha-methyldopa (10 μl), at a final concentration of 10 μM, was added to each culture tube prior to incubation with [^{14}C]-serotonin. The preparation of alpha-methyldopa involved dissolving the drug in deionized and distilled water with the addition of 0.05% HCl until fully solubilized. In addition, basal pineal [^{14}C]-serotonin metabolite levels were determined (n=5).

(iii) The determination of pineal aHT and aMT levels after in vitro beta-adrenergic stimulation of pineal glands using isoproterenol (n=5). 10 μl isoproterenol solution was added to each culture tube prior to incubation with [^{14}C]-serotonin (final isoproterenol concentration of 10 μM). In the case of basal pineal aHT and aMT determinations (n=5), 10 μl distilled water was substituted for the drug. In this experiment, no drug was administered prior to sacrifice.

(iv) The determination of pineal aHT and aMT levels after prior administration of alpha-methyldopa (50 mg/kg i.p.) and in vitro beta-adrenergic stimulation of the excised pineal glands (n=5) in culture media with isoproterenol (final concentration 10 μM). The procedure was performed exactly as in the first organ culture experiment (i), with the exception that 10 μl isoproterenol was added to each culture tube prior to incubation with [^{14}C]-serotonin. Basal pineal aHT and aMT levels (n=5) were also determined.

(v) The determination of pineal aHT and aMT levels including those of additional [^{14}C]-serotonin metabolites after the in vitro addition of phentolamine, an alpha 1- and alpha 2-receptor blocker, to pineal glands in organ culture from rats pre-treated with alpha-methyldopa (50 mg/kg i.p.) (n=5). The final concentration of phentolamine in organ culture was 10 μM. For comparative purposes, the levels of the same [^{14}C]-serotonin metabolites were determined after prior administration of alpha-methyldopa (50 mg/kg i.p.) (n=5), as described in (i).

[^{14}C]-serotonin pineal metabolite levels were also determined in organ cultures treated with 10 μM isoproterenol subsequent to the in vivo administration of alpha-methyldopa (50 mg/kg i.p.) (n=5) as described in (iv). An identical combination of treatments were given with the exception that 10 μM phentolamine was added to the organ cultures (n=5). Comparative basal [^{14}C]-serotonin pineal metabolite levels were also determined (n=5). Additional [^{14}C]-serotonin pineal metabolite levels determined were 5-hydroxyindole acetic acid (5-HIAA), 5-hydroxytryptophol (5-HTOH), 5-methoxyindole acetic acid (5-MIAA), and 5-methoxytryptophol (5-MTOH).

2.1.5.5.1 Statistics

Statistical comparisons for experiments (i), (ii), (iii) and (iv) in section 2.1.5.5 were made by using student's t test. Owing to the multiple combinations of treatment within a single experiment described in (v) (section 2.1.5.5), the Student-Newman Keuls Multiple Range Test was applied for comparison of groups. All data derived from the organ culture experiments are expressed as dpm (X10) [^{14}C]-serotonin metabolite/10 μl medium/pineal gland, calculated as the mean $\pm$ S.E.M. (n=5).

2.1.5.5.2 Results

Pineal glands from rats treated with alpha-methyldopa produced significantly elevated levels of aHT ($P< 0.001$) as well as aMT ($P< 0.025$) (Table 2.1) (Figs. 2.2, 2.3) in comparison to basal values. However, in vitro exposure of pineal glands to alpha-methyldopa (10 μM) did not alter the levels of these metabolites (Table 2.2). Exposure of pineal glands to isoproterenol 10 μM) induced a significant increase in pineal production of aHT ($P< 0.001$) and AMT ($P< 0.001$) (Table 2.1) (Figs. 2.2, 2.3) in comparison to basal levels. Pineal gland production of aHT and aMT after exposure of pineals from alpha-methyldopa-treated rats to isoproterenol were significantly raised in comparison to the raised levels obtained after alpha-methyldopa treatment alone ($P< 0.025$) (Table 2.1) (Figs 2.2, 2.3).

Pineal glands from rats treated with alpha-methyldopa and exposed in vitro to phentolamine (10 μM) produced significantly lower levels of aMT ($P< 0.05$) in comparison to levels derived from alpha-methyldopa

Table 2.1

Effect of isoproterenol, alpha-methyldopa, and isoproterenol and alpha-methyldopa treatment on rat pineal gland production of N-acetylserotonin (aHT) and melatonin (aMT) in organ culture. [DPM ^{14}C-metabolite/10μl medium/pineal gland ± S.E.M.] (n=5)

Pineal Metabolite	Treatment				Significance		
	Basal	Isoproterenol	Alpha-methyldopa (50mg/kg i.p.)	Alpha-methyldopa (50mg/kg i.p.) + Isoproterenol	Isoproterenol vs. Basal	Alpha-methyldopa vs. Basal	Alpha-methyldopa + Isoproterenol vs.
aHT	281.6 ± 60	775.5 ± 20	2712.7 ± 380	2712.7 ± 380	P< 0.001	P< 0.001	P< 0.025
aMT	2038.8 ± 290	3361.2 ± 203	5396.5 ± 598	5396.5 ± 877	P< 0.01	P< 0.025	P< 0.025

Table 2.2

Effect of alpha-methyldopa (in vitro) on rat pineal production of N-acetylserotonin (aHT) and melatonin (aMT) in organ culture. [DPM ^{14}C-metabolite/10μl medium/pineal gland ± S.E.M.] (n=5)

Pineal Metabolite	Treatment		Significance
	Basal	Alpha-methyldopa	Alpha-methyldopa vs. Basal
aHT	552.4 ± 80	580.4 ± 82	N.S.
aMT	2909.0 ± 507	2239.4 ± 253	N.S.

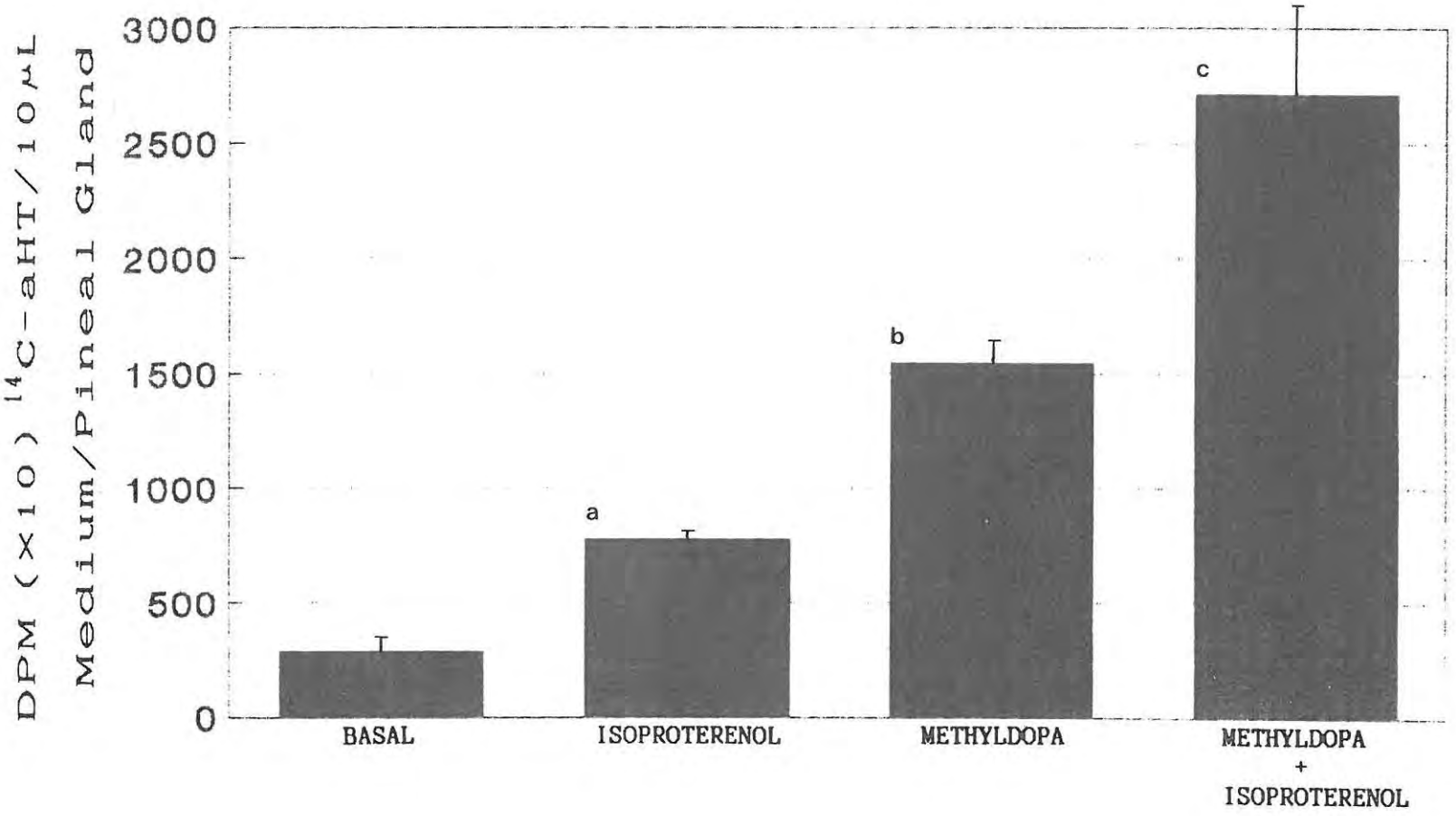

Fig. 2.2 Effect of isoproterenol, alpha-methyldopa, and isoproterenol and alpha-methyldopa on rat pineal gland production of N-acetylserotonin (aHT) ± S.E.M. a = P< 0.01 vs. basal levels. b = P< 0.001 vs. basal levels. c = P< 0.025 vs. alpha-methyldopa treatment.

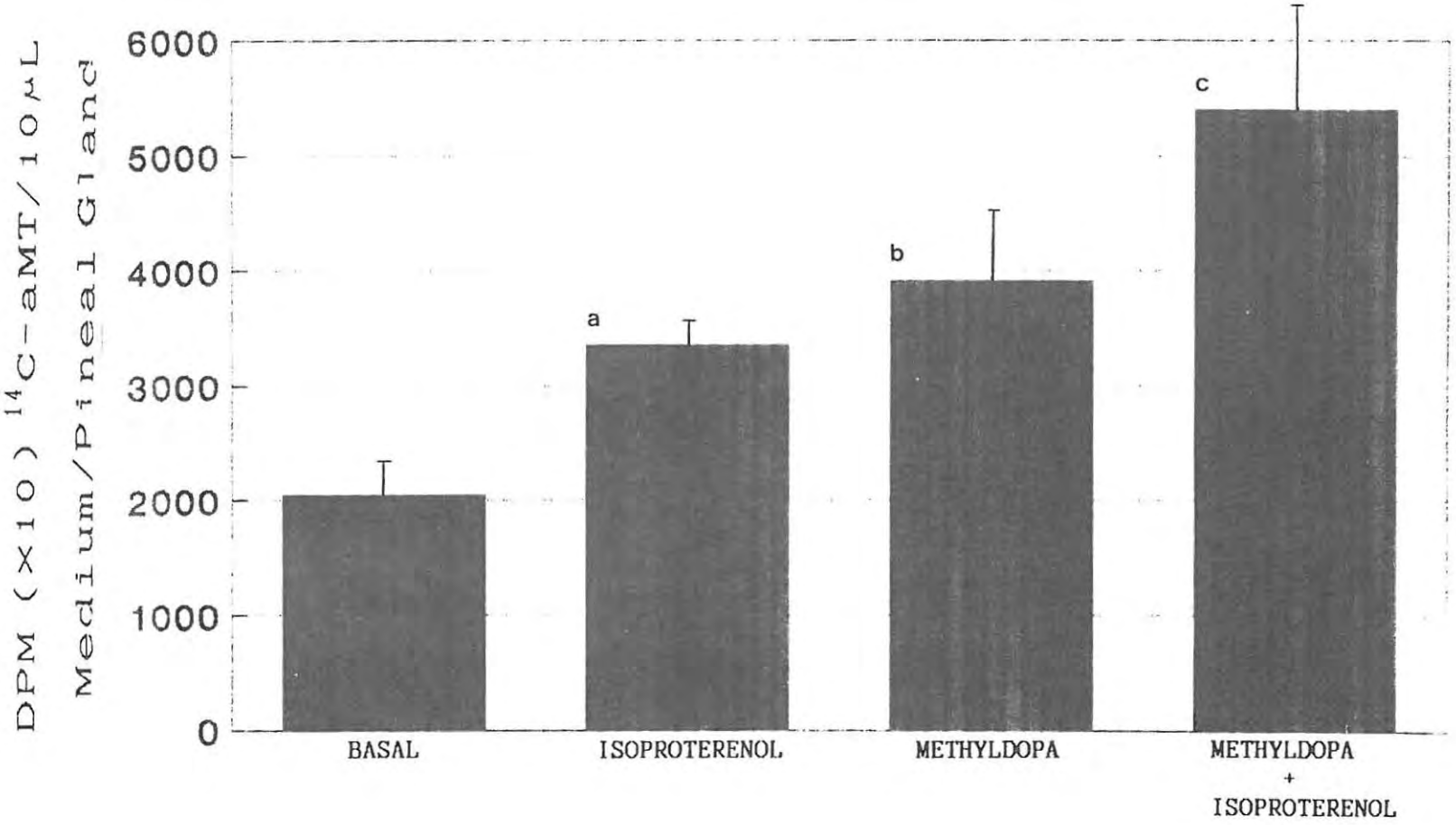

Fig. 2.3 Effect of isoproterenol, alpha-methyldopa, and isoproterenol and alpha-methyldopa on rat pineal gland production of melatonin (aMT) ± S.E.M. a = P< 0.001 vs. basal levels. b = P< 0.025 vs. basal levels. c = P< 0.025 vs. alpha-methyldopa treatment.

Table 2.3

Effect of alpha-methyldopa, alpha-methyldopa and phentolamine, alpha-methyldopa and isoproterenol, and alpha-methyldopa, isoproterenol and phentolamine treatment on rat pineal production of N-acetylserotonin (aHT), melatonin (aMT), and other pineal indole metabolites of serotonin in organ culture. [DPM ^{14}C-metabolite/10μl medium/pineal gland ± S.E.M.] (n=5). HIAA = 5-hydroxyindole acetic acid, HTOH = 5-hydroxytryptophol, MIAA = 5-methoxyindole acetic acid, MTOH = 5-methoxytryptophol.

Pineal Metabolite	Treatment					Significance		
	Basal	Alpha-methyldopa (50mg/kg i.p.)	Alpha-methyldopa (50mg/kg i.p.) + Phentolamine	Alpha-methyldopa (50mg/kg i.p.) + Isoproterenol	Alpha-methyldopa (50mg/kg i.p.) + Isoproterenol + Phentolamine	Alpha-methyldopa vs. Basal	Alpha-methyldopa + Phentolamine vs. Alpha-methyldopa	Alpha-methyldopa + Isoproterenol + Phentolamine vs. Alpha-methyldopa + Isoproterenol
aHT	531.8 ± 133	1158.2 ± 175	740.5 ± 179	2021.6 ± 293	1250.5 ± 146	P< 0.005	N.S.	P< 0.01
aMT	501.2 ± 127	1851.3 ± 284	1305.2 ± 209	2449.6 ± 133	2043.6 ± 170	P< 0.001	P< 0.05	N.S.
HIAA	9136.3 ± 3214	3776.3 ± 1895	10261.9 ± 2908	10791.2 ± 2284	12390.1 ± 4154	N.S.	N.S.	N.S.
HTOH	10271.8 ± 2264	2980.6 ± 1347	10510.9 ± 1798	8842.2 ± 1770	8569.2 ± 1428	P< 0.025	P< 0.025	N.S.
MIAA	81.1 ± 15	56.4 ± 6	77.6 ± 16	72.1 ± 5	57.4 ± 18	N.S.	N.S.	N.S.
MTOH	468.1 ± 123	202.6 ± 54	184.0 ± 47	240.8 ± 70	188.9 ± 59	P< 0.05	N.S.	N.S.

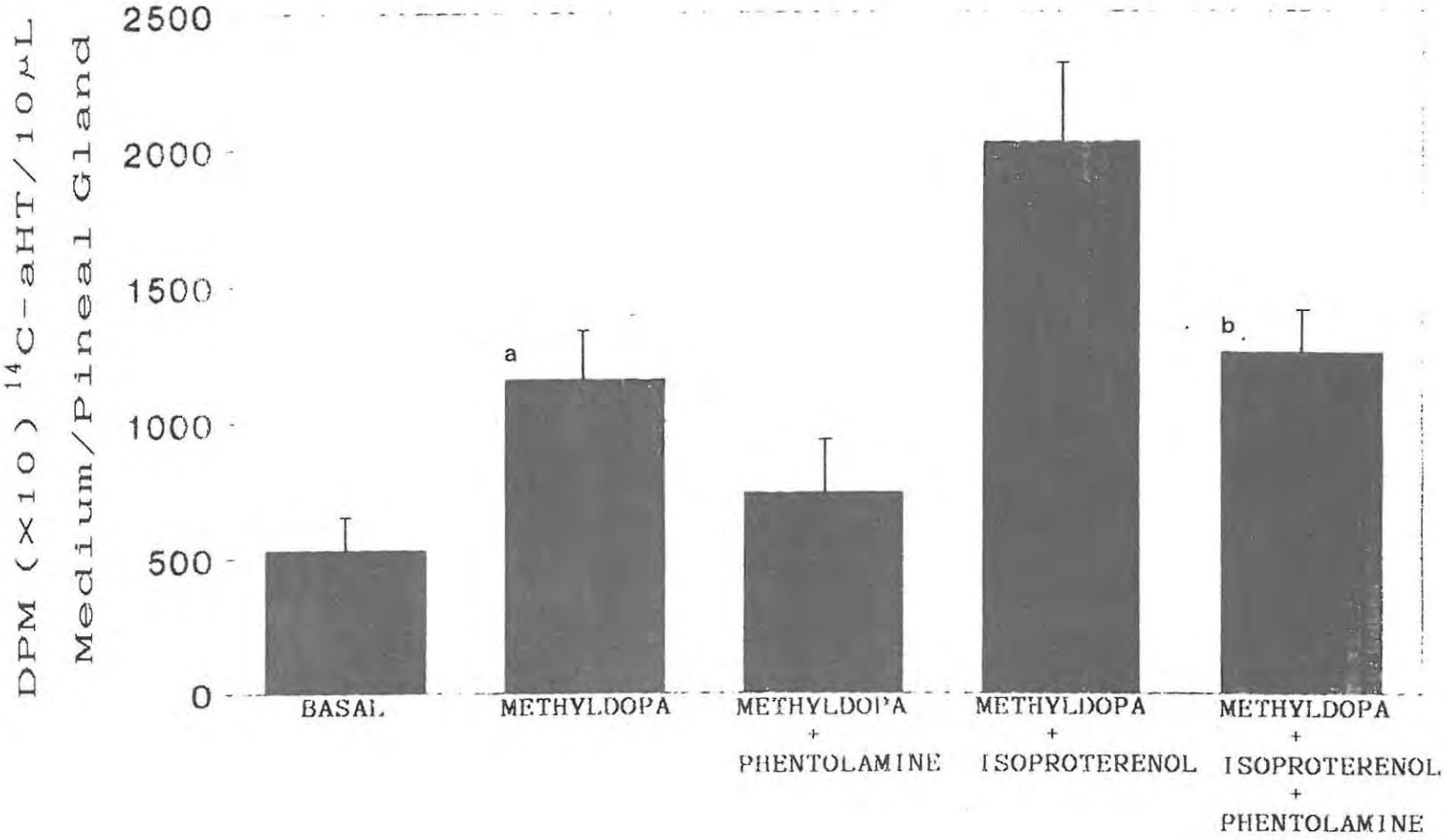

Fig. 2.4 Effect of alpha-methyldopa, alpha-methyldopa and phentolamine, alpha-methyldopa and isoproterenol, and alpha-methyldopa, isoproterenol and phentolamine treament on rat pineal production of N-acetylserotonin (aHT) ± S.E.M. a = $P < 0.05$ vs. basal levels. b = $P < 0.01$ vs. alpha-methyldopa and isoproterenol treatment.

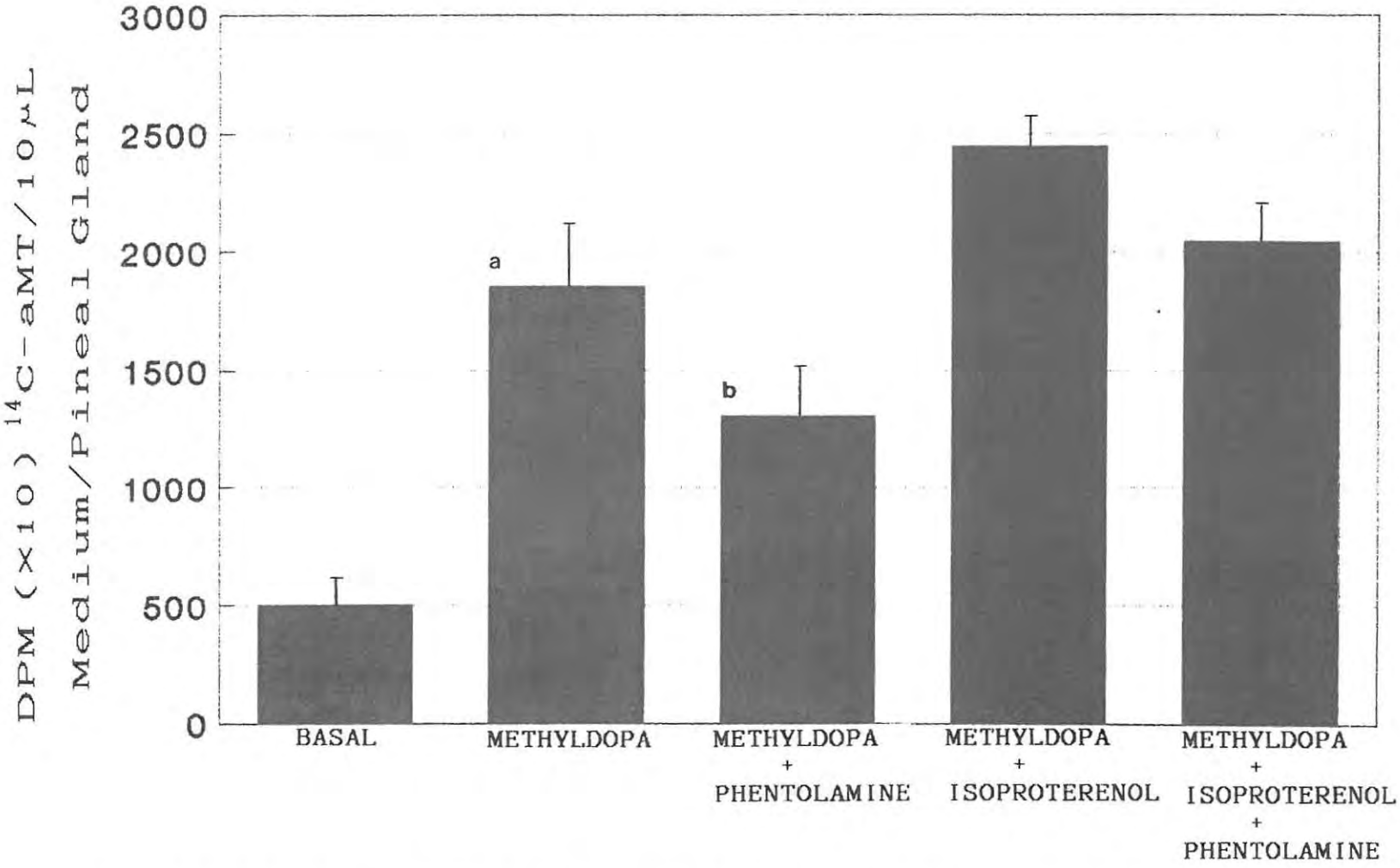

Fig. 2.5 Effect of alpha-methyldopa, alpha-methyldopa and phentolamine, alpha-methyldopa and isoproterenol, and alpha-methyldopa, isoproterenol and phentolamine treatment on rat pineal production of melatonin (aMT) ± S.E.M. a = P< 0.001 vs. basal levels. b = P< 0.05 vs. alpha-methyldopa treatment.

treatment alone. A decrease in aHT levels was also observed, though not to a significant degree (Table 2.3) (Figs. 2.4, 2.5). Combined *in vitro* treatment of pineal glands from alpha-methyldopa treated rats with isoproterenol (10 μM) and phentolamine (10 μM) produced significantly lower levels of aHT ($P< 0.01$) than pineal glands from alpha-methyldopa treated rats, treated *in vitro* with isoproterenol (10 μM) alone. Lower aMT levels were also observed in the latter group (Table 2.3) (Figs. 2.4, 2.5). In accordance with previous results (above) treatment of rats with alpha-methyldopa produced a significant increase in aHT ($P< 0.05$), and aMT ($P< 0.001$) levels in comparison to basal levels (Table 2.3) (Figs. 2.4, 2.5). Levels determined for HIAA, HTOH, MIAA and MTOH fluctuate considerably from group to group (Table 2.3). The most noticeable differences in the levels of these metabolites are between those derived from alpha-methyldopa treated rats, and basal levels. The basal levels are higher than those in the alpha-methyldopa treated group, and significantly so, in the case of HTOH ($P< 0.025$) and MTOH ($P< 0.05$) (Table 2.3). In the instance where pineals from alpha-methyldopa treated rats were treated with (10 μM) phentolamine, levels of HIAA, HTOH and MIAA were higher than the corresponding levels in alpha-methyldopa treated rats, and significantly so, in the case of HTOH ($P< 0.025$) (Table 2.3). MTOH levels, however, are lower in the former group. HTOH, MIAA and MTOH levels in rat pineal glands from alpha-methyldopa treated rats and which were treated *in vitro* with isoproterenol (10 μM) and phentolamine (10 μM) are lower than the corresponding levels in rat pineal glands from alpha-methyldopa treated rats, which were treated *in vitro* with isoproterenol (10 μM) (Table 2.3). Conversely, HIAA shows a higher level in the former group.

2.1.5.6 Discussion

Treatment of rats with alpha-methyldopa resulted in a significant increase in pineal production of aHT and aMT. *In vitro* exposure of pineal glands to alpha-methyldopa, however, did not increase the level of these two metabolites, suggesting that either alpha-methyldopa requires to be metabolized to an active metabolite or that it is unable to reach pinealocytes *in vitro*. The former supports the current theory regarding the fate of alpha-methyldopa physiologically, that alpha-methyldopa is converted to alpha-methylnorepinephrine by the action of

dopa decarboxylase. The alpha-methylnorepinephrine is hypothesized to act as a false transmitter (Rudd and Blaschke, 1985). The post ganglionic neurons which terminate in the pineal may be the site of metabolism of alpha-methyldopa. Degeneration of these endings subsequent to removal of the pineal may thus account for the failure of alpha-methyldopa to be converted in vitro to an active metabolite. An alternative consideration is that the in vivo production of aHT and aMT production is induced via the sympathetic innervation of the gland following a primary action of the drug in the brain.

The stimulation of pineal glands with isoproterenol resulted in significantly raised levels of aHT and aMT as expected. However, when isoproterenol was used to stimulate pineal glands from rats treated with alpha-methyldopa, levels of aHT and aMT were found to be raised to a significantly greater extent than with alpha-methyldopa treatment above. This is suggestive of the occurrence of a supersensitivity type phenomenon at the level of the beta-receptor, as has been shown to occur with reserpine (Cantor et al., 1981).

A possible explanation of the alpha-methyldopa potentiation of the beta-agonist isoproterenol is that the metabolite of alpha-methyldopa, alpha-methylnorepinephrine, interacts with the alpha-receptor. Melatonin production in the rat pineal is controlled by norepinephrine acting via alpha 1- and beta-adrenoreceptors on pinealocytes (Sugden et al., 1984); activation of alpha-adrenoreceptors appears to potentiate beta-adrenergic stimulation of rat pineal NAT by isoproterenol. Alpha-methylnorepinephrine might possibly also potentiate the beta-adrenergic stimulation of N-acetyltransferase by acting on the alpha-receptor. This might serve to explain the significant increases in aHT and aMT associated with alpha-methyldopa treatment, as well as the potentiation of the isoproterenol response by alpha-methyldopa. Alpha-methylnorepinephrine is thus a weak beta-agonist and possibly an alpha-receptor agonist as well.

In an attempt to confirm the alpha-agonistic activity of the physiological metabolite of alpha-methyldopa, the alpha 1- and alpha 2-receptor blocking agent, phentolamine, was used in conjunction with alpha-methyldopa. In vitro exposure of pineal glands from alpha-

methyldopa treated rats to phentolamine produced lower levels of aHT and significantly lower levels of aMT in comparison to levels of the metabolite obtained from alpha-methyldopa treatment alone. This is supportive of the idea that alpha-methylnorepinephrine possesses alpha-agonistic activity.

Combined in vitro treatment of pineal glands from alpha-methyldopa treated rats with isoproterenol and phentolamine produced lower levels of aMT and significantly lower levels of aHT than pineal glands from alpha-methyldopa treated rats, exposed in vitro to isoproterenol alone. This appears to confirm that a proportion of the adrenergic-receptor response to alpha-methylnorepinephrine is mediated via the alpha-receptor, in accordance with the hypothesis. Furthermore, it is of interest to note that although aMT levels were diminished, the decrease was not to a significant degree. There exists a hypothesis that the neural control of pineal HIOMT is mediated via a beta-adrenergic receptor (Sugden and Klein, 1983). There also exists the possibility that alpha-adrenergic activity might potentiate beta-receptor activity, and indirectly influence HIOMT. Blockade of the alpha-receptor might possibly then diminish neural control of HIOMT in the pinealocyte.

The variations in levels occurring in the other serotonin metabolites are more complex to explain. HIAA, HTOH, HIAA and MTOH levels derived from pineals in alpha-methyldopa treated rats are lower than the basal levels of these metabolites, and significantly so in the case of HTOH and MTOH. This phenomenon may possibly be explained by the fact that raised NAT levels as a result of alpha-methyldopa treatment diminishes serotonin, thus providing less substrate for MAO to produce 5-hydroxy-indoleacetaldehyde, the precursor for HIAA, HTOH, MIAA, and MTOH. In addition, less HIOMT would be available for the conversion of HTOH to MTOH and HIAA to MIAA, due to increasing levels of aHT substrate for the enzyme. This explanation would conflict with the model presented by Foley and Cairncross (1987) in which HTOH is suggested to be regulated independently, and not only by simple competition with NAT for the common substrate serotonin. In addition, the assertion by Morton (1987), that the production of methoxyindoles is dependent on the concentration of the various hydroxyindoles and their affinity for HIOMT is also suggestive of an entirely independent regulatory status,

and cannot satisfactorily explain the variations which occurred with these metabolites. The levels of HIAA, HTOH, MIAA and MTOH derived when combinations of alpha-methyldopa, isoproterenol and phentolamine were used are probably the result of complex intereactions and competition between substrates and enzymes within the pinealocyte.

2.1.6 Assaying for Serotonin-N-Acetyltransferase (NAT) Activity

The assay used for the determination of NAT activity was a modification of the method described by Deguchi and Axelrod (1972). The assay involves the acetylation of tryptamine with [^{3}H]-acetyl-coenzyme A by NAT to produce [^{3}H]-N-acetyltryptamine. Employing a different technique, [^{14}C]-labelled-acetyl-coenzyme A was also used for the assay of NAT activity. The [^{14}C]-labelled coenzyme is less sensitive than the [^{3}H]-labelled one, however it is less prone to degradation.

Addition of [^{3}H]-acetyl-CoA to pineal homogenates and incubation with tryptamine results in the enzymatic formation of radioactive acetyltryptamine, which can then be extracted into an organic solvent and measured by liquid scintillometry. The amount of radioactive acetyltryptamine/time produced is used as a measure of NAT activity.

2.1.6.1 Chemicals

[^{3}H]-Acetyl-coenzyme A (specific activity=4.0 Ci/mmol) and [^{14}C]-Acetyl-coenzyme A (specific activity 50 mCi/mmol) were obtained from Amersham (England). Tryptamine HCl was obtained from Sigma Chemical Co. (St. Louis, Mo.) and acetyl coenzyme A from Boehringer Mannheim (W. Germany). Beckman HP-B scintillation fluid was obtained from Beckman RIIC, Ltd., (Scotland).

2.1.6.2 NAT Assays

Rats were killed by neck-fracture and pineal glands were removed as described previously. Individual pineal glands were homogenized in 70 μl cold Sørensen's phosphate buffer (pH 6.5) in a glass homogenizer on ice. A 50 μl aliquot of each homogenate was transferred into a separate tube, to which was added 10 μl [^{3}H]-Acetyl-coenzyme A (specific gravity 4.0 Ci/mmol). The tryptamine solution was prepared by dissolving 19.67 mg of the substrate in 10 ml Sørensen's phosphate buffer (pH 6.5). The reaction mixtures were incubated in a water bath

at 37°C for 10 min. The reaction was stopped by the addition of 0.5 ml borate buffer (pH 10). Each reaction mixture was transferred by Pasteur pipette into a glass-stoppered test tube containing 3 ml toluene:isoamyl alcohol (97:3), and mixed on a vortex mixer for 30 s. After centrifugation at 3000 rpm for 10 min, using a Hettich Universal II centrifuge, 2 ml of the organic phase was transferred to a scintillation vial containing 3 ml scintillation cocktail, shaken for 10 min, and then counted in a scintillation counter (Beckman LS 2800).

The CPM values were converted to DPM automatically by a pre-programmed quench correction curve. DPM values were corrected to take into account the radioactive [^{3}H]-N-acetyltryptamine in the 1 ml of organic phase left in the stoppered tubes. NAT activity was expressed as fmol product/gland/hr.

A modification of this assay (Champney et al., 1984) utilizing [^{14}C]-acetyl coenzyme A as the acetyl donor was employed.

In certain instances the removed pineal glands were frozen prior to the assay, for convenience sake. The pineal glands were placed on a spatula cooled on solid CO_2, and then transferred to a microfuge tube and stored at -80°C until assay within 48 hrs.

A volume of 25 μl of PBS (0.05M, pH 6.8) was added to the pineal in the microfuge tube and sonicated at low power for approximately 5.0 s. Keeping the sonicated samples on ice, 10 μl of the homogenate was transferred to a new microfuge tube. A working solution consisting of 10 μl 5.6 mM tryptamine (5 μl/sample), 800 μM acetyl CoA (2 μl/sample), [^{14}C]-acetyl-CoA (50 mCi/mmol, 1.25 μl/sample), and 0.05M PBS pH 6.8 (1 μl/sample) was added to the side of the vial. The tube was tapped, vortexed quickly, and tapped again to combine the two drops. The reaction mixture was thus incubated for 20 minutes at 37°C in a shake bath. Chilled borate buffer (pH 10) (100 μl) was added to stop the reaction, followed by 1.0 ml chilled chloroform for extraction of [14C]-N-acetyltryptamine. The extraction mixture was centrifuged for 30 s and the supernatant aspirated using a Pasteur pipette connected to a flask (for collection of "hot" material). Borate buffer (100 μl) was

then added to wash out contamination. The mixture was shaken for 5 min, centrifuged for 30 s, and the supernatant aspirated as before. A volume of 500 μl of the extract was added to a scintillation vial and the chloroform was allowed to evaporate under a fume hood until completely dry. Beckman HP-B scintillation fluid (3 ml) was added to the tubes, which were then vortexed. The radioactivity was counted for 10 minutes in a scintillation counter (Beckman LS 2800).

2.1.6.3 The Effect of Alpha-methyldopa on Pineal Serotonin-N-acetyl-transferase (NAT) Activity

The following pineal NAT determinations were performed using [^{3}H]-acetyl CoA and the experimental procedure described above:

(i) The determination of the effect of alpha-methyldopa (50 mg/kg i.p.) on rat pineal NAT activity (n=5). Rats were dosed with alpha-methyldopa as described previously, sacrificed, and the pineals removed. Comparative basal NAT levels were also determined.

(ii) The determination of the effect of isoproterenol (5 mg/kg i.p.) on rat pineal NAT activity (n=5). Rats were injected with isoproterenol 3 h prior to sacrifice and the pineals removed. Comparative basal NAT levels were also determined.

(iii) The determination of the effect of alpha-methyldopa (50 mg/kg i.p.) and isoproterenol stimulation (5 mg/kg i.p.) on rat pineal NAT activity (n=5). Rats were dosed with alpha-methyldopa as described previously. Three hours before sacrifice, the rats were injected with isoproterenol as above.

(iv) The determination of pineal NAT activity after the in vitro addition of of phentolamine (10μM) to pineals from rats pretreated with alpha-methyldopa. Phentolamine (10μM) was also added in vitro to pineals from rats pre-treated with alpha methyldopa and 10 μM isoproterenol. The effect of alpha-methyldopa (50 mg/kg i.p.) on rat pineal NAT activity, and NAT activity in isoproterenol (5 mg/kg i.p.) treated rats was also determined for comparative purposes. [^{14}C]-acetyl CoA was used for these assays as well as the modified NAT assay procedure described above.

2.1.6.4 Statistics

Statistical comparisons for experiments (i), (ii) and (iii) in section 2.1.6.3 were made by using students' t test. The Student-Newman Keuls Multiple Range Test was applied for comparison of groups in experiment (iv) in section 2.1.6.3. All data derived from the NAT assays are expressed as moles product/gland/h calculated as the mean ± S.E.M. (n=5).

2.1.6.5 Results

Isoproterenol (5 mg/kg) i.p. induced a significant increase in pineal NAT activity ($P< 0.025$) (Table 2.4, Fig. 2.6) in comparison to basal levels. A significant increase in NAT activity ($P< 0.025$) (Table 2.4, Fig. 2.6) was also observed with alpha-methyldopa treatment i.p. These increases are in accordance with the results of the organ culture experiments.

An increase in NAT activity observed in pineals exposed to combination of alpha-methyldopa (in vivo) and isoproterenol (in vivo) was significantly greater than that shown in pineals exposed to alpha-methyldopa (in vivo) alone ($P< 0.025$) (Table 2.4, Fig. 2.6).

Phentolamine (10 μM in vitro) was observed to induce a decrease in pineal NAT activity in alpha-methyldopa treated rats in comparison to levels obtained with alpha-methyldopa (in vivo) alone, though not to a significant extent (Table 2.5, Fig. 2.7). Similarly, when pineals derived from alpha-methyldopa (i.p.) treated rats were exposed to isoproterenol (10 μM in vitro) and phentolamine (10 μM in vitro), NAT activity levels were observed to be lower than when pineals from alpha-methyldopa treated rats were exposed to phentolamine alone (Table 2.5, Fig. 2.7). These results were not evaluated as being statistically significant, however they do correlate well with data derived from the organ culture experiments.

2.1.6.6 Discussion

Raised NAT levels with alpha-methyldopa treatment were observed, as well as further enhanced levels of enzyme associated with isoproterenol stimulation of pineal glands from alpha-methyldopa treated rats. These results are in accordance with those derived from the organ culture

Table 2.4

Effect of isoproterenol, alpha-methyldopa, and isoproterenol and alpha-methyldopa on rat pineal N-acetyltransferase (NAT) activity. [fmol ^{3}H-N-acetyltryptamine/pineal gland/hour ± S.E.M.] (n=5).

	Treatment				Significance		
	Basal	Isoproterenol (5mg/kg i.p.)	Alpha-methyldopa (50mg/kg i.p.)	Alpha-methyldopa (50mg/kg i.p.) + Isoprotorenol (5mg/kg i.p.)	Isoprotorenol vs. Basal	Alpha-methyldopa vs. Basal	Alpha-methyldopa + Isoprotorenol vs. Alpha-methyldopa
NAT Activity	147.0 ± 23	417.0 ± 7	302.2 ± 25	739.4 ± 71	P< 0.025	P< 0.025	P< 0.025

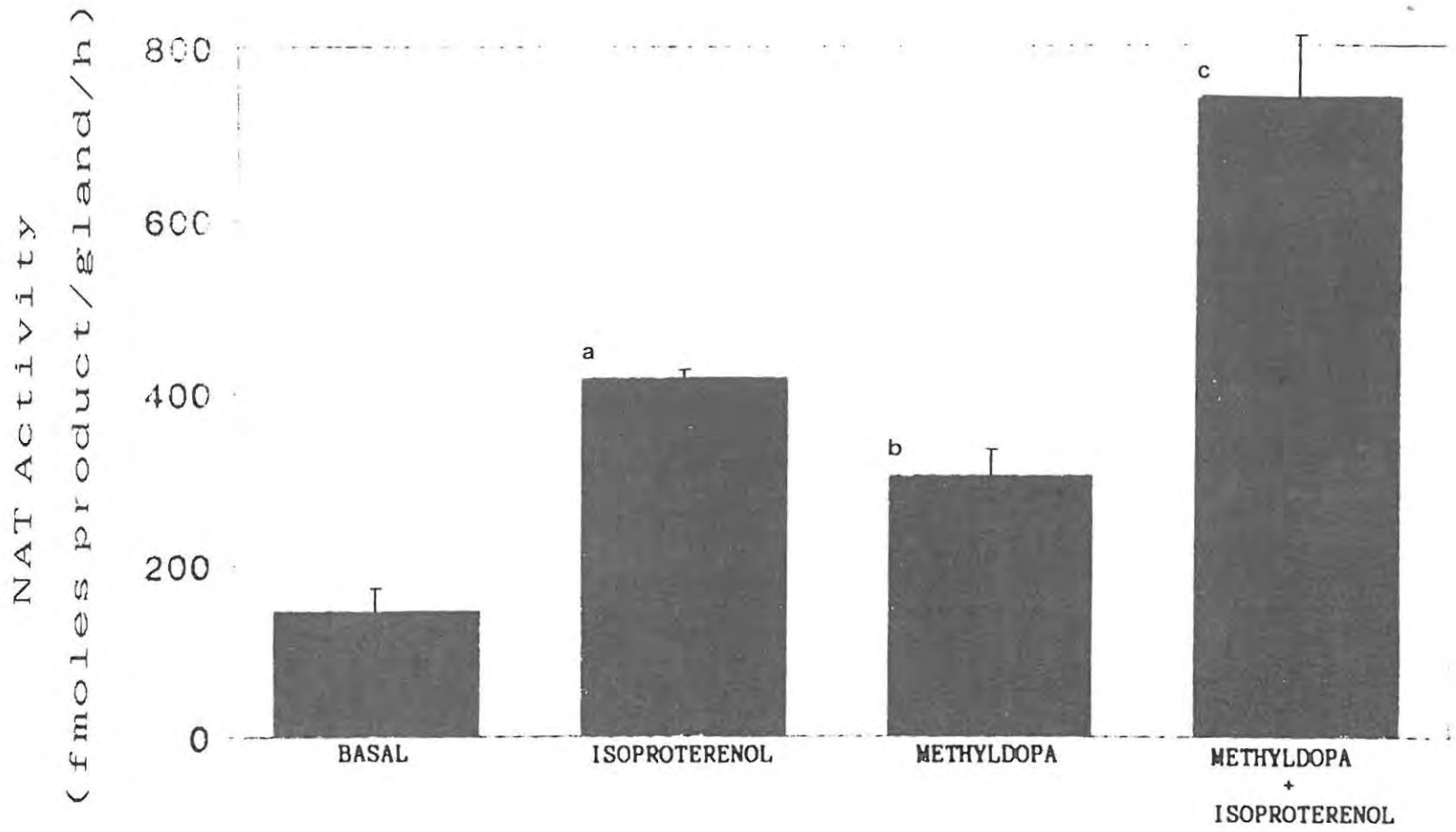

Fig. 2.6 Effect of isoproterenol, alpha-methyldopa, and isoproterenol and alpha-methyldopa on rat pineal N-acetyltransferase (NAT) activity ± S.E.M. a = P< 0.025 vs. basal levels. b = P< 0.025 vs. basal levels. c = P< 0.025 vs. alpha-methyldopa treatment.

Table 2.5

Effect of alpha-methyldopa, alpha-methyldopa and phentolamine, alpha-methyldopa and isoproterenol, and alpha-methyldopa, isoproterenol and phentolamine treatment on rat pineal N-acetyltransferase (NAT) activity. [pmoles product/gland/h ± S.E.M.] (n=5)

	Treatment					Significance		
	Basal	Alpha-methyldopa (50mg/kg i.p.)	Alpha-methyldopa (50mg/kg i.p.) + Phentolamine	Alpha-methyldopa (50mg/kg i.p.) + Isoproterenol	Alpha-methyldopa (50mg/kg i.p.) + Isoproterenol + Phentolamine	Alpha-methyldopa vs. Basal	Alpha-methyldopa + Phentolamine vs. Alpha-methyldopa	Alpha-methyldopa + Isoproterenol + Phentolamine vs. Alpha-methyldopa + Isoproterenol
NAT Activity	65.5 ± 3	80.3 ± 6	72.6 ± 1	105.0 ± 9	97.0 ± 9	N.S.	N.S.	N.S.

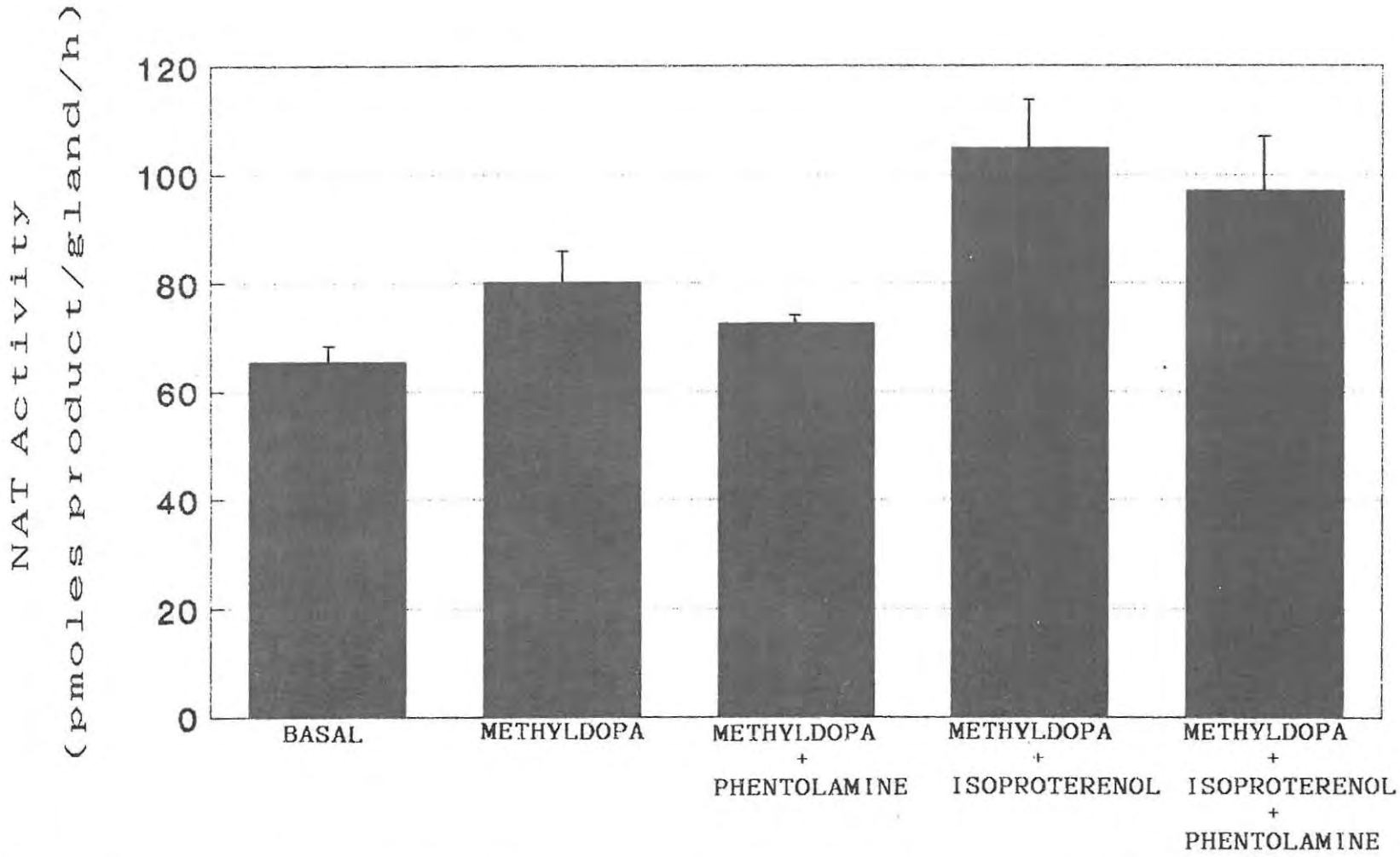

Fig. 2.7 Effect of alpha-methyldopa, alpha-methyldopa and phentolamine, alpha-methyldopa and isoproterenol, and alpha-methyldopa, isoproterenol and phentolamine treatment on rat pineal N-acetyltransferase (NAT) activity ± S.E.M.

experiments. These results lend support to the suggestion of a supersensitivity-type phenomenon occurring at the level of the beta-receptor or that of potentiation of the beta-receptor by the alpha-receptor.

Phentolamine was observed to induce a decrease in pineal NAT activity in alpha-methyldopa treated rats, in comparison to levels obtained with alpha-methyldopa alone. A decrease in NAT activity was also observed when pineals from alpha-methyldopa treated rats were exposed to a combination of isoproterenol and phentolamine, in comparison to the same regimen with the exclusion of phentolamine. These results are in accordance with those derived from the respective organ culture experiments and although not regarded as statistically significant, are supportive of the idea that alpha-methylnorepinephrine has alpha-agonistic activity which potentiates beta-adrenergic stimulation of NAT.

2.1.7. The Effect of Alpha-Methyldopa on Beta-Adrenergic Receptor Binding

The effect of alpha-methyldopa on beta-adrenergic receptor binding in rat pineal glands was investigated using [^{3}H]-CGP-12177 (specific activity 30 Ci/mmol). [^{3}H]-CGP is assumed to bind to beta-receptors as well as to other non-specific sites, giving a total binding value. Propanalol displaces [^{3}H]-CGP from the beta-binding sites selectively, providing a non-specific binding value for [^{3}H]-CGP. Specific binding was determined by subtracting the non-specific binding value from the total-binding value.

2.1.7.1 Chemicals

[^{3}H]-CGP-12177 (4-(3-t-butylamino-2-hydroxy-propoxy)-[5,7-^{3}H] benzimidazol-2-one) (specific activity 30 Ci/mmol) was obtained from Amersham (England). Propanolol was obtained from Sigma Chemical Co. (St. Louis, Mo.).

2.1.7.2 Binding Study

The method used involves a modification of a technique by Wilkinson and Wilkinson (1985), but using half pineal glands instead of the whole organ. Rats were sacrificed, the pineals removed, and bisected with a

scalpel on an upturned plastic petri dish placed on ice.

An alpha-methyldopa treated group (50 mg/kg i.p.) (n=6) was compared with a control group injected with the vehicle. Half of each group were investigated for total binding and the remainder for non-specific binding. Half pineal glands were placed in 225 μl phosphate buffered saline (PBS) pH 7.4, and 10 μl propanolol was added to 6 tubes from each group making a final concentration of 1 μM of the drug in solution. The propanalol solution was prepared by dissolving 7.40 mg of the agent in 10 ml deionized and distilled water, and diluting this solution 1:10 with the same diluent. Deionized and distilled water (10 μl) was added to each of the remaining tubes in both groups. The ligand, [3H]-CGP (15 μl) (diluted 1:1000 in 95% ethanol and PBS) was added to all the tubes making a final [3H]-CGP concentration of 2 nM. The tubes were incubated in a water-bath at 37°C for 120 min. Following this, the tubes were placed back on ice and the buffer solution was removed with a Pasteur pipette. The half-pineal glands were washed twice in ice-cold PBS (pH 7.4) (2 x 0.5 ml, 2 x 5 min), removed, and placed in scintillation vials with 2.0 ml emulsifier and scintillation cocktail (Packard Emulsifier Scint. 299 TM). The vials were shaken for 15 min before counting in a scintillation counter (Beckman LS 2800).

2.1.7.3 Statistics

Statistical comparisons were made by using Student's t test. Specific binding is expressed as CPM [^{3}H]-CGP/half-pineal gland and all data referred to were calculated as the mean ± S.E.M. (n=6).

2.1.7.4 Results

A significant decrease ($P< 0.005$) in pineal beta-receptor binding was demonstrated when rats were treated with alpha-methyldopa (50 mg/kg i.p.) (Table 2.6, Fig. 2.8).

2.1.8 Discussion

Alpha-methyldopa induces a reduction in ligand binding to the beta-receptor, as indicated by the results. The possibility of alpha-methyldopa inducing supersensitivity of the pineal beta-receptor, making the alpha-methyldopa treated rats more responsive to the beta-

Table 2.6

Effect of alpha-methyldopa on specific binding of rat pineal gland beta-receptors [CPM ^{3}H-CGP/half-pineal gland ± S.E.M.] (n=6)

	Treatment		Significance
	Basal	Alpha-methyldopa (50mg/kg i.p.)	Alpha-methyldopa vs. Basal
Specific Binding	287.0 ± 17	200.3 ± 26	P< 0.05

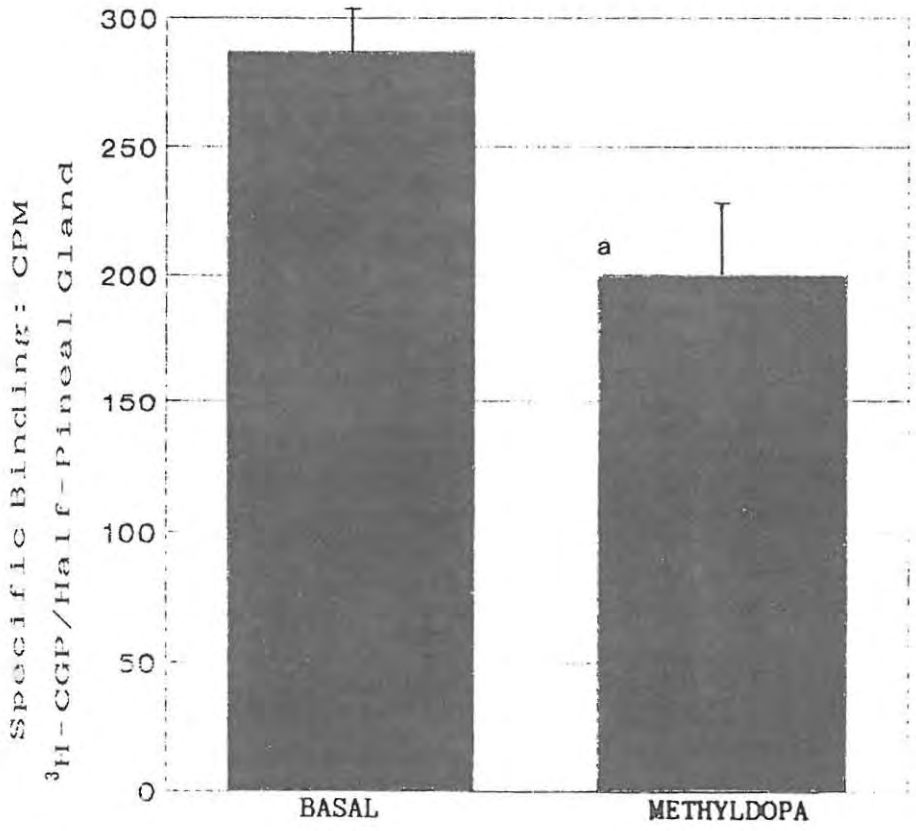

Fig. 2.8 Effect of alpha-methyldopa on specific binding of rat pineal gland beta-receptors ± S.E.M. a = P< 0.025 vs. basal specific binding.

agonist isoproterenol can hence be ruled out. These results lend support rather to alpha-adrenergic activity of the physiological metabolite of alpha-methyldopa, resulting in potentiation of the metabolite's action at the beta-receptor.

2.1.9 The Effect of Ephedrine on Pineal Serotonin Metabolism in Organ Culture

Organ culture experiments were performed in order to determine the effect of ephedrine (in vitro) on pineal serotonin metabolites in organ culture. The techniques used in these experiments with respect to collection of pineals, organ culture, separation of metabolites by TLC, and liquid scintillometry were as described previously, with the exception of the drugs used.

Levels of the metabolites aHT, aMT, HIAA, HTOH, MIAA, and MTOH were determined after exposure of rat pineal glands in vitro to ephedrine, norepinephrine, and norepinephrine and ephedrine. In addition, pineals from rats treated with reserpine (5 mg/kg i.p.) were exposed to ephedrine in vitro. In all cases, 10 μl ephedrine was added to the culture media at a final concentration of the drug of 10 μM. Norepinephrine was also added at a volume of 10 μl to make up a final concentration of the drug in the media of 10 μM. In the instances where drugs were not used, 10 μl deionized and distilled water was added.

2.1.9.1 Chemicals

Ephedrine HCl, reserpine, and norepinephrine were obtained from Sigma Chemical Co. (St. Louis, Mo.). Additional materials used were obtained from sources described earlier.

2.1.9.2 Statistics

Statistical comparisons of groups were made using the Student-Newman Keuls Multiple Range Test. All data derived from the organ culture experiments are expressed as DPM [^{14}C]-metabolite/10 μl medium/pineal gland calculated as the mean $\pm$ S.E.M. (n=5).

2.1.9.3 Results

Treatment of pineal glands in vitro with ephedrine (10 μM) resulted in

significantly raised levels of aHT ($P< 0.001$) and aMT ($P< 0.001$) in comparison to basal levels (Table 2.7, Fig. 2.9, 2.10). Similarly, *in vitro* treatment with norepinephrine (10 μM) resulted in significantly raised levels of aHT ($P< 0.001$) and aMT ($P< 0.001$) in comparison to basal levels (Table 2.7, Fig. 2.9, 2.10). Exposure of rat pineal glands *in vitro* to a combination of norepinephrine (10 μM) and ephedrine (10 μM) produced raised levels of aHT significantly greater than the metabolite levels obtained by exposure to ephedrine (*in vitro*) alone ($P< 0.001$) (Table 2.7, Fig. 2.9). In contrast, this combination of norepinephrine and ephedrine produced significantly lower levels of aMT than when pineal glands were exposed to ephedrine alone ($P< 0.001$) (Table 2.7, Fig. 2.10). These levels of aMT in pineals exposed to norepinephrine and ephedrine were however raised in comparison to basal levels of the metabolite. Exposure of pineal glands from rats treated with reserpine (5 mg/kg i.p.) to ephedrine produced aHT and aMT levels of a similar order to basal levels of these metabolites. Both metabolites were shown to be significantly lower in value than when pineals were exposed to ephedrine (10 μM) alone ($P< 0.001$) (Table 2.7, Fig. 2.9, 2.10).

An assessment of additional metabolites formed shows a significantly diminished proportion of HIAA, MIAA, and MTOH formed ($P< 0.005$) in norepinephrine treated pineal glands in comparison to basal levels (Table 2.7). Exposure of pineal glands to ephedrine results in significantly lower levels of MIAA ($P< 0.01$) and MTOH ($P< 0.05$) in comparison to basal levels of the metabolites (Table 2.7). A combination of norepinephrine and ephedrine induces significantly lower levels of HTOH ($P< 0.05$) in comparison to levels of the metabolite obtained with ephedrine treatment alone (Table 2.7).

2.1.9.4 Discussion

Exposure of pineal glands to ephedrine or norepinephrine in organ culture induced significantly raised levels of aHT and aMT in comparison to basal levels, as was expected. Combined use of norepinephrine and ephedrine produced raised levels of aHT significantly greater than the levels of the metabolite recorded after exposure of the pineal to ephedrine alone. Under these conditions, the levels of aMT were raised in relation to basal levels of the metabolite

Table 2.7

Effect of ephedrine, norepinephrine, norepinephrine and ephedrine, and ephedrine and reserpine treatment on rat pineal production of N-acetylserotonin (aHT), melatonin (aMT), and other pineal indole metabolites of serotonin in organ culture. [DPM ^{14}C-metabolite/10µl/medium/pineal gland ± S.E.M] (n=5)
HIAA = 5-hydroxyindole acetic acid, HTOH = 5-hydroxytryptophol, MIAA = 5-methoxyindole acetic acid, MTOH = 5-methoxytryptophol.

Pineal Metabolite	Treatment					Significance			
	Basal	Ephedrine	Norepinephrine	Norepinephrine + Ephedrine	Ephedrine + Reserpine (5mg/kg i.p.)	Ephedrine vs. Basal	Norepinephrine vs. Basal	Norepinephrine + Ephedrine vs. Ephedrine	Ephedrine + Reserpine vs. Ephedrine
aHT	327.1 ± 5	469.5 ± 18	870.3 ± 9	1798.0 ± 19	217.1 ± 24	P< 0.001	P< 0.001	P< 0.001	P< 0.001
aMT	1457.9 ± 16	4015.3 ± 104	2150.2 ± 58	2365.1 ± 95	1377.5 ± 60	P< 0.001	P< 0.001	P< 0.001	P< 0.001
HIAA	12772.4 ± 2888	9199.3 ± 1270	6473.7 ± 2193	5858.9 ± 861	5356.0 ± 891	N.S.	P< 0.05	N.S.	N.S.
HTOH	3064.6 ± 537	3066.8 ± 491	2860.2 ± 979	2501.5 ± 252	1470.4 ± 387	N.S.	N.S.	P< 0.05	N.S.
MIAA	315.9 ± 90	103.6 ± 17	177.8 ± 74	43.2 ± 10	34.9 ± 4	P< 0.001	P< 0.005	N.S.	N.S.
MTOH	555.5 ± 274	121.6 ± 19	250.3 ± 117	73.9 ± 13	48.7 ± 10	P< 0.005	P< 0.05	N.S.	N.S.

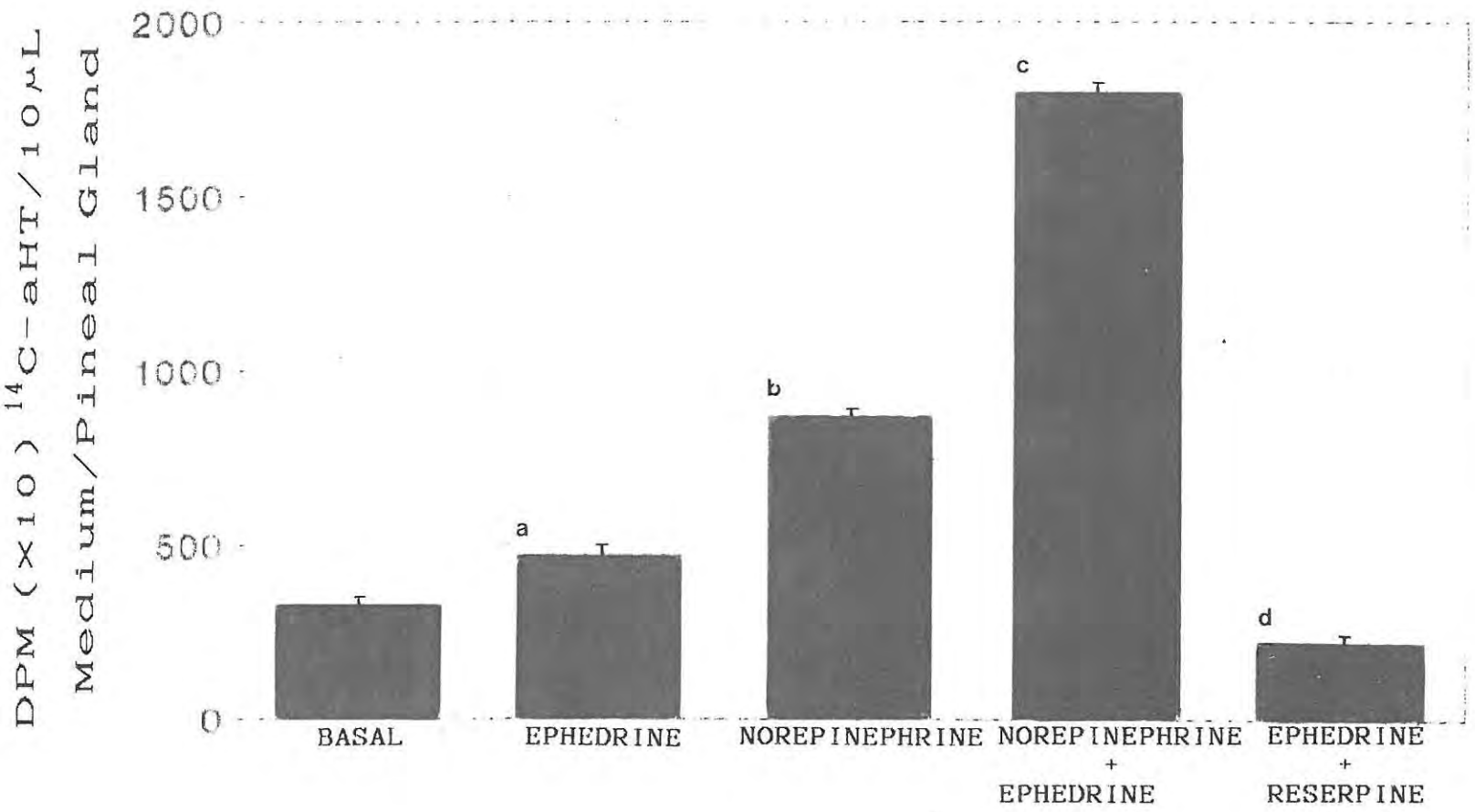

Fig. 2.9 Effect of ephedrine, norepinephrine, norepinephrine and ephedrine, and ephedrine and reserpine treatment on rat pineal production of N-acetylserotonin (aHT) in organ culture ± S.E.M.
a = P< 0.001 vs. basal levels. b = P< 0.001 vs. basal levels. c = P< 0.001 vs. ephedrine treatment. d = P< 0.001 vs. ephedrine treatment.

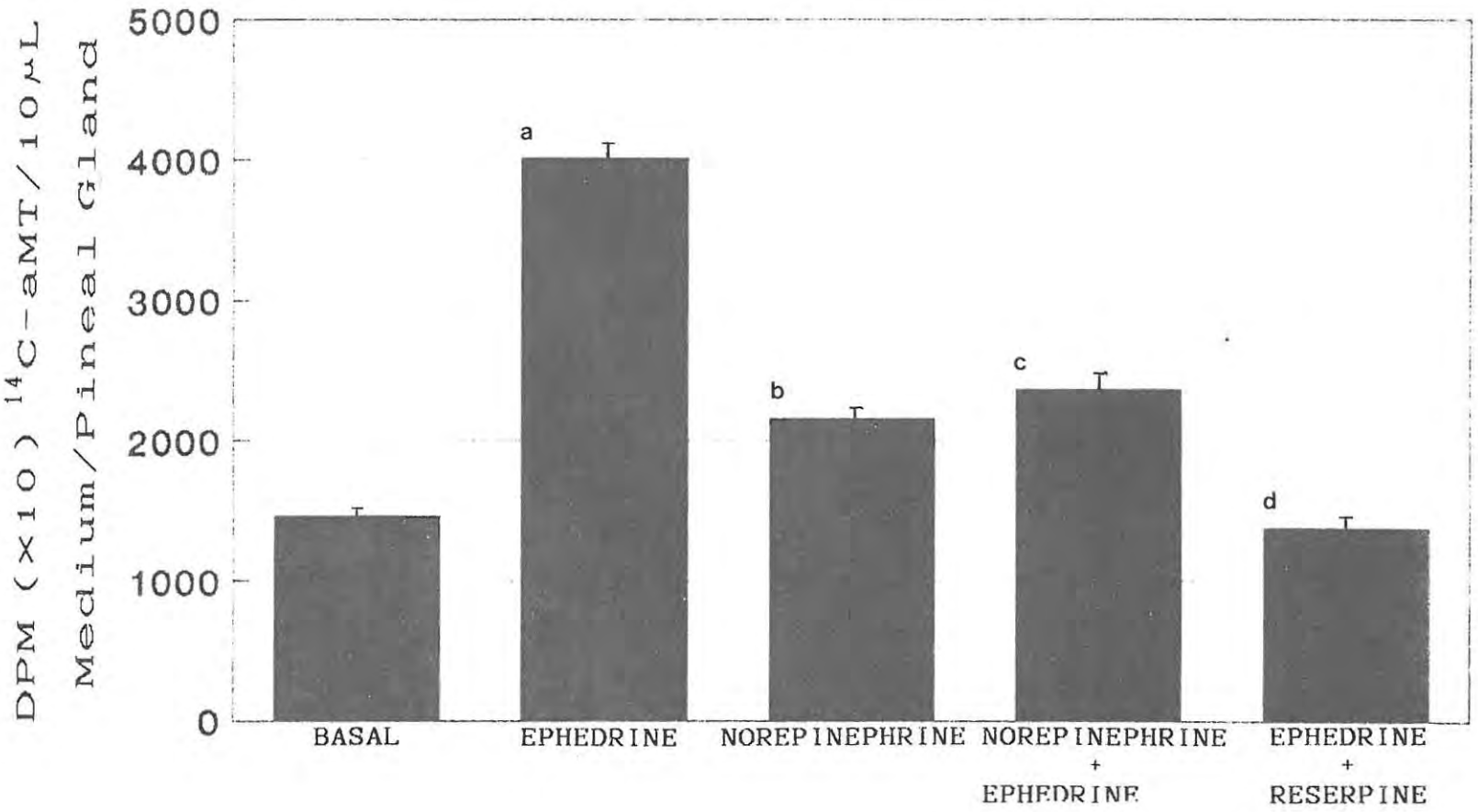

Fig. 2.10 Effect of ephedrine, norepinephrine, norepinephrine and ephedrine, and ephedrine and reserpine treatment on rat pineal production of melatonin in organ culture ± S.E.M. a = P< 0.001 vs. basal levels. b = P< 0.001 vs. basal levels. c = P< 0.001 vs. ephedrine treatment. d = P< 0.001 vs. ephedrine treatment.

but were significantly lower than with ephedrine treatment alone.

The elevated levels of aHT and aMT induced by exposure of the pineal to ephedrine, are probably the result of the inducement of the release of norepinephrine from stores in the nerve endings, and the combined action of the neurotransmitter and ephedrine on pineal alpha- and beta-receptors. Information regarding the relative contribution of ephedrine and norepinephrine with respect to activity at the alpha- and beta-receptors is however limited. The increase in aHT levels with combined exposure of the pineal to norepinephrine and ephedrine in comparison to ephedrine treatment alone, supports the theory that norepinephrine which is released as a result of ephedrine treatment, enhances the action of ephedrine, and forms part of the drug's peripheral action. Ephedrine itself may also induce supersensitivity at the beta-receptor.

In an attempt to quantify the effects of ephedrine alone in terms of its production of aHT and aMT, norepinephrine stores in the nerve endings were depleted by treatment of rats with reserpine (5 mg/kg i.p.). Exposure of pineals from reserpinized rats to ephedrine produced significantly lower levels of aHT and aMT than recorded when pineals from untreated rats were exposed to ephedrine supporting the adrenergic role of released norepinephrine in the mechanism. The levels are however of the same order as the basal levels, suggesting that ephedrine itself has little or no adrenergic activity at all. Reserpine, however, depletes stores of serotonin in many organs, including the brain (Weiner, 1985), and it is possible that the availability of [^{14}C]-5HT to NAT in the pinealocyte is interfered with, thus providing an explanation for the results. Alternatively, the beta-adrenergic component of ephedrine's activity (in the absence of norepinephrine) is minimal, and confined mainly to action at the alpha-receptor.

With respect to the other metabolites isolated from organ culture, it is apparent that enhanced pineal NAT levels as a result of ephedrine and norepinephrine treatment resulted in significantly lower levels of MIAA and MTOH in comparison to basal levels. This may be ascribed to a depletion of serotonin by raised NAT activity and hence a reduced

production of their common precursor, 5-hydroxyindoleacetaldehyde. This is also reflected in a significant reduction in HIAA levels with norepinephrine treatment and a reduction in HTOH, associated with combined norepinephrine and ephedrine treatment. These results are in accordance with those derived from the organ culture experiments involving alpha-methyldopa. In this model, adrenergic innervation induces raised levels of pineal NAT activity which depletes serotonin, thus providing less substrate for MAO to produce 5-hydroxyindole-acetaldehyde, the common precursor for HIAA, HTOH, MIAA and MTOH.

2.1.10 The Effect of Ephedrine on Pineal Serotonin-N-Acetyl-transferase (NAT) Activity

The method used for the determination of pineal NAT activity in response to exposure to ephedrine is that of Champney et al. (1984), described previously in section 2.1.6.2. All procedures employed and chemicals used were identical with the exception of drugs used.

Pineal NAT activity was determined after exposure of pineal glands to 10 μM ephedrine in vitro, 10 μM norepinephrine in vitro, and a combination of 10 μM ephedrine and 10 μM norepinephrine in vitro. In addition, pineal NAT levels were determined in reserpine treated rats (5 mg/kg i.p.) as well as in pineals from reserpine rats which were exposed to ephedrine in vitro.

2.1.10.1 Statistics

Statistical comparisons for the experiments were made by using the Student-Newman Keuls Multiple Range Test. All data derived from the NAT assays are expressed as moles product/gland/h calculated as the mean $\pm$ S.E.M. (n=5).

2.1.10.2 Results

Exposure of pineal glands to 10 μM ephedrine in vitro produced significantly raised levels of NAT activity in comparison to basal NAT levels ($P< 0.001$). Similarly, 10 μM norepinephrine in vitro also resulted in significantly elevated levels of NAT ($P< 0.001$) (Table 2.8, Fig. 2.11).

Exposure of pineal glands to a combination of 10 μM norepinephrine and

Table 2.8

Effect of ephedrine, norepinephrine, norepinephrine and ephedrine, and ephedrine and reserpine treatment on rat pineal N-acetyltransferase (NAT) activity. [pmoles/gland/h ± S.E.M.] (n=5).

	Treatment						Significance				
	Basal	Ephedrine	Norepinephrine	Norepinephrine + Ephedrine	Ephedrine + Reserpine (5mg/kg i.p.)	Reserpine (5mg/kg i.p.)	Ephedrine vs. Basal	Norepinephrine vs. Basal	Norepinephrine + Ephedrine vs. Ephedrine	Reserpine vs. Basal	Ephedrine + Reserpine vs. Ephedrine
NAT Activity	56.8 ± 1	81.3 ± 2	72.9 ± 1	92.3 ± 1	71.6 ± 0.3	61.2 ± 0.6	P< 0.001	P< 0.001	P< 0.001	N.S.	

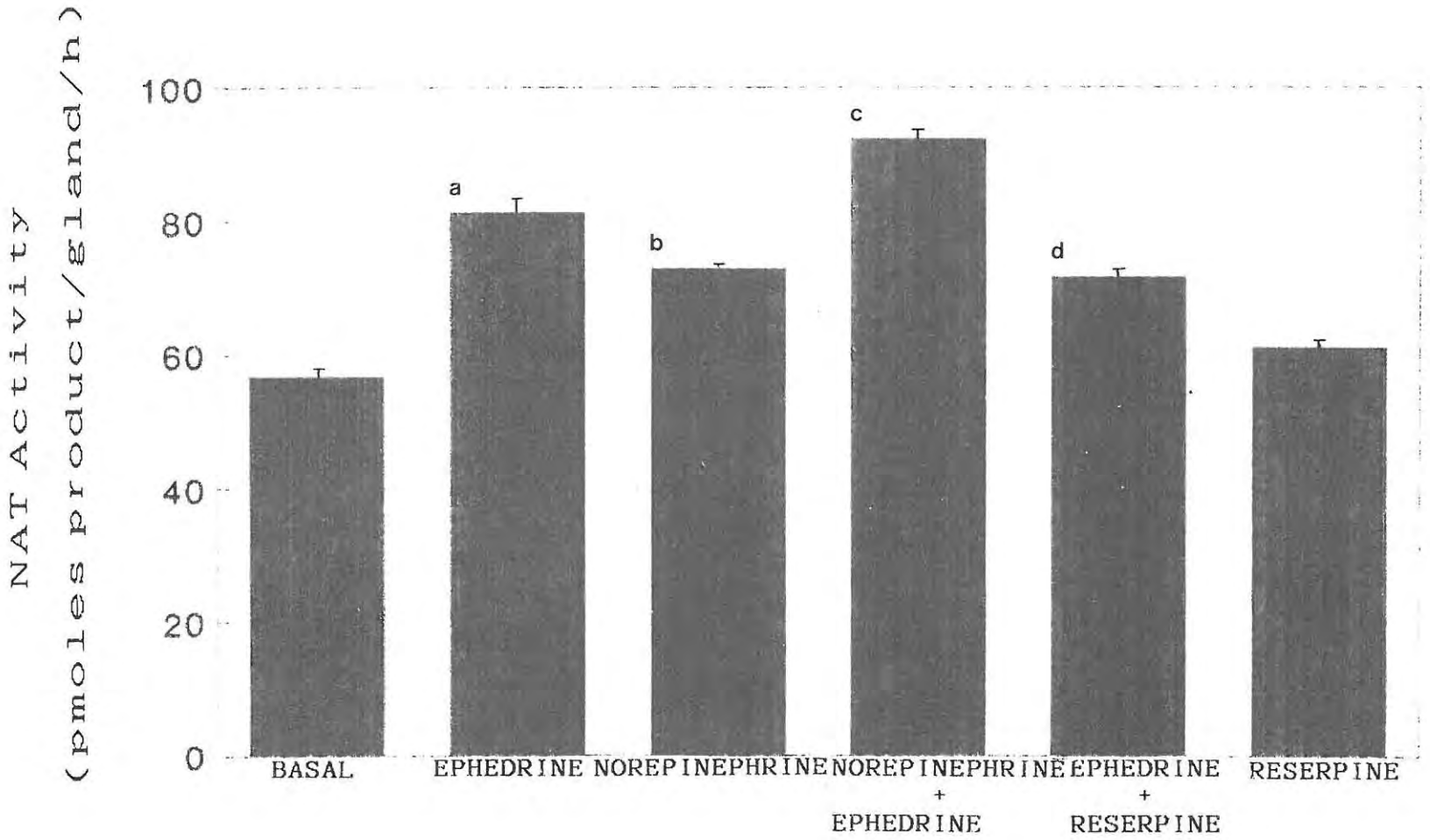

Fig. 2.11 Effect of ephedrine, norepinephrine, norepinephrine and ephedrine, ephedrine and reserpine, and reserpine treatment on rat pineal N-acetyltransferase (NAT) activity ± S.E.M. a = $P < 0.001$ vs. basal levels, b = $P < 0.001$ vs. basal levels, c = $P < 0.001$ vs. ephedrine treatment, d = $P < 0.001$ vs. ephedrine treatment.

10 μM ephedrine *in vitro* produced raised levels of NAT activity in comparison to basal levels, and significantly greater levels in comparison to ephedrine exposure alone ($P< 0.001$) (Table 2.8, Fig. 2.11).

NAT activity in pineals collected from reserpinized rats were found to be similar in activity to those in the basal group. Pineal glands from reserpinized rats, which were exposed to 10 μM ephedrine *in vitro* were found to have raised levels of NAT activity in comparison to those of the basal group, but were significantly lower than levels determined for pineals exposed to 10 μM ephedrine *in vitro* ($P< 0.001$) (Table 2.8, Fig. 2.11).

2.1.10.3 Discussion

In accordance with the organ culture results, ephedrine and norepinephrine induce significantly elevated levels of pineal NAT activity in comparison to basal levels. Exposure of pineal glands to a combination of ephedrine and norepinephrine induces elevated levels of pineal NAT activity significantly greater than the increase associated with ephedrine treatment alone. Again, the ability of norepinephrine (and specifically released norepinephrine) to produce an adrenergic effect in conjunction with ephedrine itself is demonstrated. The possibility of a supersensitivity effect induced by ephedrine at the beta-receptor is also suggested by this data. Pineal glands from reserpinized rats which were exposed to ephedrine induced significantly lower levels of NAT activity in comparison to pineals from untreated rats which were exposed to ephedrine, supporting again, the adrenergic role of released norepinephrine in the mechanism. These levels were raised in comparison to basal levels, and approximately equal to levels associated with norepinephrine treatment, conflicting in this respect with organ culture results corresponding to the same treatment,itself has substantial adrenergic activity, thus appearing to conflict with organ culture results corresponding to the same drug treatments. Reserpine treatment has been shown to induce supersensitivity at the level of the beta-receptor (Canter *et al.*, 1981), which might have induced an enhanced response to ephedrine itself in terms of NAT

activity. This raised NAT activity would not however be reflected in elevated levels of aHT and aMT if limited substrate (5-HT) was available as a result of depletion (of the substrate) by reserpine, thus offering a possible explanation.

2.1.11 The Effect of Ephedrine on Beta-Adrenergic Receptor Binding

The method used involves a modification of a technique by Wilkinson and Wilkinson (1985) described previously in section 2.1.7. All procedures and chemicals used were identical with the exception of the drugs used. Specific beta-receptor binding in response to exposure of pineal glands to 10 μM ephedrine *in vitro* was determined. Pineal glands collected from reserpinized (5 mg/kg i.p.) and untreated rats were exposed to ephedrine. Beta-receptor binding was also determined in a control group.

2.1.11.1 Statistics

Statistical comparisons were performed using the Student-Newman Keuls Multiple Range Test. Specific binding is expressed as CPM [^{3}H]-CGP/half-pineal gland and all data referred to were calculated as the mean $\pm$ S.E.M. (n=6).

2.1.11.2 Results

A significant decrease in beta-receptor binding in comparison to basal values ($P< 0.001$) was observed when pineal glands were exposed *in vitro* to 10 μM ephedrine. Furthermore, a significant decrease in beta-receptor binding occurred in pineals from reserpinized rats exposed *in vitro* to ephedrine, in comparison to basal values ($P< 0.001$) (Table 2.9, Fig. 2.12).

2.1.12 Discussion

Any possibility of ephedrine inducing a supersensitivity phenomenon at the level of the pineal beta-receptor was ruled out by data derived from the binding study. Treatment with ephedrine results in a significant decrease in ligand binding to the pineal beta-receptor. This data rules out the possibility of ephedrine inducing supersensitivity of the beta-receptors. Exposure of pineal glands from reserpinized rats to ephedrine induces a significant decrease in ligand binding to the beta-receptor, in comparison to the ligand binding

Table 2.9

Effect of ephedrine, and ephedrine and reserpine on specific binding of rat pineal gland beta-receptors [CPM ^{3}H-CGP/half pineal gland ± S.E.M.] (n=6).

	Treatment			Significance	
	Basal	Ephedrine	Ephedrine + Reserpine (5mg/kg i.p.)	Ephedrine vs. Basal	Ephedrine + Reserpine vs. Ephedrine
Specific Binding	352.0 ± 19	260.5 ± 12	139.6 ± 10	P< 0.001	P< 0.001

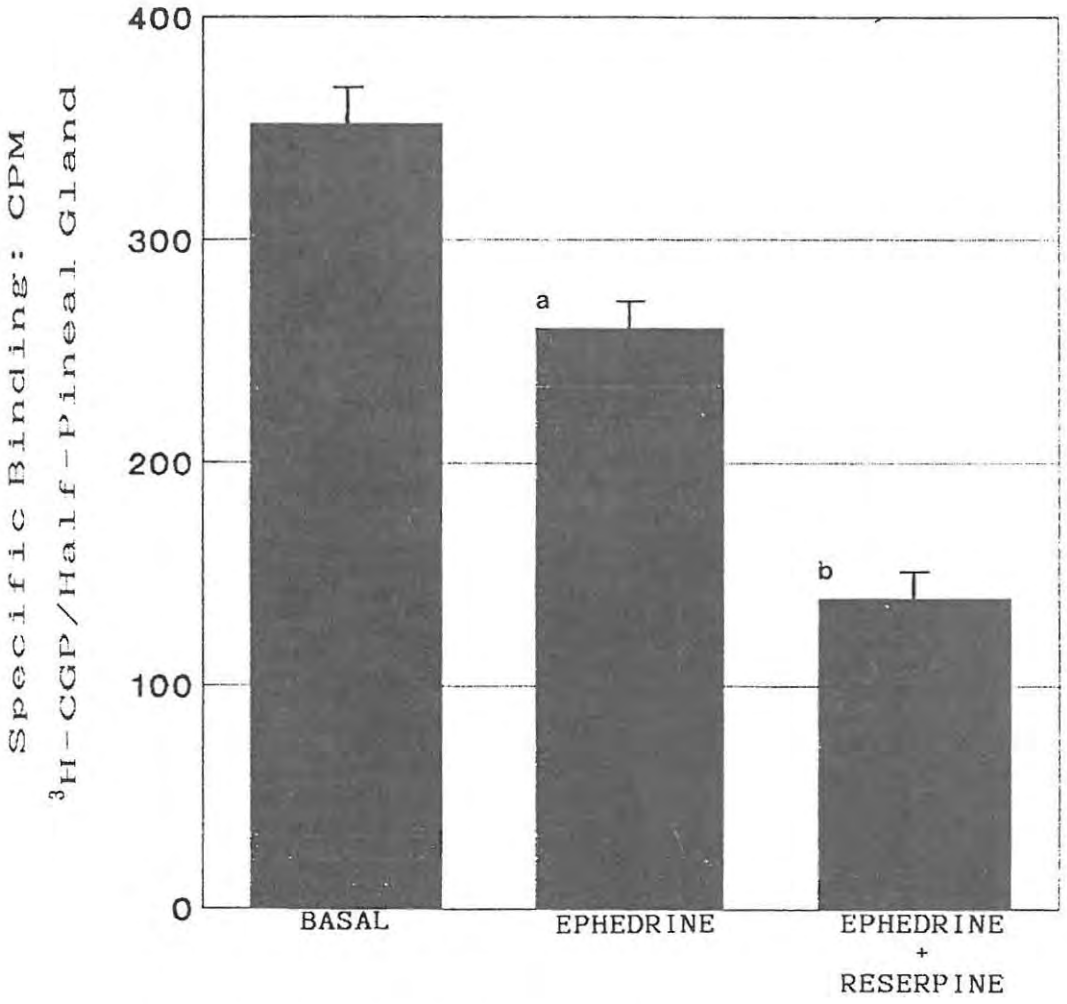

Fig. 2.12 Effect of ephedrine, and ephedrine and reserpine on specific binding of rat pineal gland beta-receptors ± S.E.M. a = P< 0.001 vs. basal. b = P< 0.001 vs. basal.

associated with ephedrine treatment of pineals from untreated rats. Ephedrine itself thus binds substantially to the beta-receptor.

2.1.13 A 16-Hour Profile of the Effect of Norepinephrine and Ephedrine on Rat Pineal Gland Synthesis of Melatonin and N-Acetylserotonin from [^{14}C]-Serotonin in Organ Culture

The effect of exogenous norepinephrine and ephedrine on the progressive formation of melatonin and serotonin was monitored over a 16 h period in organ culture. The study was undertaken in order to provide a clearer picture of the response of the pineal to ephedrine and norepinephrine over a broad time-span. The 16 h period was chosen as previous reports from this laboratory (Banoo et al., 1987) demonstrated that maximal levels of aHT and aMT are reached in organ culture over this time.

2.1.13.1 Materials and Methods

The organ culture techniques used were identical to those described previously, with the exception that at two hourly intervals, 5 μl volumes of culture medium were removed from each of the tubes, and replaced with identical aliquots containing no tissue. The [^{14}C]-aHT and [^{14}C]-aMT in the 5 μl samples were isolated by two-dimensional thin layer chromatography and measured by liquid scintillometry. Three groups of 5 pineals each were treated in this fashion. One group served as a control group, receiving no drug, and the remaining two were treated with 10 μM norepinephrine and ephedrine respectively. Chemicals and materials used were identical to those used in previous organ culture experiments involving the study of norepinephrine and ephedrine.

2.1.13.2 Statistics

Statistical comparisons were performed using Student's t-test. All data are expressed as dpm/5 μl medium and each point on the graph (Fig. 2.13) represents the mean $\pm$ S.E.M. (n=5).

2.1.13.3 Results

The formation of melatonin (aMT) and its precursor N-acetyl-serotonin (aHT) increased sharply after 2 h in norepinephrine stimulated pineals (Table 2.10, Fig. 2.13). Synthesis of aMT continued to increase

Table 2.10

16-Hour time profile of the production of N-acetylsrotonin (aHT) and melatonin by the rat pineal gland when treated with ephedrine (10μM) and norepinephrine (10μM) in organ culture. [dpm/5 μl medium ± S.E.M] (n=5).

		Time (hours)							
Treatment	Pineal Metabolite	2	4	6	8	10	12	14	16
Control	aHT	31.4 ± 4	163.2 ± 26	218.5 ± 29	273.8 ± 21	288.7 ± 27	311.2 ± 22	333.5 ± 21	355.8 ± 36
	aMT	74.4 ± 4	189.0 ± 24	216.6 ± 14	291.9 ± 32	367.8 ± 48	399.6 ± 31	454.8 ± 12	471.6 ± 28
Ephedrine	aHT	150.6 ± 21	268.8 ± 51	585.0 ± 10	645.0 ± 95	870.6 ± 104	961.5 ± 45	1170.9 ± 45	1338.0 ± 50
	aMT	27.3 ± 3	169.2 ± 10	554.5 ± 100	1044.3 ± 80	1598.7 ± 122	1729.8 ± 84	1866.9 ± 81	1886.1 ± 50
Norepinephrine	aHT	201.0 ± 11	370.6 ± 39	458.3 ± 39	595.0 ± 20	730.0 ± 37	843.0 ± 40	950.9 ± 68	1076.0 ± 93
	aMT	255.0 ± 32	484.0 ± 28	729.6 ± 24	760.5 ± 47	790.2 ± 98	841.2 ± 50	865.5 ± 70	889.8 ± 48

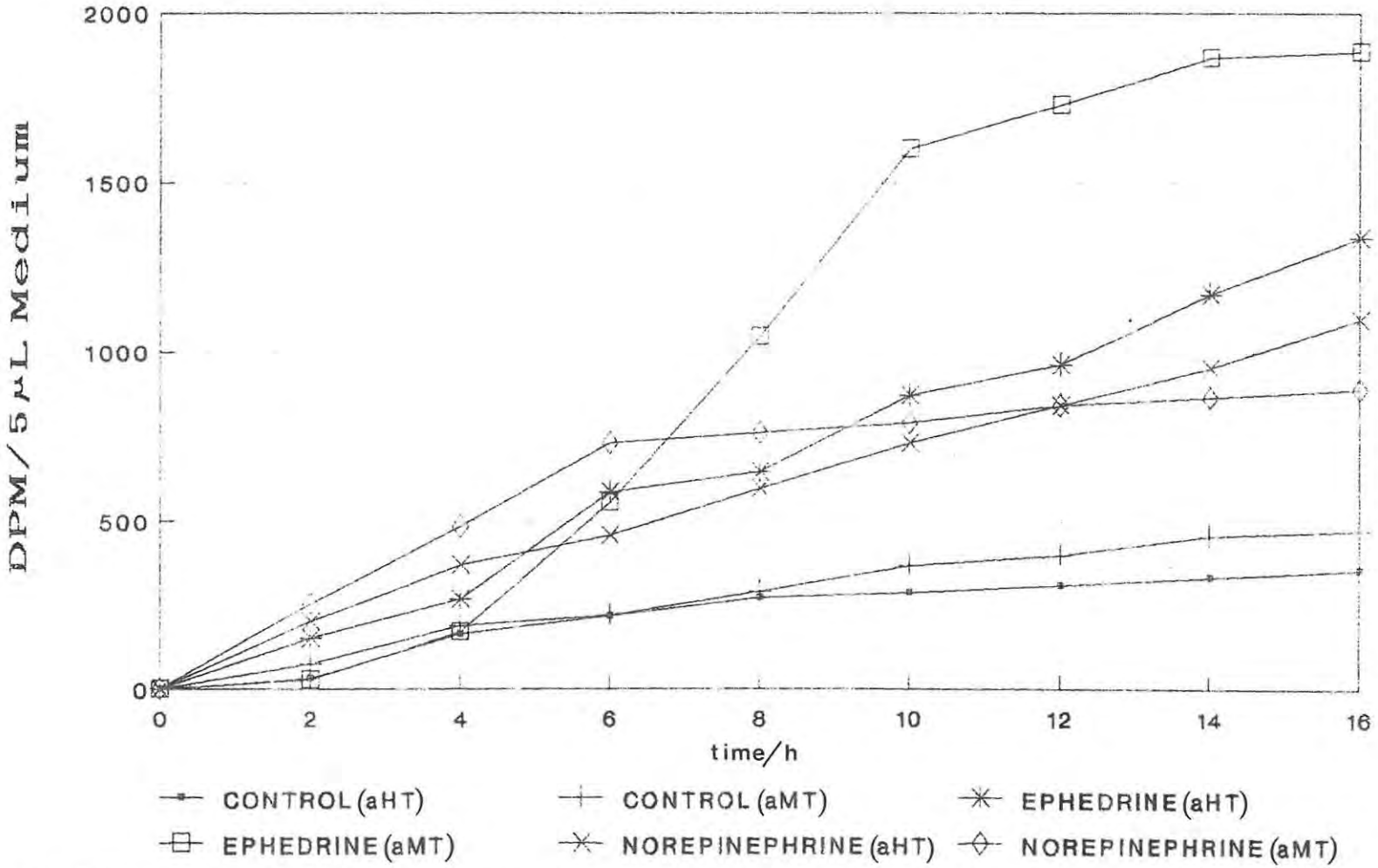

Fig. 2.13 16-Hour time profile of the production of N-acetylserotonin (aHT) and melatonin (aMT) by the rat pineal gland with ephedrine and norepinephrine treatment in organ culture.

acutely for approximately the first 6 h of incubation, after which it progressively increased in a more gradual fashion for the remainder of the incubation period. Formation of aHT progressively increased for the duration of the 16h incubation period, rising above the level of aMT in unstimulated control pineals for the duration of the 16h incubation period. In both norepinephrine stimulated and control pineals, maximal levels of aHT and aMT were observed after 16 h incubation. The maximal levels of aHT and aMT in stimulated pineals were significantly higher than the corresponding levels of aHT ($P< 0.05$) and aMT ($P< 0.025$) in the unstimulated control pineals.

Levels of aHT in ephedrine treated pineals rose steadily over the first four hours of incubation, after which the levels rose sharply to values above those of aHT in norepinephrine treated pineals (Table 2.10, Fig. 2.13). Levels of aHT then rose steadily to maximum values after 16h. Levels of aMT in ephedrine-treated rose slowly for the first 2h of the incubation period, only rising above the control aMT levels after 4h. Levels of aMT then rose sharply up until the 10 h mark to values in excess of aMT levels associated with norepinephrine treatment. From the period 10 h to 16 h, the production of pineal aMT increased at a more gradual rate, reaching a maximal level after 16 h incubation. The maximal levels of aHT and aMT in ephedrine-treated pineals were significantly higher than the corresponding levels of aHT ($P< 0.001$) and aMT ($P< 0.001$) in the unstimulated control pineals.

2.1.13.4 Discussion

Stimulation with exogenous norepinephrine causes a marked elevation in the synthesis of aMT and aHT in comparison to unstimulated control pineal glands (Fig. 2.13). This is in accordance with the general view that norepinephrine induces an increase in the levels of these indoles. These effects probably occur primarily as a result of stimulation of the activity of (NAT). NAT activity in norepinephrine-treated pineals may be deduced to be raised for the entire 16 h period of incubation, owing to the continually rising profile of aHT. The noticeable reduction, however, in the progressive increase in formation of aMT after 6 h in culture, points toward a reduction in the activity of the enzyme hydroxyindole-O-methyltransferase (HIOMT) after this time. This is in accordance with findings that norepinephrine inhibits HIOMT

activity (Weiss, 1968), and that attempts to stimulate HIOMT in organ culture with norepinephrine have not been successful (Berg and Klein, 1971). The mechanisms involved in the regulation of HIOMT have not been clearly defined. HIOMT activity has been demonstrated to be under control of the retinohypothalamic tract and the suprachiasmatic nucleus (Klein and Moore, 1979). It would appear that transmitter release is stimulated within the pineal gland in response to signals from the suprachiasmatic nucleus. Light blocks transmission of these signals, resulting in lower daily levels of HIOMT. Due to the time of collection of the pineal glands, HIOMT levels were probably relatively low at the commencement of the incubation period. Under these neurally isolated conditions in organ culture, NAT possibly served as the rate-limiting enzyme, with the existing pool of HIOMT becoming progressively diminished with time, particularly in norepinephrine stimulated pineals with enhanced NAT activity and higher levels of aHT substrate. Additionally, it could be speculated that melatonin itself may exercise a negative feedback mechanism on HIOMT. These factors might explain why, in the previous pineal organ culture experiments involving ephedrine and norepinephrine, aMT levels corresponding to pineal treatment in vitro with norepinephrine and ephedrine, were significantly lower than with ephedrine treatment alone.

The involvement, thus, of released norepinephrine in the ephedrine mechanism of action introduces complications into the interpretation of results by virtue of the response of pineal enzymes, particularly HIOMT, to the neurotransmitter. Under the experimental conditions created, NAT activity and aHT levels probably provide the most accurate reflection of the pineal gland's response to adrenergic activity of ephedrine and associated released norepinephrine. The measurement of cAMP would also have provided an accurate indication of events at the beta-adrenergic receptor.

Ephedrine treatment presents an aMT response profile markedly different from that associated with norepinephrine treatment. From 2 to 10 hours the aMT response is reflected by a sharp increase followed by a "tailing off". The diminished response after 10 hours may be the result of exhaustion of the HIOMT pool and/or possibly an inhibitory effect of released norepinephrine on the enzyme. It appears that under

these conditions NAT controls the reaction for the full period of incubation, as can be deduced from a progressively increasing level of aHT over 16 h. The strong adrenergic activity of ephedrine and released norepinephrine is conclusive.

Further studies of this nature could be executed using a perifusion technique as described by Simonneaux et al. (1989).

CHAPTER 3

3.1 The Development of a Pineal Cell Culture System

3.1.1 Introduction

The involvement of released norepinephrine in the mechanism of action of ephedrine, and the need to abolish this component in order to quantify the effects of ephedrine itself on the pinealocyte, prompted the development of a dissociated cell-line. In addition, organ (and explant) cultures have certain restrictions viz: the diffusion of oxygen and nutrients into the pineal tissue is limited, as well as the development of necrosis in oxygen and nutrient-starved tissue.

3.1.2 Chemicals and Materials

L-15 (Liebovitz) medium was obtained from Sigma Chemical Co. (St. Louis, Mo.). BGJb medium was obtained from Gibco (Europe). Minimum Essential Medium (Hank's modification) was obtained from Flow Laboratories (McLean, UA). Foetal Calf Serum (filtered and ultra-violet irradiated) was obtained from the State Vaccine Institute (RSA).

3.1.3 Methods

Pineal glands from up to twenty 3 week old rats were collected as described previously, and placed in about 2 ml of a solution containing 137mM NaCl, 5.4 mM KCl, 0.17 mM Na_2HPO_4, 0.22 mM KH_2PO_4, 55 mM glucose, and 59 mM sucrose. The accumulated glands in solution were incubated at 35-36°C for 30 min with occasional agitation with 0.25% crude trypsin. The cells were then triturated, producing a suspension of essentially single cells along with a small amount of fibrous membrane material. Foetal calf serum (virus- and mycoplasma-screened) was added (0.2 ml) and, after mixing, the suspension was centrifuged at 500 G for 2 min at room temperature. The pelleted cells were resuspended in freshly prepared L-15 "plating medium" (2 ml/pineal) (Formulation in Table 3.1). The suspension (1.0 ml portions) were then added to 7 cm^2 Falcon polystyrene dishes. The dishes were placed in sealed plastic bags pre-gassed with 95% O_2:5% CO_2, and incubated for 72 h. The medium was then substituted with an equivalent volume of Eagle's Minimum Essential Media (Hank's modification) (Formulation in Table 3.2), or BGJb medium (Fitton-Jackson Modification) (Formulation in Table 3.3) each of which contained 10% (v/v) foetal calf serum. The two types of

Table 3.1 L-15 (L4386) Medium

Formulation

Ingredients	Concentration (g/litre)
DL-Alanine	0.450
L-Arginine Free Base	0.500
L-Asparagine (Anhydrous)	0.250
L-Cysteine Free Base	0.120
L-Glutamine	0.300
L-Glycine	0.200
L-Histidine Free Base	0.250
DL-Isoleucine	0.250
L-Leucine	0.125
L-Lysine Free Base	0.075
DL-Methionine	0.150
DL-Phenylalanine	0.250
L-Serine	0.200
DL-Threonine	0.600
L-Tryptophan	0.020
L-Tyrosine Free Base	0.300
DL-Valine	0.200
Choline Chloride	0.001
Folic Acid	0.001
Flavin Mononucleotide	0.0001
Myo-Inositol	0.002
Niacinamide	0.001
DL-Pantothenic Acid Ca	0.001
Pyridoxine HCl	0.001
Thiamine Monophosphate HCl	0.001
Calcium Chloride$\cdot 2H_2O$	0.185
Magnesium Chloride$\cdot 6H_2O$	0.200
Magnesium Sulfate (Anhydrous)	0.100
Potassium Chloride	0.400
Potassium Phosphate	0.060
Sodium Chloride	8.000
Sodium Phosphate Dibasic (Anhydrous)	0.190
Galactose	0.900
Phenol Red Na	0.011
Sodium Pyruvate	0.550

Table 3.2 Minimum Essential Medium Eagle (Modified) with Hanks' Salts

Formulation

Ingredients	Concentration (mg/litre)
L-Arginine HCl	126.4
L-Cystine disodium salt	28.42
L-Glutamine	292.3
L-Histidine HCl H_2O	41.90
L-Isoleucine	52.50
L-Leucine	52.50
L-Lysine HCl	73.06
L-Methionine	14.90
L-Phenylalanine	33.02
L-Threonine	47.64
L-Tryptophan	10.20
L-Tyrosine	36.22
L-Valine	46.90
D-Ca pantothenate	1.00
Choline chloride	1.00
Folic acid	1.00
i-Inositol	2.00
Nicotinamide	1.00
Pyridoxal HCl	1.00
Riboflavin	0.10
Thiamin HCl	1.00
$CaCl_2$ $2H_2O$	185.5
KCl	400.0
KH_2PO_4	60.0
$MgSO_4$ $7H_2O$	200.0
NaCl	8000
$NaHCO_3$	350.0
Na_2HPO_4	47.50
Glucose	1000
Sodium phenol red	17.00

Table 3.3 BGJb Medium (Fitton-Jackson Modification)

Formulation

Mean tonicity = 390 milliosmoles

Ingredients	Concentration (mg/litre)
Dihydrogen sodium ortho-phosphate	90.00
Magnesium sulphate	200.00
Potassium chloride	400.00
Potassium dihydrogen phosphate	160.00
Sodium bicarbonate	3500.00
Sodium chloride	5300.00
Calcium lactate	555.00
Glucose	10000.00
Phenol red	20.00
Sodium acetate	50.00
L-Alanine	250.00
L-Arginine	175.00
L-Aspartic acid	150.00
L-Cysteine HCl	90.00
Glycine	800.00
L-Histidine	150.00
L-Isoleucine	30.00
L-Leucine	50.00
L-Lysine	240.00
L-Methionine	50.00
L-Phenylalanine	50.00
L-Proline	400.00
L-Serine	200.00
L-Threonine	75.00
L-Tryptophane	40.00
DL-Valine	65.00
Alpha tocopherol phosphate	1.00
Ascorbic acid	50.00
Biotin	0.20
Calcium pantothenate	0.20
Choline chloride	50.00
Folic acid	0.20
Inositol	0.20
Nicotinamide	20.00
Para aminobenzoic acid	2.00
Pyridoxal phosphate	0.20
Riboflavin	0.20
Thiamine hydrochloride	4.00
Vitamin B12	0.04
L-Glutamine	200mg/l
Benzyl Penicillin	100 units/ml
Streptomycin sulphate	0.1mg/ml
Amphotericin B	2.5 μg/ml

media were used in an attempt to optimise growth. Similar growth characteristics were achieved with both types. Medium changes were performed thereafter at 3-day intervals. At each media-change, cells were counted by microscopy in a counting chamber. At all times, stringent precautions were undertaken in the control of sterility. Media was supplemented with 0.06 mg/ml Benzyl Penicillin, 0.1 mg/ml Streptomycin sulphate, and 2.5 μg/ml Amphotericin B. All manipulations were performed under a displacement flow cabinet sterilized by UV light and alcohol. All equipment was pre-soaked in 95% ethanol and bottles were flamed with a bunsen burner. Plastic incubation packets and falcon dishes were exposed to UV irradiation for 3 hours prior to use. All bottles, filters and needles used for preparation and storage of media were autoclaved for 45 min prior to use. Millipore^R HA 0.45 μM filters were used for media sterilization.

3.1.4 Microscopy

In order to facilitate microscopic examination of the cultured cells, 1cm^2 plastic pallets were heat-welded to the bottom surface of the Falcon polystyrene culture dishes prior to culture preparation. After at least 5 days in culture, the pallets were cut free.

3.1.4.1 Phase Contrast Microscopy

Plastic pallets to which the layer of cells were adhered, were mounted on glass microscope slides and viewed under a Zeiss inverted phase contrast microscope at a 1000 x magnification. Photographs were made of the cultures.

3.1.4.2 Scanning Electron Microscopy

Pallets were coated with a thin film of gold or gold-palladium. The metal alloy was first heated in a tungsten basket under vacuum until melted and evaporated. The pallets were then rotated and tilted in the path of the metal vapour particles until coated. The coated specimens were viewed with a scanning electron microscope and photographs made at 8500 x magnification.

3.1.5 Results

Pineal cell cultures were grown and a cell density of 1.0 x 10^6 cells/ml was routinely obtained. Fig. 3.1 shows the phase contrast

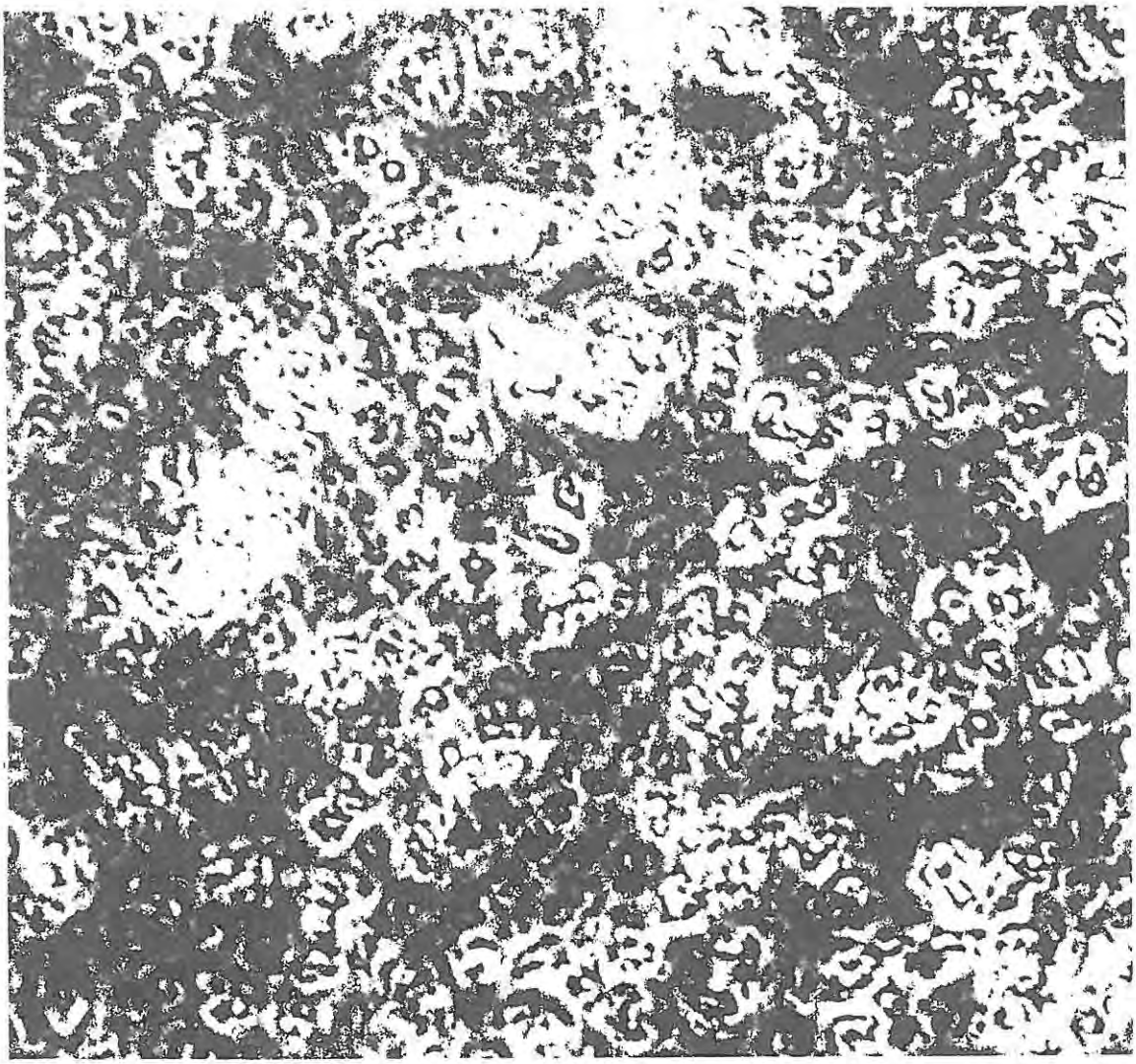

Fig. 3.1 Phase contrast microscopic morphology of cultures of cells from pineal glands. Magnification 1000 x.

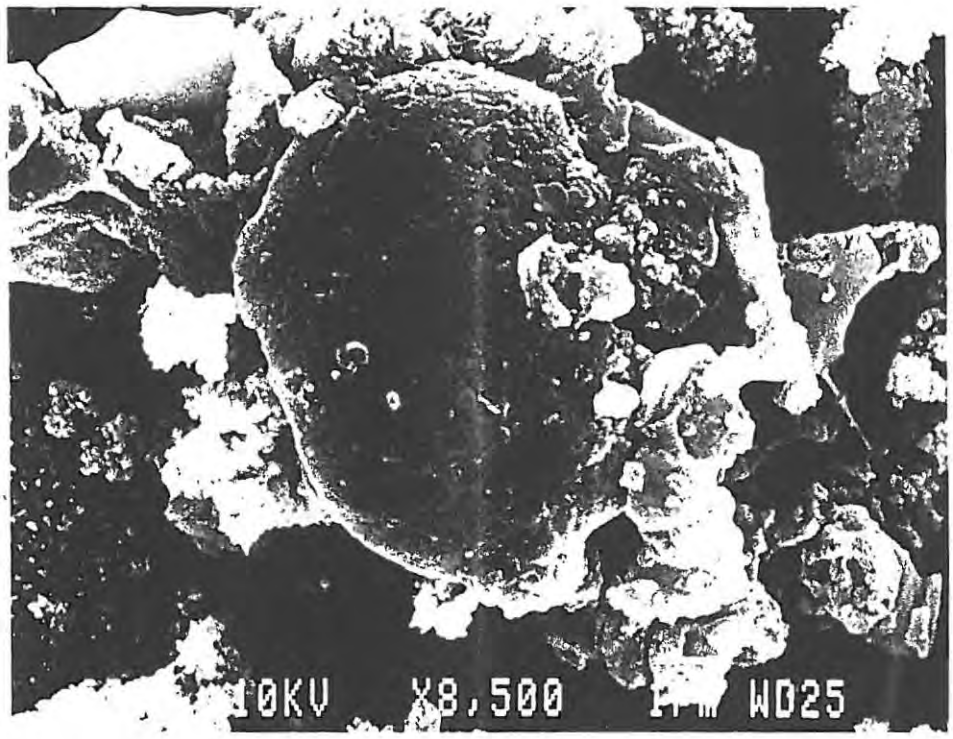

Fig. 3.2 Scanning electron micrograph of a pinealocyte cultured from dissociated pineal glands. Magnification 8500 x.

microscopic morphology of cultures of cells from pineal glands after 5 days in culture. The pinealocytes are the small, round, phase-bright cells with very little cytoplasm. Upon changing the media to MEM or BGJb, the rounded cells began to form nests of up to 10 - 100 or more cells.

Fig. 3.2 shows a single pinealocyte under S.E.M. The surrounding material and "debris" are probably constituents of the culture media and antibiotic crystals.

3.1.6 Discussion

Dissociated pinealocyte cultures can be satisfactorily grown under the conditions described. An improvement upon the procedure could have been introduced by using pineals from a large number of 2 - 3 day old pups. Facilities and finances however limited this type of approach. Distinct advantages in developing a cell-culture system are evident. It is a requirement to obtain the gland free of contamination of other tissue and the cell-culture system is ideal in this respect. The availability of pinealocytes in long-term surface cell culture could facilitate several avenues of simultaneous research at a reduced cost. The cultured cells contain organotypic enzymes, respond morphologically and biochemically to agents known to be effectors *in vivo* and in organ culture, and support the differentiation of neurons from which they are target organs. The cells can also be maintained at low cost for extended periods without loss of organotypic characteristics. A complete characterization of the pinealocytes in cell culture might provide a better understanding of the sympathetic nervous system as a whole.

3.2 Effect of Ephedrine and Norepinephrine on Serotonin N-Acetyl-transferase Activity of Pinealocytes in Cell Culture

NAT activity of dissociated pinealocytes in cell culture was determined after exposure to ephedrine and norepinephrine. Chemicals and materials used are identical to those used in cell-culture experiments and NAT-assays.

3.2.1 NAT Assay

24 hours after media change, groups of five, 5-day old cultures (in

wells) were exposed to either 10 μM ephedrine, 10 μM norepinephrine, or a combination of the two. Control NAT levels were also determined. Where drugs were not added, distilled and deionized water were substituted. A 6 h incubation period for all pharmacologic agents was chosen. After the incubation period, the media was removed according to a method described by Rowe et al. (1977) by freezing on dry ice. Before the assay, the cells were scraped from the dishes with a plastic spatula into 50 μl of 0.1M sodium phosphate buffer, pH 6.8. The suspension was homogenized and assayed according to the previously described method of Champney et al. (1984).

3.2.2 Statistics

Statistical comparisons of groups were made using the Student-Newman Keuls Multiple Range Test. All data derived from the NAT assays are expressed as pmoles product/well/h $\pm$ S.E.M. (n=5).

3.2.3 Results

Treatment with ephedrine (10 μM) produced elevated levels of NAT activity significantly greater than basal levels ($P< 0.001$) (Table 3.4, Fig. 3.3). Treatment with norepinephrine (10 μM) also induced raised NAT activity significantly greater than basal levels ($P< 0.001$) (Table 3.4, Fig. 3.3). A combination of norepinephrine (10 μM) and ephedrine (10 μM) produced raised NAT activity significantly greater than the raised NAT activity induced by ephedrine treatment alone ($P< 0.001$) (Table 3.4, Fig. 3.3).

3.2.4 Discussion

The results are in accordance with previous NAT assays performed involving ephedrine as well as with the organ culture experiments.

Norepinephrine treatment produced a significantly raised level of NAT activity in accordance with literature reports (Rowe and Parr, 1979).. Ephedrine also produced a significantly raised level of NAT activity. Owing to the fact that the dissociated cell culture possessed no norepinephrine, the results thus substantiate that ephedrine itself has beta-adrenergic activity (and possibly alpha-adrenergic activity as well). The combination of norepinephrine and ephedrine induced levels of NAT significantly greater than with ephedrine treatment alone,

Table 3.4

Effect of ephedrine, norepinephrine, and norepinephrine and ephedrine on NAT activity of pinealocytes within a dissociated cell-culture system. [pmol product/well/hr ± S.E.Ml] (n=5).

	Treatment				Significance		
	Basal	Ephedrine	Norepinephrine	Norepinephrine + Ephedrine	Ephedrine vs. Basal	Norepinephrine vs. Basal	Norepinephrine + Ephedrine vs. Ephedrine
NAT Activity	69.1 ± 13	129.6 ± 1	155.7 ± 14	209.9 ± 3	P< 0.005	P< 0.001	P< 0.001

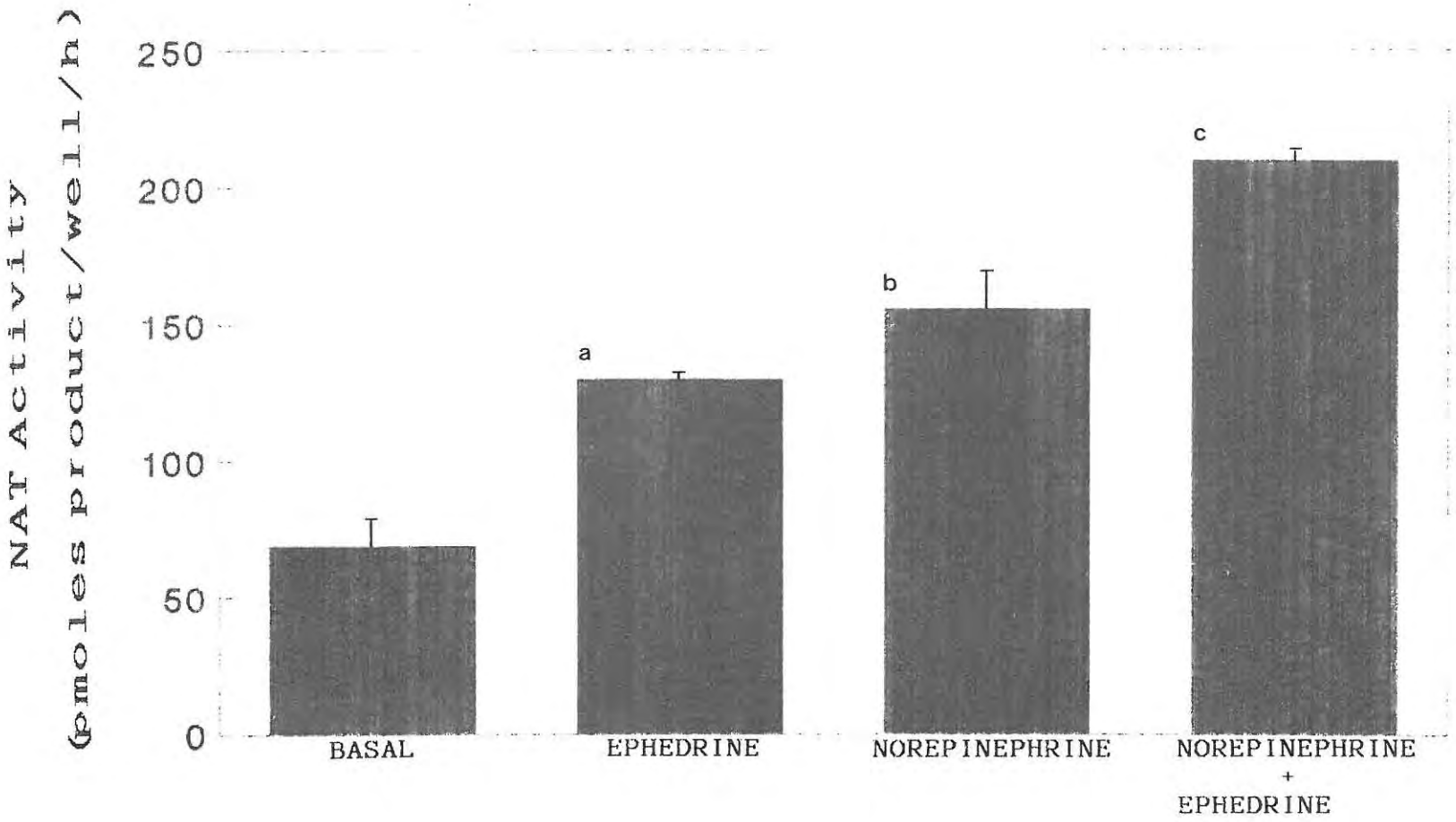

Fig. 3.3 Effect of ephedrine, norepinephrine, and norepinephrine and ephedrine on NAT activity of pinealocytes within a dissociated cell-culture system ± S.E.M. a = P< 0.001 vs. basal levels. b = P< 0.001 vs. basal levels. c = P< 0.001 vs. ephedrine treatment.

supporting previous results, and the theory that released norepinephrine is a component of the adrenergic response to ephedrine treatment, and thus active in the pharmacological activity of the drug.

CHAPTER 4

4.1 Measurement of Melatonin by High Performance Liquid Chromatography with Ultraviolet Detection

4.1.1 Introduction

High performance liquid chromatography coupled to an ultraviolet (UV) detection system was used in a brief study to measure pineal melatonin extracted from organ culture medium. Melatonin and other pineal metabolites of tryptophan have been successfully isolated by HPLC using electrochemical detection (Mefferd and Barchas, 1980), fluorometric detection (Anderson et al., 1981; Anderson et al., 1982) and dual ultraviolet and fluorometric detection (Wakabayashi et al., 1985). Taguchi et al. (1988) measured released human pineal melatonin from explants in culture medium; the melatonin was extracted from the culture medium using chloroform and nanogram quantities of the indole were measured using an electrochemical detector.

4.1.2 Chemicals and Materials

A Hewlett Packard HP 1050 HPLC incorporating a Rheodyne Model 7125 Sample Injector (Rheodyne, Cotati, CA) was used. The column used for separation was a Supelco 15.0cm x 4.6mm ID Supelcosil™ C-18 reverse phase (Supelco Inc., Bellefonte, PA). Performance tests with toluene produced a value of 213.56 theoretical plates with a peak symmetry of 1.07. The mobile phase used was a 35% methanol: 65% sodium citrate mixture. Burdick and Jackson HPLC-grade methanol was used (Burdick and Jackson, Muskegan, MI). Indomethacin (1-(p-Cholorobenzoyl)-5-methoxy-2-methylindole-3-acetic acid) was obtained from Sigma Chemical Co. (St Louis, MO).

4.1.3 Methods

Pineal glands were collected as described previously and incubated in 60 μl BGJb medium at 37°C for 24 h. After this period, pineal glands were removed and the medium was extracted with 120 μl chloroform. The upper layer of medium was removed, and the chloroform allowed to evaporate to dryness, whereupon the residue was dissolved in 100 μl of the mobile phase (35% methanol:65% sodium citrate). 10 μl of the sample was injected into the HPLC. The process was repeated 10 times. BGJb medium without tissue was treated in the same fashion and an HPLC

analysis was performed. A melatonin standard (1 mg/ml) was also prepared by dissolving melatonin in the mobile phase and injecting 10 μl (10 μg) onto the column. Melatonin was also dissolved in BGJb culture medium, extracted with chloroform, and injected into the HPLC in order to substantiate the extraction process. Indomethacin was chosen as an internal standard. 5 μg of the compound was injected into the column. Indomethacin was also included in the extracts from the pineal organ culture medium.

Retention times were recorded for methanol, indomethacin, and melatonin. The melatonin was identified by retention times and quantitated by peak height measurements.

4.1.4 Results

Melatonin standards (10 μg) were detected with the facility. The melatonin peak corresponded to a retention time (t_r) of 9.6 min. (Fig. 4.1). The methanol peak was clearly visible with a t_r of 2.3 min. The indomethacin (internal standard peak) lay extremely close to the methanol peak and had a measure t_r of 2.7 min (Fig. 4.2).

The chromatogram of chloroform extracted BGJb culture medium (no pineal tissue) (Fig. 4.3) shows the methanol peak only, as was expected. The chromatogram of chloroform extracted BGJb pineal culture medium (Fig. 4.4) shows the melatonin clearly, at a concentration of approximately 1170 picograms.

4.1.5 Discussion

It is evident from this tentative study, that the chloroform extraction process coupled with measurement by HPLC represents a viable method for quantifying melatonin levels in pineal organ culture. The UV detection system, though, is not sufficiently sensitive for the measurement for this purpose, especially for the other pineal indole metabolites such as serotonin, 5-HIAA, 5-HTOH and 5-MTOH. A fluorometric or electrochemical detection system would probably prove far more useful. A coefficient of variation was not determined due to the limited number of sample injections.

It would appear as well, that such a system combined to a more

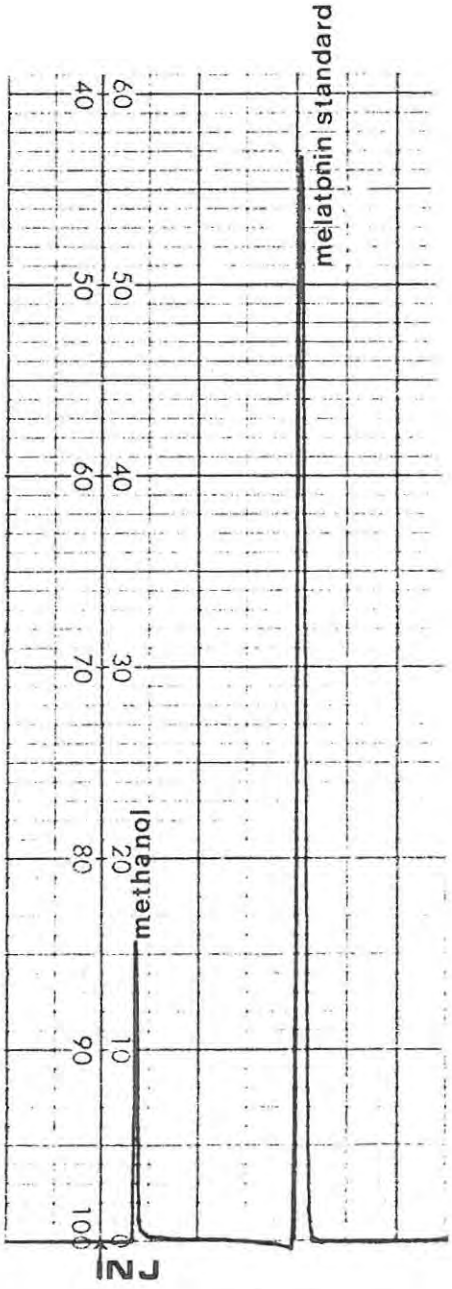

Fig 4.1 Chromatogram of melatonin standard (10 μg) with UV detection. Injection volume = 10 μl

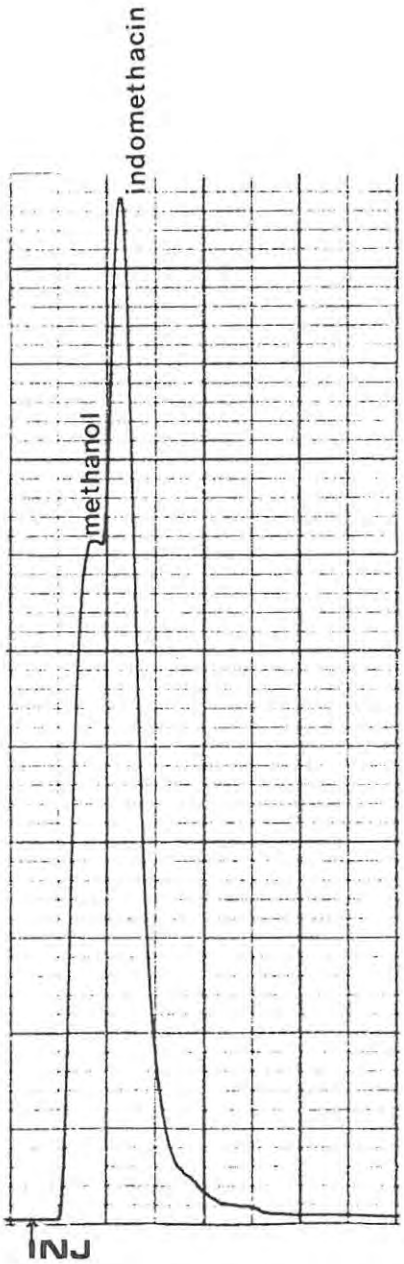

Fig. 4.2 Chromatogram of the internal standard indomethacin (5 μg) with UV detection. Injection volume = 10 μl.

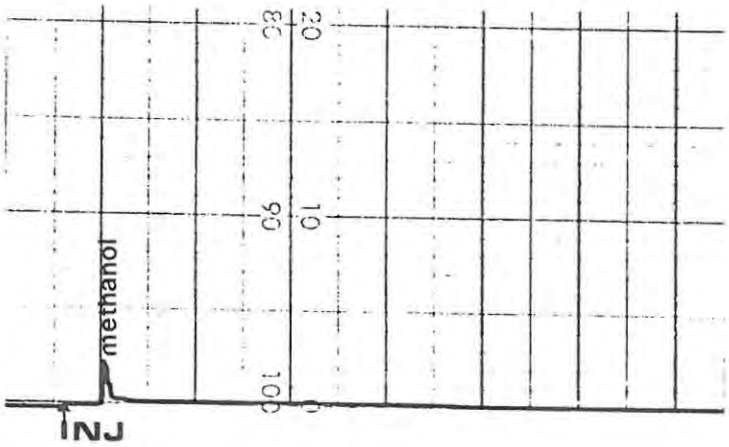

Fig 4.3 Chromatogram of chloroform extracted BGJb culture medium (no pineal tissue) showing the methanol peak. Injection volume = 10 μl.

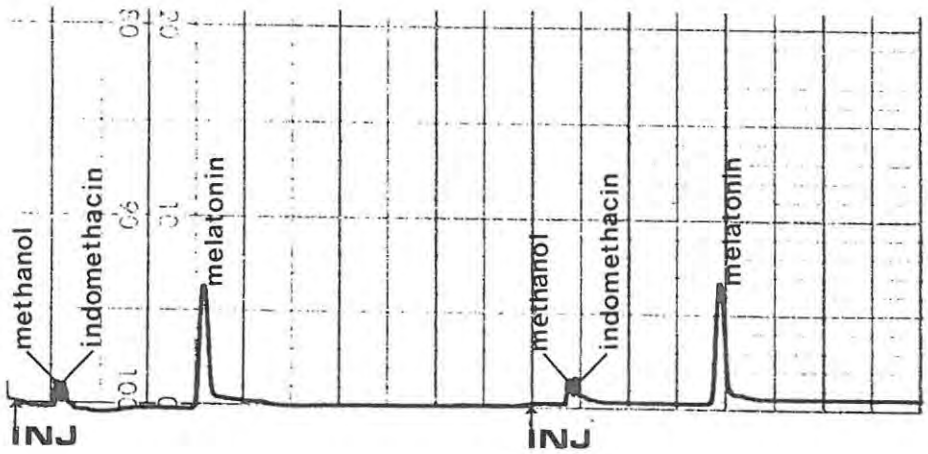

Fig. 4.4 Chromatogram of chloroform extracted BGJb pineal culture medium showing the melatonin peak. Injection volume = 10 μl.

sensitive detector could provide a useful method of measuring pinealocyte indole metabolites in cell culture. Insufficient facilities, however, prevented the investigation of such a system, which might prove to be a novel way of monitoring pineal metabolites produced in cell-culture.

CHAPTER 5

5.1 Summary of Conclusions

5.1.1 Studies Involving Alpha-Methyldopa

The organ culture experiments confirm the adrenergic activity of alpha-methyldopa in the pineal. In vitro studies suggested that alpha-methyldopa requires to be converted to a physiological metabolite in order to exert its effect. Studies involving both alpha-methyldopa and isoproterenol treatment posed the potential existence of a super-sensitivity phenomenon. Subsequent NAT activity studies were in accordance with these findings. Ligand binding to the pineal gland beta-receptors was shown, however, to decrease with alpha-methyldopa treatment. It was hypothesized that the physiological metabolite of alpha-methyldopa, alpha-methylnorepinephrine is a weak beta-agonist and possibly an alpha-agonist as well. The alpha-agonistic activity of alpha-methylnorepinephrine was confirmed using an alpha 1- and $alpha_2$-receptor blocking agent, phentolamine. It was also suggested that raised aHT and aMT levels, and NAT activity associated with alpha-methyldopa treatment was partially owing to alpha-receptor potentiation of the beta-receptor activity. Measurement of pineal HIAA, HTOH, MIAA and MTOH produced data suggestive of a mechanism in which the levels of these metabolites are dependent upon the degree of depletion of serotonin by NAT, and hence the formation of their common precursor, 5-hydroxyacetaldehyde.

5.1.2 Studies Involving Ephedrine

The results in general support the theory that part of the effect of ephedrine occurs as a result of ephedrine inducing the release of norepinephrine from stores in nerve endings. Released norepinephrine and ephedrine itself exert a combined effect, with both probably having alpha- and beta-adrenergic activity. An increase in aHT levels (derived from organ culture studies), associated with combined exposure of the pineal to norepinephrine and ephedrine, in comparison to ephedrine treatment alone, is supportive of this theory. In addition the possibility that ephedrine induces a supersensitivity of the beta-receptor was also considered. In order to quantify the effects of ephedrine itself, norepinephrine stores in the rat were depleted using reserpine, and pineals exposed to ephedrine. Levels of aHT and aMT

were significantly lower than when pineals from untreated rats were exposed to ephedrine, but were however of the same order as basal levels. A mechanism by which ephedrine itself is a weak beta-adrenergic agonist (as well as being an alpha-adrenergic agonist) was also considered. NAT activity exhibited a similar response with the same treatment, with the exception that activity levels were similar under these conditions to those recorded in response to treatment with norepinephrine, suggesting that ephedrine possesses a substantial measure of adrenergic activity.

The possibility that ephedrine induces a supersensitivity type effect at the level of the beta-adrenergic receptor was ruled out by results from a ligand binding study, in which ephedrine treatment was shown to reduce ligand binding to the beta-receptor. Furthermore, exposure of pineal glands from reserpinized rats to ephedrine resulted in a further decrease in binding at the beta-receptor.

The data presented supports current explanations of the mode of action of ephedrine in the body. Both ephedrine and released norepinephrine interact with alpha- and beta-receptors to produce their effects. A study of the nature of the interaction of the two drugs with alpha-receptors using beta-blocking agents would have been a valuable additive to these experiments, however financial restraints limited further study. Similarly it would have been useful to quantify the binding of ephedrine to beta-receptors by means of a Scatchard Analysis.

It is thus probable that ephedrine and released norepinephrine are both strong beta-adrenergic agonists and compete for beta-receptor binding sites. They are also probably alpha-adrenergic agonists, and the possibility exists within the pineal, that the alpha-activity of both drugs potentiates beta-receptor activity. The relative levels of alpha-receptor activity remains to be elucidated. It is likely that the dual action of ephedrine and released norepinephrine presents a complex interaction. The use of reserpine, as has been discussed introduces complications within the study, mainly as a direct consequence of its ability to produce a supersensitive effect at the beta-receptor. In this respect, the use of a dissociated pineal cell-

culture system overcame this limitation.

A reduction in levels of HIAA, HTOH, MIAA, and MTOH with ephedrine treatment is in accordance with the model presented to explain reductions in these metabolites associated with alpha-methyldopa treatment.

A time-profile of the production of pineal aHT and aMT with norepinephrine and ephedrine treatment provided a clearer picture of the course of action of the two agents on pineal metabolism in organ culture. The results of this study also provided potentially useful information regarding the regulation of NAT and HIOMT in the pineal. In general, the results were suggestive of a competitive interaction between ephedrine and released norepinephrine for pineal beta-receptors, and probably alpha-receptors as well.

5.1.3 Cell Culture

As a consequence of the need to create a cell system with no endogenous norepinephrine present, for the study of ephedrine, a neurotransmitter-free, dissociated cell system was developed and evaluated using phase contrast and electron microscopy. NAT assays performed on ephedrine treated pinealocytes were suggestive of a substantial beta-adrenergic activity for ephedrine and probably an alpha-adrenergic component as well. The dual mechanism of action involving both ephedrine and released norepinephrine was also confirmed.

5.1.4 Determination of Melatonin in Pineal Organ Culture by HPLC

The brief investigation into the use of HPLC coupled to a UV-detection system as a means to measure melatonin in pineal organ culture medium was moderately successful. Melatonin peaks were detected, however the UV monitor was not sufficiently sensitive and would probably not be able to measure melatonin levels accurately below the picogram range. Linking the HPLC system to a fluorometric or electrochemical detector was seen as being a more feasible combination.

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