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THE REGULATION OF SEROTONIN N-ACETYLTRANSFERASE IN THE RAT PINEAL GLAND

THESIS

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ABSTRACT

The synthesis of the pineal hormone, melatonin, is finely regulated by the pineal enzyme serotonin N-acetyltransferase (NAT). In the absence of light, the activity of NAT is markedly enhanced by the release of nor-adrenaline from sympathetic nerve endings in the pineal. Exposure of animals to light during darkness causes a sudden and dramatic reduction in the activity of NAT.

The present study investigated a possible mechanism for this sudden decline in NAT activity. These investigations included the determination of the effects of S-adenosylmethionine (SAM), adenosine nucleotides and calcium on NAT activity.

In vitro experiments using SAM showed that pineals pre-incubated with SAM prior to adrenergic stimulation did not significantly alter NAT activity or pineal indoleamine metabolism. However, measurement of pineal cyclic AMP showed that SAM exposure reduced the adrenergic-induced rise in pineal cyclic AMP.

Experiments using adenosine 5'-monophosphate (5'-AMP) showed that this nucleotide enhanced both dark- and isoproterenol-induced NAT activity. Adenosine 5'-triphosphate (ATP), on the other hand, reduced NAT activity with a concomitant reduction in pineal indoleamine metabolism. Exposure of isoproterenol-stimulated pineals in organ culture to propranolol resulted in a marked rise in ATP and adenosine 5'-diphosphate (ADP) synthesis accompanied by a decline in 5'-AMP levels as compared with pineals treated with isoproterenol alone. This then implies that exposure of animals to light could cause a change in pineal nucleotide levels.

Since nucleotide levels are also controlled by calcium, experiments were carried out to determine the effect of calcium on pineal NAT activity. These experiments showed that ethyleneglycol-bis-N,N,N,N,-tetraacetic acid (EGTA) enhanced NAT activity whilst calcium reduced the activity in pineal homogenates, implying that calcium may act directly on NAT to regulate its activity. Exposure of pineal glands in organ culture to the calmodulin antagonist R24571 caused a rise in pineal cyclic AMP levels with a concomitant decrease in

cAMP-phosphodiesterase activity. This was, however, accompanied by a decline in N-acetylserotonin and melatonin synthesis.

These findings implicate a number of factors in the regulation of pineal NAT activity. A mechanism for the regulation of pineal NAT is proposed.

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α	alpha
α-MSH	α-melanocyte-stimulatory hormone
β	beta
μCi	micro Curie
$\mu W/cm^2$	micro Watts/centimetre squared
5-ALA	5-aminolevulinic acid
5′-AMP	adenosine 5′-monophosphate
A23187	calcium ionophore
ADP	adenosine 5′-diphosphate
ATP	adenosine 5′-triphosphate
ca	circa
Ca^{2+}	calcium
cAMP	adenosine 3′,5′-monophosphate
cGMP	guanosine 3′,5′-monophosphate
CNS	central nervous system
cpm	counts per minute
DHA	dihydroalprenolol
DMSO	dimethyl sulphoxide
dpm	disintegrations per minute
DS	day study
e.g.	for example
EGTA	ethyleneglycol-bis-N,N,N,N,-tetraacetic acid
g	gram
G-protein	guanine nucleotide binding protein
h	hour
HIAA	5-hydroxyindoleacetic acid
HIOMT	hydroxyindole-O-methyltransferase
HT	serotonin
HTOH	5-hydroxytryptophol
HTp	5-hydroxytryptophan
i.e.	that is
ip	intraperitoneal
Iso	isoproterenol
Kd	equilibrium constant
Km	Michaelis constant
LD 12:12	light to dark cycle of 12 hours
MAO	monoamine oxidase
MAOP	monoamine oxidase products
mCi	milli Curie
MEL	melatonin
mg	milligram
MIAA	5-methoxyindoleacetic acid
min	minute(s)
ml	millilitre
mol	mole
MT	5-methoxytryptamine
MTOH	5-methoxytryptophol

n	nano (10^{-9})
NAS	N-acetylserotonin
NAT	serotonin N-acetyltransferase
NS	night study
p	pico (10^{-12})
PDE	cAMP-phosphodiesterase
PEI	polyethyleneimine
Prop	propranolol
R24571	calmidazolium
rpm	revolutions per minute
SADS	seasonal affective disorder syndrome
SAM	S-adenosylmethionine
SCN	suprachiasmatic nuclei
SEM	standard error of the mean
TLC	thin layer chromatography
Tp	tryptophan
VCR	vesicle-crowned rodlets
viz.	namely
W	watts
w/v	weight by volume
[^{14}C]-	carbon-14 radiolabel
[^{3}H]-	tritium radiolabel
§	section

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CHAPTER I

LITERATURE REVIEW

1.1 Pineal History

The human pineal gland was first discovered by Herophilos (325 - 280 BC) a famous anatomist at the University of Alexandria in Egypt (Ariëns-Kappers, 1979). He took the pineal to be a "tap" which regulated the stream of *pneuma* (the human spirit) from the third to the fourth ventricles of the brain.

Galen (130 - 300 AD) took the pineal to be a gland like any other. He coined the term *soma konoeides* for the pineal because, in man, the pineal is shaped like the cone of a pine tree. Berenganio da Capri (1470 - 1530) was the first to examine the human pineal gland in more detail. René Descartes (1596 - 1650) described the pineal as the "seat of the soul". This statement came at a time in history when the soul was of importance to scientists and philosophers alike. This theory of the "soul" and other superstitions lasted for many centuries (Ariëns-Kappers, 1979).

It was in the second half of the nineteenth century that a renewed interest in the anatomy, histology and embryology of the pineal gland took place. Factors promoting this interest were new techniques in the sectioning and staining of slides (Ariëns-Kappers, 1979).

The discoveries of the endocrine glands and hormone producing organs by Bernard (1813 - 1878) and Brown-Sequard (1817 - 1894) greatly stimulated the interest of physiologists in the pineal gland. It was at the end of the nineteenth century that the mammalian pineal was first considered as a serious candidate for hormonal production (Ariëns-Kappers, 1979). Later, Berlinger (1920) and Engel (1936) proposed that the pineal gland had an inhibitory effect on gonadal development. These views were later criticized when the structure and function of the hypothalamus became better known (Ariëns-Kappers, 1979).

In 1943 a landmark in pineal research was reached by Bargmann. This author published a survey on the microscopic anatomy, histology and cytology of the pineal gland, from fish to man. A further survey in 1954 by Kitay and Altschule, of all previous data on pineal physiology, initiated a steady interest in pineal research which has progressed to the present time.

1.2 Pineal Anatomy

1.2.1 Location

In the rat, the pineal gland is situated in the superior part of the *sulcus transversus cerebri* at the surface of the brain between the hemispheres anteriorly and the cerebellum posteriorly (Kappers, 1960). The pineal gland is connected to the commissural region by a thin filament-like stalk. This pineal stalk is formed by the 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 evaginations (Kappers, 1960) (**Figure 1.1**). The pineal stalk may be divided into three distinct parts, *viz.* a proximal part, a midpart and a distal part. The proximal part lies between the habenular and caudal commissures. The midpart is often extremely thin. The distal part is the largest of the three structures and is situated between the rostral and caudal collicles (Kappers, 1960).

1.2.2 Blood Supply

In 1940, Gladstone and Wakely found that the major blood supply to the pineal gland was provided by the posterior choroidal arteries. The posterior cerebral arteries also branch into the pineal gland to form a capillary network. Blood is drained from the pineal via 12 - 16 superficial connecting veins. These veins lead into the distal end of the great cerebral vein, which ultimately leads the blood into the systemic venous system (Hodde, 1979). The blood supply reaches the pineal gland at a rate of flow surpassed only by the kidney, and is greater at night than during the day (Quay, 1972; Rollag, 1988; Reiter, 1981).

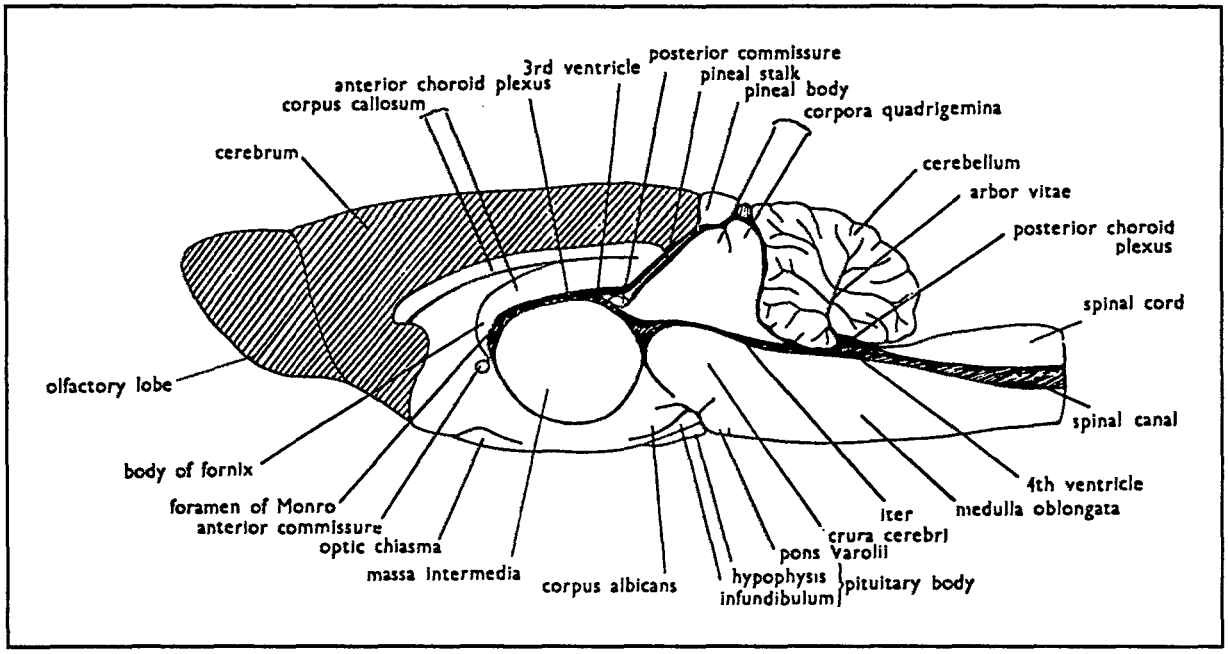

FIGURE 1.1 *Transverse section of the rat brain showing the relative position of the pineal body and pineal stalk* (Rowett, 1962).

1.2.3 Pineal Innervation

The pineal gland of mammals is heavily innervated by nor-adrenaline-containing sympathetic fibres (Pfister *et al.*, 1978; Ronnekleiv and Moller, 1978; Semm, 1978) located in the perivascular space and in the parenchyma between the pinealocytes (Matsushima *et al.*, 1981). The perikarya of these sympathetic fibres are located in the superior cervical ganglion, from which the fibres reach the pineal via the conarian nerves (Kappers, 1960). The secretory function of the mammalian pineal gland has been linked to its sympathetic innervation (Wolfe *et al.*, 1962).

The parasympathetic system is found together with the sympathetic system (Kenny, 1961; Romijn, 1975). The function of the parasympathetic nervous system in the pineal needs further investigation. The terminals of both these systems do not form synapses with the pinealocytes (Kappers, 1976), but end in the perivascular space where the neurotransmitter diffuses to receptors on the pinealocyte surface.

Aberrant commissural fibres of the habenular commissure have been shown to form hairpin loops in the pineal stalk (Kappers, 1960). Reiter (1982) suggests that stimuli from other parts of the central nervous system (CNS) may pass near to the habenular nucleus and may thereby influence its function. This suggests that pineal function may not exclusively depend on stimuli of various origins reaching the gland via its sympathetic innervation.

The pineal gland is often referred to as the "neuro-endocrine transducer organ" by virtue of its ability to synthesise and release melatonin in response to a neuronal 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, reticula formation and upper thoracic intermediolateral cell column, to the superior cervical ganglion whose post ganglionic sympathetic fibres, travelling along the tentorium cerebelli, enter the pineal gland via the conarian nerve (Ebadi *et al.*, 1986). This is diagrammatically represented in **Figure 1.2.**

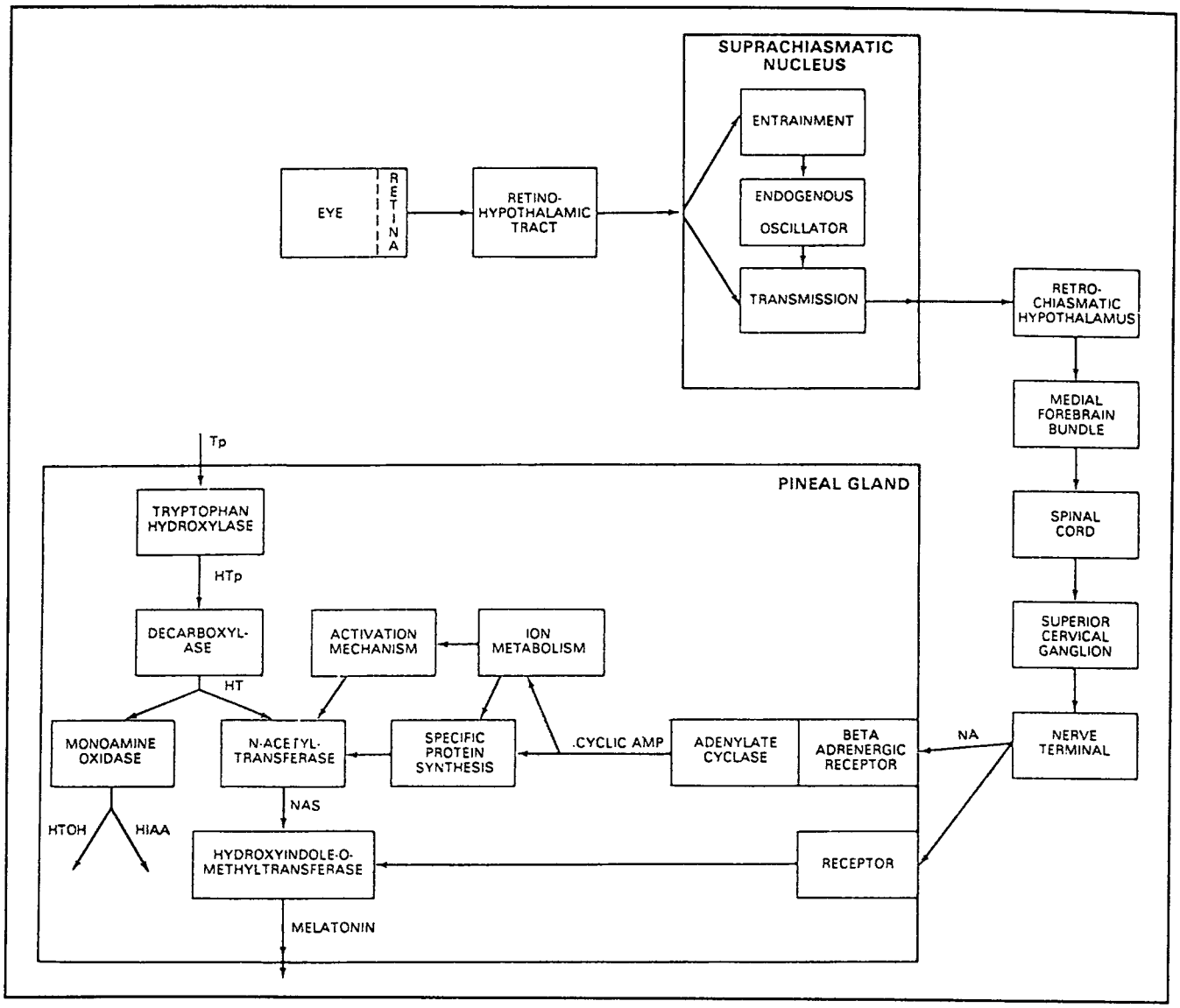

FIGURE 1.2 *Schematic representation of pineal neural control* (Adapted from Klein, 1979).

NA = nor-adrenaline; cyclic AMP = adenosine 3′,5′-cyclic monophosphate; Tp = tryptophan; HTp = 5-hydroxytryptophan; HT = Serotonin; HIAA = 5-hydroxyindoleacetic acid; HTOH = 5-hydroxytryptophol; NAS = N-acetylserotonin.

1.3 The Pineal Cells

There appears to be some confusion amongst researchers as to the nomenclature of pineal cell categories. Very often cells with different morphological characteristics are given the same name and similar cells given different names.

1.3.1 Cell Categories

1.3.1.1 Category One

These cells are characterised by the presence of granular vesicles. They have been shown to originate from the photoreceptor cells present in the pineal organ which collectively belong to the sensory cell line and are termed pinealocytes or pinealocytes *sensu stricto* (Wolfe, 1965; Collin, 1971; Pévet and Collin, 1976; Pévet *et al.*, 1977).

1.3.1.2 Category Two

The cells in this category are characterised both by their location close to the perivascular space and the absence of granular vesicles. They are usually termed interstitial or glial cells (Wolfe, 1965). There is still speculation as to whether the cells in this category originate from the same sensory cell line described by Collin (1971) (**§ 1.3.1.1**), although the opinion to date is that they do not.

The rat, cow, mule and dog have been shown to have only a single population of pinealocytes *sensu stricto* while the guinea-pig, chinchilla, mouse and rabbit have been shown to have cell groups other than the pinealocytes *sensu stricto*. These cells do not originate from the sensory cell line as described by Collin (1971), which suggests that different populations of pinealocytes could exist (Reiter, 1981).

1.3.2 Pinealocyte Ultrastructure

1.3.2.1 Nucleus

The nuclei of pinealocytes are surrounded by an envelope consisting of perinuclear cisternae bridged by nuclear pores. Chondratin strands, arranged in granular and helical masses, are found within the envelope. The nucleoli are composed of amorphous areas surrounded by a dense nucleolonema. Ribonucleoprotein granules are also present.

1.3.2.2 Cell Organelles

Concentric Lamellae: Concentric lamellae are membrane-whorls found in the cytoplasm of the pinealocyte. The functions of these lamellae are unclear. Greater numbers of lamellae are found in actively secreting cells, which suggests that their formation is in some way related to the secretory process of the cell (Pévet, 1976 and 1979).

Mitochondria and Lipid Droplets: Mitochondria are found in all eukaryotic cells. They are energy-transducing systems which recover and convert the energy contained in food to the high energy phosphate bond of adenosine triphosphate (ATP). The pinealocytes contain large numbers of mitochondria, suggesting a high requirement for energy.

Lipid droplets are also found in the cytoplasm of pinealocytes. Their role in the cell is believed to be of a metabolic nature. However, studies have shown that the number of lipid droplets fluctuates under changing conditions (Karasek, 1971), suggesting that lipid droplets could be either directly or indirectly involved in the process of synthesis and/or secretion in the pinealocyte (Pévet, 1979).

Annulate Lamellae: Annulate lamellae is a collective term given to a number of structures within the pinealocyte. These structures are found in most mammalian pineals but their function is at present unknown. According to McNeil (1977), the lamellae could be derived from a modification of the granular endoplasmic reticulum.

Vesicle-Crowned Rodlets: A vesicle-crowned rodlet consists of an electron-dense rodlet surrounded by a single layer of small clear vesicles. These rodlets lie singly or in groups in the pinealocyte cytoplasm, very often close to the cell membrane (Reiter, 1981).

A number of theories exist as to their function. Vollrath and Huss (1973) suggested that the vesicle-crowned rodlets (VCR) could be involved in intercellular communications between pinealocytes. VCR could possibly be excitatory structures alerting the physicochemical properties of neighbouring pinealocytes (Vollrath, 1973).

The number, size and location of VCR appear to be dependent upon environmental illumination. A circadian rhythm in VCR numbers has been shown in the rat by Kurumado and Wataru (1977).

Subsurface Cisterns: Subsurface cisterns are generally found close to the cell membrane in the perikarya of very active cells (Rosenbluth, 1962; Pévet, 1979). Their function is thought to be that of establishing a pathway for the exchange of metabolites or ions between neighbouring cells.

1.3.2.3 Secretory Processes

Many vesicles and organelles found within the cytoplasm of pinealocytes are responsible for the secretion of two major compounds giving the pineal gland its endocrine capabilities. These secretions usually take the form of indoleamines and peptidergic compounds (Reiter *et al.*, 1975).

There are two active secretory processes in the pinealocyte. The first involves the formation of granular vesicles by the Golgi apparatus. The second, termed the ependymal-like secretory process, involves the formation of cisternae by the granular endoplasmic reticulum (Pévet, 1979).

Granular Vesicles: Granular vesicles have been observed in the pinealocytes of practically all mammalian species (Pévet, 1979). They originate from the Golgi saccules and are found in perikarya as well as in the processes of the pinealocytes. It is generally accepted that the

granular vesicles migrate to the ends of the processes to release their contents into the perivascular space (Reiter, 1981).

It is thought that granular vesicles release their contents in a packaged form, the main product of secretion being an antigonadotrophic compound (Kappers, 1976; Benson and Krasovich, 1977; Upson and Benson, 1977; Reiter, 1978).

Ependymal-like Secretory Process: This secretory process is linked to the activity of the granular endoplasmic reticulum. This activity leads to either an accumulation of proteinaceous material in its cisterns or to the formation, by the cisterns, of vacuoles containing flocculent material (Karasek and Marek, 1978). It is proposed that the product of secretion is an antigonadotrophic compound which plays an important part in pineal physiology (Pévet, 1976).

1.4 Pineal Biochemistry

1.4.1 Circadian Rhythms

A circadian rhythm may be defined as a periodic event recurring approximately every 24 hours. Three processes in the pineal have been linked to a circadian rhythm, namely: locomotor activity (Gaston and Menaker, 1968), photoperiodic time measurement (Elliot *et al.*, 1972; Elliot, 1976) and daily colour changes (Hafeez and Quay, 1970; Bagnara and Hadley, 1978).

Serotonin, a pineal gland indole, exhibits considerable daily fluctuations (Quay, 1963; Fiske, 1964). Serotonin levels were found to be high during the day and low at night, with corresponding high levels of melatonin at night and low levels during the day. These daily fluctuations of pineal indoles have been linked to the rhythms present in the enzymes hydroxyindole-O-methyltransferase (HIOMT) and serotonin-N-acetyltransferase (NAT) (Axelrod *et al.*, 1965; Klein *et al.*, 1970; Klein, 1972; Binkley *et al.*, 1973). The presence of a HIOMT rhythm has, however, been disputed by a number of investigators.

NAT and melatonin rhythms in rats have been shown to persist for up to two weeks in constant darkness and dampen in constant light (Ralph *et al.*, 1971; Deguchi, 1975). Light intensity also affects the degree of NAT activity (Minneman *et al.*, 1976) and unexpected dawn (light) transitions imposed on rats during the dark period (night) result in a rapid decrease in NAT activity and melatonin (Klein and Weller, 1972).

1.4.2 Inorganic Constituents

The principal calcareous component of human pineal corpora anenacea is hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$). The presence of carbonite apatite ($Ca_{10}(PO_4,CO_3,OH)_6(OH)_6$) has been shown as well (Angervall *et al.*, 1958; Earle, 1964). Histochemical studies have shown the presence of iron in the superficial lamellae of the corpora anenacea. Magnesium and strontium have also been reported in small concentrations (Bayerovà and Bayer, 1960; Saliichuk, 1964; Krstić, 1976).

The pineal gland has high concentrations of copper, manganese and zinc compared with other tissues and organs. It is likely that these constituents are chiefly associated with specific proteins and enzymes (Wong and Fritze, 1969).

Recorded pineal calcium concentrations vary between different researchers, but it is understood that calcium contributes to molecular control mechanisms within the pineal (Pizarro *et al.*, 1989). These control mechanisms pertain to: sensitivity of pineal β-receptors (Wilkinson *et al.*, 1976; Wilkinson, 1978), closure of pineal canaliculi (Quay, 1974a) and the induction of enzymes participating in pineal catechol- and indoleamine synthesis. These enzymes include adenylate cyclase, cyclic-AMP phosphodiesterase, tyrosine hydroxylase and N-acetyltransferase (Cantor and Weiss, 1978; Zatz and Romero, 1978).

1.4.3 Amino Acids

The amino acids present in the pineal gland contribute to both regulatory mechanisms as well as to pathways of general metabolic importance.

Aromatic amino acids are of particular importance in the pineal gland since they include the precursors to chemical mediators of the catechol-, imidazol-, and indoleamine series.

1.4.4 Nucleotides

"Adenine nucleotides and their derivatives have diverse critical roles in both energy and biosynthetic control mechanisms within the pineal gland" (Quay, 1974b).

S-adenosylmethionine (SAM) is synthesized in pineal tissue from adenosine triphosphate (ATP) and methionine. SAM has a wide array of biological transmethylation reactions in the pineal gland (Guchait and Grau, 1978), including the methylation of biogenic amines (Deguchi and Barchas, 1971).

Of all the pineal nucleotides, cyclic adenosine 3′,5′-monophosphate (cAMP) receives the greatest attention. It is often termed the second messenger responding to particular hormonal or neurotransmitter molecules. Daily fluctuations in cAMP occur in the pineal, with peaks occurring ten hours after the onset of daily light and troughs ten hours after the onset of darkness (Ebadi *et al.*, 1971).

The synthesis of cAMP is dependent upon the availability of ATP and the activity of adenylate cyclase, a membrane bound enzyme. Nor-adrenaline stimulation of β-receptors results in increased levels of cAMP (Strada *et al.*, 1972).

1.4.5 Lipids and Carbohydrates

Lipids form between 3% and 10% of the wet weight of the pineal gland. More than half of this is phospholipid, with most of the remainder consisting of free cholesterol and glycerides. Of the pineal phospholipids, phosphatidyl choline and phosphatidyl ethanolamine form the majority (Hauser and Nijjar, 1976 and 1977).

Studies on pineal carbohydrates have concentrated on the ultrastructural localisation and changes in glycogen deposits. Radio-label studies have shown the pineal gland to behave more like an endocrine gland than brain tissue in relation to carbon dioxide production.

Where the brain uses the Embden-Meyerhof pathway, the pineal gland uses the pentose phosphate pathway (Krass and LaBella, 1966 and 1967).

1.4.6 Pineal Proteins

The pineal's content of soluble protein may be placed into two classes *viz.* endogenous and exogenous. The pineal has been found to have a high capacity for exogenous peptide uptake. Examples of peptides are: thyroid releasing hormone, luteinizing hormone-releasing hormone, α-melanocyte-stimulating hormone (α-MSH), MSH-releasing inhibiting factor, somatostatin and methionine-enkephalin (Redding *et al.*, 1973; Dupont *et al.*, 1975; Nair and Colwell, 1975; Kastin *et al.*, 1976). Other peptides identified in pineal tissue include lysine vasotocin, nerve growth factor, delta-sleep inducing peptide, S-100 protein and glial fibrillary acidic protein (Kastin *et al.*, 1978; Moller *et al.*, 1978; Perez-Polo *et al.*, 1978).

1.5 Pineal Indole Compounds

1.5.1 Tryptophan

Tryptophan concentrations in the pineal gland are a function of the tryptophan in circulation which is controlled by the liver's cytosolic enzyme, tryptophan pyrrolase (Badaway, 1979). The administration of 5-aminolevulinic acid (5-ALA) was shown to increase tryptophan pyrrolase activity in rats with a resultant decrease in tryptophan levels in forebrain regions (Daya *et al.*, 1989). Pineal gland studies, however, showed that tryptophan levels remained unaffected by 5-ALA administrations (Daya *et al.*, 1989). Deguchi and Barchas (1972) demonstrated that injections of tryptophan could result in elevated pineal tryptophan levels.

1.5.2 Serotonin

Serotonin in the pineal gland is synthesised predominately in the pinealocyte (Fuller and Perry, 1977). Three factors which regulate the concentration of serotonin in the pineal gland are:

- the synthesis-loading of the pinealocyte with tryptophan (which increases serotonin)
- the rate at which serotonin is converted to oxidised products, and

- the rate at which serotonin is converted to N-acetylserotonin by the enzyme serotonin N-acetyltransferase (NAT).

Increasing or decreasing the activity of NAT has a direct reciprocal effect on serotonin concentrations (Klein *et al.*, 1973) (**Figure 1.3**).

1.5.3 N-Acetylserotonin

The levels of N-acetylserotonin (NAS) are directly controlled by NAT. When NAT activity is high, as is the case at night, NAS levels are elevated. The opposite occurs during the day (Brownstein *et al.*, 1973; Klein and Weller, 1973).

1.5.4 Melatonin

In 1917 McCord and Allen reported that amphibians fed with bovine pineal extracts exhibited skin lightening. It was not until 1957 that the active compound was isolated by Lerner and co-workers, and was termed 5-methoxy-N-acetyltryptamine (Lerner *et al.*, 1958). The name was later changed to melatonin because of its blanching effect on melanophores (Lerner *et al.*, 1959; Lerner and Case, 1960).

Melatonin was originally thought to be synthesised only in the pineal gland. The basis for this speculation was the distribution of the enzyme HIOMT (Axelrod *et al.*, 1965). However, research has shown that melatonin is synthesised in other tissues, but to a lesser extent. These tissues are the retina, intestine and Harderian gland (Cardinali and Rosner, 1972; Cardinali and Wurtman, 1972; Bubenik *et al.*, 1977; Pang *et al.*, 1980).

Melatonin is very lipophilic and is found to permeate many parts of the body, such as the brain, peripheral nerves and sympathetic nerve chain (Wurtman *et al.*, 1964).

Once synthesised, melatonin is released into the circulation where most of it is found associated with serum albumin (Cardinali *et al.*, 1972; Arendt, 1978). In the liver, melatonin is metabolised to 6-hydroxymelatonin.

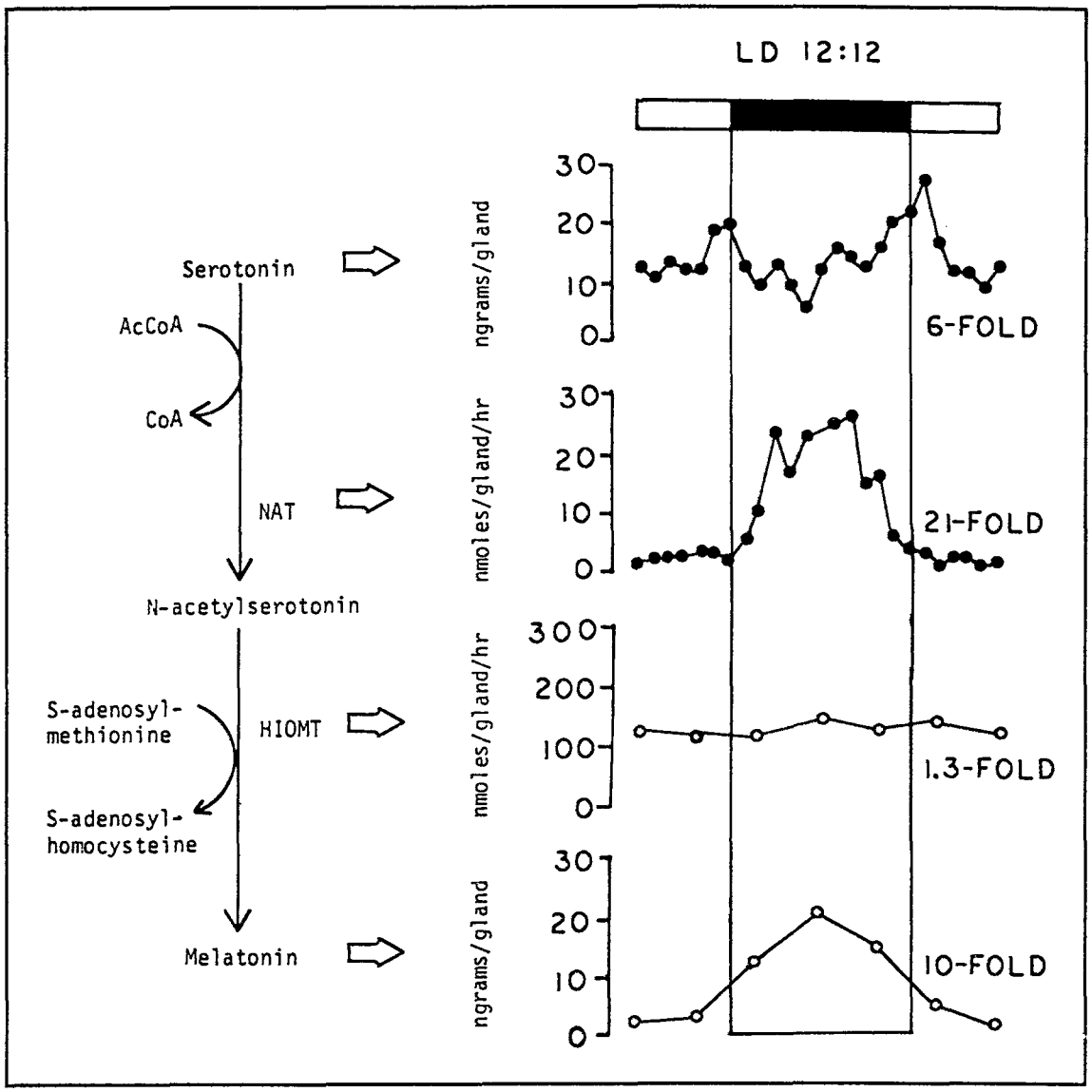

FIGURE 1.3 *Daily rhythms in serotonin metabolism in the rat pineal gland (Reiter, 1981).*

Melatonin is found in most mammals, indeed most vertebrates. It's synthesis follows a 24 hour cycle, with a peak being reached at night. Seasonal physiological adjustments, such as those involved in breeding patterns and hibernation seem to be the major function of the hormonal response of the pineal gland (Arendt, 1986; Reiter, 1988a).

Melatonin receptor populations have been found in the hypothalamus (Reppert *et al.*, 1988), pars tuberalis of the pituitary gland (Morgan and Williams, 1989) and the hipocampus (Zisapel, 1988). This indicates that melatonin could play a role in the regulation of these tissues. These discoveries were made possible by the use of 2-iodomelatonin, a potent melatonin analogue (Vakkuri *et al.*, 1984).

1.5.4.1 Melatonin Hypotheses

Reiter (1987) suggested three main hypotheses regarding hormonal rhythms, namely:

- the duration hypothesis
- the coincidence hypothesis, and
- the amplitude hypothesis.

The **duration hypothesis**, as the name implies, takes into account the duration of nocturnal melatonin levels. According to this hypothesis, as seasons change, the animal is exposed to different durations of melatonin *i.e.* longer exposure during winter (long nights) than during summer (short nights) (Reiter, 1991).

This hypothesis has been extended to "take into account the direction of change of elevated nocturnal melatonin *i.e.* whether the period of elevated levels is becoming longer as the season changes to winter, or shorter, as summer approaches" (Brown, 1992).

The **coincidence hypothesis** implies that, although the nocturnal highs of melatonin are important they are immaterial unless the organ is receptive to those levels of melatonin at that particular point in time (Carter and Goldman, 1983; Stetson and Watson-Whitmyre, 1986).

The **amplitude hypothesis** examines the magnitude of the nocturnal rise of melatonin (Berga *et al.*, 1988). The importance of this hypothesis lies in its ability to be used as a diagnostic

tool *i.e.* "humans often show altered melatonin levels in the presence of certain disease states" (Brown, 1992).

1.5.4.2 Reported Influences of Melatonin

As reported by Brown (1992) melatonin has been shown to be implicated in:

i. Reproduction: melatonin causes a marked reduction in sperm motility and at high concentrations, which are still within normal physiological values, completely abolishes movement (Oosthuizen *et al.*, 1986).

ii. Hormonally dependent tumours *e.g.* women with breast cancer often have enlarged pineals (Blask, 1984). Pinealectomised rats show little resistance to cancer growth while melatonin administration reduces cancer growth (Reiter, 1989).

iii. Immunocompetence: melatonin administrations have been shown to enhance the immune response (Pierpaoli and Maestroni, 1987).

iv. Stress and its effects *e.g.* stress related ulcers. Prior melatonin administrations caused a significant reduction in gastric ulcerations in stressed male rats (Khan *et al.*, 1990).

v. Sudden infant death syndrome (Sparks and Hunsaker, 1988).

vi. Changes in environmental light *e.g.* "jet-lag". Melatonin administration appears to shorten the re-entrainment of the circadian rhythm (Turek, 1987).

vii. Ageing. Relative melatonin deficiencies with persistent serotonin have been reported to promote ageing (Rozencwaig *et al.*, 1987).

viii. Seasonal affective disorder syndrome (SADS). In the Northern Hemisphere patients may experience a depressive state of mind between December and February, which when treated with light (which impliedly reduces levels of plasma melatonin) causes an improvement (Ebadi and Govitrapong, 1986).

1.6 Pineal Indole Metabolism

The pathways involved in the metabolism of pineal indoles are schematically represented in **Figure 1.4.**

Serotonin is synthesised from tryptophan in a process involving two enzymes, namely tryptophan hydroxylase and aromatic amino acid decarboxylase (Lovenberg *et al.*, 1967). Tryptophan is first converted to 5-hydroxytryptophan by tryptophan hydroxylase in the mitochondria (Hori *et al.*, 1976). This appears to be the rate-limiting step in the synthesis of serotonin from tryptophan. Tryptophan hydroxylase, like other mammalian aromatic hydroxylases, requires a pteridine co-factor to function. High concentrations of pteridine co-factors are found in the pineal gland (Levine *et al.*, 1979).

5-Hydroxytryptophan is converted to serotonin by the enzyme aromatic amino acid decarboxylase. This enzyme is non-specific and is found in most tissues (Christenson *et al.*, 1972). Within the pineal, the enzyme is located in the cytosol and requires a pyridoxal phosphate to function (Snyder *et al.*, 1965a).

The next step involves the acetylation of serotonin to N-acetylserotonin by the enzyme serotonin N-acetyltransferase (NAT) with the co-factor acetyl coenzyme A being the acetyl donor (Weissbach *et al.*, 1960). N-acetylserotonin is converted to melatonin by O-methylation in the 5-position by the enzyme hydroxyindole-O-methyltransferase (HIOMT).

Serotonin may be metabolised by way of a second pathway, involving deamination and oxidation reactions. Serotonin is oxidised to 5-hydroxyindole acetaldehyde by the enzyme monoamine oxidase (Axelrod *et al.*, 1969). The acetaldehyde is then converted to 5-hydroxyindoleacetic acid by aldehyde dehydrogenase (Wurtman and Larin, 1968). Part of the 5-hydroxyindole acetaldehyde is also converted to 5-hydroxytryptophol by alcohol dehydrogenase (McIsaac and Page, 1959) and is then methoxylated by HIOMT to form 5-methoxytryptophol (Wurtman and Axelrod, 1967).

In the final reaction, serotonin may also be methoxylated by HIOMT to form 5-methoxytryptamine.

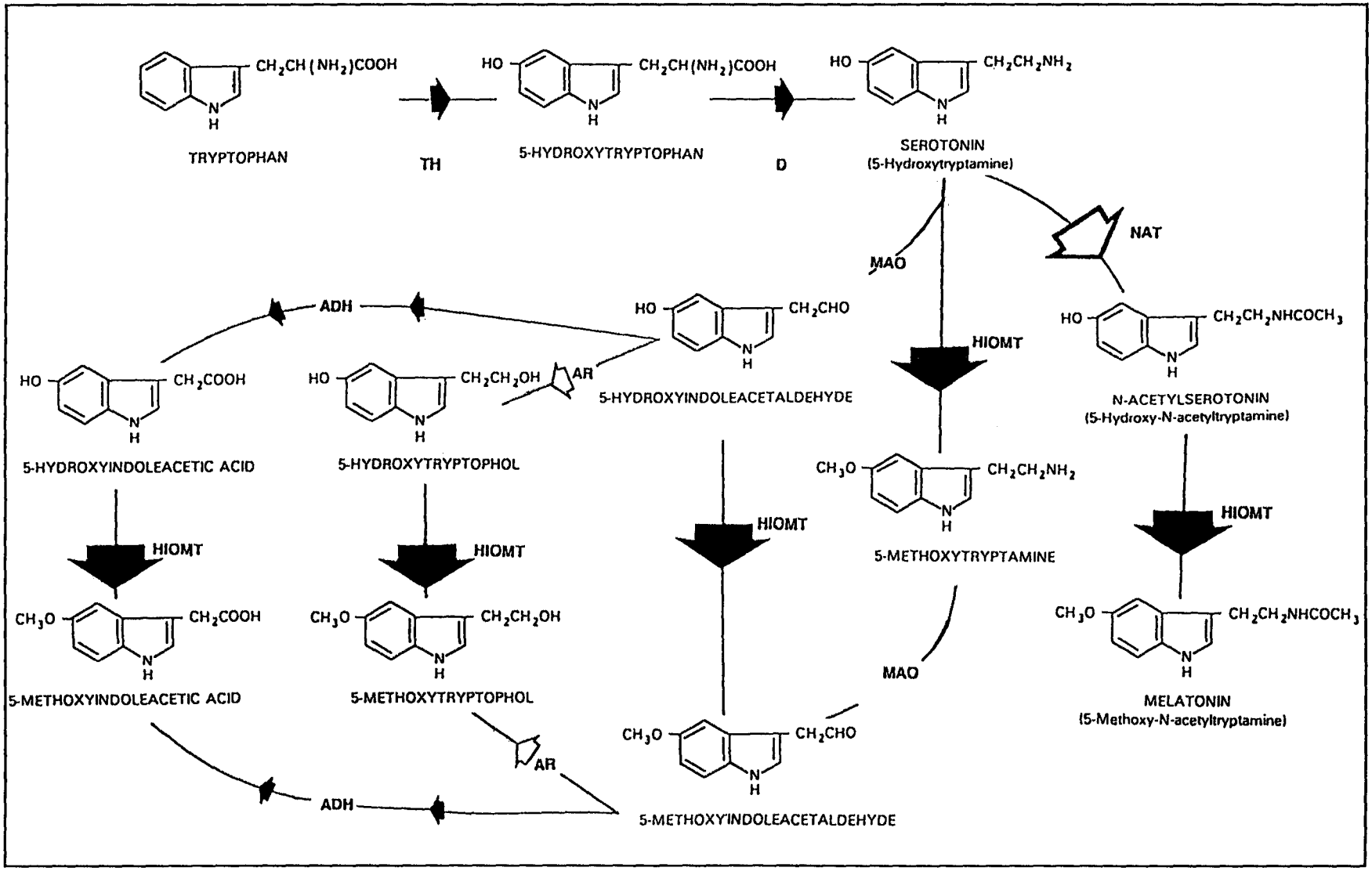

FIGURE 1.4 *Schematic representation of pineal indole metabolism* (Smith and Hauser, 1981).

1.7 The Pineal Beta-Adrenergic Receptor

The earliest characterisation of the pineal β-receptor was made using indirect methods. These methods involved the measuring of adenylate cyclase activity, an enzyme linked to the β-adrenergic receptor, in the presence of β-receptor agonists and antagonists (Weiss and Costa, 1968). Further studies confirmed the presence of β-receptors and that nor-adrenaline acted via these receptors (Deguchi, 1973; Klein and Weller, 1973; Klein and Yuwiler, 1973).

With the advent of direct receptor binding techniques it became possible to measure the number and affinity of the β-receptors (Kebabian *et al.*, 1975; Zatz *et al.*, 1976). The use of [^{3}H]-dihydroalprenolol (DHA), a specific β-receptor radio-ligand, allowed the measurement of receptor numbers. Further DHA studies showed that β-receptor numbers changed as a function of adrenergic stimulation. Animals maintained under constant light, or which were treated with adrenergic agonists during times when this stimulation was not naturally present, showed either a 50% fall or rise in receptor numbers. This phenomenon of a rise in receptor numbers during the day and a fall in numbers at night is referred to as subsensitivity and supersensitivity respectively. As a result of these changes, a diurnal rhythm in receptor numbers exists (Kebabian *et al.*, 1975).

Direct binding studies clearly indicate that the changes in receptor numbers have no effect on the receptor's affinity for agonists (Romero *et al.*, 1975; Zatz *et al.*, 1976). Receptor numbers have been shown to be affected by age *i.e.* receptor numbers decrease with an increase in age. The large day-night differences found in younger rats decrease in ageing animals (Greenberg and Weiss, 1978; Weiss *et al.*, 1979).

The effect of a β-receptor agonist is conveyed into the cell via the synthesis of cAMP from ATP (Weiss and Costa, 1968). The enzyme adenylate cyclase catalyses the above reaction and has been shown to be a separate protein to the β-receptor. Adenylate cyclase is linked to the β-receptor by a regulatory G protein which functions as a coupling transducer. G proteins are divided into two groups, namely G_s (stimulatory G protein) and G_i (inhibitory G protein) according to their α subunits (α_s and α_i) (Taylor and Merritt, 1986). In the pineal, the stimulation of β-adrenergic receptors results in the activation of adenylate cyclase via a stimulatory guanine nucleotide-binding regulatory protein (G_s) (Spiegel, 1989). This

receptor/enzyme complex is located entirely in the plasma membrane, with the G protein binding to it at the cytosolic face of the membrane (Pfeuffer, 1977; Cassel and Pfeuffer, 1978; Ross *et al.*, 1978; Kaslow *et al.*, 1979).

The concentration of cAMP in the pinealocyte is controlled primarily by its rate of synthesis and its rate of destruction. Large daily fluctuations in the cAMP concentration have been linked to stimulation of β-receptors. Shortly after β-adrenergic receptor stimulation the amount of cAMP in the pineal gland increases about 100-fold (Strada *et al.*, 1972; Deguchi, 1973; Klein *et al.*, 1978). A peak is reached five to 15 minutes after stimulation, but it is not sustained. The gradual decrease in cAMP concentration is related not to the continued presence or absence of β-receptor agonists, but rather to a decrease of adenylate cyclase activity.

Cyclic AMP is inactivated to 5′-adenosine monophosphate (5′-AMP) by the enzyme pineal cAMP-phosphodiesterase. This enzyme has been demonstrated to have both a low- and a high-Km form (Oleshansky and Neff, 1975). Minneman and Iverson (1976) showed that the amount of phosphodiesterase activity was a function of adrenergic stimulation. Isoproterenol stimulation resulted in a 50% increase in the low-Km enzyme, with the activity returning to basal levels some five hours after stimulation was terminated (Oleshansky and Neff, 1975). Minneman and Iverson (1976) further showed a diurnal change in phosphodiesterase activity, with higher activity at night.

The current concept of cAMP's mode of action is that it acts through the activation of an enzyme, protein kinase A, which phosphorylates other proteins and enzymes. In this way, enzymes involved in pineal indole metabolism are activated, resulting in the synthesis of melatonin.

1.8 The Pineal Alpha-Adrenergic Receptor

The presence of α-receptors in the pineal gland was originally demonstrated by Vacas *et al.*, (1980) and later confirmed by Sugden and Klein (1984). Smith *et al.* (1979), demonstrated that α-receptors were located post-synaptically. Sugden and Klein (1985) further showed that, unlike β-adrenergic receptors, the α-receptors showed no circadian rhythm. It was

demonstrated that these receptors were under neural control and that chronic treatment of the receptors with agonists failed to change the receptor numbers. The change in β-receptor numbers was suggested by Stadel *et al.* (1983) to be a process involving internalisation and recycling rather than a slower process of synthesis and degradation. On the other hand it is believed that α-receptor numbers vary as a result of their synthesis and degradation (Sladeczek and Bockaert, 1983; Sugden and Klein, 1985).

α2-Receptors are also detectable in rat pineal glands and are situated pre-synaptically. Noradrenaline release has been shown to be regulated via a feedback mechanism mediated by these receptors (Pelayo *et al.*, 1977).

1.9 Enzymes Involved in Pineal Indole Metabolism

1.9.1 Tryptophan Hydroxylase

Tryptophan hydroxylase catalyses the conversion of tryptophan to 5-hydroxytryptophan in the mitochondria of pinealocytes (Romijn *et al.*, 1977). The enzyme requires a pteridine co-factor, probably tetrahydrobioterin, to function (Levine *et al.*, 1979). The half-life of the enzyme has been found to be 75 minutes which suggests that the enzyme is being continually synthesised in the cell in order to maintain the levels at which it occurs (Sitaram and Lees, 1978).

The enzyme was successfully purified to a 1 000-fold purification by Nukiwa *et al.* (1974) and was shown to have a molecular weight of 30 000. The Km for tryptophan was shown to be 16 μM.

Pineal tryptophan hydroxylase differs from brain tryptophan hydroxylase. The most significant difference is related to the circadian rhythm of the enzyme. At night, pineal tryptophan hydroxylase almost doubles, whereas there is relatively little daily change in the activity of this enzyme in the various regions of the brain, including the hypothalamus (Shibuya *et al.*, 1978).

It has been suggested that pineal tryptophan hydroxylase is regulated by its diurnal rhythm which appears to be endogenously generated and persists in darkness. The enzyme's rhythm is suppressed in constant light. Propranolol blocks the nighttime increase in activity of this enzyme, suggesting that nor-adrenaline may be the neurotransmitter involved in its regulation (Shibuya *et al.*, 1978).

Substrate concentration is an important regulatory mechanism for this enzyme. An increase in tryptophan was shown to result in an increase in the rate of serotonin formation in the pineal gland (Deguchi and Barchas, 1972; Bensinger *et al.*, 1974).

1.9.2 Aromatic-l-Amino Acid Decarboxylase

Aromatic-l-amino acid decarboxylase is a non-specific enzyme found in all body tissues, but is present in higher concentrations in the pineal gland (Lovenberg *et al.*, 1962; Christenson *et al.*, 1972) where it is located in the cytoplasm (Snyder and Axelrod, 1964). Pyridoxal phosphate is required by the enzyme as a co-factor in its decarboxylation of 5-hydroxytryptophan to serotonin (Lovenberg *et al.*, 1962).

The activity of the enzyme has been shown by Snyder *et al.* (1965b) to be influenced by environmental lighting acting through the sympathetic nerves. Animals exposed to constant light showed an increase in enzyme activity whereas those kept in constant darkness did not. This suggests that adrenergic stimulation results in a depression of enzyme activity (Snyder *et al.*, 1964).

1.9.3 Monoamine Oxidase

Indoleamines in the pineal gland are oxidatively deaminated, as in other tissues, by a process initiated by the action of monoamine oxidase. This enzyme converts monoamines to their corresponding aldehydes. The aldehydes are immediately converted to either acid or alcohol by aldehyde dehydrogenase or alcohol dehydrogenase respectively (Feldstein and Williamson, 1968; Keglevic *et al.*, 1968).

Monoamine oxidase is situated within the mitochondria of pinealocytes and in the nerve endings of nerves innervating the pineal gland (Goridis and Neff, 1971; Yang *et al.*, 1972). In the nerve endings its function is to inactivate neurotransmitter amines such as noradrenaline and adrenaline.

The enzyme differs between the pinealocyte and nerve endings. It has thus been classified into two types namely, type A and type B (Johnson, 1968). Type A is primarily found in the sympathetic nerve terminals and is inhibited by the inhibitor clorgyline. The enzyme is relatively heat stable and is easily inactivated by trypsin. Type A accounts for about 15% of the pineal monoamine oxidase and acts on tryptamine, tyramine and serotonin (Yang *et al.*, 1972).

Type B is found predominantly in the pinealocyte and is heat labile but is not as readily inactivated by trypsin or inhibited by clorgyline (Yang *et al.*, 1972).

1.9.4 Alcohol Dehydrogenase

Alcohol dehydrogenase converts the 5-hydroxyindoleacetaldehyde formed by the action of monoamine oxidase to 5-hydroxytryptophol. The enzyme requires a reduced pyridine co-factor for the above reaction (Feldstein and Williamson, 1968; Keglevic *et al.*, 1968; Tabakoff, 1977).

1.9.5 Aldehyde Dehydrogenase

Aldehyde dehydrogenase converts aldehydes into acids, such as 5-hydroxyindoleacetaldehyde into 5-hydroxyindoleacetic acid. In catalysing this reaction, the enzyme requires an oxidised pyridine co-factor (Feldstein and Williamson, 1968; Keglevic *et al.*, 1968; Tabakoff, 1977).

1.9.6 Hydroxyindole-O-Methyltransferase

Hydroxyindole-O-methyltransferase (HIOMT) belongs to a group of enzymes that transfer methyl groups from S-adenosylmethionine to acceptor molecules. In the pineal gland the acceptor molecules are 5-hydroxyindoles (Axelrod and Weissbach, 1961).

HIOMT is found in high concentrations in the pineal gland and in lower concentrations in the retina, Harderian gland and intestine of mammals (McIsaac *et al.*, 1965; Jackson and Lovenberg, 1971).

In the pineal gland HIOMT catalyses the conversion of N-acetylserotonin to melatonin and to a lesser extent converts 5-hydroxyindoleacetic acid to methoxyindoleacetic acid; hydroxytryptamine to methoxytryptamine; and hydroxytryptophol to methoxytryptophol (McIsaac *et al.*, 1965; Jackson and Lovenberg, 1971).

HIOMT is located entirely in the cytosol of the pinealocyte. The enzyme is abundant within the cell and accounts for approximately 2% - 4% of its soluble protein. The enzyme has a molecular weight of approximately 78 000 and appears to have two sub-units, each with a molecular weight of 39 000 (Karahasanoglu and Ozand, 1972; Kuwano and Takahashi, 1978).

HIOMT follows a daily rhythm (Klein, 1972) and is regulated *in vivo* by two mechanisms, namely light acting to modulate neural stimulation and by circulating steroids altering the activity of the enzyme (Preslock, 1977; Klein and Moore, 1979).

A change in environmental lighting slowly changes pineal HIOMT activity (Axelrod and Wurtman, 1978). Exposure of animals to constant darkness increases the activity of the enzyme through an increase in HIOMT molecules (Yang and Neff, 1975). Conversely, exposure to constant light reduces enzyme activity (Wurtman *et al.*, 1963; Axelrod *et al.*, 1965). This has however been disputed by many authors. The neural pathway regulating this enzyme involves the retinohypothalamic projection which innervates the suprachiasmatic nucleus, the retrochiasmatic hypothalamus and peripherally, the superior cervical ganglia (Klein and Moore, 1979).

Circulating gonadal steroids have been shown to participate in HIOMT regulation (Preslock, 1977). Testosterone increases HIOMT activity at low concentrations and decreases the enzyme activity at high concentrations. Estradiol at nanomolar concentrations increases HIOMT activity by between 50 % and 80 % (Mizobe and Kurokawa, 1976).

1.9.7 Serotonin N-Acetyltransferase

Serotonin N-acetyltransferase (NAT) is the enzyme responsible for the conversion of serotonin to N-acetylserotonin (Weissbach *et al.*, 1960). NAT has a diurnal rhythm, with its activity being 30 - 70 fold higher at night (in the dark) than during the day (Deguchi and Axelrod, 1972a; Ellison *et al.*, 1972; Romero *et al.*, 1978). These large changes in enzyme activity are believed to be controlled via a photic-neural pathway (Klein, 1979).

Voisin *et al.* (1984) established the presence of two distinct NAT enzymes in the rat pineal gland. The enzymes were termed arylamine N-acetyltransferase and arylalkylamine N-acetyltransferase as a result of the arylamines and arylalkylamines they respectively acetylate. Isoproterenol treatment of arylalkylamine N-acetyltransferase increased its activity 100-fold without increasing arylamine N-acetyltransferase activity. The two enzymes were found to become inactivated at different rates at 4°C, while on size exclusion chromatography, the enzymes were resolved in two different bands. Voisin *et al.* (1984) observed that arylalkylamine N-acetyltransferase was independently regulated and was primarily responsible for the physiological conversion of serotonin to melatonin via the intermediate N-acetylserotonin.

1.10 Serotonin N-Acetyltransferase : Regulatory Mechanisms

1.10.1 Circadian Control of NAT

The rat pineal NAT rhythm is truly circadian as it persists even in a non-periodic environment *i.e.* constant darkness (Klein and Weller, 1970). The rhythm is abolished by denervation of the gland through bilateral superior cervical ganglionectomy (Klein *et al.*, 1971). The rhythm is thus controlled by a circadian pacemaker located outside the gland, most probably in the suprachiasmatic nuclei (SCN) of the hypothalamus (Klein and Moore, 1979). Signals sent from the SCN stimulate a phase of NAT activity at night (Binkley *et al.*, 1974; Illnerovà, 1974).

In constant darkness the endogenous pacemaker controlling the NAT rhythm may free-run with its own period close to 24 hours. The external light-dark cycle generated by the earth's

rotation may serve as an entraining agent and may force circadian systems to run with a period of 24 hours. Light in each light-dark cycle must change the period of the pacemaker by an amount proportional to either a phase-advance in short daylight days (winter) or a phase-delay in long daylight days (summer). Thus, the entrainment of the circadian system to the 24 hour day is achieved by a phase-shifting of the pacemaker by light (Illnerovà *et al.*, 1989; Illnerovà and Humlovà, 1990).

Neural regulation of the NAT rhythm occurs via signals, originating in the SCN of the hypothalamus, being transmitted to the pineal by a neural circuit which includes superior cervical ganglia of the sympathetic nervous system (Kappers, 1960). Sympathetic nerve endings in the pineal release the neurotransmitter nor-adrenaline, which, through β-adrenergic and partly α-adrenergic receptors and the cAMP system, induces and activates N-acetyltransferase (Klein *et al.*, 1983). Nor-adrenaline release is found to be high at night and low during the day (Brownstein and Axelrod, 1974) **(Figure 1.5)**.

1.10.1.1 The Suppressive Effect of Light on the NAT Rhythm

The rat pineal NAT rhythm is abolished by the exposure of animals to constant light (Klein and Weller, 1970). The pineal melatonin pathway is affected by an abrupt light exposure at night. Serotonin concentrations in the pineal were shown to increase from a nighttime low to a daytime high, within a 14 minute period, after exposure of animals to a one minute light burst (Illnerovà, 1971). Similarly, melatonin concentrations decrease with a half-time of approximately five minutes after light exposure (Illnerovà *et al.*, 1978). NAT was also shown to decline rapidly after sudden light exposure at night (Deguchi and Axelrod, 1972a; Klein and Weller, 1972).

The sensitivity of NAT to light is characterised by the colour and intensity of the light. White, blue and green light have been shown to markedly inhibit NAT activity, while red light appears to exert no effect on the enzyme's activity (Cardinali *et al.*, 1972). Light intensities ranging between full moon (0.03 - 0.05 $\mu W/cm^2$) and full sunlight (50 000 $\mu W/cm^2$) have been shown to reduce NAT activity (Biberman *et al.*, 1966). However, maximal inhibition of NAT was found with light at an intensity less than 15 $\mu W/cm^2$ (Minneman *et al.*, 1976).

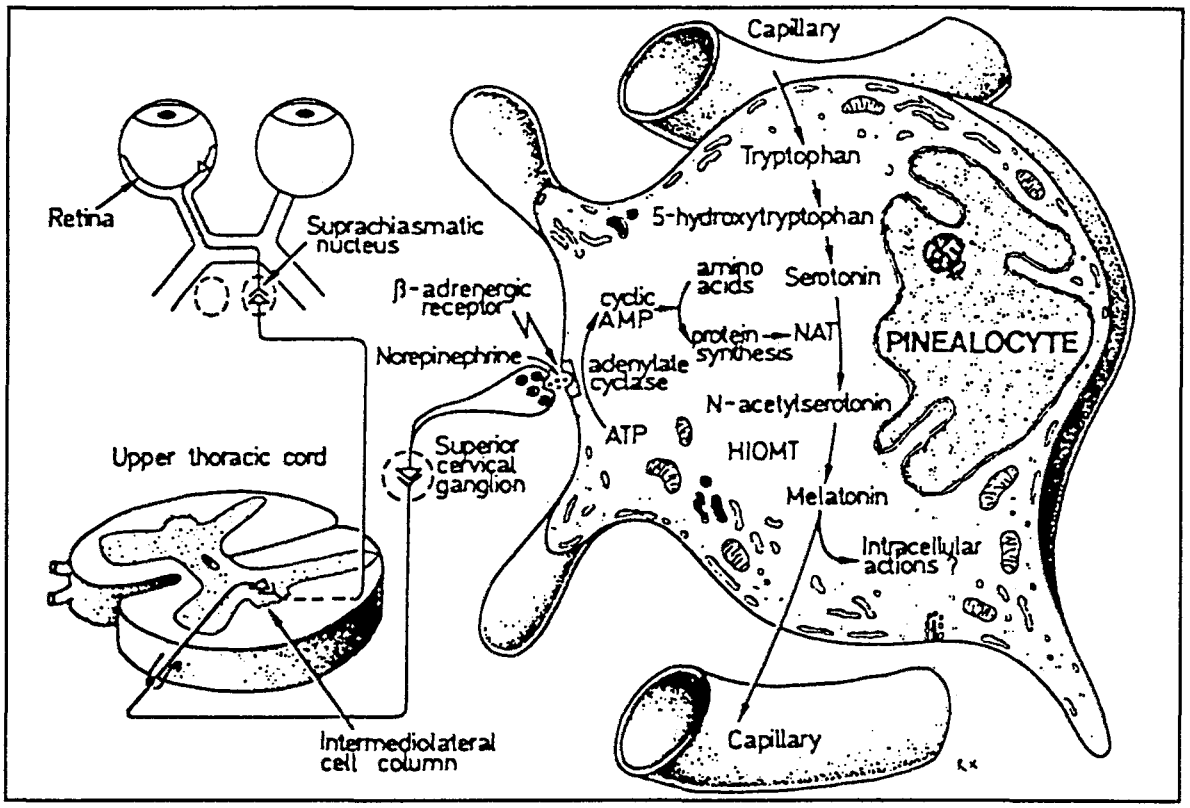

FIGURE 1.5 *Diagrammatical representation of neural connections between the eyes and a pinealocyte* (Reiter, 1988b).

The mechanism for NAT's loss of activity is not fully understood. However, a study by Illnerovà and Vanecek (1979) on rats exposed to light at night showed a rapid decline in NAT activity over the first 12 minutes to 10% of its former value half-time of 3.8 minutes and then slowly with a half-time of 70 minutes.

The same biphasic decline was found in rats injected with the β-adrenergic blocker, trimepranol, at night in darkness (Illnerovà and Vanecek, 1979). It was thus concluded that light exposure at night caused a reduced or complete "shut-off" of nor-adrenaline release from nerve endings in the pineal gland. Deguchi and Axelrod (1972b) reported a similar result to Illnerovà and Vanecek (1979), for the decrease in NAT activity at night after the blockage of protein synthesis. It was thus postulated that light interrupts the synthesis of the enzyme.

In contrast to rat pineal NAT, chick pineal NAT rhythm is not affected by bilateral denervation of the superior cervical ganglion or by injections of catecholamines (Binkley *et al.*, 1975; Ralph *et al.*, 1975).

Organ culture experiments involving chick pineal glands demonstrated that chick pineal NAT activity exhibited a circadian change comparable with that of the rhythm *in vivo* (Binkley and Mosher, 1989). When cultured in darkness, pineal NAT activity autonomously increased at midnight and decreased during the day. The pattern was found to be similar in the chick pineal regardless of the time of sacrifice, which indicated a possible timekeeping system. Further studies showed that the rhythmical rise and fall of NAT activity, *in vivo*, only occurred for one cycle in constant darkness. This indicated that either chick pineals could keep the schedule for only one cycle in the absence of a light-dark cycle, or that the timekeeping system in the chick pineals could desynchronize between pineal glands or even between pinealocytes within a given gland (Deguchi, 1979).

Recent work involving neonatal animals (Bronstein *et al.*, 1990) showed that these animals reacted differently to a one minute light exposure at night. Animals six days old or older showed a suppression of pineal NAT activity after a one minute light exposure at night. This suppression was, however, not found in animals between three and five days of age even though they displayed clear light-dark differences in pineal NAT activity. Nighttime levels of NAT activity in seven day old animals, which had been bilaterally enucleated, were not

suppressed when the animals were exposed to a nocturnal light stimulus. This suggests that the effect is retinally mediated.

1.10.2 The Effect of Inorganic Constituents on NAT Activity

The stimulation of both transcription and translation is required for the nocturnal induction of pineal NAT activity (Romero *et al.*, 1978). Inhibitors of both transcription and translation have been shown to block the nocturnal rise in enzyme activity (Romero *et al.*, 1978). The most important inorganic constituents in the regulation of NAT activity are potassium, lithium and calcium.

Work by Namboodiri *et al.* (1979) has shown that inorganic compounds such as sodium chloride, sodium phosphate, sodium fluoride, potassium fluoride, ammonium acetate and magnesium chloride prevent the loss of NAT activity over a 30 minute period. The decrease in inactivation caused by these salts appears to be due to an increase in ionic strength rather than an increase in molarity. The salts appear to have a non-specific effect on NAT and it is suspected that they affect the conformation of the enzyme (Namboodiri *et al.*, 1979).

1.10.2.1 Potassium

High concentrations of potassium have been shown to inhibit the induction of NAT activity by nor-adrenaline (Parfitt *et al.*, 1975). The effect of potassium appears to be secondary to the post-synaptic depolarization caused by elevated potassium or the prevention of the hyperpolarisation caused by nor-adrenaline.

1.10.2.2 Lithium

Lithium, at therapeutic concentrations, has been shown to inhibit NAT activity by inhibiting intracellular increases in cAMP (Zatz, 1979). It is thought that lithium inhibits cyclic nucleotide synthesis by interfering with the role of divalent cations (Zatz, 1979). Thus, lower cellular levels of cAMP prevent NAT induction.

1.10.2.3 Calcium

Of all the inorganic constituents of the pineal, calcium is by far the most important. This is evident in the role it plays in the decalcification of the pineal gland with age (Tapp and Huxley, 1972) and in the "calcium signal" as a component of nor-adrenergic regulation of the synthetic and secretory activity of the pineal gland (Sugden *et al.*, 1987).

Early work on the relationship between calcium ions and the pineal gland showed that extracellular calcium was necessary for complete induction of NAT activity in rats (Wilkinson, 1976; Zatz and Romero, 1978). Chelation of extracellular calcium led to a reduction in NAT activity by an indirect effect on protein synthesis (Zatz and Romero, 1978), and stimulation of β-adrenergic receptors required extracellular calcium for full expression of the activity of the acetylating enzyme (Wilkinson, 1976).

Wilkinson (1976) further showed that long term exposure to elevated calcium concentrations led to a reduction in NAT activity, thus indicating a possible inhibitory role for calcium in addition to its stimulatory activity. The inhibitory effect of elevated intracellular calcium on rat pineal NAT has been suggested to be the result of activation of a NAT inhibitory substance in the pineal (Chan and Ebadi, 1981; Khoory and Schloot, 1986; Lerchl *et al.*, 1991).

Research has shown that α-adrenergic receptors are involved in the regulation of the intracellular calcium ion concentration (Sugden *et al.*, 1986 and 1987). In these studies the authors treated rat pinealocytes with a variety of adrenergic agonists and antagonists. The results strongly suggested the involvement of an α- rather than β-receptor.

Calcium channel activity in the pinealocyte has also been shown to affect NAT activity (Zatz and Mullen, 1988; Zatz, 1989; Zawilska and Nowak, 1990). Blockade of calcium channels during the night resulted in the inhibition of normal nocturnal increase in NAT activity. This effect is prevented by the administration of calcium channel agonists (Zatz and Mullen, 1988; Zatz, 1989; Zawilska and Nowak, 1990). The enhancement of nocturnal NAT activity by calcium channel agonists suggests that the α-adrenergic augmentation of β-adrenergically-stimulated NAT induction is the result of enhanced calcium channel-mediated calcium influx.

Recent work on rat and chick pineal gland homogenates has revealed that EGTA, a non-specific ion chelator, prevents the loss of NAT activity during pre-incubation before substrate addition (Rodriguez-Cabello *et al.*, 1990a and 1990b).

Calmodulin's role in calcium's action: Calcium has increasingly been shown to play an important role in pineal function (Sugden *et al.*, 1986 and 1987). Its role within the cell has been shown to be linked to the action of nor-adrenaline (Vanecek *et al.*, 1985 and 1986). Research has shown that α_1-adrenergic receptor stimulation together with β-receptor stimulation potentiates cAMP production (Reiter, 1991). This potentiation is thought to act through a calcium/phospholipid-dependent protein kinase and cGMP (Sugden *et al.*, 1986).

Meyer *et al.* (1964) were the first to consider the possibility that calcium might require a binding protein to exert its effect within the cell. Calmodulin was found to be that binding protein. It consists of a single polypeptide chain containing 148 amino acids. The protein weighs 16 500 daltons and is heat stable. "Calmodulin has been implicated in conferring calcium sensitivity (*i.e.* Ca^{2+}-dependent activation) to approximately 30 different target proteins (for reviews see Cheung, 1980; Means and Dedman, 1980; Stoclet, 1981; Manalan and Klee, 1984) and thus plays the role of the multi-functional intracellular calcium receptor" (Brown, 1992).

Calmodulin contains four calcium binding sites (Dedman *et al.*, 1978) with a dissociation constant of approximately 10^{-6}M (Klee, 1977; Wolff *et al.*, 1977). Large conformational changes occur to calmodulin when it binds four calcium ions. These result in the exposure of a hydrophobic binding site, which in turn binds to target proteins and enzymes (LaPorte *et al.*, 1980; Johnson and Wittenauer, 1983).

Calmodulin's mode of action was first shown in the phosphodiesterase system (Haiech *et al.*, 1981). Calmodulin is activated by the binding of four calcium ions to the calcium binding sites. The active form (more helical conformation) then binds to an inactive apo-enzyme of PDE, resulting in the formation of an active holo-enzyme (Haiech *et al.*, 1981).

The role played by calmodulin indirectly affecting NAT activity became apparent when it was found that both adenylate cyclase and cAMP-phosphodiesterase were activated by calmodulin.

Adenylate cyclase is activated at a much lower concentration of calcium than is cAMP-phosphodiesterase. The result is that once the optimal calcium concentration for cAMP-phosphodiesterase activation is reached, adenylate cyclase is already being inhibited. This is diagrammatically represented in **Figure 1.6.**

1.10.3 Hormonal Influence on NAT Activity

1.10.3.1 Estradiol

NAT activity was found to be insensitive to estradiol treatment in rats (Illnerovà, 1975; Preslock, 1975; Cardinali and Vacas, 1978). Estradiol administration also disturbed neither the light-dark cycles of NAT nor its induction by isoproterenol (Illnerovà, 1975). Protein synthesis in the pineal gland is affected by estradiol (Cardinali *et al.*, 1975) but this synthesis is unrelated to that of NAT.

1.10.3.2 Progesterone

"Progesterone, like estradiol, does not affect pineal NAT activity and therefore probably does not modulate melatonin production by affecting NAT. Although progesterone has been shown to decrease protein synthesis in the pineals of ovariectomized rats (Cardinali and Vacas, 1978) it does not seem to affect the rise in NAT activity which depends on synthesis of NAT molecules" (Daya, 1982).

1.10.3.3 Castration and Testosterone

Castration affects both the biochemistry and morphology of the pineal gland. The nocturnal rise of pineal NAT activity is depressed by castration (Pavlinov and Isachenkov, 1979; Rudeen and Reiter, 1980). An explanation for this is that castration decreases the pineal concentration of cAMP. Cyclic AMP is essential for the induction of NAT, thus a decrease in cAMP in the pineal results in a decrease in NAT activity (Karasek *et al.*, 1978).

Testosterone restores the nocturnal rise in NAT activity in castrated animals, which indicates that this hormone influences NAT activity (Rudeen and Reiter, 1980). The maintenance of

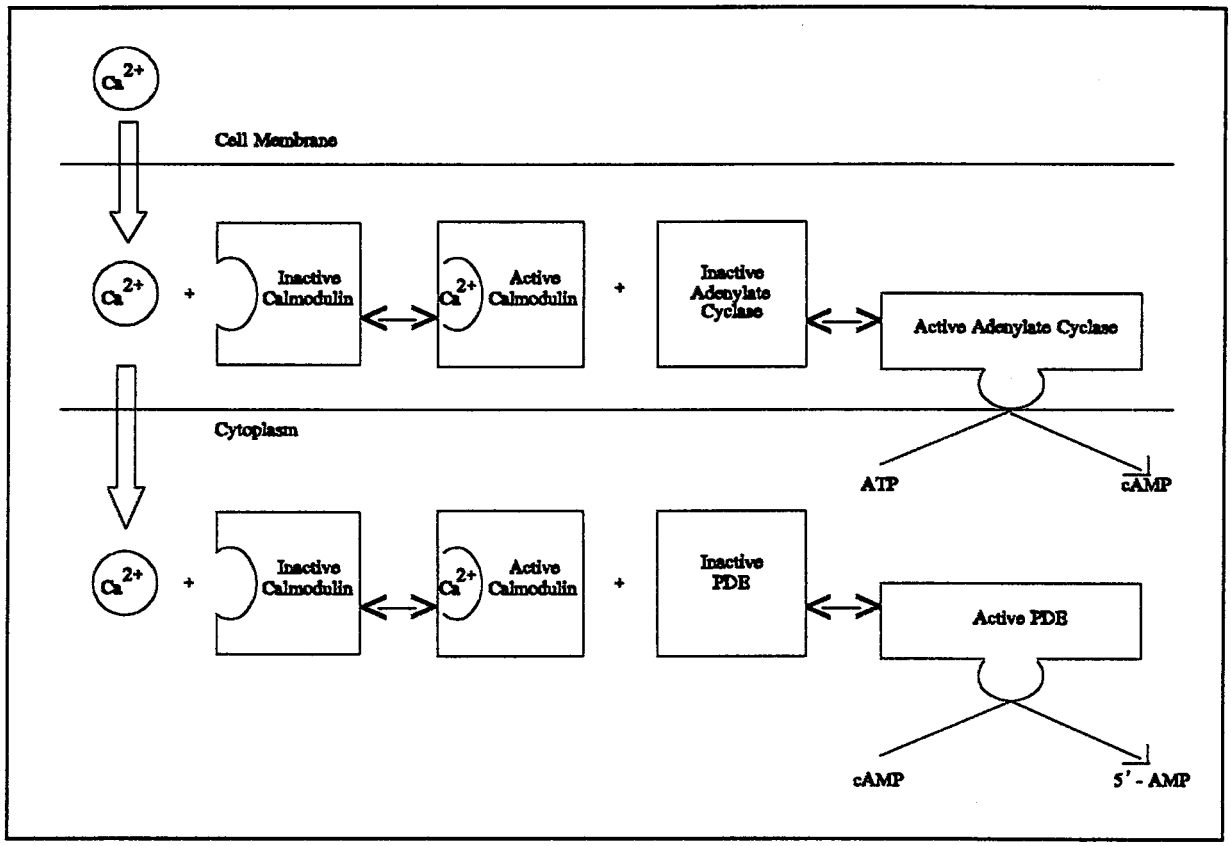

FIGURE 1.6 *The sequential activation of adenylate cyclase and cAMP-phosphodiesterase (PDE) in response to Ca^{2+} influx through the plasma membrane and into the cytoplasm* (Adapted from Cheung, 1982; Brown, 1992).

the diurnal rhythm has also been linked to the action of testosterone on the pineal gland by Daya (1982), who proposed that testosterone affected the release or synthesis of nor-adrenaline by the sympathetic nerves of the pineal gland.

1.10.3.4 Prostaglandins

Recent studies have suggested that prostaglandins may play a role in the regulation of NAT (Ritta *et al.*, 1981). α-Adrenergic receptor stimulation of pineal gland explants resulted in a release of prostaglandin E_2 (Cardinali and Vacas, 1987). Prostaglandin synthesis inhibitors were found to block the nocturnal rise in NAT activity, and *in vitro* prostaglandin E_2 increased pineal NAT activity (Cardinali and Vacas, 1987).

1.10.4 Effect of Pineal Indoles on NAT Activity

The synthesis of melatonin from tryptophan occurs for the greater part in darkness. Only the synthesis of serotonin is high during the daytime and low during darkness (Pellegrino de Iraldi and Rodriguez de Lores Arnaiz, 1964; Snyder *et al.*, 1965b). Thus, serotonin concentrations during the daytime are crucial for the amount of N-acetylserotonin synthesised by NAT during the night (Quay, 1963). The daytime concentration of serotonin has a dual function. Firstly, serotonin serves as the substrate for the synthesis of N-acetylserotonin which will ultimately be converted into melatonin. Secondly, serotonin exerts an inhibitory effect on nor-adrenaline induced activation of adenylate cyclase, thus having an indirect inhibitory effect on NAT activity and its own synthesis (Balemans, 1979).

Bade and co-workers (1977) demonstrated that the inhibition of daytime monoamine oxidase increased nocturnal serotonin levels. These high levels of serotonin were found, in turn, to increase nighttime NAT activity, suggesting an enzyme-substrate related regulatory mechanism for NAT. The opposite was shown by King *et al.* (1982), who demonstrated that low levels of serotonin enhanced nocturnal and agonist-stimulated NAT activity.

Tryptophan administrations increase serotonin levels in rat pineal glands (Snyder *et al.*, 1965b). These administrations have also been shown to inhibit normally elevated nocturnal NAT activity (King *et al.*, unpublished results). Enzyme kinetic studies (enzyme-substrate

interactions) have demonstrated that the enhanced levels of serotonin caused by the administration of tryptophan play no part in the tryptophan inhibition of NAT. Further studies showed that the blocking of serotonin receptors with cyproheptadine HCl blocked the tryptophan induced inhibition of NAT activity. This suggests that NAT inhibition by high concentrations of serotonin is receptor mediated and not substrate related (King *et al.*, unpublished results).

Melatonin treatment of rats was initially found to partially counteract the light-induced depletion of pineal lipids (Ebels and Prop, 1965; Fiske and Huppert, 1978). Subsequent investigation showed that melatonin increases the activity of the pineal enzymes, NAT and HIOMT, in a dose-dependant manner, revealing the lack of a feedback inhibition mechanism to melatonin (Cardinali and Wurtman, 1972).

The other pineal gland indoles have, as yet, not been shown to exhibit direct effects on NAT activity.

1.10.5 External Factors Affecting NAT Activity

1.10.5.1 Drugs

Stimulation of the sympathetic nerves and/or post-synaptic β-adrenergic receptor innervating the pineal gland is essential for pineal NAT activity. The neurotransmitter nor-adrenaline is involved in this action. The administration of any analogue of nor-adrenaline will result in an increase in NAT activity. The drug most used in pineal research in this regard is isoproterenol. Likewise, a compound such as desmethylimipramine, which blocks the uptake of nor-adrenaline from the synaptic space, effects an increase in NAT activity (Moyer *et al.*, 1979). Pargyline, a compound that prevents the inactivation of nor-adrenaline by inhibiting monoamine oxidases, also causes an increase in NAT activity (Deguchi and Axelrod, 1972b; Mata *et al.*, 1976).

Recent work by Attia *et al.* (1990 and 1991b) has shown that lindane, a chlorinated hydrocarbon pesticide, enhances the nighttime rise in NAT activity. Lindane's site of action was shown to be the pineal β-receptor. This site of action was elucidated with experiments

involving propranolol, a specific β-receptor blocker, in the presence of lindane. Propranolol blocked the stimulatory effect of lindane, pointing to lindane's β-receptor specificity. Further work by Attia *et al.* (1991a) on carbamate insecticides showed that carbaryl also increased NAT levels in the pineal gland and had a similar mode of action to that of lindane.

Drugs known to decrease NAT activity are those such as propranolol, which block β-receptors. Compounds such as cycloheximide, that block protein synthesis, also result in a decrease in NAT activity by preventing the synthesis of the enzyme (Deguchi and Axelrod, 1972b; Klein, 1979). Depolarising drugs, such as ouabain, also inhibit the induction of NAT activity by blocking the transmission of the neural impulse from the sympathetic nerves to the pinealocytes (Parfitt *et al.*, 1975).

1.10.5.2 Stress

Stress causes a minor increase in NAT activity (Lynch *et al.*, 1973; Klein, 1979). In the presence of catecholamine re-uptake blockers, stress raises NAT activity to levels equivalent to those seen at night (Parfitt and Klein, 1976; Vaughan *et al.*, 1978).

1.10.5.3 Photoperiods

The effect of photoperiods on pineal NAT activity are considerable. As previously discussed in **§ 1.10.1**, exposure of animals to light at night results in a rapid decrease in NAT activity (Klein and Weller, 1970; Alphs *et al.*, 1980). This decrease is probably due to a cessation of nor-adrenaline release, the uptake of extracellular nor-adrenaline and the dissociation of nor-adrenaline from the β-adrenergic receptors, which result in a decrease in cAMP production and inactivation of the enzyme (Parfitt *et al.*, 1975).

1.10.6 Effect of Purine Nucleotides on NAT Activity

In the pinealocyte, nucleotide concentrations are primarily controlled by the rate of synthesis (neural stimulation affecting synthesis) and the rate of degradation.

The most abundant neurotransmitter released from the nerves innervating the pineal gland is nor-adrenaline. The release of nor-adrenaline has been shown to exhibit a circadian rhythm, with highest release during the dark (Pellegrino de Iraldi and Zieher, 1966; Wurtman and Axelrod, 1966). Nor-adrenaline acts on α_1-, α_2-, β_1- and β_2-adrenergic receptors, but shows more selectivity for α_1- and β_1-receptors (Ariëns *et al.*, 1979; Fain and Garcia-Sainz, 1980). Both receptor types have been fully characterised and shown to play an important role in cAMP, guanosine 3′,5′-monophosphate (cGMP) and NAT synthesis (Auerbach *et al.*, 1981; Klein *et al.*, 1983; Sugden and Klein, 1984; Vanecek *et al.*, 1985).

1.10.6.1 Cyclic AMP

The binding of β-receptor agonists to β-receptors results in an approximately 10 fold increase in cAMP concentration. The increase in cAMP occurs as a result of the activation of adenylate cyclase, a membrane bound enzyme, which converts ATP to cAMP in a process involving the hydrolysis of pyrophosphate. The activity of adenylate cyclase has been shown to be influenced by age, sex and environmental lighting (Strada *et al.*, 1972).

Nor-adrenaline has been shown to act via both α_1- and β-receptors. Where both receptors are stimulated, the cAMP concentration is increased between 30 and 100 fold. α_1-Receptor stimulation alone was found to elicit no change in cellular cAMP concentrations. Thus, it may be concluded that α_1-receptor stimulation potentiates β-receptor stimulation of pineal cAMP (Klein, 1985; Reiter, 1991). A peak in cAMP concentration is reached about five to 15 minutes after β-receptor stimulation. This peak is sustained for a period of approximately 30 minutes (Deguchi, 1973). The eventual decrease in cAMP concentration is not related to the continued presence or absence of β-receptor agonist, but rather to a decrease in adenylate cyclase activity (Klein *et al.*, 1978; Vanecek *et al.*, 1985).

The pineal gland is a rich source of cAMP-dependent protein kinase. The elevated cAMP levels result in the activation of protein kinase. The protein kinase, in turn, causes the phosphorylation of other cellular proteins responsible for protein synthesis, resulting in the synthesis of NAT (Morrissey and Lovenberg, 1978; Benfenati *et al.*, 1986; Reiter, 1991).

Cyclic AMP is inactivated by the enzyme pineal cAMP-phosphodiesterase. The enzyme's activity is influenced by intracellular calcium concentrations (Brown *et al.*, 1989a and 1989b). The net result of increased intracellular concentrations of cAMP is an increase in NAT activity.

1.10.6.2 Cyclic GMP

Cyclic GMP and cAMP are controlled by a similar mechanism (Vanecek *et al.*, 1985). The difference in their regulation is found at the site of control. Regulation of cAMP is post-synaptic, while cGMP is regulated pre-synaptically (O'Dea and Zatz, 1976; Zatz and O'Dea, 1977).

The role of cGMP in the regulation of pineal function remains to be determined. It has been proposed that intracellular cGMP may complement or counteract the effects of cAMP (Strada *et al.*, 1976). Thus, cAMP may be responsible for the activation of NAT and, by another mechanism, cGMP may counteract that activation.

A study by Wilkinson (1976) suggests that NAT induction can be inhibited by the addition of dibutyryl cGMP. However, these findings have not been independently verified.

Stimulation of pre-synaptic α-receptors has been shown to inhibit nor-adrenaline release in the pineal (Pelayo *et al.*, 1977). The administration of dibutyryl cGMP also inhibits nor-adrenaline release (Pelayo *et al.*, 1978). Thus, cGMP may be involved in the regulation of pineal pre-synaptic function (O'Dea and Zatz, 1976).

1.10.7 Effect of Lipids and Amino Acids on NAT Activity

1.10.7.1 Lipids

Lipids, of which more than half are phospholipids, form between 3% and 10% of the wet weight of the pineal gland. Pinealocyte phospholipids have thus far been shown to exhibit an indirect effect on NAT activity.

Nordio *et al.* (1989) showed that phosphatidylserine stimulated NAT activity. The mechanism for this stimulation is thought to take place via stimulation of the α_1-adrenergic receptor (Nordio *et al.*, 1989).

Nor-adrenaline stimulation of β-receptors on the pinealocyte cell surface results in an increased intracellular cAMP concentration which in turn induces NAT activity. α_1-Receptor stimulation by nor-adrenaline has been shown to potentiate the above effect (Klein, 1985; Reiter, 1991), which is thought to take place via the activation of a phosphodiesterase to generate diacylglycerol and inosital phosphate, with the former activating protein kinase C (Berridge, 1986). Essential co-factors for protein kinase C activation are calcium and phosphatidylserine. Protein kinase C has been implicated in many processes including phosphorylation of proteins essential for pinealocyte protein synthesis, thereby influencing NAT synthesis (Bazzi and Nelsestuen, 1987).

Phospholipids have also been shown to exert an indirect effect on NAT through calcium. Diacylglycerol, formed by α_1-receptor stimulation, is phosphorylated by a diacylglycerol kinase to form a phosphatidic acid which has been implicated as an ionophore to gate calcium across the plasma membrane (Putney *et al.*, 1980).

1.10.7.2 Amino Acids

The most important amino acid affecting NAT activity is taurine. Taurine is synthesised from cysteine and is located entirely in the pinealocyte (Wheler *et al.*, 1979). A daily rhythm in the concentration at the start of the dark period and peak levels during the middle of the light period have been shown (Grosso *et al.*, 1978).

Wheler and Klein (1979 and 1980) found that treatment of pineals with nor-adrenaline or β-receptor agonists effected a large increase in the release of taurine, mediated by β-receptor and cAMP.

High concentrations of taurine increase NAT activity. The stimulatory effect is blocked by propranolol, indicating that taurine is acting through β-adrenergic receptors (Wheler *et al.*, 1979). The functional importance of taurine is not currently clear. However, it is believed

that molecules of taurine released from the pinealocytes could interact with β-receptors on the pre-synaptic, nor-adrenaline-containing, nerve terminals and thereby modify the release of nor-adrenaline (Klein *et al.*, 1981).

CHAPTER II

EXPERIMENTAL PROCEDURES

2.1 Animals

Adult male Wistar rats weighing between 200 and 250 g were used throughout the study. The animals were housed under artificial illumination with a daily photoperiod of 12 hours (lights on at 06h00). The light intensity was approximately 300 W/cm^2 provided by cool white fluorescent tubes. The animal-house temperature was maintained at a constant 20°C to 24°C and an extractor fan ensured the constant removal of stale air. The rats were housed three to four per cage with food and water *ad libitum*.

When required, rats were sacrificed by neck fracture and decapitated. The brain was exposed by making an incision through the bone on either side of the parietal suture, from the foramen magnum to near the orbit and by removing the calvarium. The pineal glands were swiftly removed with sterile forceps and used in subsequent assays.

For dark phase experiments, pineal glands were collected in a darkroom with the aid of a dim photo-safe red light (Phillips, 20W). Previous studies have shown that the activity of pineal enzymes sensitive to white light is not affected by red light (Cardinali *et al.*, 1972).

2.2 Indole Metabolism by Pineal Organ Cultures

2.2.1 Introduction

The origins of organ culture may be traced back, over a hundred years, to Claude Bernard (1856) who stated that experimental techniques should be devised to allow previously invisible physiological occurrences to take place outside the body for man to witness (Brendel *et al.*, 1987).

The Strangeways Laboratory, some 60 years ago, was the first to practically implement the thoughts and ideas of Bernard through the culturing of embryonic rudiments (Strangeways and Fell, 1926; Fell and Robinson, 1929). Trowell (1959) successfully employed the organ culture technique to sustain fully differentiated organs or parts thereof, and introduced the concept of stabilizing the culture medium's alkalinity by including 5% carbon dioxide (CO_2) with the oxygen (O_2). Using this technique, pineal glands were kept viable for a minimum period of six days (Klein, 1969). This technique has been modified by other investigators to suit their individual requirements.

Pineal glands in culture are able to utilise radio-labelled precursors such as tryptophan, 5-hydroxytryptophan or serotonin, to synthesise various indole metabolites.

Klein and Rowe (1970) and Skene (1985) showed that 95% of radioactive indoles synthesised from exogenous radio-labelled precursors are secreted out of the pineal gland into the culture medium. The radio-labelled indoles found in the culture medium may then be separated by employing bi-dimensional thin layer chromatography (TLC) and quantified by liquid scintillometry (Klein and Notides, 1969; Balemans *et al.*, 1978).

2.2.2 Materials and Method

Animals: Male Wistar rats weighing between 200 and 250 g were used in these experiments and were maintained as described in **§ 2.1**. The animals were sacrificed between 11h00 and 11h30 during the daytime and between 23h00 and 23h30 during the nighttime. At night, the procedure was performed in a darkroom with the aid of a dim photo-safe red light.

Chemicals and Reagents: 5-Hydroxy [side chain-2-^{14}C]-tryptamine creatine sulphate (specific activity 55 mCi/mmol) and L-[methyl-^{14}C]-tryptophan (specific activity 54 mCi/mmol) were purchased from Amersham (England), synthetic pineal indoles from Sigma Chemical Co. (USA), aluminium TLC plates coated with silica gel 60 F_{254} (0.20 mm) from Merck (West Germany), BGJb culture medium (Fitton-Jackson modification) from Gibco (Europe) and Kimble glass organ culture tubes (10 x 75 mm) (USA). All other chemicals and reagents were purchased from local commercial sources.

Pineal Gland Cultures: Wistar rats were sacrificed and the pineal glands rapidly removed according to the method described in **§ 2.1**. The glands were cleaned of adhering connective tissue and individually placed into sterile organ culture tubes containing 52 μl of BGJb medium. The BGJb medium was further supplemented with 0.06 mg/ml benzylpenicillin, 0.1 mg/ml streptomycin sulphate and 2.5 μg/ml amphotericin B. The composition of the BGJb medium is shown in **Table 2.1**. To the 52 μl of medium, 8 μl of substrate (either [^{14}C]-serotonin or [^{14}C]-tryptophan) was added. In the case of pharmacological manipulations, drugs were added in 5 and/or 10 μl volumes to yield a final experimental volume of 70 μl. The tubes were then aerated with carbogen (95% O_2 : 5% CO_2) and sealed with Parafilm®. The culture tubes containing the pineal glands were then placed in a dark incubator at 37°C for 24 hours.

TLC Analysis: The TLC technique used is based on the method developed by Klein and Notides (1969). The culture tubes were removed from the incubator after the 24 hour incubation period and the reaction terminated by the removal of the glands from the medium. Ten microlitres of medium was removed from each tube and spotted onto a TLC plate (silica gel 60 F_{254}, 0.20 mm x 10 cm x 10 cm aluminium plates). Following this, a solution containing synthetic unlabelled standards of all the pineal indoles was spotted on top of the culture medium spot. A total of 10 μl of a solution containing 0.2 mg/ml of each standard was spotted. The indole standard solution was prepared as follows: 1 mg of each of the eight standards (listed in **Figure 2.1**) were dissolved together in 2.5 ml of 95% ethanol. To this, 2.5 ml of a solution of 1% ascorbic acid in 0.1 N HCl was added. Klein and Notides (1969) included ascorbic acid in their standard to protect the indoles from oxidation. The pineal indole standards were stored, in the dark, at -20°C. The TLC plates were spotted under subdued light.

TABLE 2.1 *The composition of BGJb culture medium* (Fitton-Jackson modification).

CONTENTS	CONCENTRATION (mg/l)
Amino Acids	
L-Alanine	250.00
L-Arginine	175.00
L-Aspartic Acid	150.00
L-Cysteine HCl	90.00
L-Glutamine	200.00
Glycine	800.00
L-Histidine	150.00
L-Isoleucine	30.00
L-Leucine	50.00
L-Lysine HCl	240.00
L-Methionine	50.00
L-Phenylalanine	50.00
L-Proline	400.00
L-Serine	200.00
L-Threonine	75.00
L-Tryptophan	40.00
L-Tyrosine	40.00
DL-Valine	65.00
Vitamins	
α-Tocopherol Phosphate	1.00
Ascorbic Acid	50.00
Biotin	0.20
Calcium Pantothenate	0.20
Choline Chloride	50.00
Folic Acid	0.20
Inosital	0.20
Nicotinamide	20.00
Para Aminobenzoic Acid	2.00
Pyridoxal Phosphate	0.20
Riboflavin	0.20
Thiamine HCl	4.00
Vitamin B_{12}	0.04
Inorganic Salts	
Dihydrogen Sodium Ortho Phosphate	90.00
Magnesium Sulphate $7H_2O$	200.00
Potassium Chloride	400.00
Potassium Dihydrogen Phosphate	160.00
Sodium Bicarbonate	3 500.00
Sodium Chloride	5 300.00
Other Components	
Calcium Lactate	555.00
Glucose	10 000.00
Phenol Red	20.00
Sodium Acetate	50.00

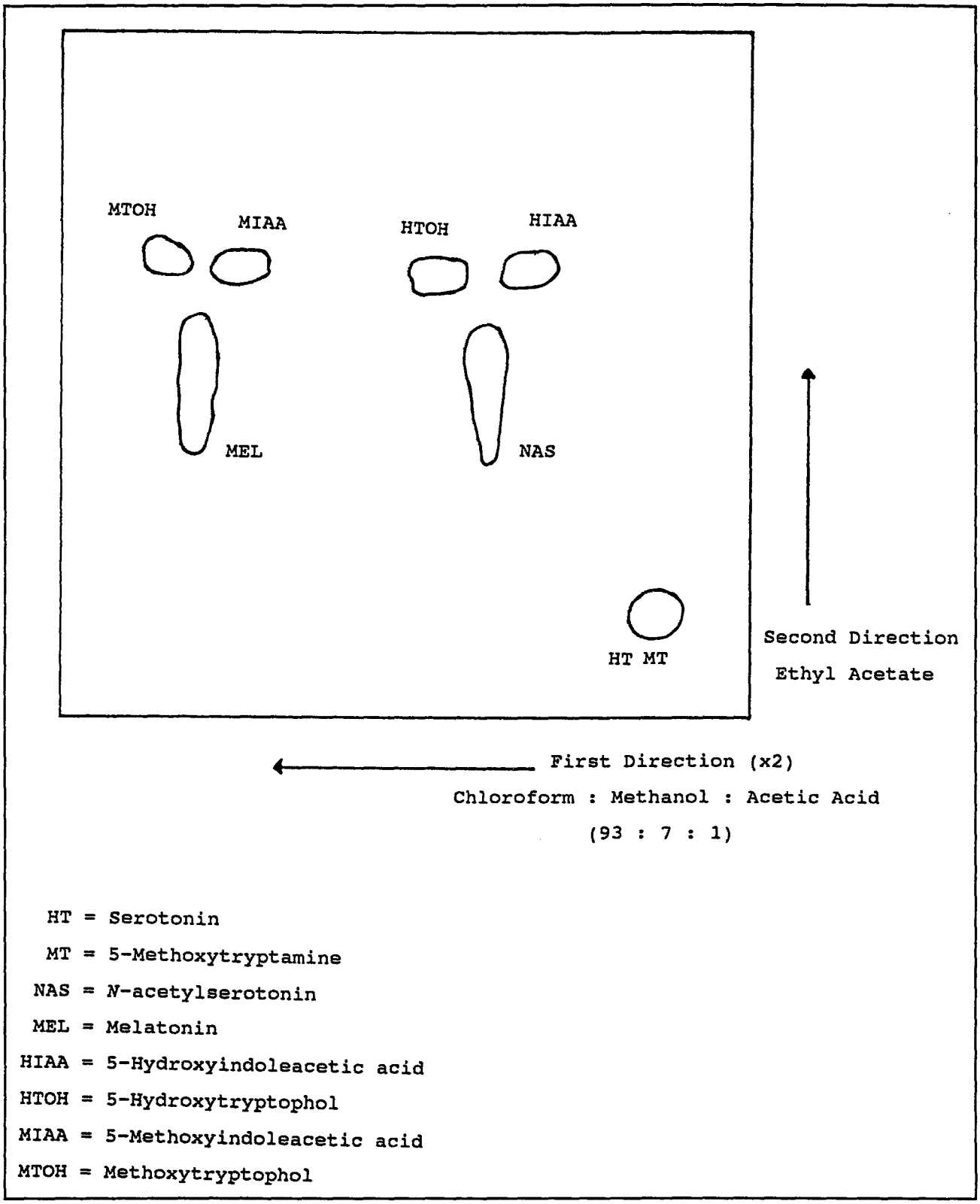

FIGURE 2.1 *Diagrammatic representation of a 2-dimensional TLC plate showing the separation of pineal indoles.*

A gentle stream of nitrogen was used to dry the spots, which were no larger than 0.5 cm, to protect the indole metabolites from atmospheric oxidation. Klein and Notides (1969) proposed the spotting and running of the plates in the dark to protect the indoles. Morton and Potgieter (1982) found no differences between plates spotted and run in normal light and dark. Each spotted plate was then placed in a TLC tank and developed twice, in the same direction, with solvent A composed of chloroform : methanol : acetic acid (93 : 7 : 1). The solvent front was allowed to advance 8 cm up the plate, whereupon the plate was removed from the tank and dried under a stream of nitrogen. Once dry, the plate was placed back into the same tank and allowed to develop in the same direction for a second time. Following this, the plates were dried under a stream of nitrogen, rotated 90° and placed into a second TLC tank containing solvent B (ethyl acetate). This time the solvent was allowed to advance to a height of 6 cm. The plates were removed from the tank, dried again with nitrogen, and sprayed with Van Urk's reagent (1 g of 4-dimethylaminobenzaldehyde dissolved in 50 ml of 25% HCl, followed by the addition of 50 ml of 95% ethanol). After spraying, the plates were dried in an oven at 60°C for 20 minutes to visualise the spots. The spots were then cut out and placed individually into plastic scintillation vials containing 1 ml of absolute ethanol and allowed to stand. After five minutes, 3 ml of Ready-Solv HP scintillation cocktail was added to each vial, whereafter the vials were shaken for 15 minutes and the radioactivity quantified by liquid scintillometry.

2.2.3 Results

A bi-directional thin layer chromatogram of the indoles is presented in **Figure 2.1**. Two pineal indoles, namely serotonin and 5-methoxytryptamine, were found to remain at the origin.

2.2.4 Discussion

Banoo (1991) showed that unlabelled indole standards exerted no significant interference on the radioactive counts of the labelled indoles. The values of the standards were similar to the blank value and accounted for between 0.02% and 0.05% of the total radioactivity present.

2.3 Pineal Serotonin N-Acetyltransferase Activity Assay

2.3.1 Introduction

Serotonin N-acetyltransferase (NAT) (EC 2.3.1.87) is the enzyme responsible for the conversion of serotonin to N-acetylserotonin, the precursor of melatonin. This conversion of serotonin to N-acetylserotonin has often been referred to as the rate-limiting step in melatonin synthesis (Klein *et al.*, 1981; Reiter, 1991).

The exact mechanism by which NAT is regulated is still unknown. The main reason for this, is the inability of the enzyme to be purified without losing its activity. Research has shown that the enzyme is affected by many factors, such as: light, stress, hormones, proteins, inorganic constituents and drugs, to name but a few.

The cascade effect produced by β-receptor stimulation has been shown to be the primary route for NAT regulation. The cascade effect occurs as follows: nor-adrenaline is released in relatively large quantities at night by the sympathetic nerves innervating the pineal gland. Nor-adrenaline binds to the β-receptors, resulting in the activation of a membrane-bound enzyme, adenylate cyclase, which converts ATP to cAMP (Deguchi and Axelrod, 1975). The increased intracellular cAMP concentration activates phosphorylating enzymes and proteins. These, in turn, promote protein synthesis, resulting in the *de novo* synthesis of NAT.

As a result of its "rate-limiting status", NAT is often used as a measure of pineal activity. The most commonly used assay method for this enzyme was developed in 1972 by Deguchi and Axelrod. This method was modified by Champney *et al.* (1984), and this modification has been used in all subsequent experimental work.

The principle of the assay is based on the acetylation of tryptamine (substrate) with radio-labelled acetyl coenzyme A by endogenous pineal NAT, to form a radio-labelled product, N-acetyltryptamine. The radio-labelled N-acetyltryptamine formed is then extracted into an organic solvent and quantified by liquid scintillometry.

2.3.2 Materials and Method

Animals: Male Wistar rats weighing between 200 and 250 grams were used in these experiments. The animals were maintained as described in **§ 2.1.** The animals were sacrificed between 11h00 and 11h30 during the daytime and between 23h00 and 23h30 during the nighttime. At night this was performed in a darkroom with the aid of a dim photo-safe red light.

Chemicals and Reagents: [^{14}C]-Acetyl coenzyme A (specific activity 4.0 mCi/mmol) was purchased from Dupont Chemical Co. (USA). Tryptamine HCl and l-isoproterenol were purchased from Sigma Chemical Co. (USA). Unlabelled acetyl coenzyme A was purchased from Merck (West Germany). All other chemicals and reagents were purchased from local sources.

Buffer Solutions:

a) The phosphate buffer was prepared as follows:

Solution A: 1.19 g of $Na_2HPO_4 \cdot 2H_2O$ was dissolved in 100 ml of distilled water.

Solution B: 908 mg of KH_2PO_4 was dissolved in 100 ml of distilled water.

Solution C: 49.2 ml of solution A and 50.8 ml of solution B were added together to give a phosphate buffer with pH 6.8.

b) The borate buffer was prepared as follows:

Solution A: 746 mg of KCl and 620 mg of H_3BO_3 were dissolved in 100 ml of distilled water.

Solution B: 50 ml of solution A was then adjusted to pH 10 using 0.1 N NaOH.

Working Solution: The working solution, as shown in **Table 2.2**, contained 5.6 mM tryptamine HCl, 800 μM acetyl coenzyme A, 40 nCi [^{14}C]-acetyl coenzyme A and chilled phosphate buffer pH 6.8 in a final volume of 10 μl, per sample. The working solution was prepared immediately before the assay and kept chilled on a bed of crushed ice.

TABLE 2.2 *Scheme of NAT working solution.*

REAGENTS	**VOLUME (μl/sample)**
Tryptamine HCl (5.6 mM)	5
Acetyl Coenzyme A (800 μM)	2
[^{14}C]-Acetyl Coenzyme A (40 nCi)	2
Phosphate Buffer	1
Total Volume	10

Assay Procedure: Rats were sacrificed by neck fracture and the pineal glands rapidly removed. Each pineal was individually placed into a small glass homogeniser containing 100 μl of ice-cold 50 mM phosphate buffer pH 6.8 and homogenised for 30 seconds.

The assay was carried out, in duplicate, in 1 500 μl Eppendorf microfuge tubes. Homogenates (20 μl aliquots) from each pineal gland were transferred to the bottom of the microfuge tubes. A 10 μl aliquot of working solution was then pipetted onto the side of each tube. The surface tension was adequate in keeping the working solution, on the side of the tube, and the pineal homogenate, at the bottom of the tube, separate. Once all tubes had been properly prepared, they were tapped on the work bench which caused a mixing of the working solution and pineal homogenate. The tubes were further vortexed to ensure proper mixing. The tubes were then incubated at 37°C in a shaking water bath for 20 minutes. Blank incubations were performed in which only the enzyme (NAT in pineal homogenate) was omitted.

After incubation, the reaction was terminated by the addition of 100 μl of ice-cold borate buffer pH 10 to each tube. The radio-labelled N-acetyltryptamine formed was extracted by

the addition of 1 ml of toluene : isoamyl alcohol (97 : 3) to each tube. The tubes were shaken for five minutes and the resultant emulsion was centrifuged at 4 500 rpm for 30 seconds. The tubes were again opened and washed with a second 100 μl aliquot of ice-cold borate buffer, shaken and centrifuged. A 0.5 ml aliquot of the organic phase from each tube was transferred to plastic scintillation vials containing 3 ml of scintillation cocktail (Ready-Solv HP, Beckman, USA) and the radioactivity quantified by liquid scintillometry.

Calculation of NAT Activity: The results of the NAT assay are expressed as NAT activity/pineal gland/hour.

$$\frac{(DPM_{sample} - DPM_{blank}) \times m \times p \times 2 \times 3}{DPM_{total}} = \frac{pmol\ NAT\ Activity}{pineal\ gland/hour}$$

where:
- m = fraction of pineal homogenate assayed (1 for whole pineal *i.e.* 100 μl; 5 for 20 μl *etc*),
- p = pmoles of acetyl coenzyme A per sample,
- 2 = factor for counting only half the toluene : isoamyl alcohol used for the extraction of [^{14}C]-N-acetyltryptamine, and
- 3 = factor for incubating the sample for 20 minutes (Khan, 1989).

***In Vitro* Studies:** For *in vitro* studies of NAT activity, groups of five pineal glands were incubated in sterile 12 mm glass culture tubes containing 300 μl of BGJb culture medium. Alternatively, pineals were incubated individually in sterile glass tubes containing 60 μl of BGJb medium. In either case, a 10 μl aliquot of test drug or compound, was added to each tube. The tubes were incubated at 37°C in a humidified atmosphere of 95% O_2 and 5% CO_2 for a specified time period. Following incubation, the glands were removed, homogenised and the enzyme activity assayed as described above.

2.4 Pineal Cyclic AMP Assay

2.4.1 Introduction

A variety of techniques have been employed to measure cAMP levels. Early assay techniques involved the measurement of adenylate cyclase activity as an index of cAMP levels (Krishna *et al.*, 1968).

Gilman (1970) developed the first direct assay method, using a purified protein from bovine muscle. Brown *et al.* (1971) developed a saturation assay for cAMP, using purified bovine adrenal proteins as the specific binding protein. This binding protein had been identified earlier by Gill and Garren (1969) as a protein kinase.

The saturation assay involves the incubation of tissue sample together with binding protein and tritiated cAMP. Competition arises between endogenous cAMP and the tritiated cAMP for the binding sites on the binding protein. The binding protein is later separated and counted. A standard cAMP curve is generated for each experiment, from which the endogenous cAMP of the tissue sample is determined.

2.4.2 Materials and Method

Animals: Male Wistar rats weighing between 200 and 250 g were used in these experiments. The animals were sacrificed between 11h00 and 11h30 during the daytime and between 23h00 and 23h30 during the nighttime. At night, the procedure was performed in a darkroom with the aid of a dim photo-safe red light.

Chemicals and Reagents: [8-^{3}H]-Adenosine 3′,5′-cyclic phosphate, ammonium salt (specific activity 30 Ci/mmol) was purchased from Amersham (England). Standard cAMP was purchased from Boehringer Mannheim (West Germany). Theophylline was purchased from Sigma Chemical Co. (USA) and activated charcoal from Merck (West Germany). All other chemicals and reagents were purchased from local sources.

Preparation of cAMP Binding Protein: Bovine adrenal glands were collected from freshly slaughtered animals and transported on ice to the laboratory. The glands were stripped of adhering tissue and the medullas discarded. The adrenal cortices were homogenised in 1.5 volumes of an ice-cold medium comprising 0.25 M sucrose, 50 mM Tris-HCl buffer pH 7.4, 25 mM potassium chloride and 5 mM magnesium chloride. The homogenate was centrifuged at 2 000 g for 15 minutes at 4°C. The supernatant was re-centrifuged at 5000 g for 15 minutes at 4°C. The resultant supernatant was stored in 0.5 ml aliquots at -20°C. The preparation was thawed and diluted as required with 50 mM Tris-HCl buffer pH 7.4 containing 8 mM theophylline and 6 mM 2-mercaptoethanol.

Preparation of Pineal Tissue: Male Wistar rats were sacrificed and the pineal glands rapidly removed. The pineals were dissected free of adhering connecting tissue and placed individually into 100 μl of ice-cold phosphate buffer pH 6.8. The pineal glands were homogenised and 50 μl of the homogenate was used for cAMP determination.

Cyclic AMP Assay Procedure: The assay was carried out according to the scheme represented in **Table 2.3**. Briefly, the assay was carried out in 1.5 ml Eppendorf microfuge tubes containing known amounts of standard cAMP (0 - 8 pmol/assay tube) or 50 μl of sample, 100 μl of diluted binding protein and 50 mM Tris-HCL buffer pH 7.4 containing 8 mM theophylline and 6 mM 2-mercaptoethanol, to a final volume of 350 μl. The tubes were gently agitated and then placed on ice at 4°C for 100 minutes. After incubation, 100 μl of a 10% w/v suspension of charcoal in buffer containing 2% w/v bovine serum albumin was added to each reaction tube. The tubes were vortexed for 10 seconds each and then centrifuged at 1 200 g for 15 minutes at 4°C. A 100 μl aliquot of the supernatant was removed from each tube and placed into a plastic scintillation vial containing 3 ml of Ready-Solv HP (Beckman, USA) scintillation fluid. The vials were agitated and the radioactivity quantified by means of liquid scintillometry in a Beckman LS 2500 scintillation counter at an efficiency of 60% - 65%. Counting efficiency was determined by external channel ratio method of quench correction. A correction factor was built into the quench correction program to allow for tritium decay. A calibration curve was plotted in terms of standard concentration of nucleotide (pmol) against the Co/Cx ratio. The Co/Cx ratio is obtained by dividing the radioactivity (cpm) of the zero standard (Co) by the radioactivity of the higher

TABLE 2.3 *Scheme of the cyclic AMP assay.*

	CYCLIC AMP STANDARDS (pmol)								
REAGENTS (μl)	**BLANK**	**0**	**0.25**	**0.5**	**1.0**	**2.0**	**4.0**	**8.0**	**SAMPLE**
Buffer*	250	150	150	150	150	150	150	150	150
Standard	---	---	50	50	50	50	50	50	50
Sample	---	---	---	---	---	---	---	---	50
[^{3}H]-cAMP	50	50	50	50	50	50	50	50	50
Diluted Protein	---	100	100	100	100	100	100	100	100
INCUBATE ON ICE FOR 100 MINUTES									
Charcoal	100	100	100	100	100	100	100	100	100
CENTRIFUGE AT 1 200 g FOR 15 MINUTES AT 4°C									
Supernatant	100	100	100	100	100	100	100	100	100
ADD TO 3 ml SCINTILLATION FLUID AND COUNT RADIOACTIVITY									

* Tris-HCl (50 mM, pH 7.4) containing 8 mM theophylline and 6 mM 2-mercaptoethanol

standards (Cx) after subtraction of the blank value. The amount of unknown cAMP in the samples was determined with reference to the standard curve in each assay.

2.4.3 Results

Dilution of Binding Protein: Serial dilutions of binding protein in 50 mM Tris-HCl buffer pH 7.4 containing 8 mM theophylline and 6 mM 2-mercaptoethanol were prepared according to the scheme reflected in **Table 2.4**, together with the general method of cAMP assay explained in **§ 2.4.2.** Each dilution was assayed in duplicate and the radioactivity (cpm) plotted against the dilution.

TABLE 2.4 *Binding protein dilution curve protocol.*

REAGENTS	VOLUME (μl)
[^{3}H]-cAMP	50
Standard cAMP	50
Diluted Binding Protein	100
Buffer*	150
Total Volume	350

* Tris-HCl buffer (50 mM, pH 7.4) containing 8 mM theophylline and 6 mM 2-mercaptoethanol.

The results obtained from the dilution curve (**Figure 2.2**) showed that a 1 : 9 dilution with assay buffer provided adequate binding. In all subsequent cAMP assays, a dilution of 1 : 9 for the binding protein was used. Each time a new batch of binding protein was prepared, a new dilution curve was determined.

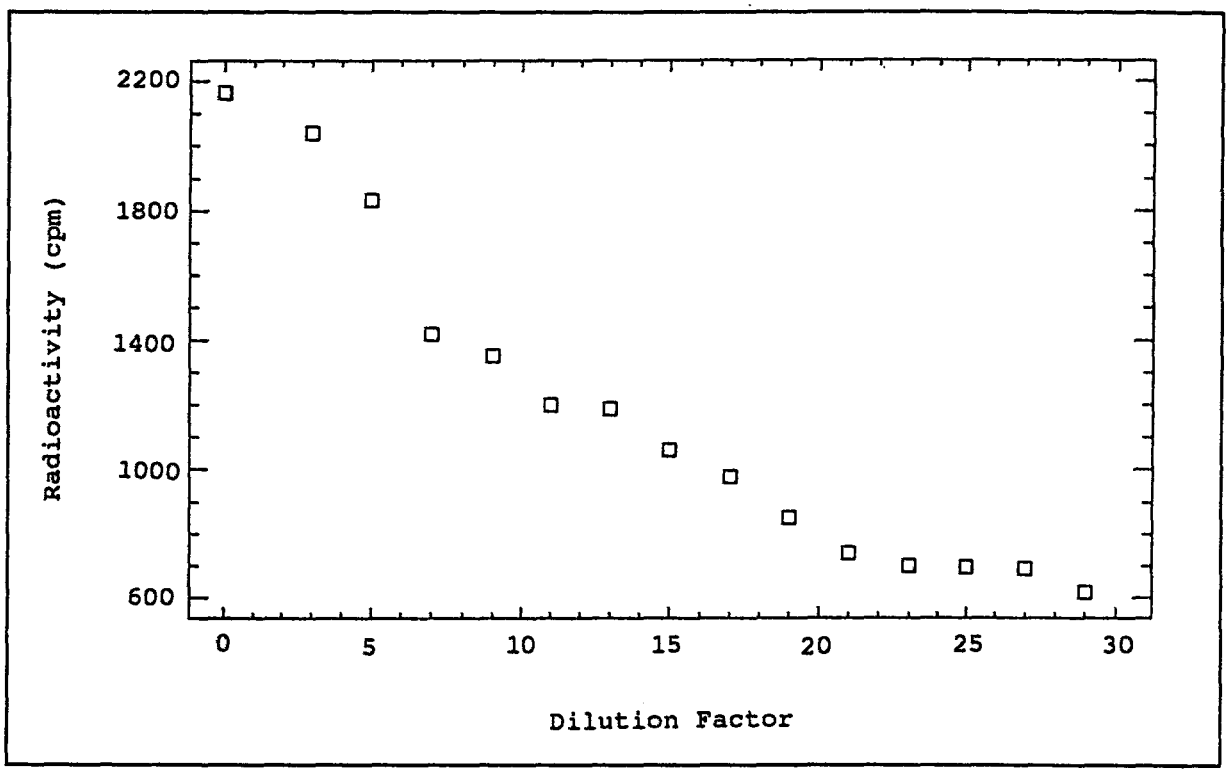

FIGURE 2.2 *Cyclic AMP binding protein dilution curve.*

Each point represents the mean of duplicate determinations.

Standard cAMP Curve: The standard curve was plotted in terms of concentration of nucleotide (pmol/tube) against the Co/Cx ratio, where:

Co = cpm zero standard - cpm blank and

Cx = cpm standard - cpm blank.

The result was a linear standard curve between 0 and 8 pmol cAMP (**Figure 2.3**, linear regression analysis, $r^2 = 0.998$).

Interference by Non-cAMP Material: The sensitivity of a saturation assay method is influenced by a number of factors, the most prominent being interference by non-cAMP material. To test the extent of this interference, pineal tissue homogenates were incubated with commercially purchased phosphodiesterase (Boehringer Mannheim) for one hour at 37°C to destroy cAMP. Samples incubated without phosphodiesterase acted as controls. The results showed no significant difference between the samples incubated with or without phosphodiesterase, indicating that interference by non-cAMP material is negligible in this assay.

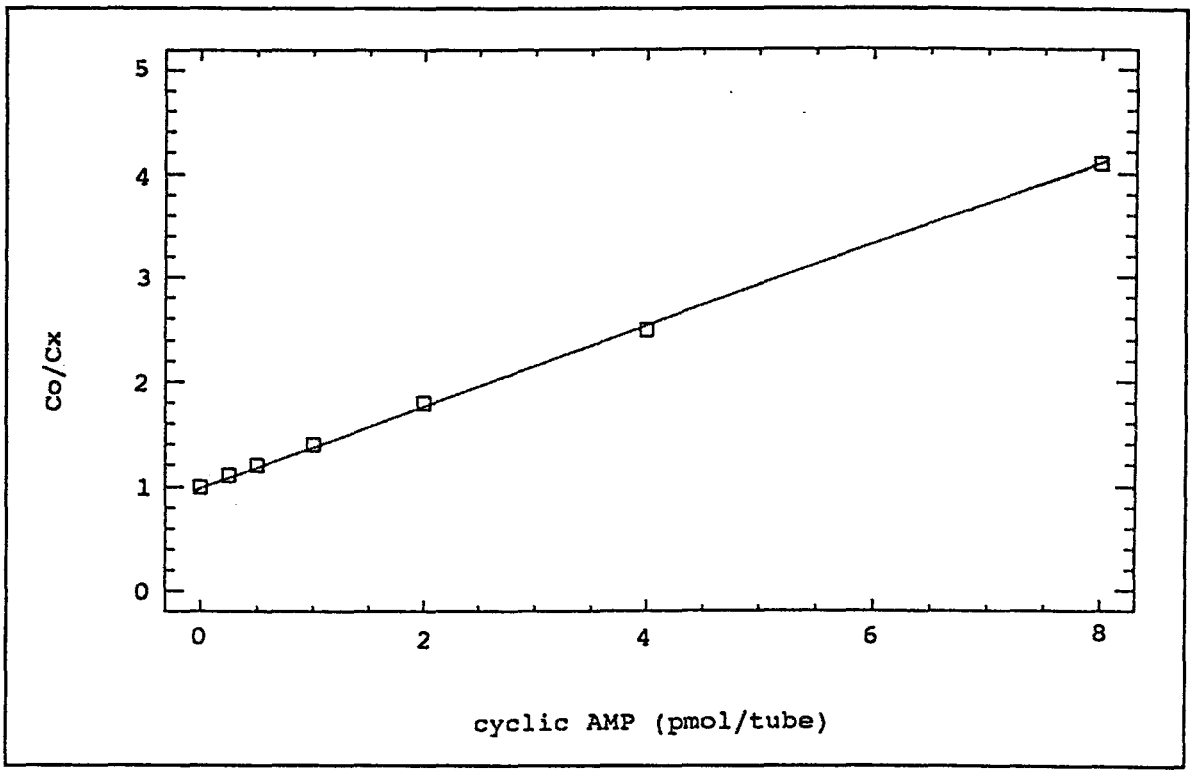

FIGURE 2.3 *Cyclic AMP standard curve.*

Each point represents the mean of duplicate determinations ($r^2 = 0.998$).

2.5 Pineal Cyclic AMP-Phosphodiesterase Assay

2.5.1 Introduction

Cyclic nucleotides play an important role in the control of intracellular events, and their concentrations are delicately regulated (Teo *et al.*, 1973). These intracellular concentrations are primarily controlled by the rate of synthesis and degradation of the nucleotides. Degradation is catalysed by phosphodiesterase enzymes which hydrolytically cleave the cyclic nucleotides.

Phosphodiesterase was first discovered by Sutherland and Rall (1958). The enzyme was partially purified from bovine heart in 1967 by Butcher and Sutherland. The activity of the enzyme was found to be dependent on the presence of an activator protein (Cheung, 1970; Kakiuchi *et al.*, 1970). This protein was later named calmodulin (Cheung, 1980).

Calmodulin has been termed "a universal receptor of the Ca^{2+} signal" in cells by Klee and Haiech (1980) due to its ability to be modified by intracellular calcium concentrations. Drugs have been shown to affect phosphodiesterase activity through modification of calmodulin activity.

The separation of cyclic nucleotides from their nucleosides has been shown to be easier than separating them from their monophosphate precursors. A number of methods, including batch or column anion-exchange resins (Thompson and Appleman, 1971; Lynch and Cheung, 1975), paper chromatography (Nakai and Brooker, 1975), precipitation (LeDonne and Coffee, 1979) and TLC (Neuman, 1983), have been developed to monitor phosphodiesterase activity.

In this study the TLC method developed by Neuman (1983) was used.

2.5.2 Materials and Method

Animals: Male Wistar rats weighing between 200 and 250 g were used in these experiments and were maintained as described in **§ 2.1**. The animals were sacrificed at 11h00 during the

daytime and at 23h00 during the nighttime. At night the procedure was performed in a darkroom with the aid of a dim photo-safe red light.

Chemicals and Reagents: [8-^{3}H]-Adenosine 3′,5′-cyclic phosphate, ammonium salt (specific activity 30 Ci/mmol) was purchased from Amersham (England). Phosphodiesterase (EC 3.1.4.17, specific activity *ca* 0.31 U/mg), adenosine 3′,5′-cyclic phosphate and adenosine 5′-monophosphate were purchased from Boehringer Mannheim (West Germany). TLC plates (Alugram Sil G/UV$_{254}$, 0.025 x 20 x 20 cm) were purchased from Macherey-Nagel (West Germany). All other chemicals and reagents were purchased from local sources.

Assay Procedure: The assay was performed according to the scheme represented in **Table 2.5**. Rats were sacrificed by neck fracture and the pineal glands rapidly removed. The glands were cleaned of adhering connective tissue and placed individually into glass homogenisers containing 100 μl of Tris-HCl buffer (40 μM, pH 8 containing 5 μM $MgCl_2$). The glands were homogenised for 30 seconds (approximately 15 strokes). Duplicate 10 μl aliquots of each pineal homogenate were transferred to reaction vessels containing 10 μl cAMP (1 μM), 10 μl [^{3}H]-cAMP and 20 μl buffer. The blank contained all the above mentioned reagents with the exception of the pineal homogenate. The tubes were then incubated at 37°C for 30 minutes. The reaction was terminated by the addition of 30 μl of 10% trichloroacetic acid, and the [^{3}H]-5′-AMP formed was separated from the remaining [^{3}H]-cAMP by TLC.

Thin Layer Chromatography: Ten microlitre aliquots of each sample were spotted 15 mm from the bottom of the plates. Standard solutions containing 0.2 mg/ml authentic cAMP or 5′-AMP dissolved in distilled water were spotted at the ends of the plate in order to monitor the mobility of cAMP and 5′-AMP in the samples. The plates were developed once with an ascending solvent front composed of isopropylalcohol : 25% water of NH_4OH : water (7 : 1 : 2). Following development, the plates were dried under a stream of nitrogen. The spots were visualized under short wave ultraviolet light and the radioactivity collected by scraping each spot into a separate plastic scintillation vial containing 400 μl 0.01 M KOH and 3 ml of scintillation cocktail (Ready-Solv HP, Beckman, USA). The vials were shaken for 20 minutes and the radioactivity quantified in a Beckman LS 2800 scintillation counter.

TABLE 2.5 *Scheme of cAMP-phosphodiesterase assay* (Neuman, 1983).

REAGENTS (μl)	**SAMPLE**	**BLANK**
Pineal Gland Homogenate	10	---
cAMP	10	10
[^{3}H]-cAMP	10	10
Buffer*	20	30
INCUBATE AT 37°C FOR 30 MINUTES		
Trichloroacetic Acid (10%)	30	30
REMOVE 10 μl FROM EACH TUBE FOR TLC		

* Tris-HCl Buffer (40 μM, pH 8) containing 5 μM $MgCl_2$

2.5.3 Results

Figure 2.4 illustrates the hydrolysis of cAMP by phosphodiesterase with time. The hydrolysis of cAMP followed an almost linear fashion for the 30 minute period, resulting in the use of 30 minutes as the incubation time for the assay. The activity of phosphodiesterase is expressed as pmoles of [^{3}H]-5′-AMP formed per gland per hour. Blank values were found to be consistently less than 0.45% of the monitored radioactivity.

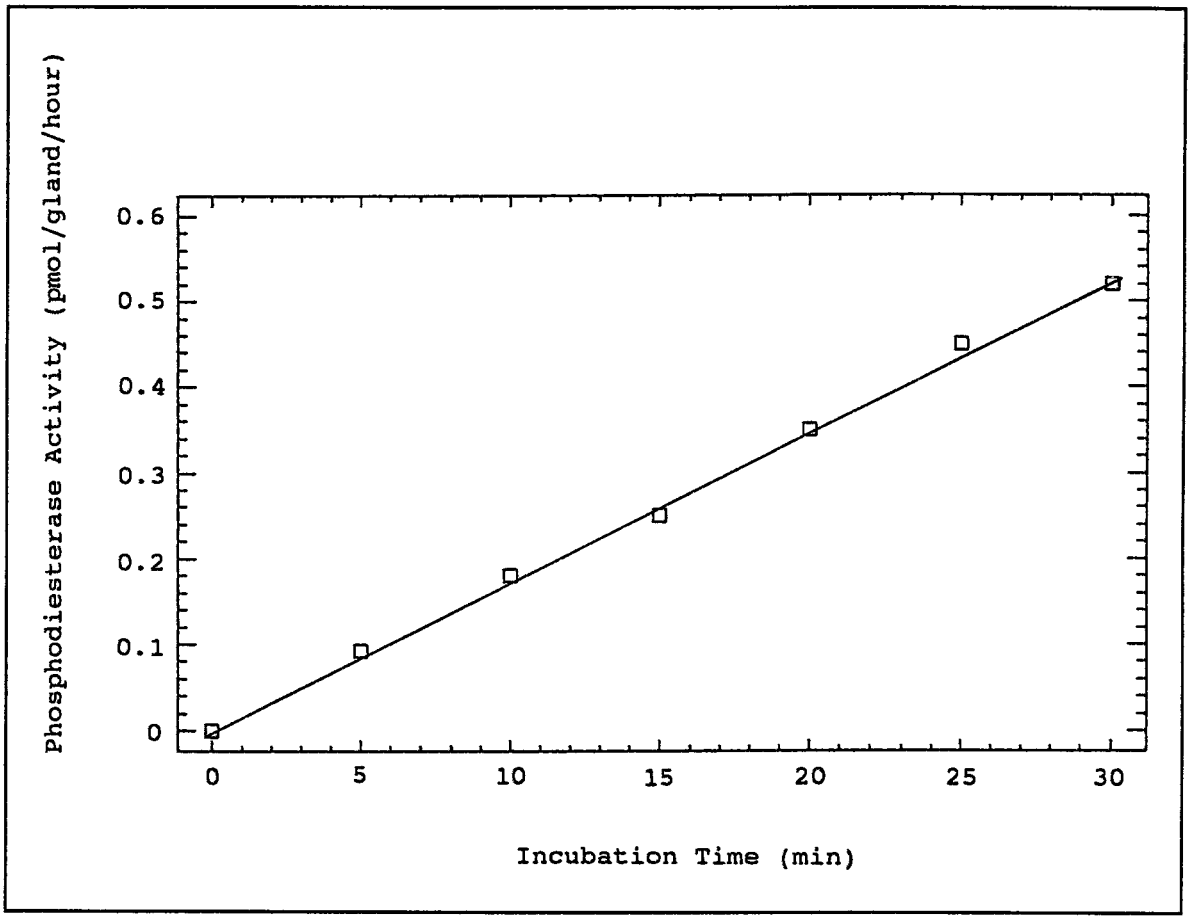

FIGURE 2.4 *The effect of incubation time on phosphodiesterase-mediated cAMP hydrolysis.* Each point represents the mean of duplicate determinations.

2.6 The Separation of Pineal Adenosine Nucleotides

2.6.1 Introduction

The separation of nucleotides, nucleosides and nucleic acid bases has been of interest to scientists for many decades. During the early to mid 1960's, thin layer chromatographic (TLC) methods were developed to separate and quantify these compounds. The advantages of these TLC techniques were the simplicity of use together with their sharp resolution and great sensitivity (Randerath and Randerath, 1965).

The separation of nucleotides was found to be most effective on anion-exchange layers. Of the many compounds tested, polyethyleneimine (PEI) cellulose was shown to have superior resolution and a versatile applicability. The migration of nucleotides on PEI-cellulose layers is affected predominantly by: the cellulose material, the amount of PEI used and the concentrations of aqueous electrolyte solutions used as solvents (Bohme and Schultz, 1967).

In these studies the technique described by Bohme and Schultz (1967) was used.

2.6.2 Materials and Method

Animals: Male Wistar rats weighing between 200 and 250 g were used in this study and were maintained as described in **§ 2.1.** The animals were sacrificed by neck fracture at 11h00.

Chemicals and Reagents: [2-^{3}H]-Adenosine (specific activity 26 Ci/mmol) was purchased from Amersham (England). Unlabelled adenosine 5′-triphosphate, adenosine 5′-diphosphate, adenosine 5′-monophosphate and cyclic adenosine 3′, 5′-monophosphate were purchased from Boehringer Mannheim (West Germany). l-Isoproterenol and propranolol were purchased from Sigma Chemical Co. (USA). Plastic TLC plates (5 cm x 20 cm) coated with polyethyleneimine cellulose (Polygram®, Cel 300 PEI/UV_{254}) 0.1 mm were purchased from Macherey-Nagel (West Germany). BGJb culture medium (Fitton-Jackson modification) was purchased from Gibco (Europe). All other chemicals and reagents were purchased from local commercial sources.

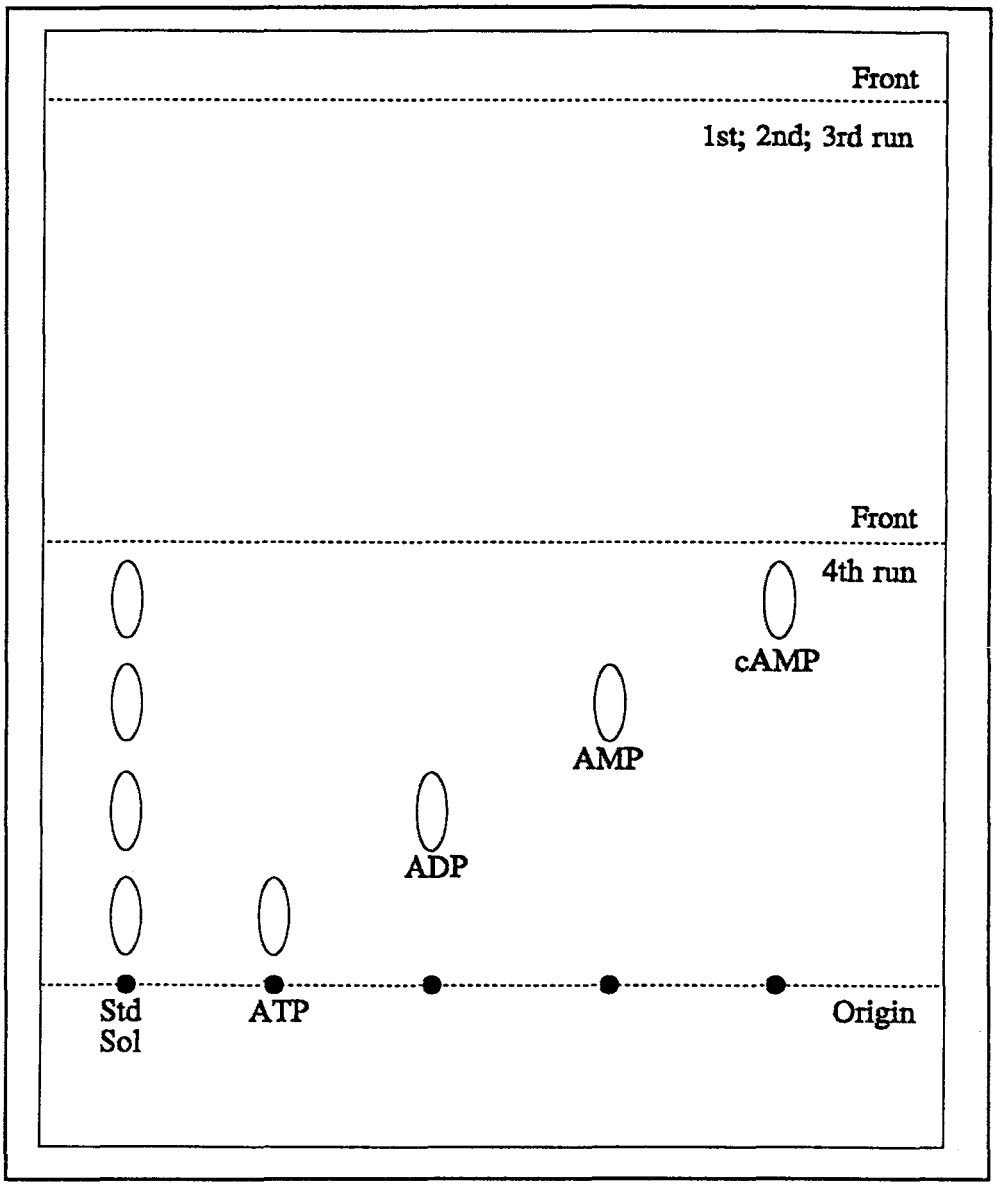

FIGURE 2.5 *The separation of adenosine nucleotides from each other in a standard nucleotide solution containing ATP, ADP, 5'-AMP and cAMP, alongside individual nucleotide markers.*

CHAPTER III

RAT PINEAL GLAND INDOLEAMINE METABOLISM

3.1 Introduction

The ability of the rat pineal gland to produce indole metabolites from exogenous precursors such as tryptophan, 5-hydroxytryptophan and serotonin was shown some 60 years ago (Strangeways and Fell, 1926). Based on this principle, Klein and Notides (1969) developed a method to examine the metabolism of indoles in pineal cultures.

The advantages of this technique are that it:

- provides a quantitative picture of pineal indole metabolism,
- is sensitive to pharmacological manipulations (Klein and Rowe, 1970; Balemans *et al.*, 1983; Voisin *et al.*, 1983; Daya and Potgieter, 1985), and
- adequately reflects changes in pineal indole metabolism.

In 1986, Daya and Fata were the first to describe a method which allowed the progressive measurement of indole formation in pineal glands over a 24 hour period. The advantages of this technique compared to that of Klein and Notides (1969) are that it:

- provides information on the progressive formation of indole metabolites at any stage of the culture period, and
- could indicate the ideal time to terminate the culture period (Banoo, 1991).

Both methods have been used in subsequent studies to investigate the metabolism of [^{14}C]-tryptophan to its metabolites over a 24 hour period and the differences between serotonin and tryptophan metabolism.

3.2 Experiment 1: A 24 Hour Profile of [^{14}C]-Tryptophan Metabolism by Organ Culture of Rat Pineal Glands.

3.2.1 Introduction

Pineal organ culture assays have shown little deviation from the practical method described by Klein and Notides (1969). One of the variations to this method was that of Daya and Fata (1986), who were able to show the progressive metabolism of pineal indoles. This method was later used by Banoo *et al.* (1987) to describe the progressive formation of pineal indoles from [^{14}C]-serotonin in the presence of β-receptor agonist.

The purpose of the present study was to investigate the effect of nor-adrenaline, the neurotransmitter found in the nerve terminals innervating the pineal gland, on the progressive metabolism of exogenous radio-labelled tryptophan to its radio-labelled metabolites.

3.2.2 Materials and Method

Male Wistar rats weighing between 200 and 250 g were used in this study. The animals were maintained as described in **§ 2.1.** The animals were sacrificed by neck fracture at 11h00.

Chemicals and Reagents: L-methyl-[^{14}C]-tryptophan (specific activity 54 mCi/mmol) was purchased from Amersham (England) and nor-adrenaline from Sigma Chemical Co. (USA). All other chemicals and reagents were obtained from previously acknowledged sources (**§ 2.2.2**).

Organ Culture: After sacrifice, the pineal glands (n = 5) were rapidly removed and placed individually into sterile culture tubes containing BGJb medium, [^{14}C]-tryptophan and 10 μM nor-adrenaline (to a final volume of 70 μl) at 37°C in an atmosphere of 95% O_2 and 5% CO_2 as previously described (**§ 2.2.2**). Control pineal glands (n = 5) were incubated in the absence of nor-adrenaline. At two-hourly intervals, 5 μl aliquots of culture medium were removed from each tube, the tubes being immediately replenished with an equivalent volume from an identical set of tubes containing no tissue. The indoles in the 5 μl aliquots were

isolated by two dimensional TLC and quantified by liquid scintillometry as described in § **2.2.2.**

All data were statistically compared using Student's t test and expressed as dpm/5 μl medium/pineal gland.

3.2.3 Results

The nor-adrenaline stimulated pineals showed a progressive increase in N-acetylserotonin and melatonin with peak levels being reached after 16 and 20 hours respectively (**Figures 3.1** and **3.2**). The peaks reached in the nor-adrenaline stimulated pineals were significantly higher than those in the unstimulated controls ($p < 0.05$). N-acetylserotonin levels were significantly higher than melatonin levels in both stimulated and unstimulated pineals.

When compared to the controls, the other indole metabolites, 5-hydroxyindoleacetic acid (HIAA), 5-hydroxytryptophol (HTOH), 5-methoxyindoleacetic acid (MIAA) and 5-methoxytryptophol (MTOH) progressively increased for 16 to 20 hours (**Figures 3.3** to **3.6**).

From the onset of the experiment, HIAA was the only indole to show a sharp significant rise ($p < 0.05$) when compared with the control. As HIAA and HTOH levels increased with time (**Figures 3.3** and **3.4**), MIAA and MTOH levels were respectively found to decrease and remain constant compared with control levels (**Figures 3.5** and **3.6**).

3.2.4 Discussion

β-Adrenergic receptor stimulation by nor-adrenaline, the neurotransmitter and β-receptor agonist found in the sympathetic nerves innervating the pineal gland, caused a marked rise in the synthesis of N-acetylserotonin and melatonin as compared with the unstimulated controls (**Figures 3.1** and **3.2**). The initial rise of N-acetylserotonin and melatonin was found to be gradual when compared with the results of Banoo *et al.* (1987). Using serotonin as the precursor in their experiments, Banoo *et al.* (1987) showed a rapid increase in N-acetylserotonin and melatonin synthesis within the first few hours of stimulation.

The higher N-acetylserotonin levels as compared with melatonin levels could be the result of changes in hydroxyindole-O-methyltransferase (HIOMT) activity. Nor-adrenaline stimulates tryptophan hydroxylase to convert tryptophan to 5-hydroxytryptophan (Wurtman *et al.*, 1971; Shibuya *et al.*, 1978). 5-Hydroxytryptophan is converted to serotonin which is acted upon by N-acetyltransferase (NAT) to form N-acetylserotonin. NAT activity increases between 30- and 100-fold in the presence of β-adrenergic receptor agonist (Klein and Berg, 1970; Klein *et al.*, 1970; Klein *et al.*, 1978). Thus, nor-adrenaline causes the production of vast quantities of N-acetylserotonin. On the other hand, HIOMT activity has been shown to be increased at a much slower rate than NAT (Klein and Berg, 1970; Sugden and Klein, 1983). As a result of a slow conversion of N-acetylserotonin to melatonin, a build-up of N-acetylserotonin could arise.

The conversion of 5-hydroxyindoles to 5-methoxyindoles by HIOMT was limited in nor-adrenaline stimulated pineals. This was probably due to substrate competition for the enzyme. The high levels of 5-hydroxyindoleacetic acid in nor-adrenaline stimulated pineals points to the possibility that its synthesis by monoamine oxidase (MAO) could be regulated via a β-adrenergic receptor mechanism.

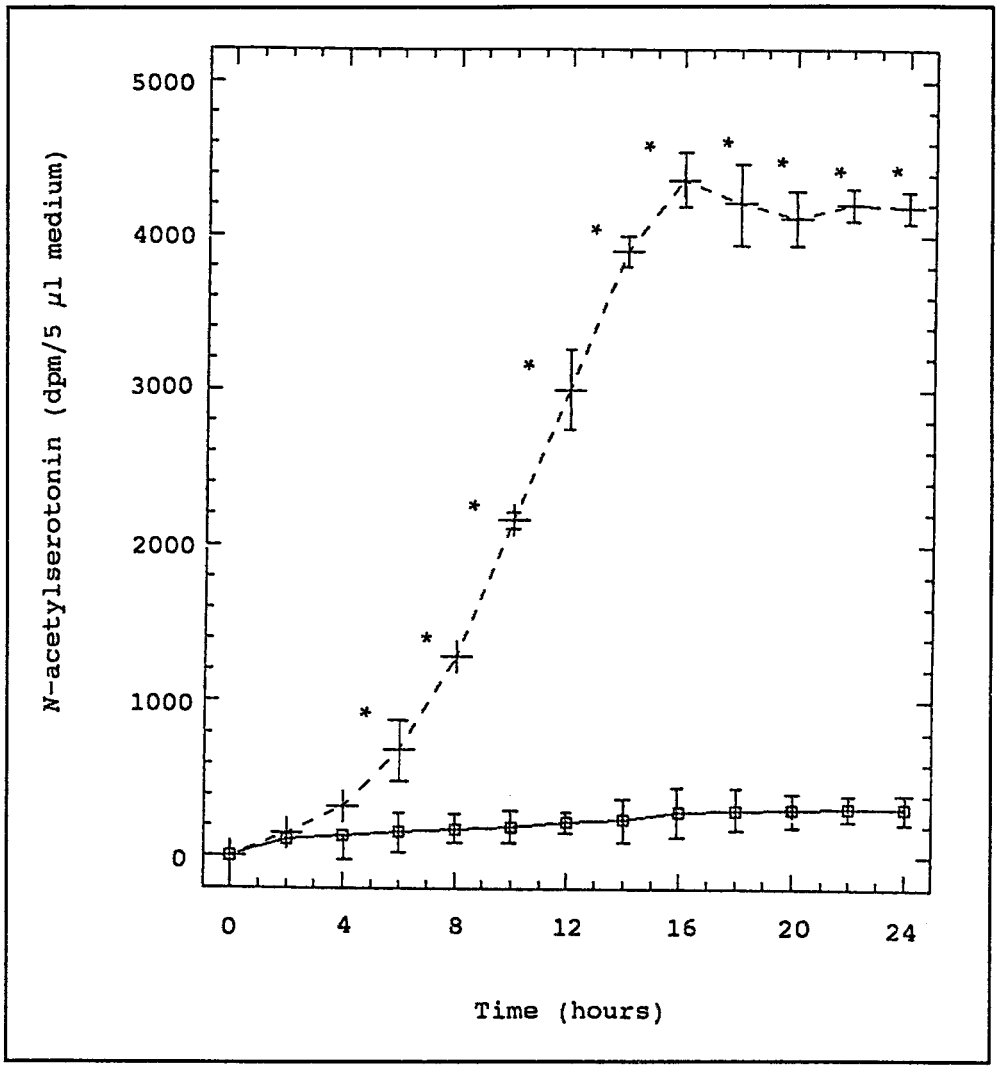

FIGURE 3.1 *A 24 hour profile of tryptophan metabolism to N-acetylserotonin in noradrenaline stimulated* (---+---) *and control* (———□———) *pineal glands (means ± SEM, n = 5).*

* as compared with the control, $p < 0.05$, Student's t test.

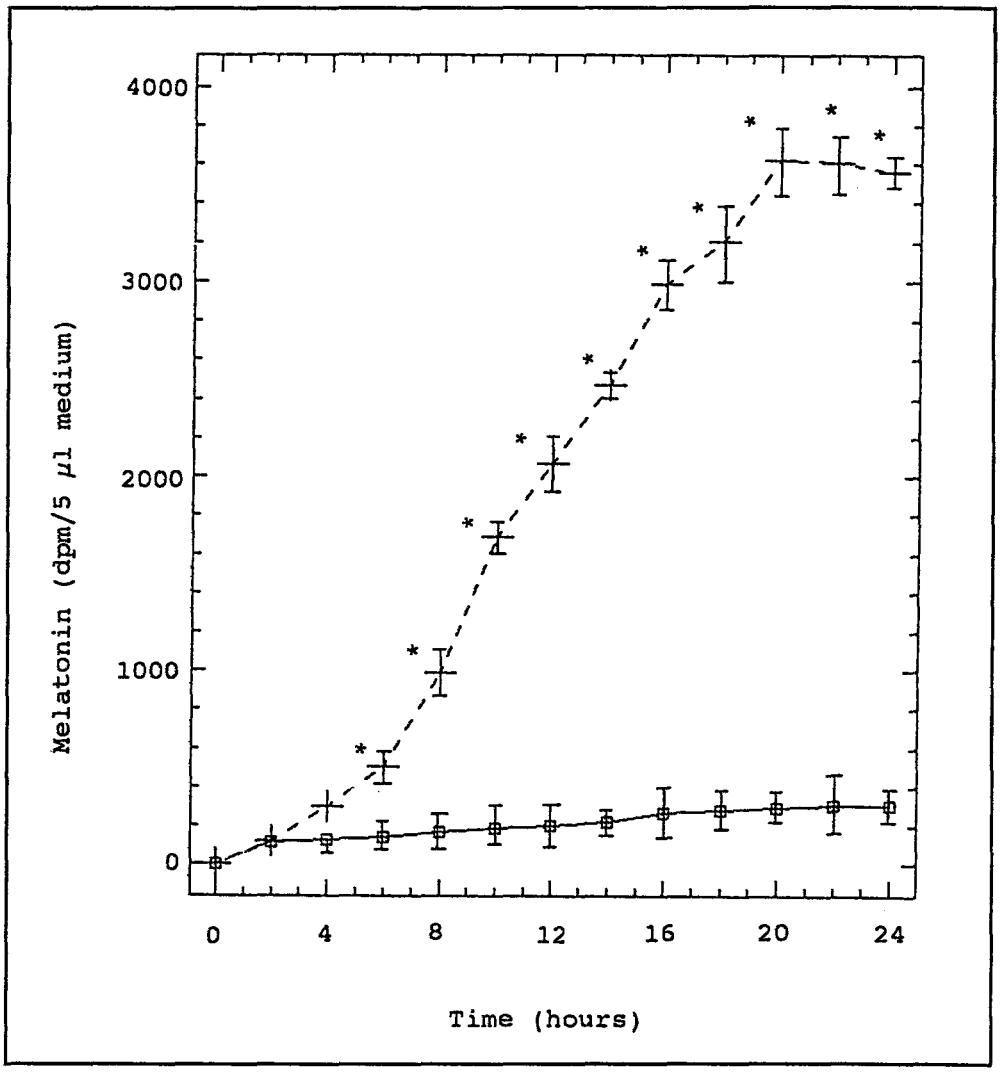

FIGURE 3.2 *A 24 hour profile of tryptophan metabolism to melatonin in nor-adrenaline stimulated* (---+---) *and control* (———□———) *pineal glands (means ± SEM, n = 5).*

* as compared with the control, $p < 0.05$, Student's t test.

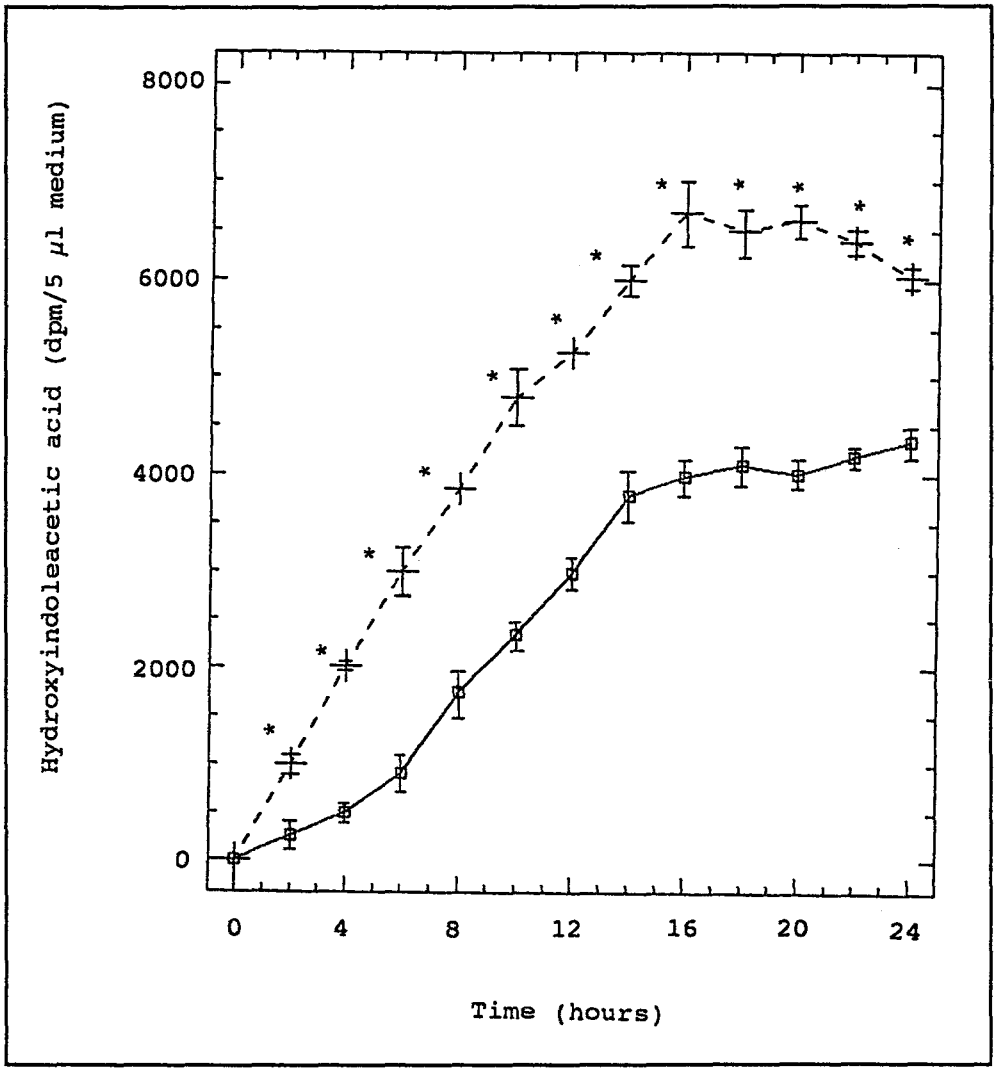

FIGURE 3.3 *A 24 hour profile of tryptophan metabolism to 5-hydroxyindoleacetic acid in nor-adrenaline stimulated* (---+---) *and control pineal glands* (———□———) *(means ± SEM, n = 5).*

* as compared with the control, $p < 0.05$, Student's t test.

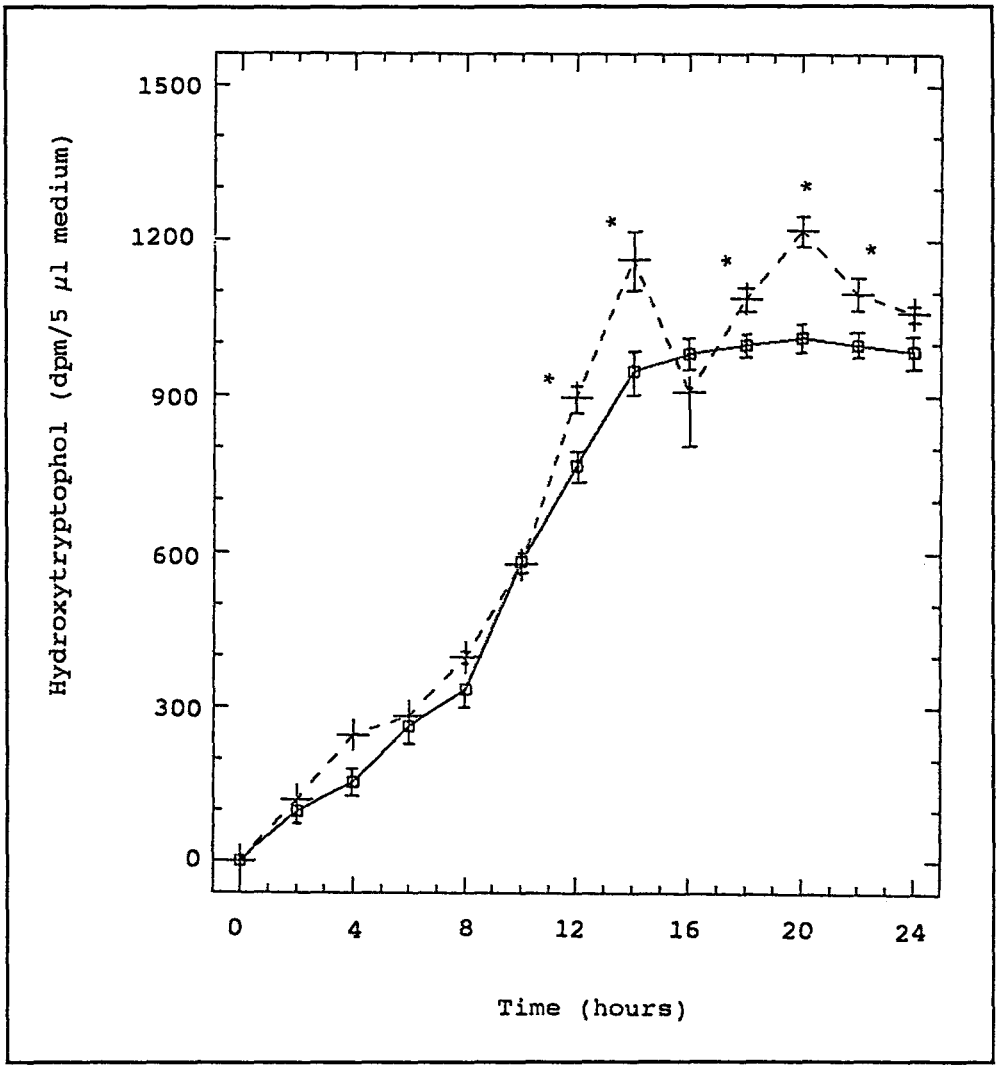

FIGURE 3.4 *A 24 hour profile of tryptophan metabolism to hydroxytryptophol in noradrenaline stimulated* (---+---) *and control* (———□———) *pineal glands (means ± SEM, n = 5).*

* as compared with the control, $p < 0.05$, Student's t test.

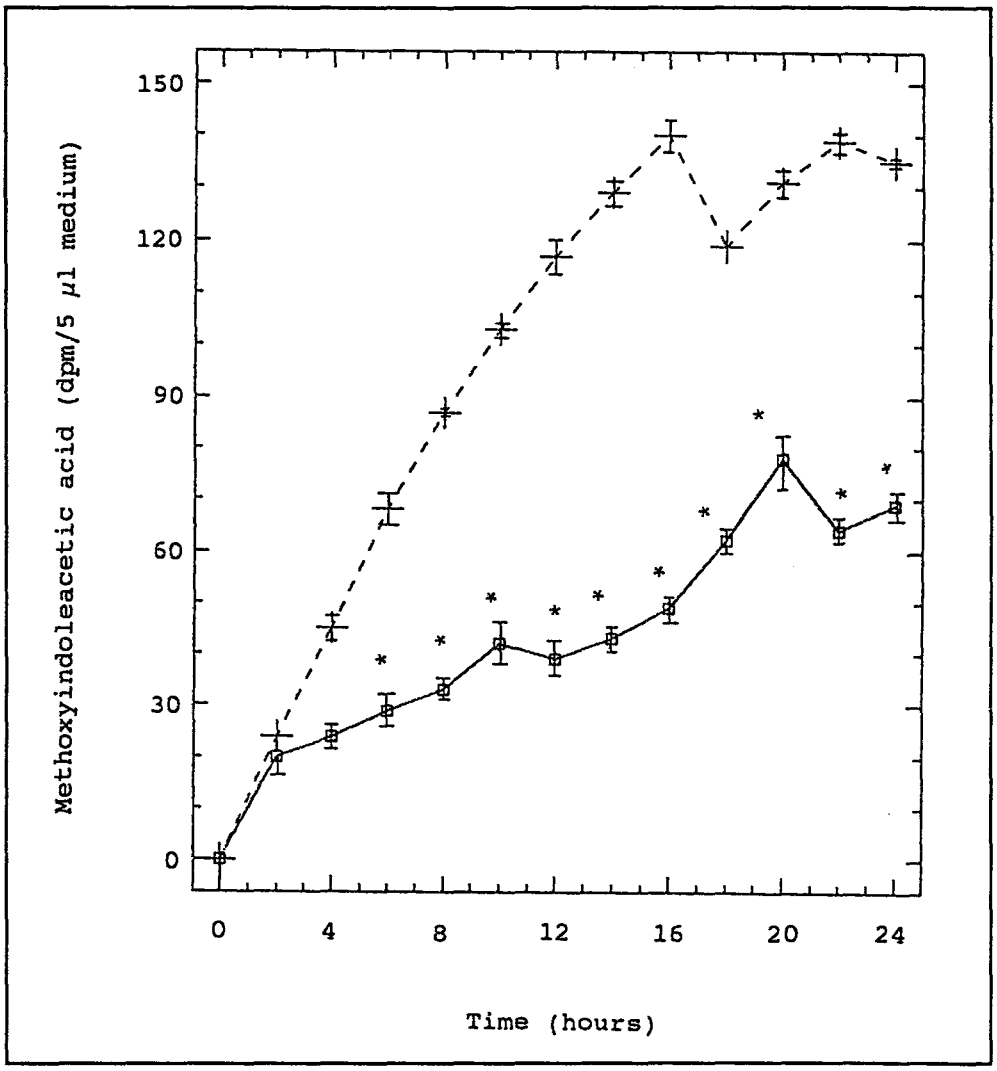

FIGURE 3.5 *A 24 hour profile of tryptophan metabolism to 5-methoxyindoleacetic acid in nor-adrenaline stimulated* (———□———) *and control* (---+---) *pineal glands (means ± SEM, n = 5).*

* as compared with the control, $p < 0.05$, Student's t test.

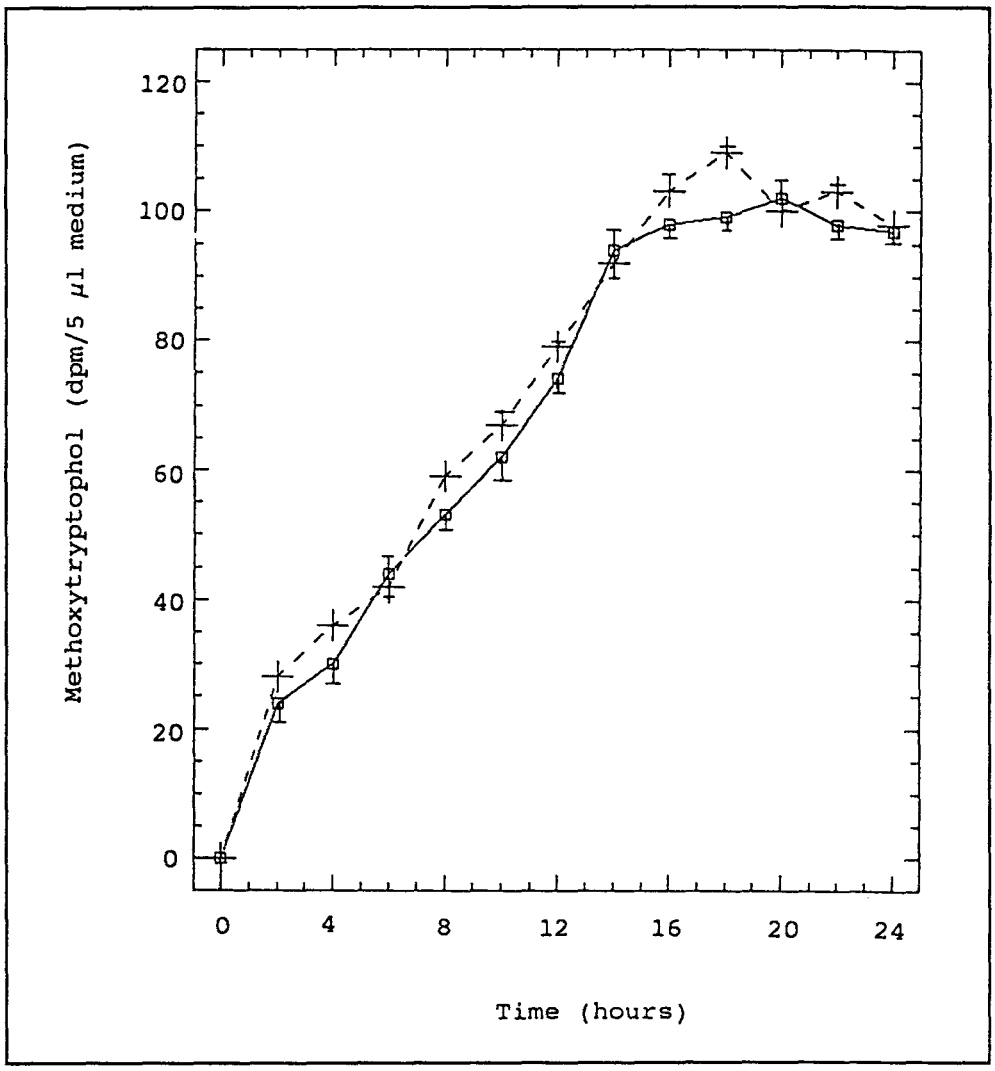

FIGURE 3.6 *A 24 hour profile of tryptophan metabolism to methoxytryptophol in noradrenaline stimulated* (---+---) *and control* (———□———) *pineal glands (means ± SEM, n = 5).*

3.3 Experiment 2: Comparison of [^{14}C]-Tryptophan and [^{14}C]-Serotonin Metabolism by Organ Culture of Rat Pineal Glands.

3.3.1 Introduction

Pineal glands of rats housed under diurnal lighting conditions exhibit a diurnal rhythm in the synthesis of the pineal hormone, melatonin (Brownstein and Axelrod, 1974; Romero, 1976). This is effected by the release of nor-adrenaline at night from the sympathetic nerve terminals innervating the gland (Relkin, 1976). Nor-adrenaline, acting via the β-adrenergic mechanism, stimulates the activity of the pineal enzyme N-acetyltransferase (NAT) resulting in enhanced conversion of the high levels of serotonin accumulated during the day to N-acetylserotonin (NAS). NAS is subsequently converted to melatonin by the enzyme hydroxyindole-O-methyltransferase (HIOMT). Consequently, the rat pineal concentrations of serotonin and melatonin follow an inverse relationship (Wurtman *et al.*, 1968; Wolstenholme and Knight, 1971; Axelrod, 1974; Quay, 1974a).

It is well established that the rat pineal gland in organ culture is able to convert radio-labelled tryptophan to melatonin (**§ 3.1**). Serotonin formed as an intermediate in this pathway is acted upon by the enzyme monoamine oxidase (MAO) to produce a number of indole metabolites. There appear to be two distinct pools of serotonin in the pineal. One of these is the pinealocyte proper, which accounts for 30% of the MAO activity of the gland (Goridis and Neff, 1971), and the other is the nerve terminals of the fibres from the superior cervical ganglia, which accounts for 70% of the MAO activity of the gland (Goridis and Neff, 1971).

In chick pineal glands, it has been shown that marked differences exist in the metabolism of tryptophan and serotonin (Wainwright, 1977). The major metabolite of serotonin metabolism is 5-hydroxyindoleacetic acid (HIAA) whereas the major metabolite of tryptophan metabolism is melatonin.

The purpose of this study was to compare the metabolism of exogenous radio-labelled tryptophan and serotonin by organ culture of rat pineal glands.

3.3.2 Materials and Method

Twenty four male Wistar rats weighing between 200 and 250 g were used in this experiment. The animals were maintained as described in **§ 2.1.** The animals were sacrificed by neck fracture at 11h00 for the day study and at 23h00 for the night study. The collection of pineals for the night study was performed according to **§ 2.1.**

Chemicals and Reagents: L-methyl-[^{14}C]-tryptophan (specific activity 54 mCi/mmol) and 5-hydroxy [side chain-2-^{14}C]-tryptamine creatine sulphate (specific activity 57 mCi/mmol) were purchased from Amersham (England). All other chemicals and reagents were obtained from previously acknowledged sources (**§ 2.2.2**).

Organ Culture: The technique of pineal organ culture and the analysis of labelled indoles was performed as outlined in **§ 2.2.2.** Briefly, after sacrifice, the pineal glands were swiftly removed and placed individually into sterile glass tubes each containing 60 μl BGJb culture medium in which either 0.4 μCi of [^{14}C]-serotonin (n = 6) or 0.4 μCi of [^{14}C]-tryptophan (n = 6) was present. The tubes were then incubated at 37°C for 24 hours in a humidified atmosphere of 95% O_2 and 5% CO_2.

In the night study, animals were sacrificed in darkness, whereafter pineals were removed and placed in organ culture as described above.

The incubation was terminated after 24 hours and 10 μl aliquots of culture medium were removed from each tube for analysis. The indoles were isolated using TLC and quantified by liquid scintillometry as described in **§ 2.2.2.**

All data were statistically compared using Student's t test and expressed as the mean of six separate incubations $\pm$ SEM.

3.3.3 Results

Tryptophan metabolism was similar in pineals removed during both the day and the night (**Table 3.1**). Melatonin formation by pineals removed during the dark phase was

approximately twice as great ($p < 0.05$) as that of pineals removed during the light phase (**Figure 3.7**). Contrary to expectations, N-acetylserotonin levels were slightly higher during the day study (**Figure 3.8**). 5-Hydroxyindoleacetic acid and 5-methoxyindoleacetic acid showed no significant difference between day and night studies (**Figures 3.9** and **3.10**).

The metabolism of serotonin on the other hand yielded a significant difference between the day and night study (**Table 3.2**). Approximately six times more 5-hydroxyindoleacetic acid was synthesised by serotonin metabolism than by tryptophan metabolism. In addition, the percentage of melatonin and monoamine oxidase products formed from serotonin was greater than with tryptophan as the precursor. The metabolism of serotonin to melatonin, N-acetylserotonin and 5-hydroxyindoleacetic acid by pineals removed at night was significantly greater ($p < 0.05$) than by pineals removed during the daytime (**Figures 3.11** to **3.13**). 5-Methoxyindoleacetic acid levels decreased in the night study compared with the day study, but not significantly (**Figure 3.14**).

3.3.4 Discussion

Radio-labelled serotonin appears to be metabolised to a greater extent than radio-labelled tryptophan. The major metabolite of both precursors is 5-hydroxyindoleacetic acid (HIAA). However, approximately six times more HIAA is produced as a result of serotonin metabolism compared to that of tryptophan. A possible reason for this is that a large amount of serotonin is taken up by the sympathetic nerve terminals and becomes subjected to monoamine oxidase degradation, whereas the tryptophan is preferentially taken up by the pinealocytes proper (Goridis and Neff, 1971).

Considering that both precursors were of similar specific activity and the same radioactive concentration, significantly more serotonin was converted to N-acetylserotonin and melatonin than was tryptophan. This is probably due to the fact that serotonin only has to undergo a two-step conversion to melatonin whereas tryptophan enters the pathway at an earlier stage. In the nighttime study, tryptophan metabolism did not differ much from the daytime study except that melatonin production was higher in the nighttime study. Since N-acetylserotonin levels remained unchanged, it is possible that melatonin synthesis in the nighttime tryptophan study was due to conversion of methoxytryptamine to melatonin.

Compared with the daytime study, significantly more melatonin, N-acetylserotonin and 5-hydroxyindoleacetic acid were produced during serotonin metabolism in the nighttime study. The rise in N-acetylserotonin and melatonin levels is expected, since N-acetyltransferase activity is known to be high during the dark phase in the intact rat pineal (Klein, 1979). However, the total monoamine oxidase products, including 5-hydroxyindoleacetic acid, were also elevated in the nighttime study of serotonin metabolism. At present, there is no evidence to indicate that nor-adrenaline stimulates pineal monoamine oxidase activity. These results suggest that nor-adrenaline activated monoamine oxidase activity, possibly in the sympathetic nerve terminals.

TABLE 3.1 *Metabolism of [^{14}C]-tryptophan by organ culture of rat pineal glands (means ± SEM, n = 6).*

	DPM recovered as:					% of total counts	
	MEL	NAS	HIAA	MIAA	Total MAOP	MEL	MAOP
Tryptophan (DS)	436 ± 101	539 ± 64	3 010 ± 159	371 ± 82	4 726	0.34	3.6
Tryptophan (NS)	761[1] ± 159	525 ± 91	3 148 ± 273	267 ± 43	4 791	0.59	3.7

(DS) Day Study; (NS) Night Study.

MEL = melatonin, NAS = N-acetylserotonin, HIAA = 5-hydroxyindoleacetic acid, MIAA = 5-methoxyindoleacetic acid, MAOP = monoamine oxidase products.

[1] as compared with day study, $p < 0.05$, Student's t test.

TABLE 3.2 *Metabolism of [^{14}C]-serotonin by organ culture of rat pineal glands (means ± SEM, n = 6).*

	DPM recovered as:					% of total counts	
	MEL	NAS	HIAA	MIAA	Total MAOP	MEL	MAOP
Serotonin (DS)	1 364 ± 92	1 146 ± 107	18 428 ± 819	332 ± 68	25 962	1.1	20.1
Serotonin (NS)	5 605[1] ± 348	3 422[1] ± 829	29 581[1] ± 1 103	267 ± 15	40 888	4.3	31.6

(DS) Day Study; (NS) Night Study.

MEL = melatonin, NAS = N-acetylserotonin, HIAA = 5-hydroxyindoleacetic acid, MIAA = 5-methoxyindoleacetic acid, MAOP = monoamine oxidase products.

[1] as compared with day study, $p < 0.05$, Student's t test.

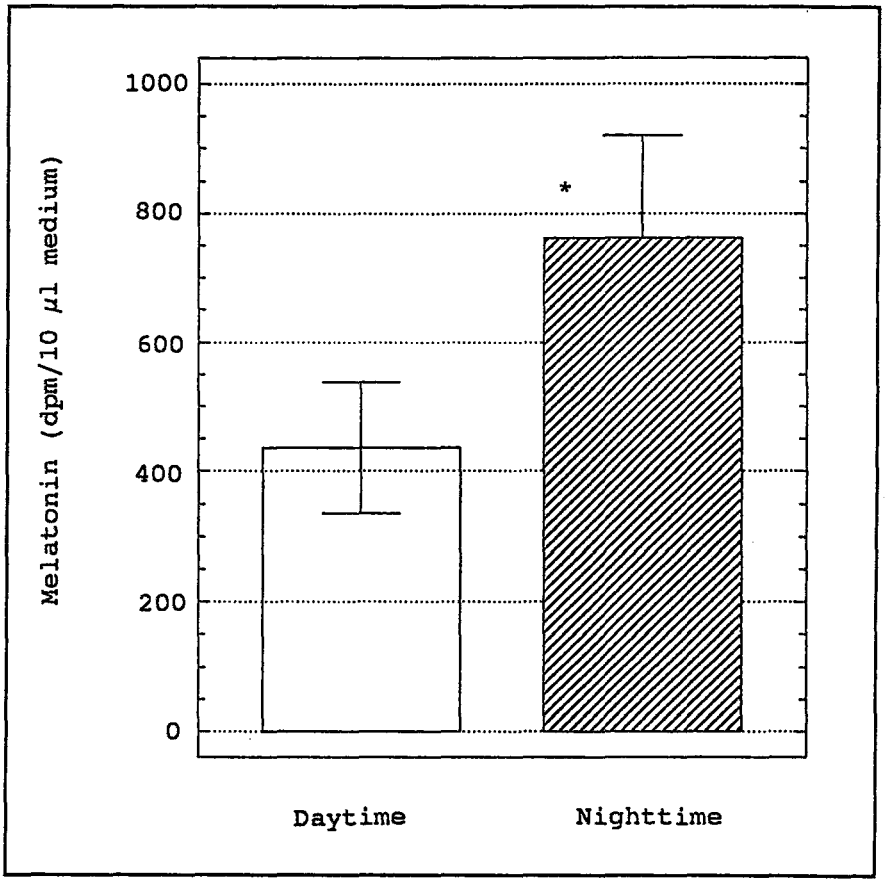

FIGURE 3.7 *The formation of melatonin from [^{14}C]-tryptophan by pineal glands in organ culture (means ± SEM, n = 6).*

* as compared with the day study, $p < 0.05$, Student's t test.

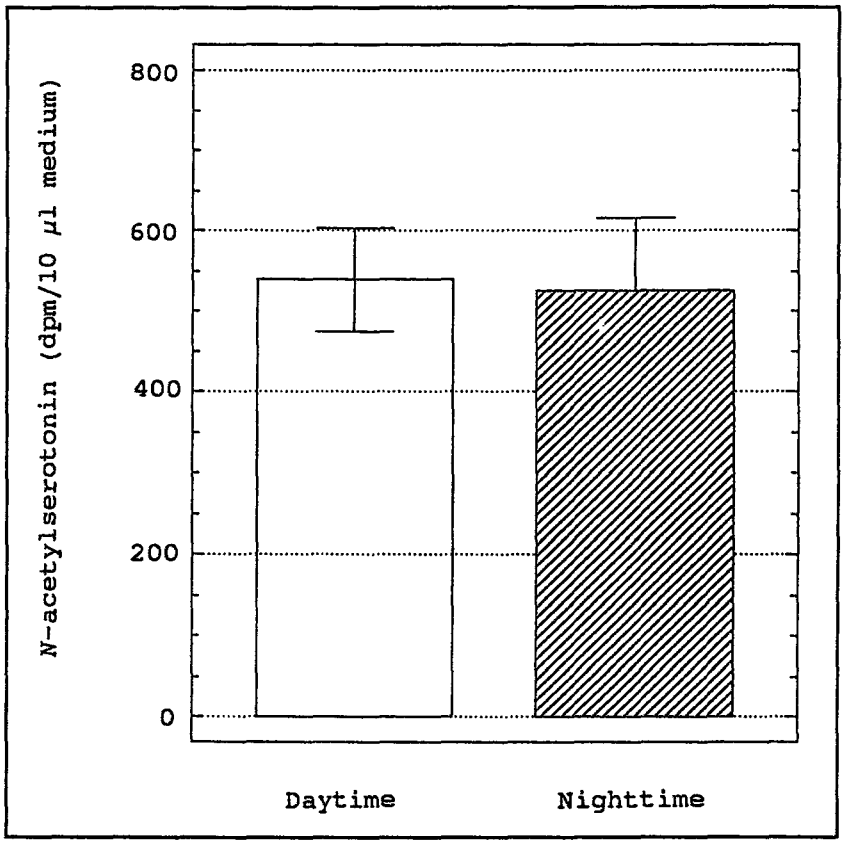

FIGURE 3.8 *The formation of N-acetylserotonin from [^{14}C]-tryptophan by pineal glands in organ culture (means ± SEM, n = 6).*

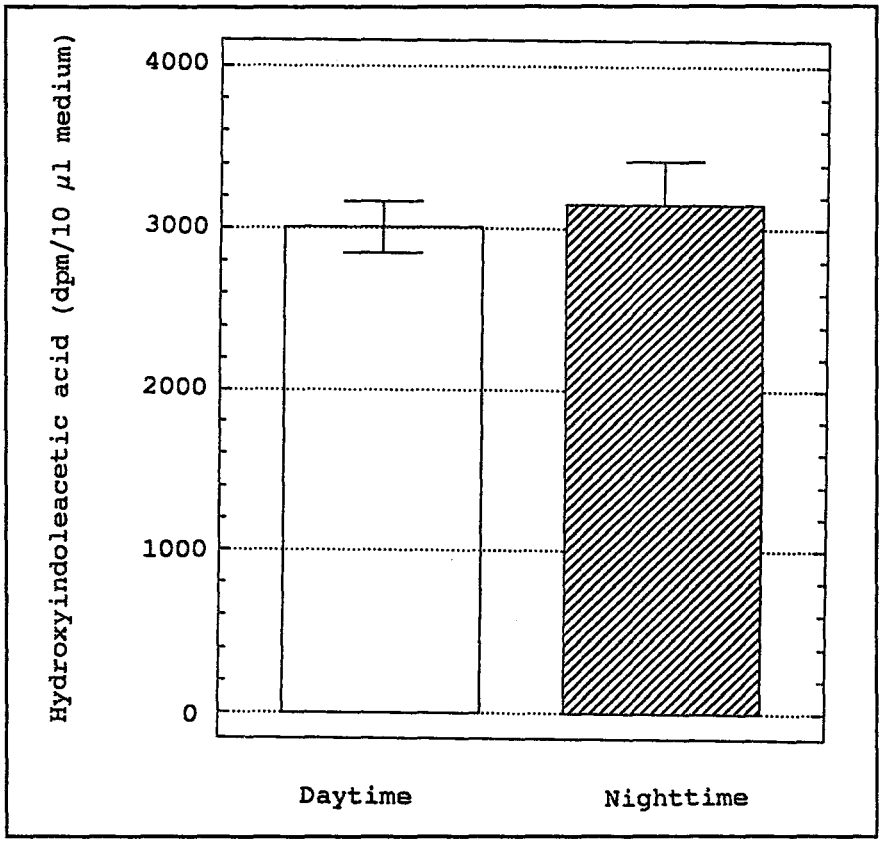

FIGURE 3.9 *The formation of 5-hydroxyindoleacetic acid from [^{14}C]-tryptophan by pineal glands in organ culture (means ± SEM, n = 6).*

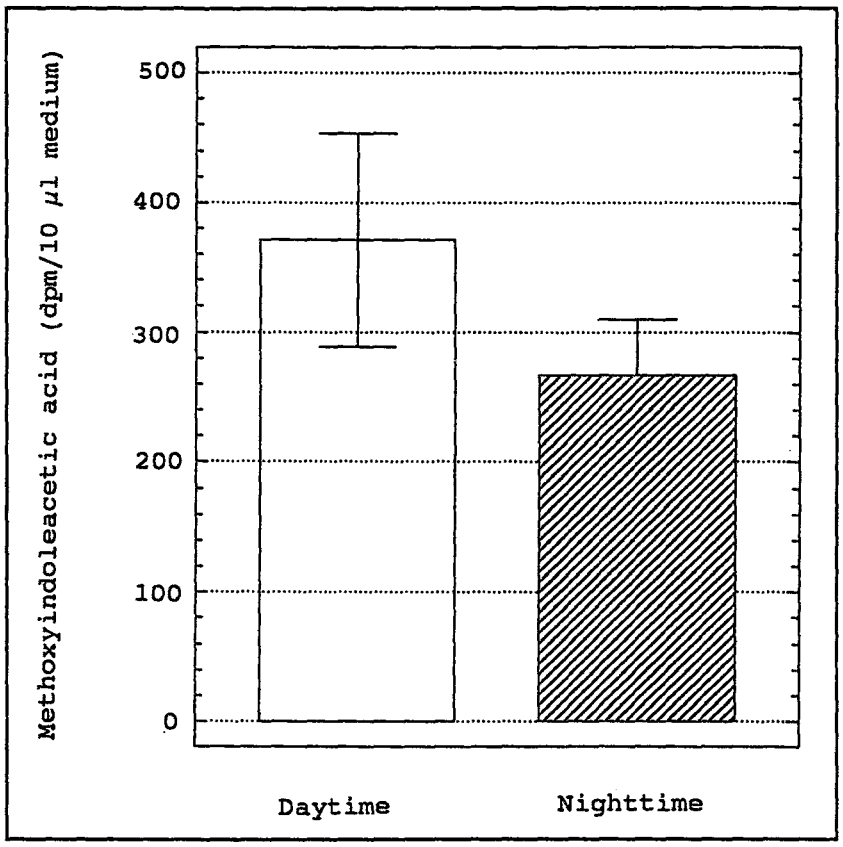

FIGURE 3.10 *The formation of 5-methoxyindoleacetic acid from [^{14}C]-tryptophan by pineal glands in organ culture (means ± SEM, n = 6).*

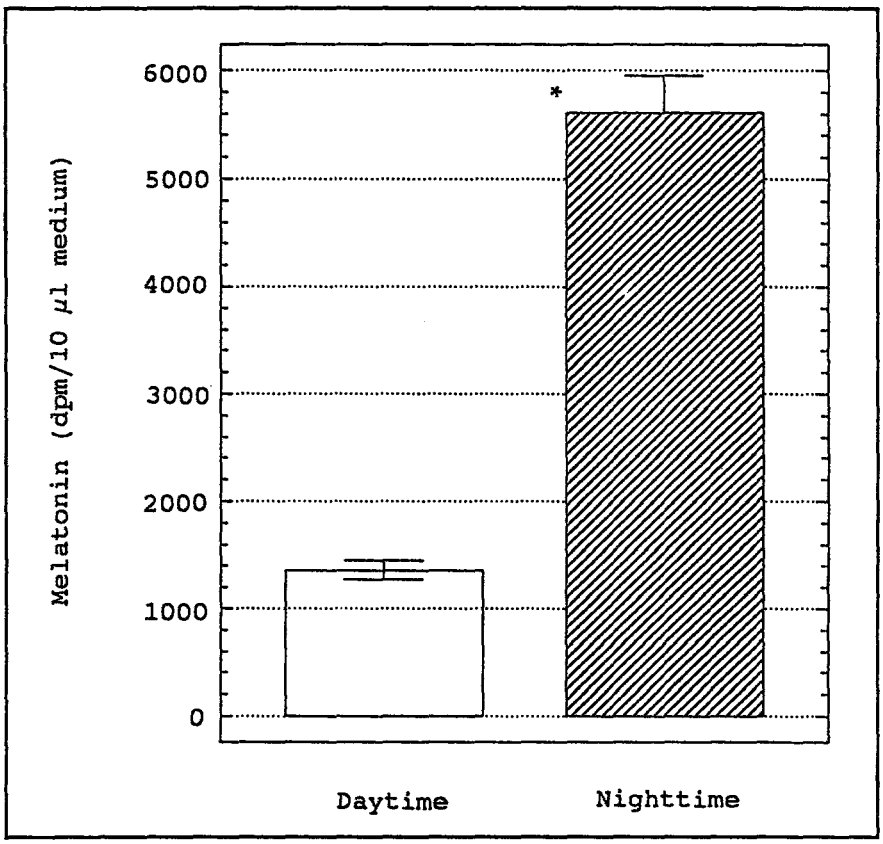

FIGURE 3.11 *The formation of melatonin from [^{14}C]-serotonin by pineal glands in organ culture (means ± SEM, n = 6).*

* as compared with the day study, $p < 0.05$, Student's t test.

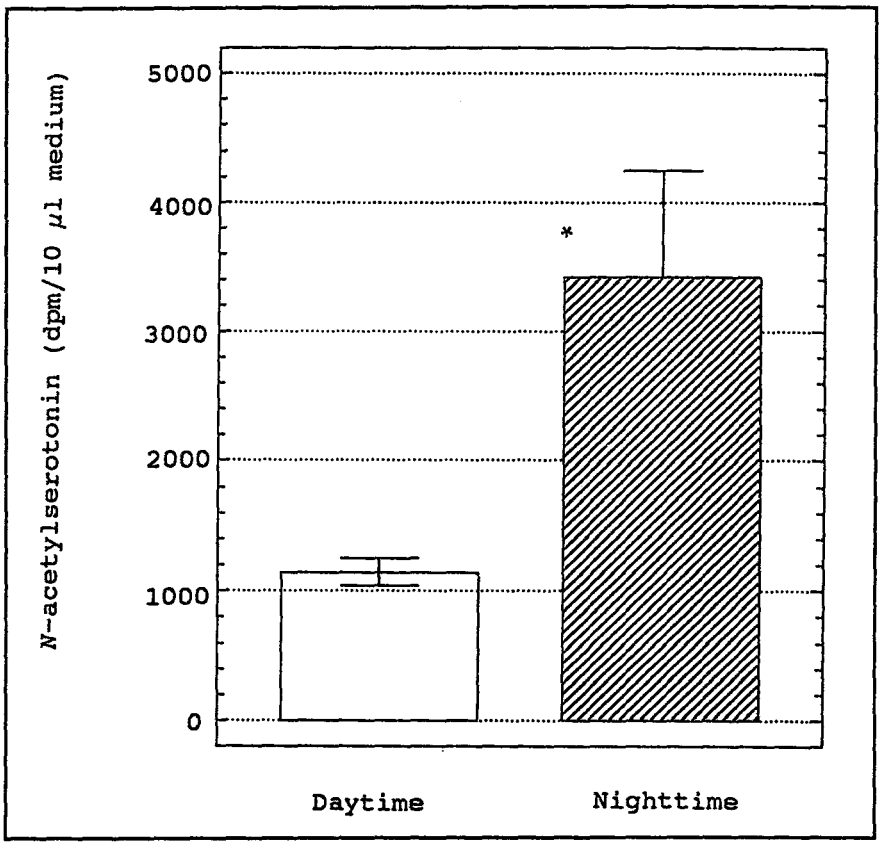

FIGURE 3.12 *The formation of N-acetylserotonin from [^{14}C]-serotonin by pineal glands in organ culture (means ± SEM, n = 6).*

* as compared with the day study, $p < 0.05$, Student's t test.

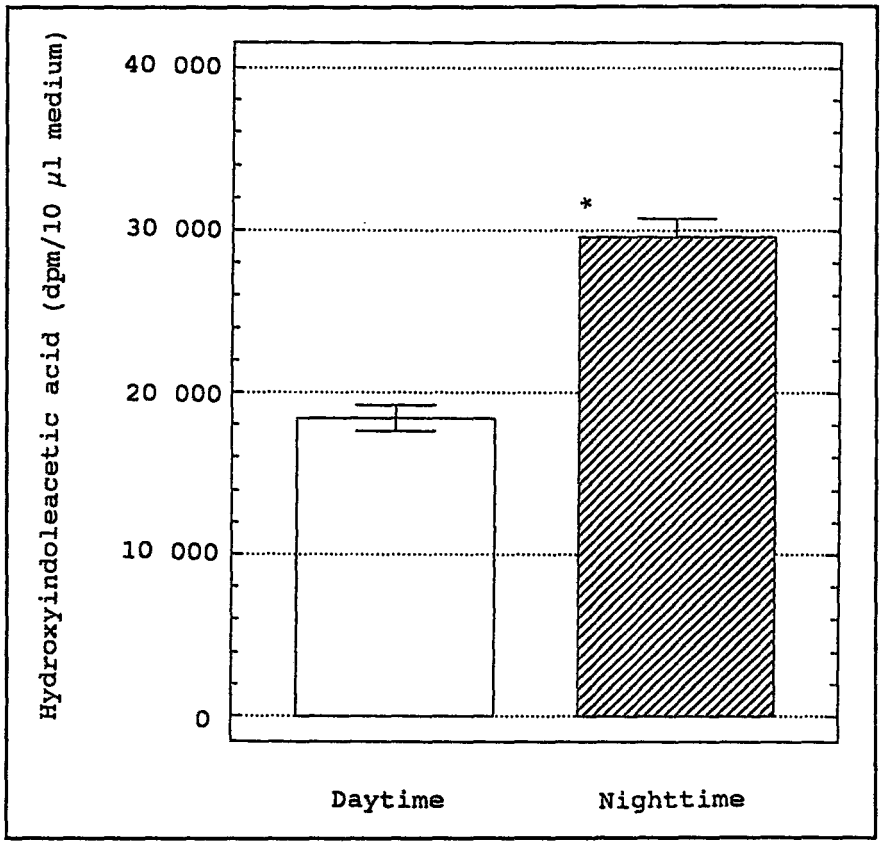

FIGURE 3.13 *The formation of 5-hydroxyindoleacetic acid from [^{14}C]-serotonin by pineal glands in organ culture (means ± SEM, n = 6).*

* as compared with the day study, $p < 0.05$, Student's t test.

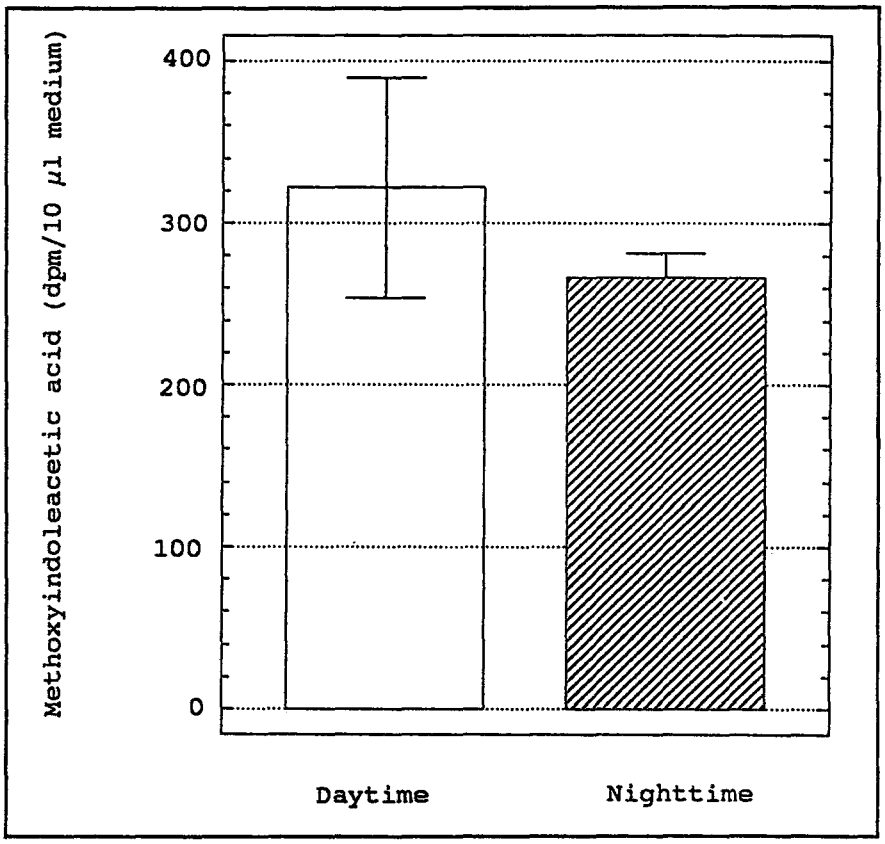

FIGURE 3.14 *The formation of 5-methoxyindoleacetic acid from [^{14}C]-serotonin by pineal glands in organ culture (means ± SEM, n = 6).*

3.4 Conclusion

The results of the tryptophan profile show that nor-adrenaline stimulates *in vitro* pineal indole synthesis. It appears that N-acetyltransferase is stimulated to a greater extent than hydroxyindole-O-methyltransferase, resulting in an accumulation of N-acetylserotonin. 5-Hydroxyindole levels were found to be high in comparison with 5-methoxyindole levels, suggesting possible β-adrenergic receptor involvement in monoamine oxidase regulation.

The results show serotonin to be more readily metabolised to N-acetylserotonin and melatonin than tryptophan. This is probably due to the fact that serotonin only has to undergo a two-stage conversion to melatonin, whereas tryptophan enters the pathway at an earlier stage. Once again, 5-hydroxyindoleacetic acid was found to be a major metabolite, pointing to monoamine oxidase involvement.

CHAPTER IV

THE EFFECT OF S-ADENOSYL-L-METHIONINE ON PINEAL FUNCTION

4.1 Introduction

S-adenosylmethionine (SAM) is found in large quantities in pineal tissue (38.4 $\pm$ 2.7 μg/g tissue) (Baldessarini and Kopin, 1966). It is synthesised through the transfer of an adenosyl group from ATP to the sulphur atom of methionine. The methyl group of the methionine unit is activated, for donation, by the positive charge on the adjacent sulphur atom (Guchait and Grau, 1978). SAM is said to be the most important methyl donor in biological transmethylation reactions, including the methylation of biogenic amines such as melatonin (Quay, 1981). A closely related derivative, S-adenosylhomocysteine, which is formed either from homocysteine or by the loss of the active methyl group from SAM, inhibits the methylation of biogenic amines (Deguchi and Barchas, 1971).

Recently, Zhong *et al.* (1990) showed that brain slices pre-incubated with SAM before being stimulated with nor-adrenaline or isoproterenol, potentiated cAMP production. This effect has not been demonstrated in the pineal gland in the literature investigated to date.

The present study was carried out to determine SAM's effect on pineal function. For this investigation three assay methods were used, namely: organ culture assay, NAT assay and cAMP assay.

4.2 Experiment 1: The Effect of SAM on Pineal Indoleamine Metabolism.

4.2.1 Introduction

Research thus far has been restricted to examining the role of SAM in transmethylation reactions within the pineal gland. A recent report by Zhong *et al.* (1990), showed that SAM potentiated cAMP production in brain slices, stimulated via the β-adrenergic receptor.

The effect of SAM on the pineal gland was investigated using organ culture studies. This technique provides a holistic view of pineal activity through the measurement of pineal indoleamine levels.

4.2.2 Materials and Method

Male Wistar rats weighing between 200 and 250 g were used in this study. The animals were maintained as described in **§ 2.1.** The animals were sacrificed by neck fracture at 11h00 for daytime studies and at 23h00 for nighttime studies. The collection of pineals for the nighttime studies was performed according to **§ 2.1.**

Chemicals and Reagents: 5-Hydroxy [side chain-2-^{14}C]-tryptamine creatine sulphate (specific activity 57 mCi/mmol) was purchased from Amersham (England). 5-Adenosyl-l-methionine was purchased from Boehringer Mannheim (West Germany) and l-isoproterenol from Sigma Chemical Co. (USA). All other chemicals and reagents were purchased from local commercial sources.

Organ Culture: The pineal organ culture technique and the analysis of the radio-labelled indoles formed were performed as outlined in **§ 2.2.2.**

Experiment A: After sacrifice, the pineal glands were swiftly removed and placed individually into sterile glass tubes, each containing 52 μl BGJb culture medium in the presence of (n = 5) and absence of (n = 5) 10 μl of SAM at a final concentration of 10^{-5}M. All tubes were incubated at 37°C in a humidified atmosphere of 95% O_2 and 5% CO_2 for

three hours, whereafter 8 μl of [^{14}C]-serotonin was added to all tubes. The tubes were then re-incubated under the same conditions for 21 hours.

In the case of isoproterenol-stimulated pineals, tubes were pre-incubated in the presence of (n = 5) and absence of (n = 5) 10^{-5}M SAM at 37°C in a humidified atmosphere of 95% O_2 and 5% CO_2. After three hours, isoproterenol at a final concentration of 10^{-5}M and [^{14}C]-serotonin were added to all tubes, whereafter they were all re-incubated for 21 hours.

The procedures mentioned above were followed for both daytime and nighttime studies.

Experiment B: After sacrifice, the pineal glands were swiftly removed and placed individually into sterile glass tubes, each containing 52 μl BGJb culture medium in the presence of (n = 5) and absence of (n = 5) 10 μl of SAM at a final concentration of 10^{-5}M. All tubes were incubated at 37°C in a humidified atmosphere of 95% O_2 and 5% CO_2 for three hours, whereafter 8 μl of [^{14}C]-serotonin was added to all tubes. The tubes were then re-incubated under the same conditions for four hours.

In the case of isoproterenol-stimulated pineals, tubes were pre-incubated in the presence of (n = 5) and absence of (n = 5) 10^{-5}M SAM at 37°C in a humidified atmosphere of 95% O_2 and 5% CO_2. After three hours, isoproterenol at a final concentration of 10^{-5}M and [^{14}C]-serotonin were added to all tubes, whereafter they were all re-incubated for four hours.

The procedures mentioned above were performed for daytime studies only.

After the incubation period (Experiment A, 24 hours and Experiment B, four hours) a 10 μl aliquot of culture medium was removed from each tube for analysis. The indoles in the 10 μl aliquots were isolated by TLC and quantified by liquid scintillometry as described in § **2.2.2.**

All data were statistically compared using Student's t test and expressed as the mean of five separate incubations $\pm$ SEM.

4.2.3 Results

Experiment A: SAM showed no significant effect on daytime indole metabolism in both unstimulated (**Table 4.1**) and stimulated (**Table 4.2**) pineal glands. Similarly, SAM showed no effect on nighttime indole metabolism in both unstimulated (**Table 4.3**) and stimulated (**Table 4.4**) pineal glands.

Significant differences ($p < 0.05$) were however found between stimulated and unstimulated glands in both day and nighttime studies .

Experiment B: After the four hour incubation the results followed a similar trend to that of Experiment A. No significant effect was shown by SAM on both unstimulated (**Table 4.5**) and stimulated (**Table 4.6**) pineal glands.

Significant differences ($p < 0.05$) were however found between stimulated and unstimulated glands.

4.2.4 Discussion

SAM showed no effect on the metabolism of serotonin to N-acetylserotonin and melatonin by pineal glands in organ culture. This was observed in both day and nighttime experiments. The expected rise in the metabolism of indoles to N-acetylserotonin and melatonin in pineals stimulated with isoproterenol was observed in both day and nighttime studies. These findings support those of other workers (Axelrod *et al.*, 1969; Deguchi, 1973) who demonstrated the role of β-adrenergic receptor stimulation in indole metabolism.

The absence of a significant effect in the 24 hour organ culture experiments promoted further investigations involving SAM. These investigations examined the role SAM played during the initial period of pineal indoleamine metabolism, *i.e.* the first four hours of metabolism. The early stage of metabolism is characterised by a sharp rise in melatonin and N-acetylserotonin levels with peaks being reached after approximately 16 hours (Banoo *et al.*, 1987).

SAM was once again found to exert no significant effect on the early stages of indoleamine metabolism in both stimulated and unstimulated pineal glands. The rise in pineal indoles was observed to be greater in those pineals stimulated with isoproterenol.

TABLE 4.1 *The effect of S-adenosyl-l-methionine pre-incubation on daytime unstimulated 24 hour pineal indole metabolism (means ± SEM; n = 5).*

	DPM recovered as:			
	MEL	**NAS**	**MAOP**	**Total Counts**
Control	1 687 ± 101	1 455 ± 86	32 095	35 237
SAM	1 676 ± 98	1 592 ± 93	30 444	33 712

SAM = S-adenosyl-l-methionine; MEL = melatonin; NAS = N-acetylserotonin; MAOP = monoamine oxidase products.

TABLE 4.2 *The effect of S-adenosyl-l-methionine pre-incubation on daytime isoproterenol-stimulated 24 hour pineal indole metabolism (means ± SEM; n = 5).*

	DPM recovered as:			
	MEL	**NAS**	**MAOP**	**Total Counts**
Control	3 319 ± 391	3 865 ± 610	28 662	35 846
SAM	3 465 ± 472	3 876 ± 560	37 989	45 330

SAM = S-adenosyl-l-methionine; MEL = melatonin; NAS = N-acetylserotonin; MAOP = monoamine oxidase products.

TABLE 4.3 *The effect of S-adenosyl-l-methionine on nighttime unstimulated 24 hour pineal indole metabolism (means ± SEM; n = 5).*

	DPM recovered as:			
	MEL	**NAS**	**MAOP**	**Total Counts**
Control	6 210 ± 1 260	5 999 ± 840	53 304	65 513
SAM	6 160 ± 941	5 554 ± 902	56 489	68 203

SAM = S-adenosyl-l-methionine; MEL = melatonin; NAS = N-acetylserotonin; MAOP = monoamine oxidase products.

TABLE 4.4 *The effect of S-adenosyl-l-methionine pre-incubation on nighttime isoproterenol-stimulated 24 hour pineal indole metabolism (means ± SEM; n = 5).*

	DPM recovered as:			
	MEL	**NAS**	**MAOP**	**Total Counts**
Control	7 836 ± 920	10 116 ± 1 480	47 871	65 823
SAM	6 004 ± 1 290	9 016 ± 1 321	47 174	62 194

SAM = S-adenosyl-l-methionine; MEL = melatonin; NAS = N-acetylserotonin; MAOP = monoamine oxidase products.

TABLE 4.5 *The effect of S-adenosyl-l-methionine on daytime unstimulated four hour pineal indole metabolism (means ± SEM; n = 5).*

	DPM recovered as:			
	MEL	**NAS**	**MAOP**	**Total Counts**
Control	395 ± 26	700 ± 83	13 591	14 686
SAM	368 ± 53	543 ± 51	17 400	18 311

SAM = S-adenosyl-l-methionine; MEL = melatonin; NAS = N-acetylserotonin; MAOP = monoamine oxidase products.

TABLE 4.6 *The effect of S-adenosyl-l-methionine pre-incubation on daytime isoproterenol-stimulated four hour pineal indole metabolism (means ± SEM; n = 5).*

	DPM recovered as:			
	MEL	**NAS**	**MAOP**	**Total Counts**
Control	688 ± 34	997 ± 93	24 627	26 312
SAM	779 ± 71	969 ± 84	20 275	22 023

SAM = S-adenosyl-l-methionine; MEL = melatonin; NAS = N-acetylserotonin; MAOP = monoamine oxidase products.

4.3 Experiment 2: The Effect of *In Vitro* SAM Pre-incubation on Pineal NAT Activity.

4.3.1 Introduction

Serotonin N-acetyltransferase (NAT) is thought to be the rate limiting step in pineal indole metabolism (Klein *et al.*, 1978). Interest in NAT regulation has grown due to its function as the enzyme responsible for the synthesis of N-acetylserotonin, the precursor of the pineal hormone melatonin.

The reliance of NAT on cAMP for its synthesis and activation has been shown by many researchers. It is this fact and recent work by Zhong *et al.* (1990) which showed SAM to potentiate isoproterenol stimulation of cAMP, that led to this study.

4.3.2 Materials and Method

Male Wistar rats weighing between 200 and 250 g were used in this study. The animals were maintained as described in § **2.1**. The animals were sacrificed by neck fracture at 11h00.

Chemicals and Reagents: [1-^{14}C]-Acetyl coenzyme A (specific activity 4.0 mCi/mmol) was purchased from Dupont Chemical Co. (USA). Tryptamine HCl and l-isoproterenol were purchased from Sigma Chemical Co. (USA) and unlabelled acetyl coenzyme A from Merck (West Germany). All other chemicals and reagents were purchased from local commercial sources.

NAT Assay: After sacrifice, the pineal glands were swiftly removed and individually placed into sterile glass tubes each containing 60 μl of culture medium and SAM (n = 5) to a final concentration of 10^{-5}M. Controls (n = 5) contained only culture medium. The tubes were incubated at 37°C in a humidified atmosphere of 95% O_2 and 5% CO_2 for three hours. After the incubation period, pineal glands were removed from their tubes, pooled, homogenised in 500 μl of buffer and assayed for NAT activity according to § **2.3.2**.

In isoproterenol-stimulated pineal glands, the pineals were initially pre-incubated in the presence of (n = 5) and absence of (n = 5) 10^{-5}M SAM for three hours prior to 10^{-5}M isoproterenol administration. Tubes were re-incubated for three hours, whereafter the pineals were removed from their tubes, pooled, homogenised in 500 μl of buffer and assayed for NAT activity according to **§ 2.3.2.**

All data were statistically compared using the Student's t test and expressed as NAT activity (pmol/gland/hour).

4.3.3 Results

SAM exhibited no significant effect on both unstimulated (**Figure 4.1**) and stimulated (**Figure 4.2**) pineal NAT activity. A significant difference ($p < 0.05$) was however found between stimulated and unstimulated groups.

4.3.4 Discussion

The pre-incubation of pineal glands with 10^{-5}M SAM had little effect on pineal NAT activity in both stimulated and unstimulated glands. The expected rise in NAT activity in the presence of isoproterenol, a β-receptor agonist, was observed. This rise was significant ($p < 0.05$) when compared with unstimulated values.

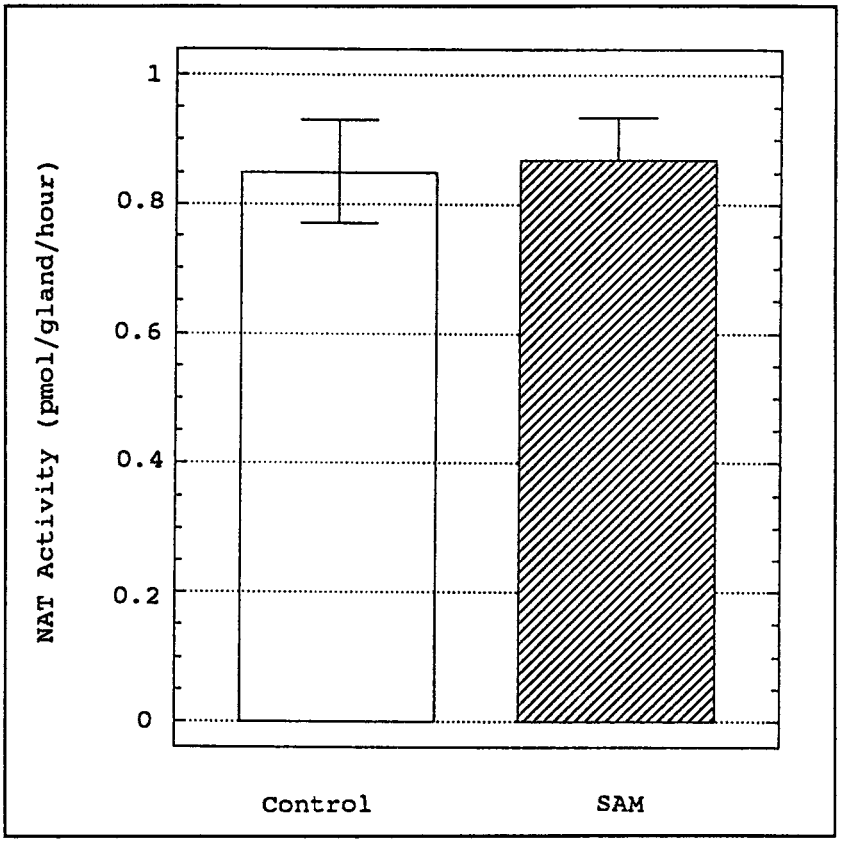

FIGURE 4.1 *The effect of 10^{-5}M SAM on daytime unstimulated rat pineal NAT activity (means ± SEM, n = 5).*

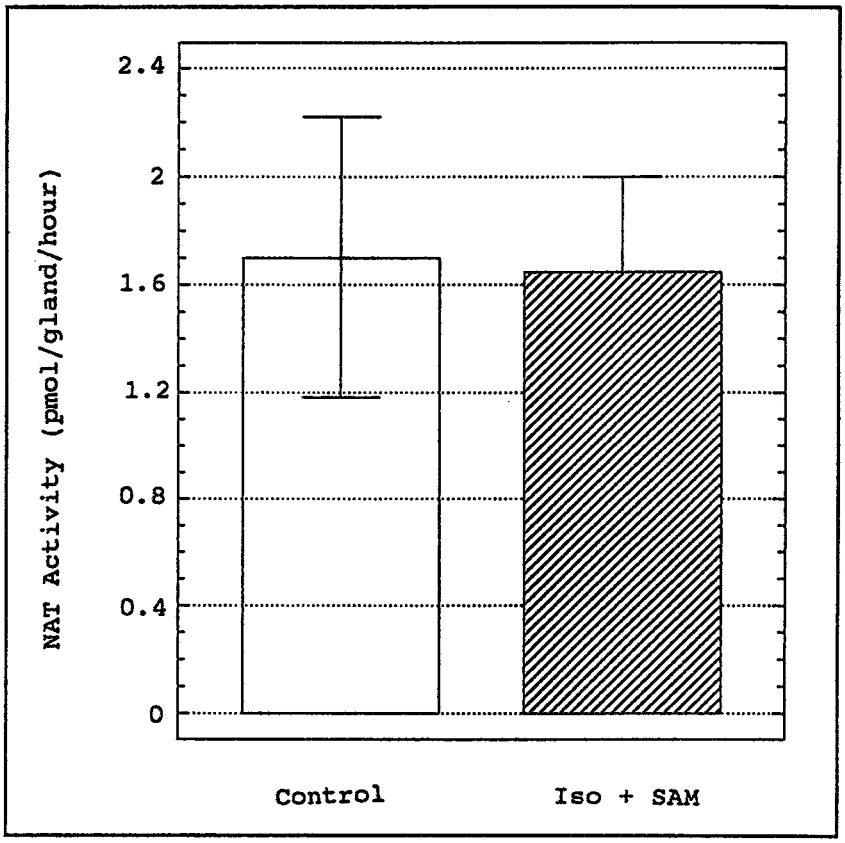

FIGURE 4.2 *The effect of 10^{-5}M SAM on daytime isoproterenol-stimulated rat pineal NAT activity (means ± SEM, n = 5).*

4.4 Experiment 3: The Effect of *In Vitro* SAM Pre-incubations on Pineal cAMP Levels.

4.4.1 Introduction

The existence of cAMP was first shown by Sutherland and Rall (1958). Sutherland further showed that cAMP was synthesised from ATP via a membrane bound enzyme, adenylate cyclase, and was metabolised to AMP via the enzyme cAMP-phosphodiesterase (Sutherland *et al.*, 1968).

The importance of cAMP as a second messenger within the cell and its effect on NAT have been well documented. Stimulation of β-adrenergic receptors results in an increase in cAMP levels in both brain and pineal tissue (Axelrod, 1974). SAM has been shown to increase brain receptor-mediated cAMP production *in vitro* (Zhong *et al.*, 1990). The mechanism for this effect in brain tissue is not known but both phospholipid methylation (Hirata and Axelrod, 1980) and protein carboxy methylation (Buckland and Aksamit, 1988) by SAM have been proposed as important steps in the signal transduction.

This study investigated the effect of *in vitro* SAM pre-incubations on pineal cAMP levels.

4.4.2 Materials and Method

Male Wistar rats weighing between 200 and 250 g were used in this study. The animals were maintained as described in **§ 2.1**. The animals were sacrificed by neck fracture at 11h00.

Chemicals and Reagents: Tritiated cAMP (specific activity 30 Ci/mmol) was purchased from Amersham (England), standard cAMP from Boehringer-Mannheim (West Germany), theophylline and l-isoproterenol from Sigma Chemical Co. (USA) and activated charcoal form Merck (West Germany). All other chemicals and reagents were purchased from local commercial sources.

Cyclic AMP Assay: The preparation of bovine cAMP-binding protein, pineal tissue and assay procedure were carried out as described in **§ 2.4.2**.

Briefly, rats were sacrificed and the pineal glands rapidly removed. Pineals were placed into sterile glass tubes containing 300 μl of BGJb culture medium in the presence and absence of 10^{-5}M SAM. The tubes were pre-incubated at 37°C for three hours in a humidified atmosphere of 95% O_2 and 5% CO_2. After the pre-incubation, Group 1 (n = 5) did not receive any further treatment and was used as the control. Group 2 (n = 5), which had been exposed to SAM (10^{-5}M), did not receive any further treatment. Group 3 (n = 5) received isoproterenol at a final concentration of 10^{-5}M and Group 4 (n = 5), which had been exposed to SAM (10^{-5}M), received isoproterenol at a final concentration of 10^{-5}M. After the above treatments, all pineals were re-incubated for five minutes at 37°C in a humidified atmosphere.

Two further experiments were performed as described above with the exception that pineals were re-incubated for 15 and 30 minutes respectively.

The incubations were terminated after five, 15 or 30 minutes respectively by homogenising each pineal individually in 100 μl of ice-cold phosphate buffer, pH 6.8. The assay was then carried out as outlined in **§ 2.4.2** and **Table 2.3.**

All data were compared using Student's t test and expressed as cAMP concentration (pmol/gland).

4.4.3 Results

Cyclic AMP levels showed little variation in daytime unstimulated pineal glands in the presence or absence of SAM. This held true for all incubation times (**Figures 4.3, 4.4** and **4.5**).

The opposite was found in daytime isoproterenol-stimulated pineal glands. At incubation times of five and 15 minutes (**Figures 4.3** and **4.4** respectively) SAM pre-incubation was found to significantly ($p < 0.05$) reduce the stimulatory effect of isoproterenol on cAMP levels. This was, however, not the case after the 30 minute incubation (**Figure 4.5**) where cAMP levels were found to be similar in the presence or absence of SAM. No significant differences were observed between the SAM pre-incubated isoproterenol-stimulated groups.

4.4.4 Discussion

The stimulation of β-adrenergic receptors is essential for cAMP production (Axelrod, 1974). cAMP levels climb rapidly in the first 10 to 15 minutes of receptor stimulation and return to base-line levels after approximately 30 minutes (Deguchi, 1973). The results of the control groups in this experiment are consistent with the above. On the other hand, the pre-incubation of pineals with SAM was found to have a profound effect on isoproterenol-stimulated cAMP levels. The characteristic climb of cAMP levels during the first 15 minutes of stimulation was markedly reduced. This inhibitory effect was, however, not observed after 30 minutes, which is typically marked by a return to base-line levels of cAMP. This phenomenon has been shown to be related to a decrease in adenylate cyclase activity (Deguchi, 1973).

Interestingly, the inhibitory effect of SAM was found to be limited. Cyclic AMP levels were at all times greater than the control levels, suggesting that even in the presence of SAM β-receptor stimulation was still taking place. This, in turn, suggests that SAM could be exerting its inhibitory effect distal to the β-adrenergic receptor *i.e.* either at the G protein, adenylate cyclase or on the enzyme cAMP-phosphodiesterase (by increasing its activity).

These speculations need further investigation.

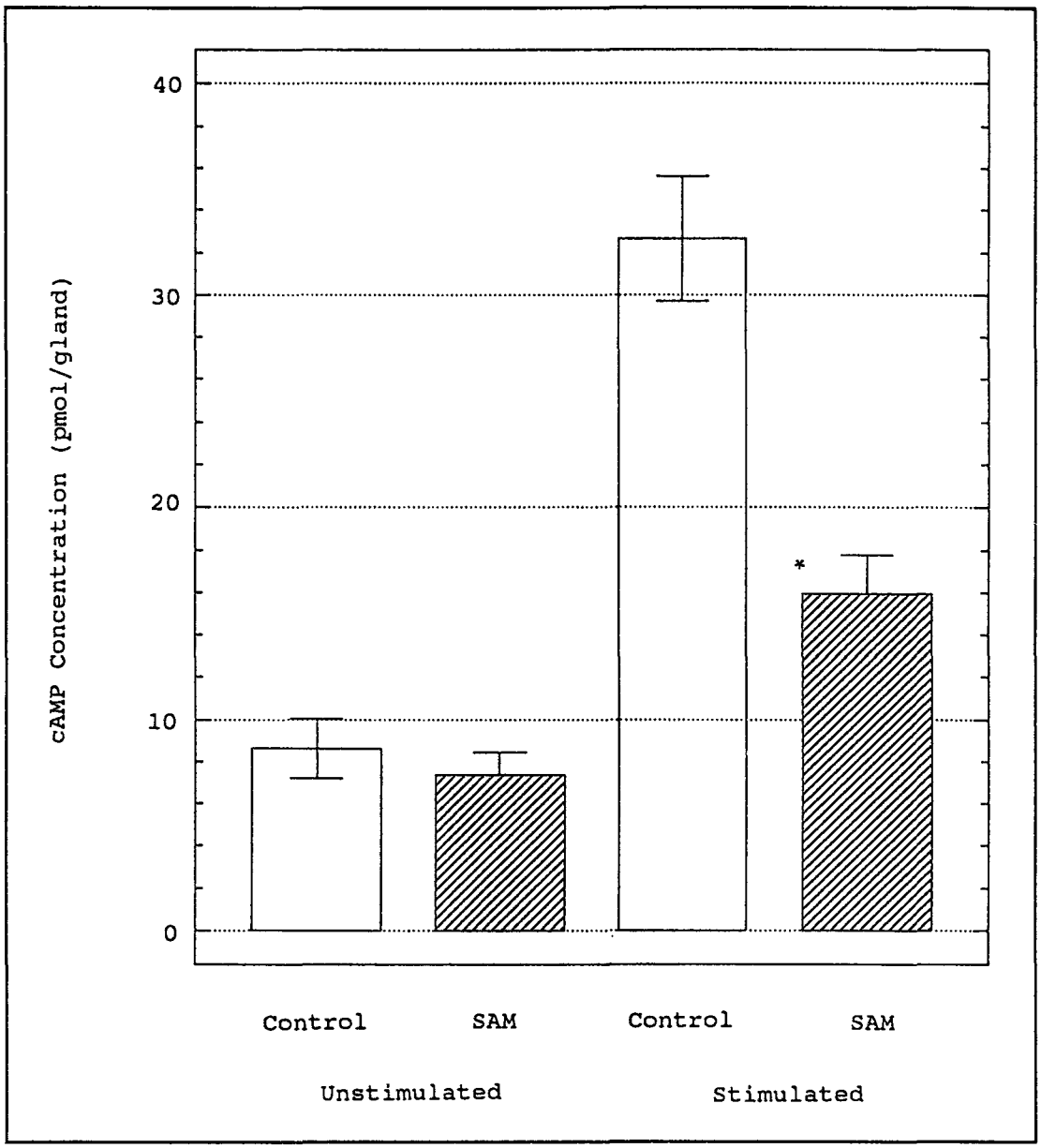

FIGURE 4.3 *The effect of in vitro SAM pre-incubation on five minute pineal cAMP levels, in the presence and absence of isoproterenol stimulation (means ± SEM, n = 5).*

* as compared with the isoproterenol-stimulated control, $p < 0.05$, Student's t test.

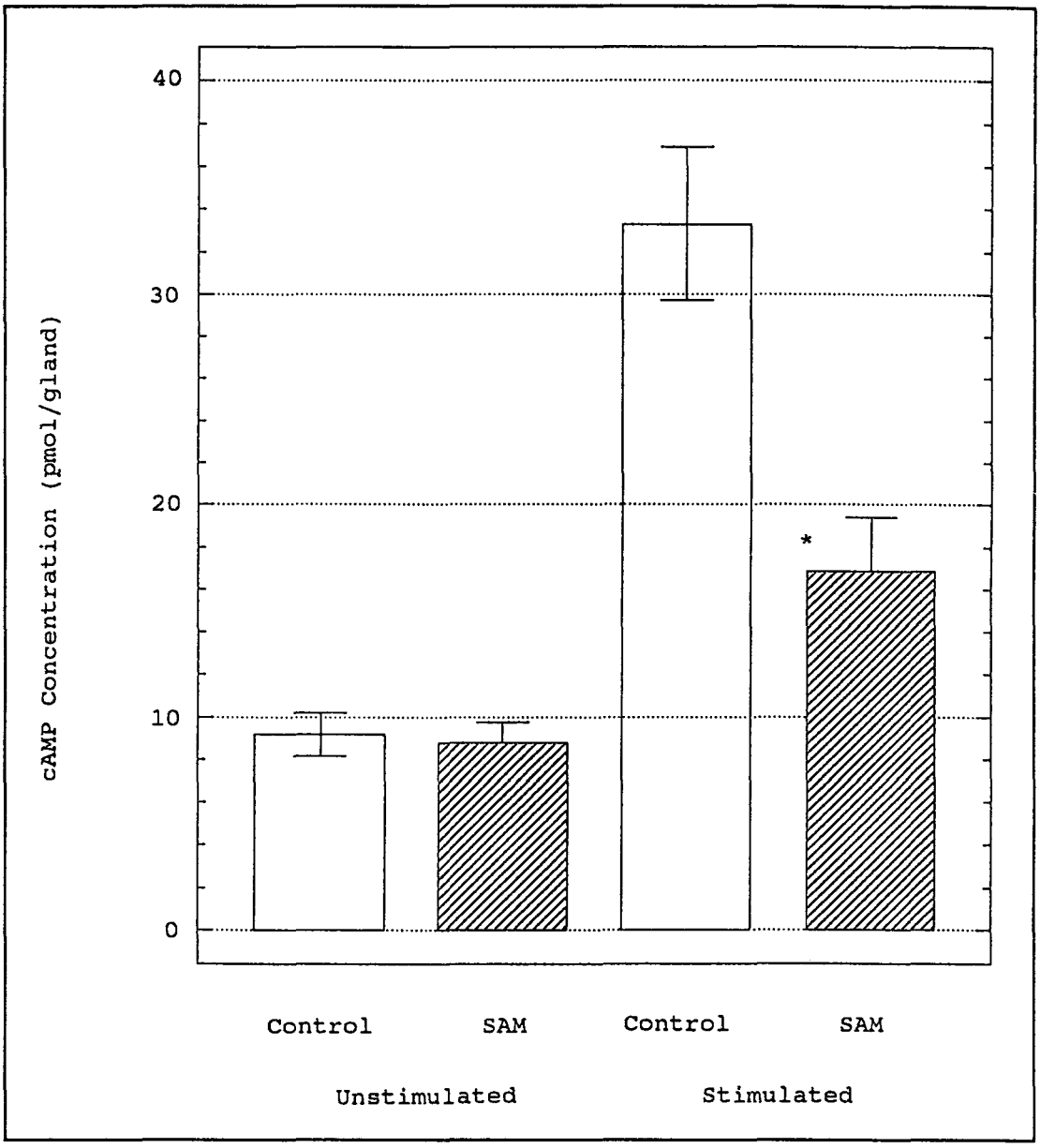

FIGURE 4.4 *The effect of in vitro SAM pre-incubation on 15 minute pineal cAMP levels in the presence and absence of isoproterenol stimulation (means ± SEM, n = 5).*

* as compared with the isoproterenol-stimulated control, $p < 0.05$, Student's t test.

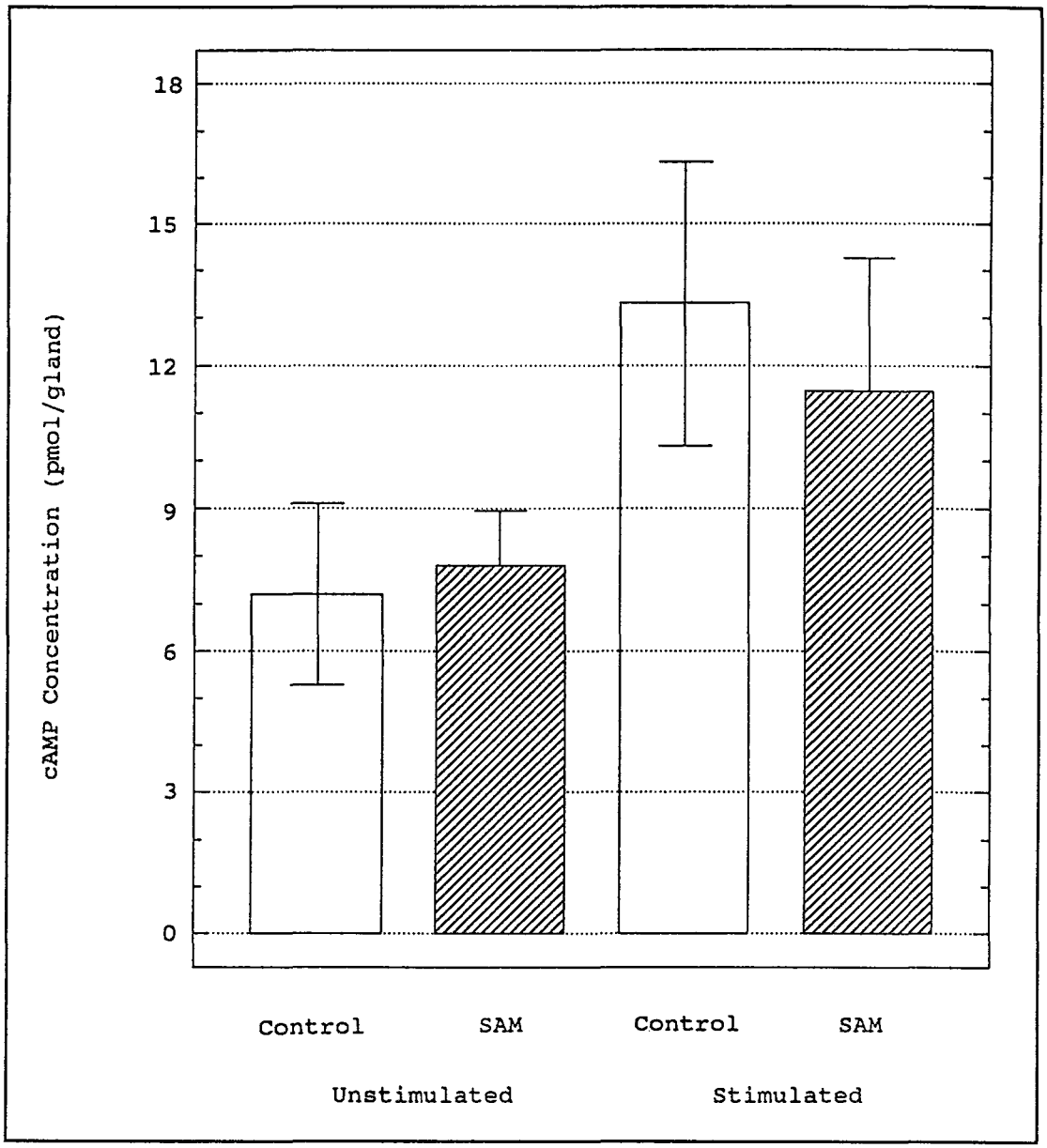

FIGURE 4.5 *The effect of in vitro SAM pre-incubation on 30 minute pineal cAMP levels in the presence and absence of isoproterenol stimulation (means ± SEM, n = 5).*

4.5 Conclusion

The rate-limiting enzyme in pineal indole metabolism, N-acetyltransferase (NAT), has been shown to be reliant on cAMP for its synthesis and activation (Klein *et al.*, 1978; Reiter, 1991). Isoproterenol stimulation of pineal glands results in an increase in NAT activity, which in turn results in increased indole metabolism (Deguchi and Axelrod, 1972b; Mata *et al.*, 1976).

Pre-incubation of pineal glands with SAM, in 24 hour organ cultures, was found to have no significant effect on daytime isoproterenol-stimulated metabolism. Studies by Banoo *et al.* (1987) showed that pineal indole metabolism increased sharply in the first four hours of incubation. The experiment was repeated with the incubation time changed to four hours. The purpose of the shorter incubation time was to investigate the possibility that SAM might exert an effect during the early stages of pineal metabolism. The results showed that SAM exhibited no significant effect in the early stages of pineal indole metabolism.

Further investigation into the effect of SAM on NAT found that the enzyme's activity remained unchanged in both the presence and absence of SAM. This was found in both stimulated and unstimulated pineal glands. These results thus support the findings of the organ culture assay.

Having performed the organ culture and NAT assays first and finding that SAM exerted no significant effects, it was presumed that cAMP levels would remain unchanged. This was however not the case.

Cyclic AMP levels were found to be significantly inhibited during the initial period of cAMP synthesis *i.e.* the first 15 minutes. These results are in direct contrast to those of Zhong *et al.* (1990) on brain tissue and to those of the organ culture and NAT assays performed earlier in this chapter.

An explanation for the conflicting results in this study could be that:

> in the cAMP studies, the inhibitory effect produced by SAM was limited (*i.e.* the inhibition was not great enough to reduce cAMP levels to below control values),

suggesting that β-receptor stimulation was still taking place in the presence of SAM. This could explain the attenuated β-receptor response since the cAMP concentration could still be enough to allow normal indole metabolism as shown in the organ culture and NAT assays.

This however does not explain the limited reduction in cAMP levels. It is possible that SAM affects cAMP levels by affecting regions distal to the β-receptor such as adenylate cyclase, the regulatory G-proteins or cAMP-phosphodiesterase. In these situations, SAM may reduce adenylate cyclase activity or increase cAMP-phosphodiesterase activity and thereby alter pineal cAMP concentrations.

These speculations, however, need further investigation.

CHAPTER V

PURINE NUCLEOTIDE ACTION ON PINEAL ACTIVITY

5.1 Introduction

Exposure of rats to light during darkness (Klein and Weller, 1972) or the blocking of β-adrenergic receptors in isoproterenol-stimulated rat pineal glands during darkness (Deguchi and Axelrod, 1972b) results in a rapid decline in N-acetyltransferase (NAT) activity. Maintenance of a high level of NAT activity requires continuous stimulation of the β-adrenergic receptors on the pinealocytes. It appears unlikely that the precipitous fall in NAT activity is linked to cyclic AMP (cAMP) since pineal cAMP returns to baseline levels 30 minutes after stimulation of the gland with β-adrenergic receptor agonists (Deguchi, 1973).

The exact mechanism by which pineal NAT is inactivated remains unexplained. However, a number of agents have been shown to improve the stability of stimulated pineal NAT activity by protecting against its inactivation. For example, Binkley *et al.* (1976) demonstrated that 4 mM acetyl coenzyme A protected against heat-induced inactivation of pineal NAT activity at 37°C. This finding was confirmed by Namboodiri *et al.* (1979) who also showed that high concentrations (20 mM) of nucleotide triphosphates and pyrophosphate protect against inactivation of NAT. However, it is unlikely that such concentrations could be reached in the pineal *in vivo*.

5.2 Experiment 1: The Effect of Adenosine 5′-Monophosphate (5′-AMP) on Pineal NAT Activity

5.2.1 Introduction

This study investigates the possibility that the product of cAMP inactivation, *viz.* adenosine 5′-monophosphate (5′-AMP), may be involved in the regulation of pineal NAT.

5.2.2 Materials and Method

Male Wistar rats weighing between 200 and 250 g were used in this study. The animals were maintained as described **§ 2.1**. The animals were sacrificed by neck fracture at 11h00 for daytime experiments and 23h00 for nighttime experiments.

The collection of pineal glands for the nighttime experiments was performed according to **§ 2.1**.

Chemicals and Reagents: [1-^{14}C]-Acetyl coenzyme A (specific activity 4.0 mCi/mmol) was purchased from Dupont Chemical Co. (USA). [8-^{3}H]-Adenosine 3′,5′-cyclic phosphate (specific activity 30 Ci/mmol) was purchased from Amersham (England). 5′-AMP and cAMP were purchased from Boehringer Mannheim (West Germany). l-Isoproterenol and theophylline were purchased from Sigma Chemical Co. (USA). All other chemicals and reagents were purchased from local commercial sources.

Experiment A: The following experiment was performed to examine the effect of 5′-AMP on pineal NAT activity. Animals were sacrificed during the day (n = 10) and during the night (n = 10). After sacrifice, in each light phase, pineals were swiftly removed and individually homogenised in small glass homogenisers containing 100 μl of phosphate buffer and assayed for NAT activity as described in **§ 2.3.2** in the absence of (n = 5) and presence of (n = 5) 10^{-5}M 5′-AMP.

Experiment B: The following experiment was performed to examine the effect of 5′-AMP on isoproterenol-stimulated pineal NAT activity during the daytime. Animals were injected

intraperitoneally (ip) with isoproterenol (5 mg/kg) three hours before sacrifice. After sacrifice the pineals were individually homogenised in 100 μl of phosphate buffer and assayed for NAT activity as described in **§ 2.3.2**, in the presence of (n = 5) and absence of (n = 5) 10^{-5}M 5′-AMP.

Experiment C: The following experiment was performed to examine the effect of a range of 5′-AMP concentrations on isoproterenol-stimulated pineal NAT activity. Animals were injected ip with isoproterenol (5 mg/kg) three hours before sacrifice. After sacrifice the pineals were pooled and homogenised in 500 μl of phosphate buffer and assayed for NAT activity as described in **§ 2.3.2** in the presence of various concentrations of 5′-AMP ranging from 10^{-8}M to 10^{-3}M.

Experiment D: The following experiment was performed to examine the effect of an inhibitor of 5′-AMP formation, *viz.* theophylline, on pineal NAT and cAMP levels. Animals housed in darkness were injected ip with theophylline (10 mg/kg) at 21h00 with the aid of a dim, photo-safe red light. The animals were sacrificed at 23h00, in the dark, whereafter the pineals were collected and assayed for NAT activity and cAMP as described in **§ 2.3.2** and **§ 2.4.2** respectively. In order to perform the above experiment, a cAMP-phosphodiesterase assay (**§ 2.5.2**) was performed to investigate the diurnal variation in phosphodiesterase activity.
For the phosphodiesterase assay, the animals were sacrificed at 11h00 during the day and at 23h00 during the night.

Statistical differences between groups were determined using Student's t test.

5.2.3 Results

Experiment A: 5′-AMP was found to have no effect on daytime NAT activity (**Figure 5.1**). However, in the nighttime study, 5′-AMP caused a significant ($p < 0.05$) enhancement of the dark-induced rise of this enzyme compared to controls (**Figure 5.2**).

Experiment B: 5′-AMP (10^{-5}M) was found to potentiate the isoproterenol-induced rise in NAT activity (**Figure 5.3**).

Experiment C: The isoproterenol-induced rise in NAT activity observed in Experiment B (**Figure 5.3**) was also found to increase with increasing concentrations of 5′-AMP ranging between 10^{-8}M and 10^{-3}M (**Figure 5.4**).

Experiment D: Pineal cAMP-phosphodiesterase activity showed a significant ($p < 0.05$) increase during the dark phase (**Figure 5.5**). The administration of theophylline (10 mg/kg) two hours prior to sacrifice, in the early part of the dark phase, caused a significant ($p < 0.05$) increase in pineal cAMP (**Figure 5.6**). This increase was accompanied by a significant ($p < 0.05$) decline in pineal NAT activity (**Figure 5.7**).

5.2.4 Discussion

It has been established that for pineal NAT activity to remain high, continual stimulation of the pineal β-adrenergic receptor is a prerequisite (Deguchi, 1973). Failure to stimulate the β-adrenergic receptor by exposure of animals to light (Klein and Weller, 1972) or blockage of the β-adrenergic receptor with antagonists in stimulated pineals (Deguchi and Axelrod, 1972b) results in a rapid and precipitous fall in NAT activity. This rapid inactivation of NAT does not appear to be linked to cAMP (Deguchi, 1973), since pineal cAMP levels return to baseline levels within 30 minutes of β-receptor stimulation. It is presently not known what factors are responsible for the rapid inactivation of NAT. Binkley *et al.* (1976) and Namboodiri *et al.* (1979) have shown that acetyl coenzyme A, nucleotide triphosphates and pyrophosphates protect NAT against inactivation at high concentrations (20 mM and 25 mM respectively).

The results of the present study show that 5′-AMP at a final concentration of 10^{-5}M further enhances dark-induced as well as isoproterenol-induced NAT activity, and that a prerequisite for this effect is the stimulation of the pineal β-adrenergic receptor. In addition, 5′-AMP enhances isoproterenol-induced NAT activity in concentrations as low as 10^{-8}M to concentrations as high as 10^{-3}M. It appears therefore that 5′-AMP plays a regulatory role in the activation of pineal NAT. This is further supported by the fact that theophylline, a cAMP-phosphodiesterase inhibitor which reduces the conversion of cAMP to 5′-AMP, caused an expected rise in pineal cAMP levels with a concomitant decline in pineal NAT activity

during the dark phase. It is thus possible for a lower availability of 5′-AMP to be responsible for the reduction in NAT activity.

The inactivation of NAT through a rapid removal of pineal 5′-AMP by, for example, phosphorylation to ADP and ATP when animals are exposed to light at night is a possibility that remains to be investigated.

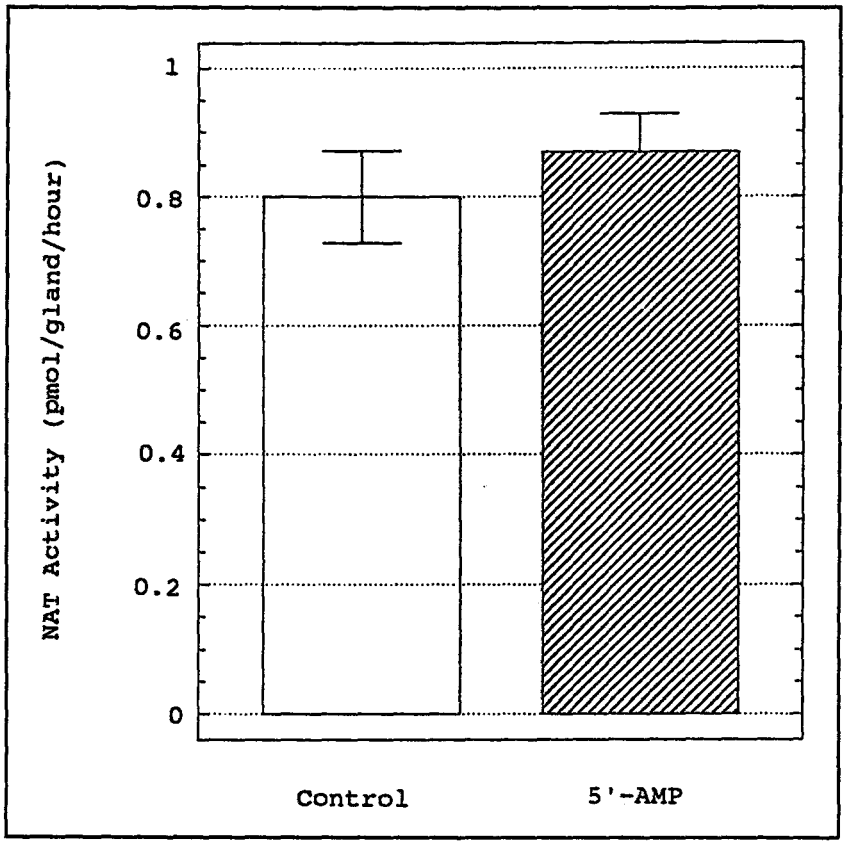

FIGURE 5.1 *The effect of 10^{5}M 5'-AMP on daytime unstimulated rat pineal NAT activity (means ± SEM, n = 5).*

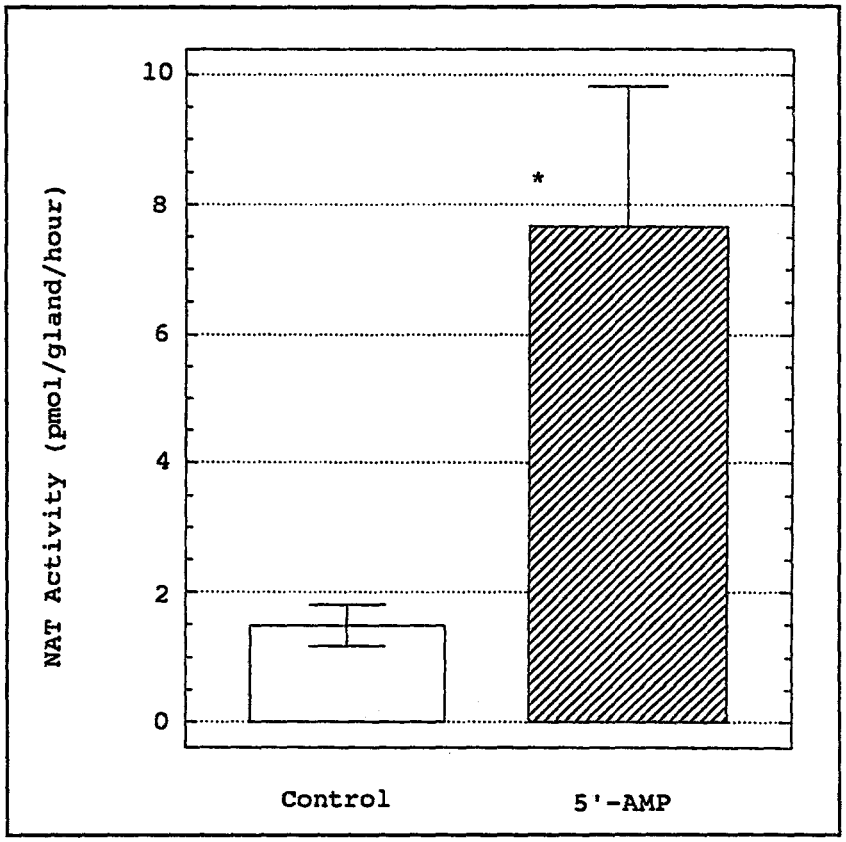

FIGURE 5.2 *The effect of 10^{-5}M 5'-AMP on dark stimulated rat pineal NAT activity (means ± SEM, n = 5).*

* as compared with the 5'-AMP free control, $p < 0.05$, Student's t test.

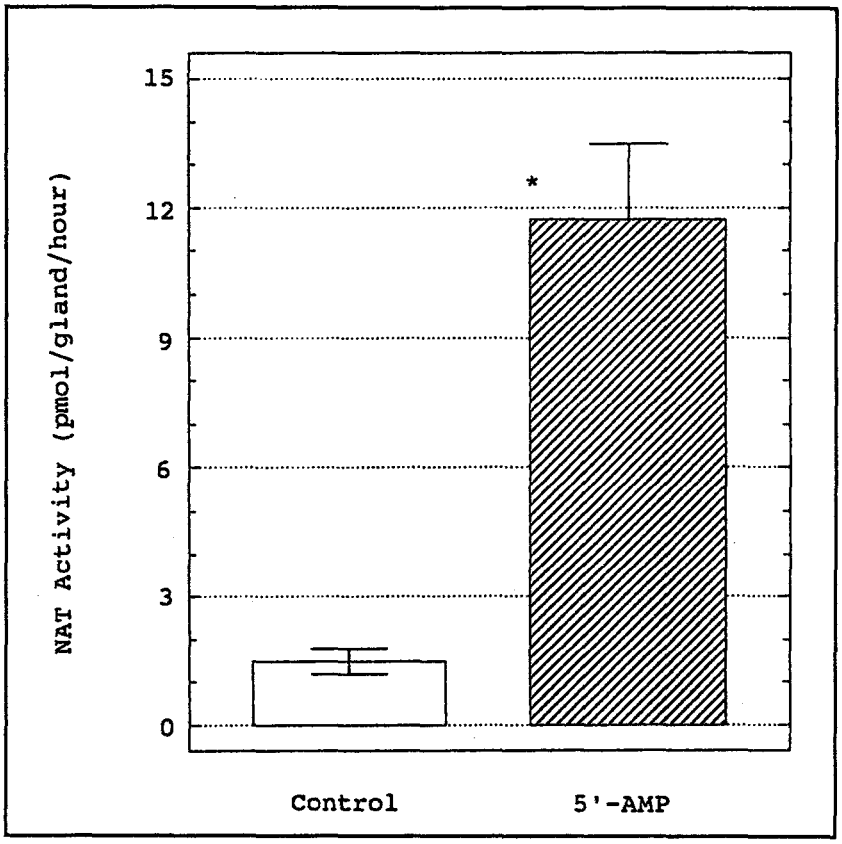

FIGURE 5.3 *The effect of 10^{-5}M 5'-AMP on daytime isoproterenol-stimulated rat pineal NAT activity (means ± SEM, n = 5).*

* as compared with the 5'-AMP free control, $p < 0.05$, Student's t test.

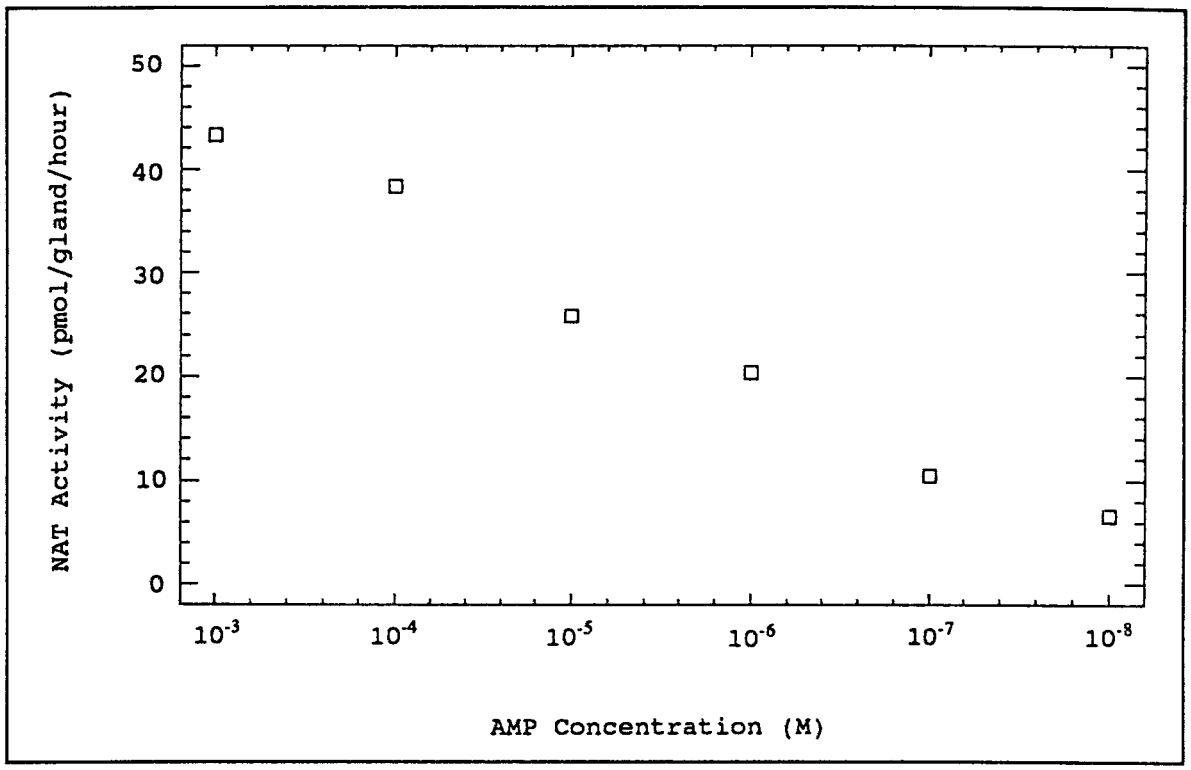

FIGURE 5.4 *The effect of 10^{-3} - 10^{-8}M 5'-AMP on daytime isoproterenol-stimulated rat pineal NAT activity (means ± SEM, n = 5).*

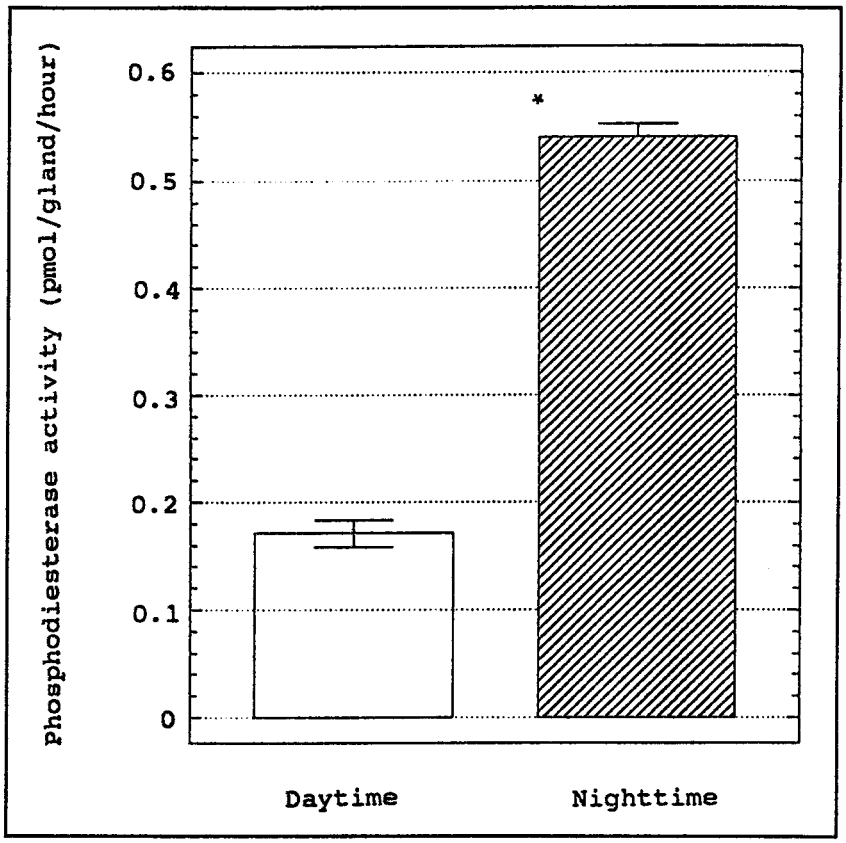

FIGURE 5.5 *The diurnal variation of pineal cAMP-phosphodiesterase activity. Animals were sacrificed at 11h00 for the day study and in the dark at 23h00 for the night study (means ± SEM, n = 5).*

* as compared with the day study, $p < 0.05$, Student's t test.

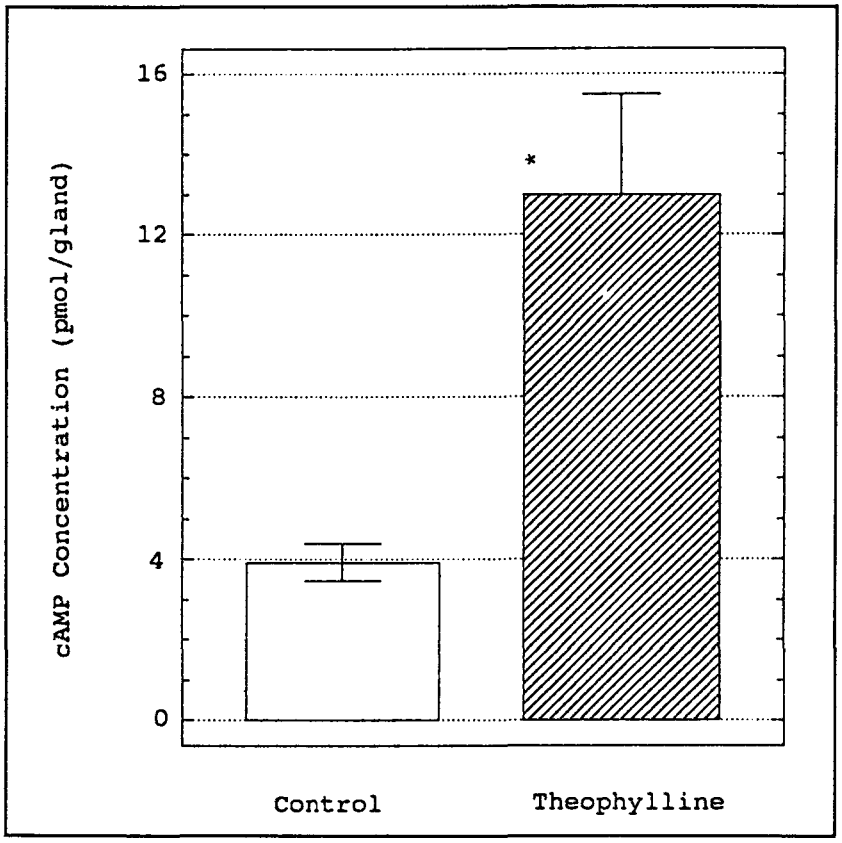

FIGURE 5.6 *The effect of a theophylline injection (10 mg/kg) on nocturnal levels of pineal cyclic AMP (means ± SEM, n = 5).*

* as compared with the theophylline free control, $p < 0.05$, Student's t test.

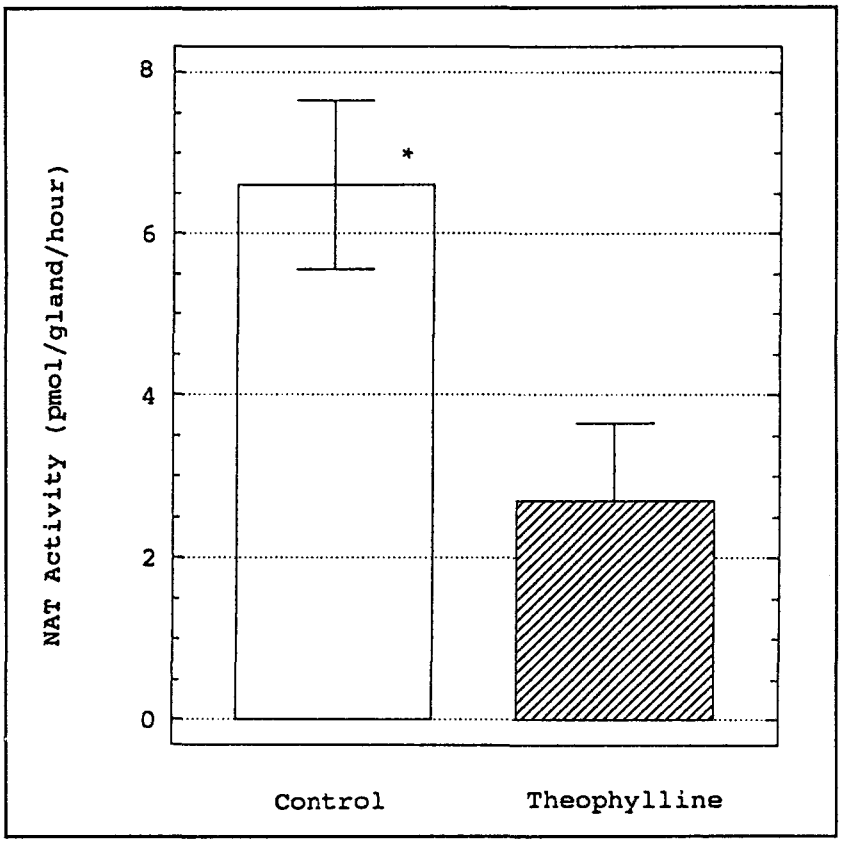

FIGURE 5.7 *The effect of a theophylline injection (10 mg/kg) on nocturnal levels of rat pineal NAT activity (means ± SEM, n = 5).*

* as compared with the theophylline group, $p < 0.05$, Student's t test.

5.3 Experiment 2: The Effect of Adenosine 5'-Triphosphate (ATP) on Pineal NAT Activity and Indoleamine Metabolism.

5.3.1 Introduction

Since the early 1970's a school of thought has emerged which proposes that ATP could act as a co-transmitter with nor-adrenaline in sympathetic nerve transmissions. The evidence for this to date is largely restricted to excitatory responses in smooth muscle. These responses are known to be elicited via the P_{2X} purinoceptor sub-type (Kennedy, 1990).

Adenosine, a related compound, has been shown to increase NAT activity in the pineal (Gharib *et al.*, 1988). Nordio *et al.* (1989) were however unable to demonstrate this increase in NAT activity in similar experiments. The administration of ATP (20 mM) to pineal gland homogenates has been shown to protect the enzyme from inactivation over a 20 minute period. Furthermore, ATP at a concentration of 25 mM activates NAT in pineal homogenates (Namboodiri *et al.*, 1979).

The effect of ATP at more physiological concentrations on pineal indole metabolism and NAT activity remains unclear. This experiment investigates the effect of ATP on both NAT activity and pineal indoleamine metabolism.

5.3.2 Materials and Method

Male Wistar rats weighing between 200 and 250 g were used in this study. The animals were maintained as described in **§ 2.1** and were sacrificed by neck fracture at 11h00.

Chemicals and Reagents: [1-^{14}C]-Acetyl coenzyme A (specific activity 4.0 mCi/mmol) was purchased from Dupont Chemical Co. (USA). 5-Hydroxy [side chain-2-^{14}C]-tryptamine creatine sulphate (specific activity 57 mCi/mmol) was purchased from Amersham (England). ATP was purchased from Boehringer Mannheim (West Germany) and l-isoproterenol was purchased from Sigma Chemical Co. (USA). All other chemicals and reagents were purchased from local commercial sources.

Experiment A: The following experiment was performed to examine the effect of ATP on pineal NAT activity. Animals (n = 10) were injected ip with isoproterenol (5 mg/kg) three hours before sacrifice. After sacrifice, the pineals were swiftly removed and individually homogenised in small glass homogenisers containing 100 μl of phosphate buffer and assayed for NAT activity as described in **§ 2.3.2** in the absence of (n = 5) and presence of (n = 5) 10^{-5} M ATP.

Experiment B: The following experiment was performed to examine the effect of ATP on pineal indoleamine metabolism. Animals (n = 10) were sacrificed at 11h00 whereafter the pineal glands were swiftly removed and placed individually into sterile glass culture tubes each containing BGJb culture medium, [^{14}C]-serotonin, 10^{-5}M isoproterenol (final concentration) in the absence of (n = 5) and presence of (n = 5) ATP at a final concentration of 10^{-5}M. All tubes were incubated at 37°C for 24 hours in a humidified atmosphere of 95% O_2 and 5% CO_2.

After the incubation period, a 10 μl aliquot of the culture medium was removed from each tube for analysis. The indoles were isolated by TLC and quantified by liquid scintillometry as described in **§ 2.2.2.**

All data were statistically compared using Student's t test and expressed as the mean of five separate incubations ± SEM.

5.3.3 Results

Experiment A: ATP caused a slight decrease in isoproterenol-stimulated NAT activity. However, this was not statistically significant **(Figure 5.8)**.

Experiment B: ATP was found to inhibit isoproterenol-stimulated pineal indole metabolism. Both melatonin and N-acetylserotonin synthesis were significantly ($p < 0.05$) reduced when compared with the ATP free control **(Table 5.1)**.

5.3.4 Discussion

The presence of ATP receptors that co-transmit with nor-adrenaline has been shown in tissues innervated by the sympathetic nervous system (Kennedy, 1990). The pineal gland is also heavily innervated by the sympathetic nervous system. This fact lends itself to the possibility that ATP may be involved in the regulation of pineal cellular processes.

Organ culture experiments have shown themselves to be reliable methods for investigating pineal cellular activity. The results of this experiment showed that ATP (at a final concentration of 10^{-5}M) in the presence of isoproterenol inhibited both N-acetylserotonin and melatonin synthesis. The reduction in N-acetylserotonin levels, the precursor to melatonin, points to the possibility that ATP may exert its inhibitory action either on the enzyme NAT, thereby reducing NAS levels, or at a point before the enzyme.

The results of the NAT assay remain inconclusive in that no significant inhibition of NAT was observed in the presence of ATP. Further studies, particularly receptor studies, are necessary to investigate the presence of ATP receptors on the pineal cell surface. Present studies have, however, indicated an inhibitory interaction between ATP and the pineal gland.

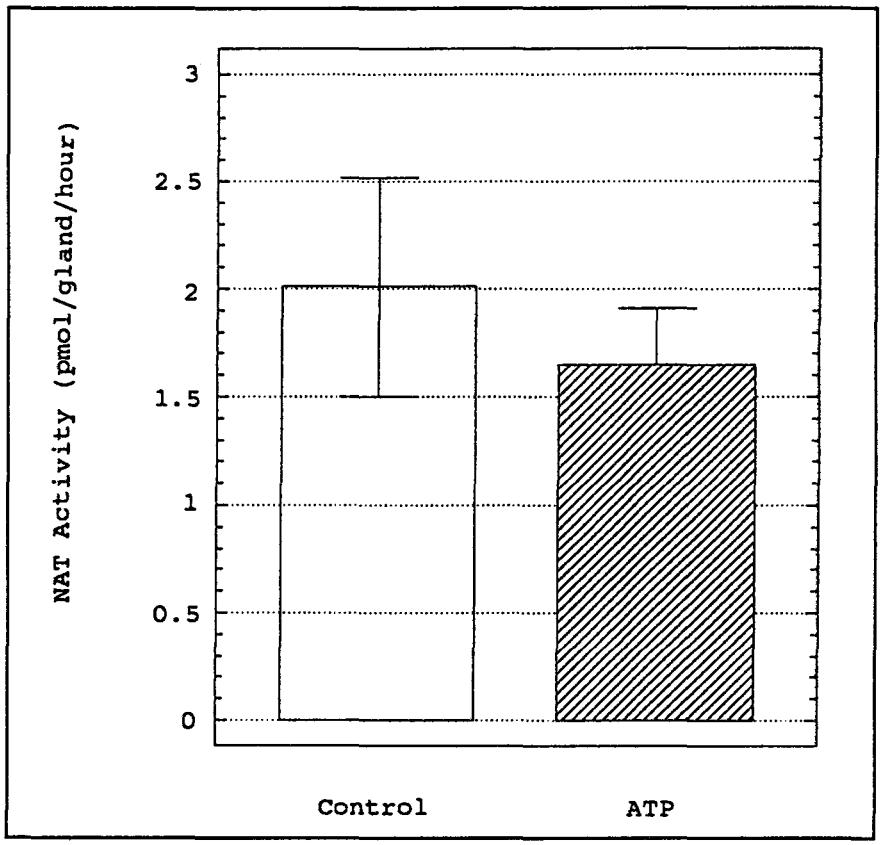

FIGURE 5.8 *The effect of ATP (10^{-5}M) on daytime isoproterenol-stimulated NAT activity (means ± SEM, n = 5).*

TABLE 5.1 *The effect of ATP (10^{-5}M) on daytime isoproterenol-stimulated pineal indole metabolism (means ± SEM, n = 5).*

	DPM recovered as:			
	MEL	NAS	MAOP	Total Counts
Control	4 884[1] ± 786	3 083[1] ± 465	45 374	53 341
ATP	2 139 ± 563	525 ± 91	26 750	30 378

ATP = adenosine 5'-triphosphate; MEL = melatonin; NAS = N-acetylserotonin; MAOP = monoamine oxidase products.

[1] as compared with the ATP group, $p < 0.05$, Student's t test.

5.4 Experiment 3: The Effect of Propranolol on Pineal Adenosine Nucleotide Levels in Three Hour Isoproterenol-Stimulated Cultures.

5.4.1 Introduction

The synthesis of N-acetylserotonin, the precursor of melatonin, is regulated by the activity of NAT (Klein, 1979). NAT follows a diurnal rhythm with its activity being highest at night, in the dark (Deguchi and Axelrod, 1972a; Klein and Weller, 1972). The neurological components involved in the synthesis and activation of NAT also follows a diurnal rhythm. These components are thought to interact as follows: nor-adrenaline, released in greater quantities at night, binds to β-adrenergic receptors on the pineal cell surface (Deguchi and Axelrod, 1975). This binding results in increased levels of cellular cAMP through the activation of adenylate cyclase. Cyclic AMP activates protein synthesis which results in the synthesis of NAT. The activity of this NAT is dependent on the continued presence of nor-adrenaline at the β-receptor (Axelrod, 1983; Ebadi, 1984).

Purine nucleotides have repeatedly been shown to be important in the activity of the pineal. For example:

- cyclic AMP's essential role in the synthesis and activation of NAT,
- ATP's role as a cellular free energy source and its ability to protect NAT from inactivation (Namboodiri *et al.*, 1979), and
- 5'-AMP's ability to stimulate NAT activity (**§ 5.2.3**).

It is therefore possible that these nucleotides act collectively in affecting pineal function.

The purpose of this study was to investigate the effect of propranolol on nucleotide synthesis in three hour isoproterenol-stimulated organ cultures. Propranolol was used in this study to mimic the effect of light by competing with isoproterenol for the β-receptor.

5.4.2 Materials and Method

Male Wistar rats weighing between 200 and 250 g were used in this study. The animals were maintained and sacrificed as described in **§ 2.1.**

Chemicals and Reagents: [2-^{3}H]-Adenosine (specific activity 26 Ci/mmol) was purchased from Amersham (England). Unlabelled adenosine 5′-triphosphate, adenosine 5′-diphosphate, adenosine 5′-monophosphate and cyclic adenosine 3′,5′-monophosphate were purchased from Boehringer Mannheim (West Germany). l-Isoproterenol and propranolol were purchased from Sigma Chemical Co. (USA). Plastic TLC plates (50 mm x 200 mm) coated with polyethyleneimine cellulose (Polygram®, Cel 300 PEI/UV_{254}) 0.1 mm were purchased from Macherey-Nagel (West Germany). BGJb culture medium (Fitton-Jackson modification) was purchased from Gibco (Europe). All other chemicals and reagents were purchased from local commercial sources.

Assay: The assay was carried out as described in **§ 2.6.2.** Briefly, rats were sacrificed and the pineal glands rapidly removed and scored with a sterile scalpel. The pineals were placed in groups of five into sterile glass culture tubes containing [^{3}H]-adenosine and BGJb culture medium to a final volume of 300 μl. The tubes were pre-incubated at 37°C in a humidified atmosphere of 95% O_2 and 5% CO_2 for three hours in the presence or absence of 10^{-5}M isoproterenol. After the pre-incubation, Group 1, which had not been exposed to isoproterenol, did not receive any further treatment and was used as the control. Group 2, which had been exposed to isoproterenol (10^{-5}M) for three hours, did not receive any further treatment. Group 3, which had been exposed to isoproterenol (10^{-5}M), received propranolol at a final concentration of 10^{-4}M. The tubes were then re-incubated for one hour at 37°C in a humidified atmosphere of 95% O_2 and 5% CO_2.

After the incubations, the pineals were removed and individually homogenised in small glass homogenisers containing 100 μl distilled water. The homogenates were centrifuged at 6 000 rpm for one minute, whereafter 10 μl of the supernatant was removed from each tube for TLC analysis. After separation, the spots were scraped into plastic scintillation vials containing 3 ml scintillation cocktail (Ready-Solv HP, Beckman) and shaken for five minutes. After shaking, the radioactivity was quantified by liquid scintillometry.

5.4.3 Results

Results are presented in **Table 5.2.**

ATP levels were found to be similar between the unstimulated control and the isoproterenol-stimulated groups. A significant difference ($p < 0.001$) was however found in the isoproterenol + propranolol group when compared with both the control and isoproterenol groups (**Figure 5.9**).

ADP levels varied significantly ($p < 0.001$) between groups. Recorded levels were highest in the isoproterenol group and lowest in the isoproterenol + propranolol group (**Figure 5.10**).

5′-AMP and cAMP levels (**Figures 5.11** and **5.12** respectively) followed inverse relationships within each experimental group, with the isoproterenol group showing both the highest 5′-AMP level and the lowest cAMP level.

5.4.4 Discussion

The nocturnal release of nor-adrenaline precipitates a cascade of events within the pineal, one of which is the activation of adenylate cyclase. This activation results in increased intracellular cAMP levels (Deguchi, 1973). Isoproterenol, a β-receptor agonist, produces the same effect as nor-adrenaline during the day and also stimulates cAMP-phosphodiesterase activity (Brown *et al.*, 1989b).

The results of the present study show that nucleotide levels remain constant in unstimulated daytime pineals. The isoproterenol-stimulated group produced high 5′-AMP and ADP levels whereas the isoproterenol + propranolol group produced high ATP and cAMP levels and low 5′-AMP and ADP levels.

The high levels of 5′-AMP in the isoproterenol group were predictable, considering that isoproterenol stimulates both cAMP production and its metabolism to 5′-AMP through adenylate cyclase and cAMP-phosphodiesterase, respectively. The high levels of ADP found

within the isoproterenol group could result from increased re-phosphorylation due to high levels of the substrate, 5′-AMP.

The stimulatory effect of isoproterenol appears to be reduced in the pineals incubated with propranolol. The high levels of ATP and lower levels of 5′-AMP found within the isoproterenol + propranolol group compared with the isoproterenol group could be ascribed to a reduction in both adenylate cyclase and cAMP-phosphodiesterase activity. This could be due to the presence of the β-receptor antagonist and to a lower requirement for ATP during unstimulated periods. Low ADP levels could result from a lower rate of re-phosphorylation owing to low substrate levels.

The differences observed between the different experimental groups lends itself to speculation on its significance in relation to pineal function.

During unstimulated periods (daytime) the requirement of the pineal for nucleotides is limited, resulting in an equilibrium or steady state between the nucleotides. Alternatively, during stimulated periods (nighttime) cAMP and 5′-AMP levels are raised due to a higher β-receptor occupancy. This increase disrupts the equilibrium and tilts it to the left, thereby activating cellular processes. The shifting of the balance to the right *i.e.* increasing ATP and ADP levels while reducing cAMP and 5′-AMP levels, could produce an inhibitory effect, acting as a switch to "turn off" the activated cellular processes. It is therefore possible that a "see-saw" effect is present within the pineal which produces stimulatory or inhibitory responses depending on which side the nucleotide balance is tilted.

TABLE 5.2 *The effect of propranolol on adenosine nucleotide levels in three hour isoproterenol-stimulated pineal cultures (means ± SEM, n = 5).*

	Nucleotide Concentrations (fmol/gland)			
	ATP	ADP	5'-AMP	cAMP
Control	54.6 ± 1.9	65.6[2] ± 1.4	65.0 ± 1.2	84.1[4] ± 2.3
Isoproterenol	52.2 ± 1.8	107.8[3] ± 3.0	222.2[3] ± 7.8	48.3 ± 1.9
Isoproterenol + Propranolol	75.8[1] ± 1.7	47.5 ± 0.8	78.7 ± 1.8	83.8[4] ± 1.8

ATP = adenosine 5'-triphosphate; ADP = adenosine 5'-diphosphate; 5'-AMP = adenosine 5'-monophosphate; cAMP =adenosine 3',5'-cyclic phosphate.

[1] as compared with both the control and isoproterenol groups, $p < 0.001$
[2] as compared with the isoproterenol + propranolol group, $p < 0.001$
[3] as compared with both the control and the isoproterenol + propranolol groups, $p < 0.001$
[4] as compared with the isoproterenol group, $p < 0.001$

All groups were statistically compared using, one-way ANOVA followed by the Bonferroni multiple range test.

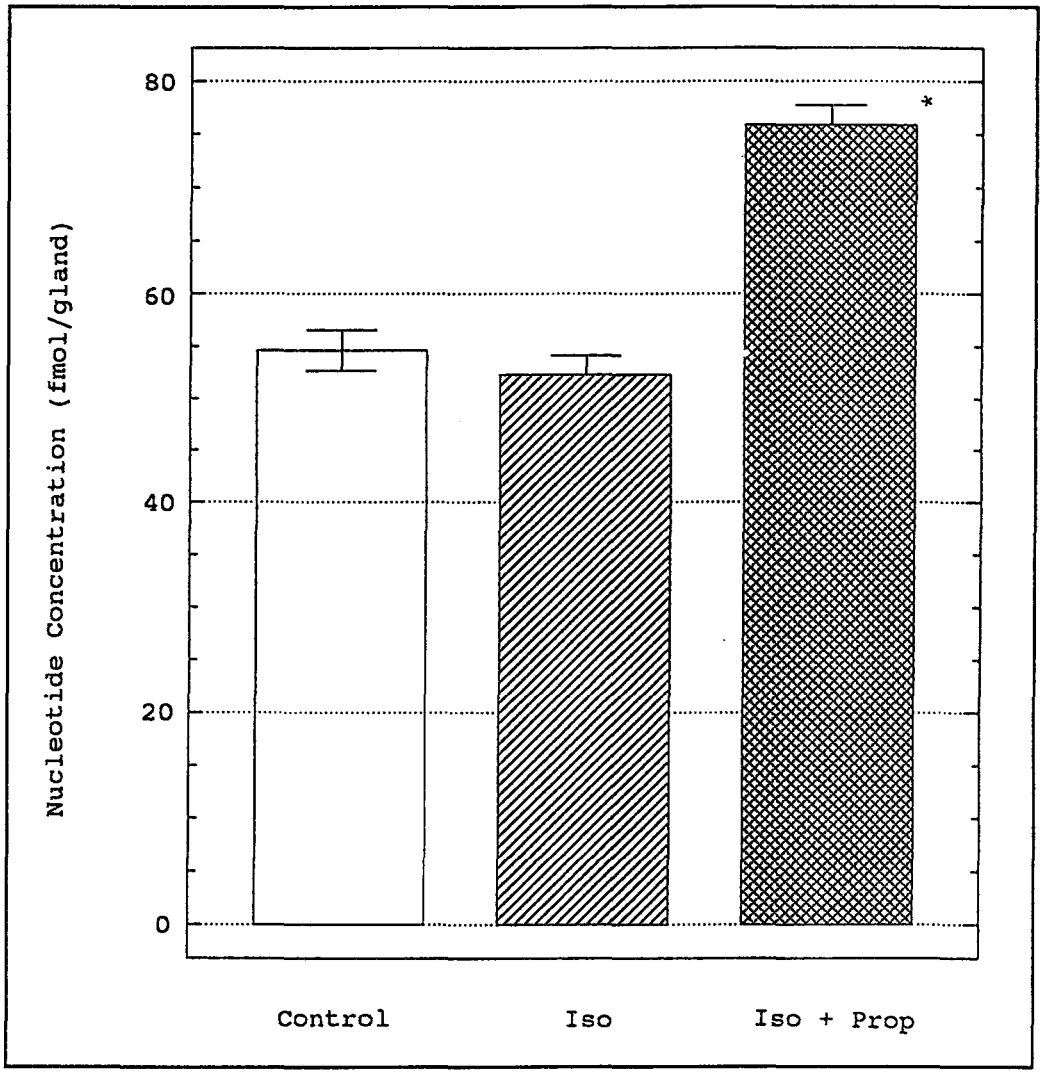

FIGURE 5.9 *The effect of isoproterenol (Iso) and isoproterenol + propranolol (Iso + Prop) on daytime adenosine 5'-triphosphate (ATP) concentrations in three hour pineal cultures (means ± SEM, n = 5).*

* as compared with both the isoproterenol free control and isoproterenol groups, $p < 0.001$, one-way ANOVA followed by the Bonferroni multiple range test.

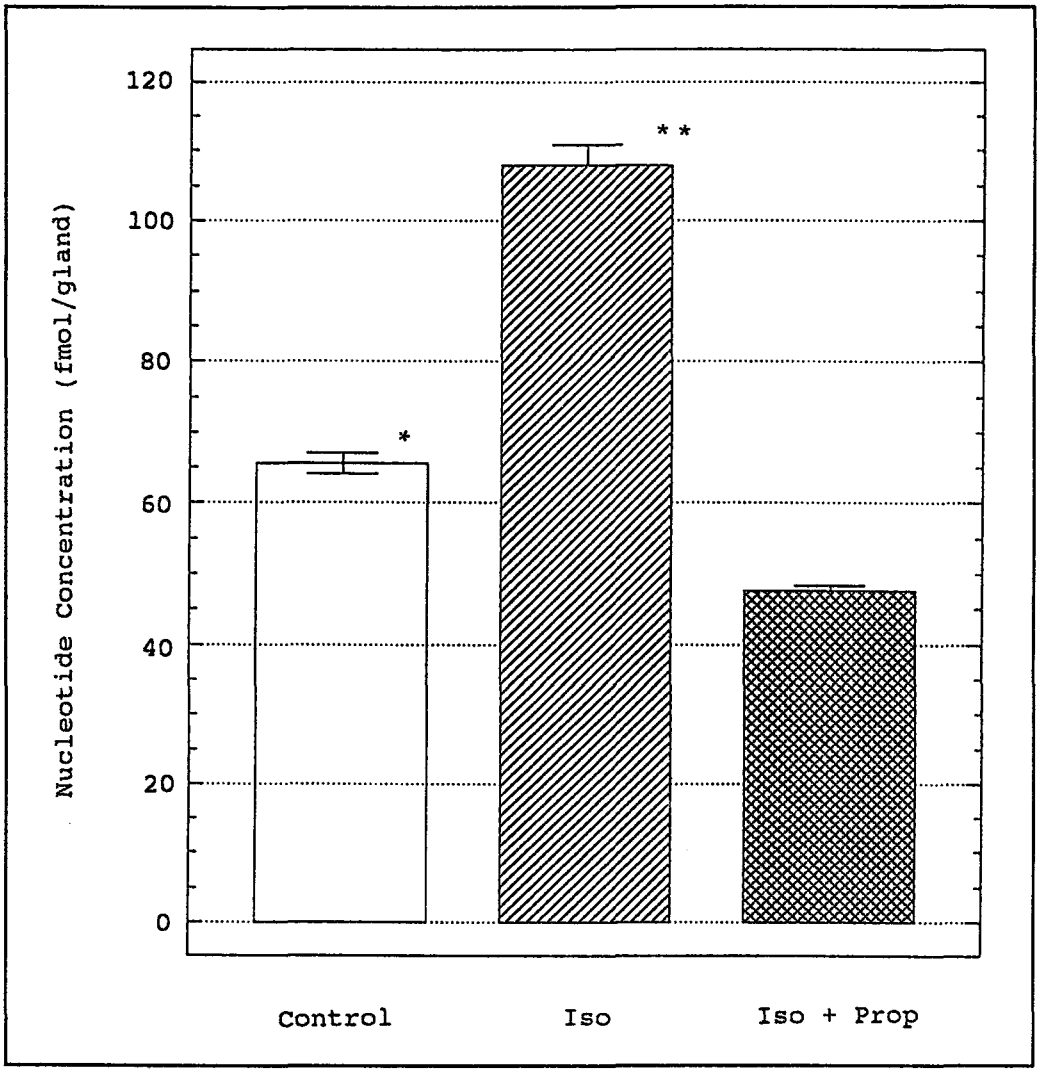

FIGURE 5.10 *The effect of isoproterenol (Iso) and isoproterenol + propranolol (Iso + Prop) on daytime adenosine 5'-diphosphate (ADP) concentrations in three hour pineal cultures (means ± SEM, n = 5).*

* as compared with the isoproterenol + propranolol group, $p < 0.001$

** as compared with both the isoproterenol free control and isoproterenol + propranolol groups, $p < 0.001$, one-way ANOVA followed by the Bonferroni multiple range test.

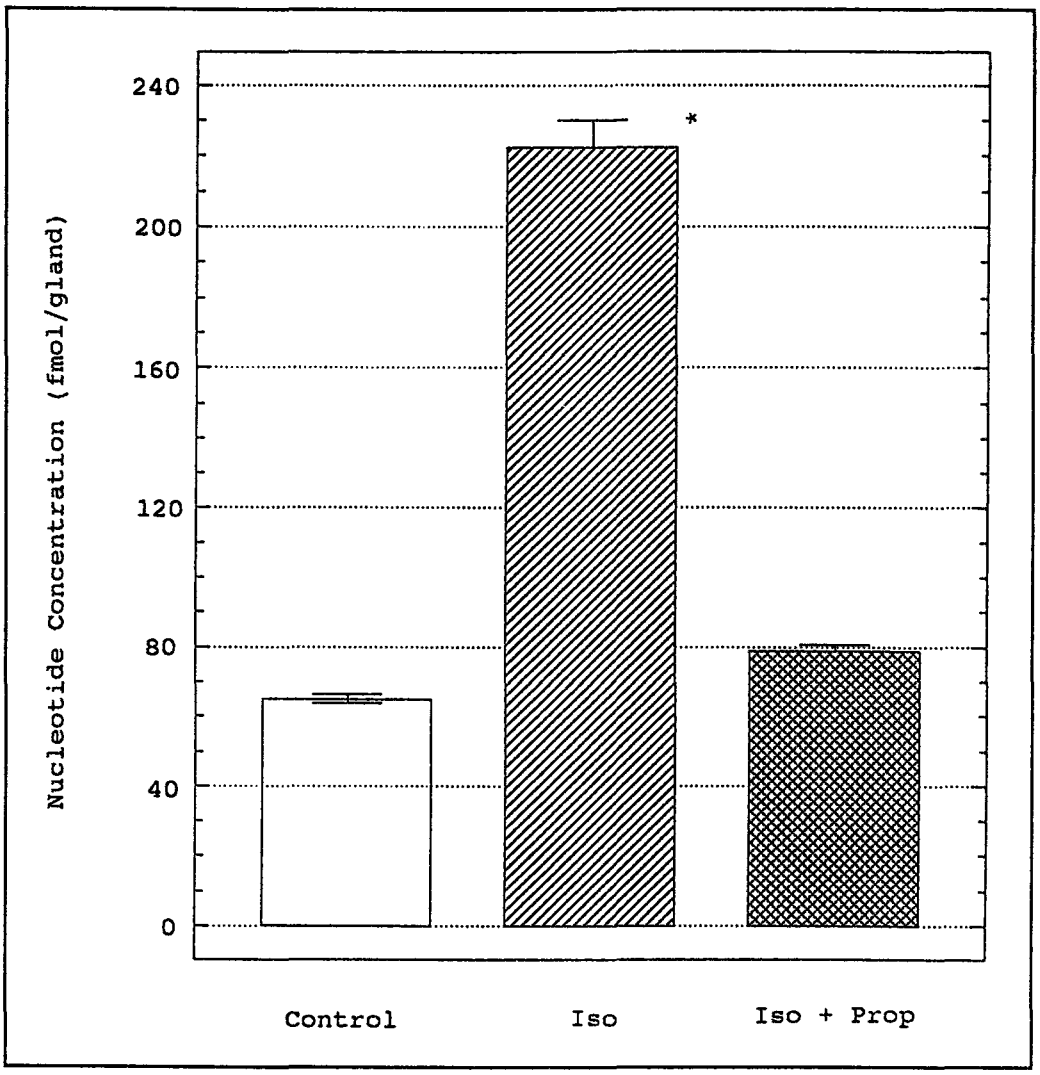

FIGURE 5.11 *The effect of isoproterenol (Iso) and isoproterenol + propranolol (Iso + Prop) on daytime adenosine 5'-monophosphate (5'-AMP) concentrations in three hour pineal cultures (means ± SEM, n = 5).*

* as compared with both the isoproterenol free control and isoproterenol + propranolol groups, $p < 0.001$, one-way ANOVA followed by the Bonferroni multiple range test.

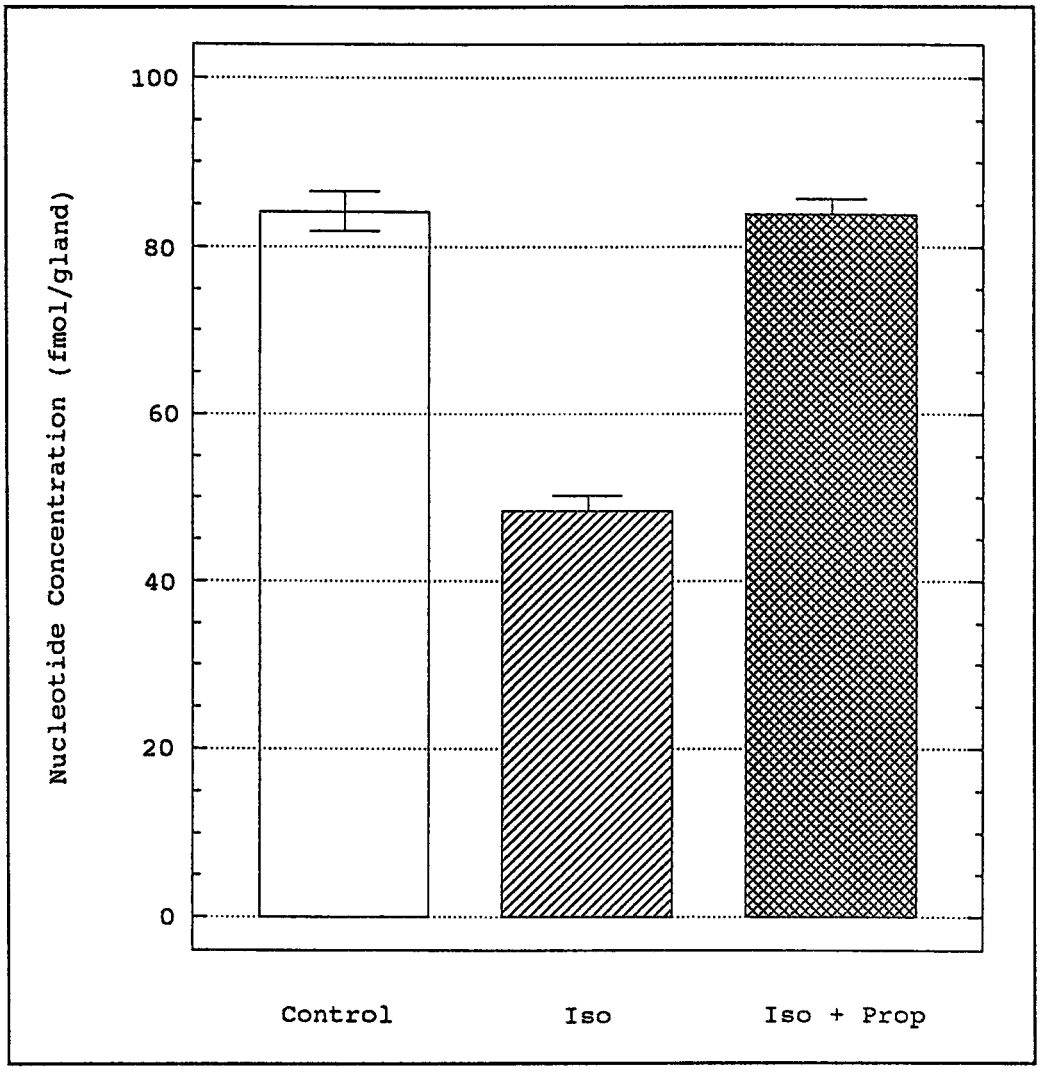

FIGURE 5.12 *The effect of isoproterenol (Iso) and isoproterenol + propranolol (Iso + Prop) on daytime cyclic adenosine 3',5'-monophosphate (cAMP) concentrations in three hour pineal cultures (means ± SEM, n = 5).*

* as compared with both the isoproterenol free control and the isoproterenol + propranolol groups, $p < 0.001$, one-way ANOVA followed by the Bonferroni multiple range test.

5.5 Conclusion

The investigation into the effect of 5′-AMP on NAT activity revealed that 5′-AMP stimulated NAT activity in both dark- and isoproterenol-stimulated pineals. 5′-AMP's stimulatory effect appeared to be inhibited in the presence of a cAMP-phosphodiesterase inhibitor, reaffirming the idea that 5′-AMP, rather than cAMP, is important for sustained NAT activity in the presence of a β-receptor agonist. However, ATP was found to inhibit indoleamine metabolism and also appeared to reduce NAT activity in the presence of β-receptor agonist. It therefore appears that 5′-AMP and ATP act antagonistically in the regulation of NAT activity.

The above results led to further investigations into the collective effect of adenosine nucleotides on pineal function. These investigations demonstrated a trend in pineal nucleotide levels at different metabolic states. At rest (daytime) the pineal appeared to fix its nucleotide levels according to its requirements for them. Stimulation resulted in a demand for cAMP and 5′-AMP, thereby depleting ATP levels. The sudden blockade of β-receptors by propranolol in stimulated pineals, resulted in the depletion of high 5′-AMP levels and an increase in ATP, possibly via a re-phosphorylation of 5′-AMP through ADP to ATP. It is therefore possible that a "switch" is produced by the levels of nucleotides within the pineal relative to one another. Furthermore, this "switch" may be turned "on/off" via external stimuli *e.g.* light acting through the β-adrenergic receptor.

If the above holds true, the sudden inactivation of NAT at night after exposure of animals to light may be explained by a shift in nucleotide levels towards ATP and a depletion of cAMP and 5′-AMP.

CHAPTER VI

THE EFFECT OF CALCIUM MANIPULATIONS ON THE RAT PINEAL GLAND

6.1 Introduction

Calcium plays an important role in the regulation of many cellular processes, such as the regulation of enzymes (Cohen, 1982) and the secretion of hormones (Katz, 1969; Baker and Knight, 1981). Within the pineal gland, calcium fluxes affect the secretion of melatonin and pinealocyte enzyme activity (Brown *et al.*, 1989b).

Calcium cellular concentrations vary between different cell types, with resting pinealocyte calcium concentrations estimated at approximately 0.05 to 0.5 μM (Reuter, 1983). It is important to note that these concentrations represent the free cellular calcium *i.e.* that found in the cytoplasm. Within the cytoplasm, calcium has been shown to aggregate into precipitates (Pizarro *et al.*, 1989). Higher calcium concentrations have been detected in pinealocyte mitochondria and nuclei of animals sacrificed at night. This could possibly indicate a change in the functional state of the cell during nocturnal calcium fluxes (Pizarro *et al.*, 1989).

The calcium signal is an important component of the nor-adrenergic regulation of the synthetic and secretory activities of the pineal gland (Sugden *et al.*, 1987). Adrenergic stimulation of the pineal results in increased cellular levels of cAMP (Klein *et al.*, 1989). Cyclic AMP regulates the synthesis of serotonin N-acetyltransferase, the rate-limiting enzyme in the synthesis of melatonin (Axelrod, 1974). *In vitro* studies by Sugden *et al.* (1985) demonstrated the action of calcium at the receptor level within this mechanism, where its presence is required by both α- and β-receptors in cAMP synthesis. The α-receptor is reliant on calcium for its potentiation of β-receptor cAMP synthesis, through protein kinase C (Sugden *et al.*, 1985).

6.2 Experiment 1: The Effect of Calcium on NAT Activity in Rat Pineal Gland Homogenates.

6.2.1 Introduction

The synthesis of the pineal hormone, melatonin, is regulated primarily by a classic β-adrenergic receptor system where calcium plays an important role (Brown *et al.*, 1989b). During the synthesis of melatonin in the pineal gland, the enzyme N-acetyltransferase catalyses the conversion of serotonin to N-acetylserotonin, the precursor of melatonin (Weissbach *et al.*, 1960; Axelrod and Weissbach, 1961). The activity of NAT is presently used as an indicator of pineal activity (Rodriguez-Cabello *et al.*, 1990b). The exposure of rats to light during the dark phase of the diurnal light cycle induces a rapid decline in pineal NAT activity (Klein and Weller, 1972). Such a decline has also been observed during homogenisation of stimulated pineal glands (Binkley *et al.*, 1976).

Previous studies have shown that calcium channel blockers reduce the nocturnal rise in pineal NAT activity (Zawilska and Nowak, 1991). It has yet to be established whether calcium is directly involved in the synthesis of *de novo* NAT protein, or whether it is an activator of NAT in the pineal. However, it has been shown that chelation of extracellular calcium results in a reduction of NAT activity due to reduced β-adrenergic receptor sensitivity and reduced NAT protein synthesis (Zatz and Romero, 1978). A recent study using the chelating agent ethyleneglycol-bis-N,N,N,N,-tetraacetic acid (EGTA) in pineal homogenates, from isoproterenol-treated rats, has shown that EGTA reduces the rate of deactivation of NAT (Rodriguez-Cabello *et al.*, 1990a).

The purpose of this study was to investigate the effect of calcium as well as its chelation on NAT activity in pineal gland homogenates.

6.2.2 Materials and Method

Male Wistar rats weighing between 200 and 250 g were used in this study. The animals were maintained as described in **§ 2.1**. The animals were sacrificed by neck fracture at 11h00.

Chemicals and Reagents: [1-^{14}C]-Acetyl coenzyme A (specific activity 4.0 mCi/mmol) was purchased from Dupont Chemical Co. (USA). EGTA, l-isoproterenol and tryptamine HCl were purchased from Sigma Chemical Co. (USA). Cold acetyl coenzyme A was purchased from Boehringer Mannheim (West Germany). Calcium chloride was purchased from BDH Chemicals Ltd (England). All other chemicals and reagents were purchased from local commercial sources.

Experiment A: The following experiment was performed to investigate the effect of a range of calcium concentrations on daytime isoproterenol-stimulated NAT activity. Briefly, animals (n = 5) were injected ip with isoproterenol (5 mg/kg) three hours prior to sacrifice. The pineals were swiftly removed, pooled and homogenised in 500 μl phosphate buffer pH 6.8. The homogenate was assayed for NAT activity (as described in § **2.3**) in the presence of various final concentrations of calcium chloride ranging from 10^{-3}M to 10^{-8}M.

Experiment B: The following experiment was performed to investigate the effect of calcium on the loss of NAT activity. Briefly, animals (n = 10) were injected ip with isoproterenol (5 mg/kg) three hours prior to sacrifice. Pineal glands were removed and pooled into two groups and homogenised. Aliquots from the two homogenates were incubated on ice for 0, 5, 10, 15, 20, 25 and 30 minutes without the addition of NAT substrate, in the presence of (n = 5) and absence of (n = 5) calcium chloride at a final concentration of 10^{-3}M. After the pre-incubation period, NAT substrate was added and the NAT activity assayed as described in § **2.3**.

Experiment C: The following experiment was performed to investigate the effect of EGTA on the loss of NAT activity in pineal gland homogenates. Briefly, animals (n = 10) were injected ip with isoproterenol (5 mg/kg) three hours prior to sacrifice. The pineal glands were removed, pooled and homogenised in phosphate buffer pH 6.8. Tubes were incubated on ice in the presence of EGTA (either 1 mM or 10 mM, final concentration) or in the absence of EGTA for varying time periods (0, 5, 10, 15, 20, 25 and 30 minutes) in the absence of NAT substrate. After the designated time, NAT substrate was added and NAT activity assayed as described in § **2.3**.

6.2.3 Results

Experiment A: NAT activity increased from 1.21 ± 0.05 pmol/gland/hour at a 10^{-3}M calcium concentration to 1.25 ± 0.08 pmol/gland/hour at a 10^{-8}M calcium concentration (**Figure 6.1**). It appears that calcium at a concentration as low as 10^{-8}M, reduces NAT activity as compared with the calcium free control (1.4 ± 0.05 pmol/gland/hour).

Experiment B: The loss of NAT activity in the presence of 10^{-3}M calcium is shown in **Figure 6.2**. In the absence of calcium, NAT activity dropped from 1.89 ± 0.02 to 1.03 ± 0.03 pmol/gland/hour over a period of 30 minutes. However, in the presence of calcium, NAT activity fell from 1.15 ± 0.04 to 0.7 ± 0.02 pmol/gland/hour over the same period.

Experiment C: Both 1 mM and 10 mM EGTA prevented the loss of NAT activity over a 30 minute period when compared with the EGTA free control. Of the two concentrations, 1 mM EGTA gave the highest overall value (**Figure 6.3**). Comparing initial NAT activities at zero time, the 10 mM EGTA gave the most NAT activity protection (2.83 ± 0.26 pmol/gland/hour) (**Figure 6.4**).

6.2.4 Discussion

Chelation of extracellular calcium has been shown to reduce NAT activity by reducing both protein synthesis and β-adrenergic receptor sensitivity (Zatz and Romero, 1978).

On the other hand, chelation of calcium in pineal homogenates resulted in an increase in the initial NAT activity and a reduction in the rate of deactivation of the enzyme (Rodriguez-Cabello *et al.*, 1990a). The results of this investigation support the view that EGTA, a chelating agent, protects NAT from inactivation and further suggests that EGTA enhances NAT activity. One millimolar EGTA appeared to provide the greatest overall protection over a 30 minute period, while 10 mM EGTA provided the highest initial NAT activity (at zero time). The addition of calcium was found to significantly reduce NAT activity in a concentration-dependent manner.

The mechanism for the activation or inactivation of NAT in the presence of both EGTA and calcium remains unclear. However, it is possible that calcium may affect a NAT inhibitory compound (Chan and Ebadi, 1981; Khoory and Schloot, 1986; Lerchl *et al.*, 1991) which may be responsible for the regulation of NAT.

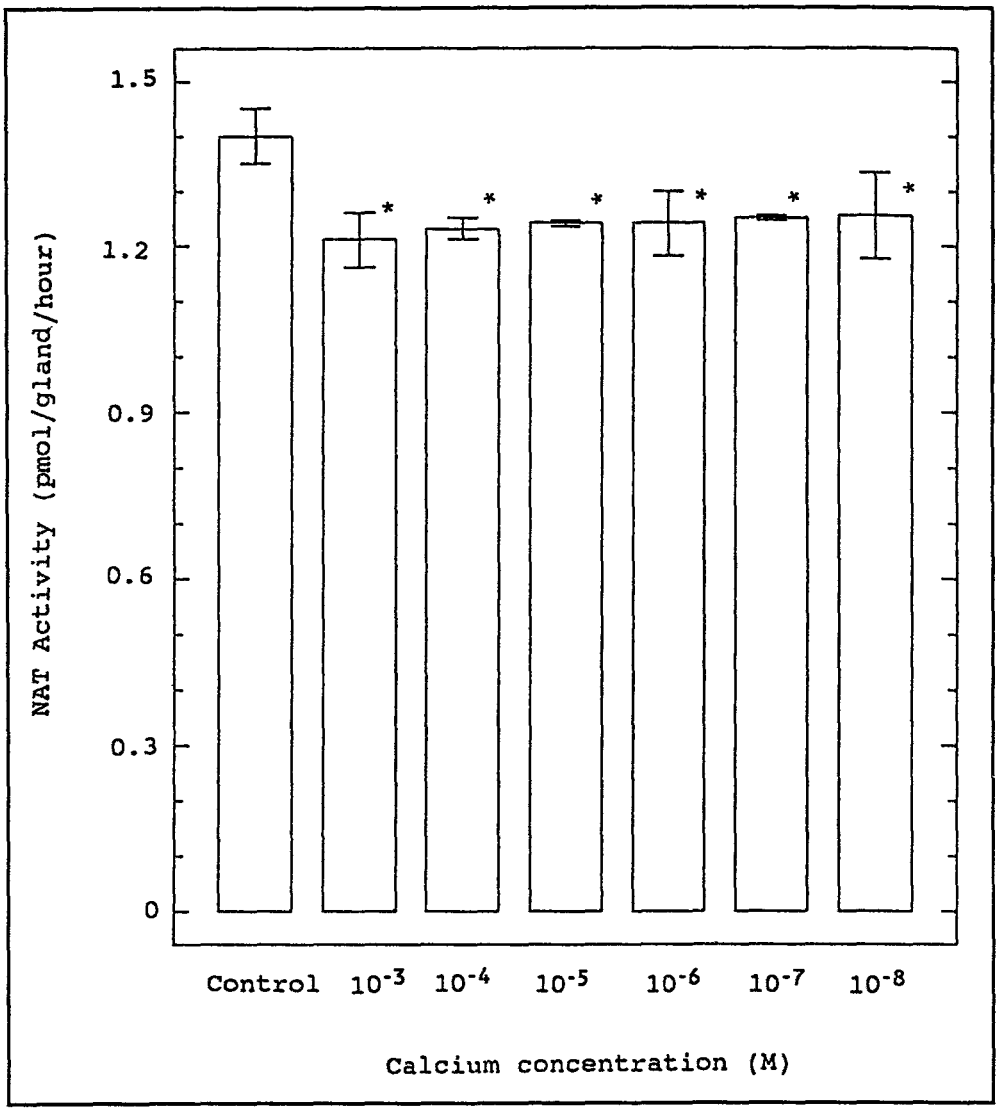

FIGURE 6.1 *The effect of 10^{-3} - 10^{-8}M calcium chloride on isoproterenol-stimulated rat pineal NAT activity (means ± SEM; n = 5).*

* as compared with the calcium free control, $p < 0.05$, Student's t test.

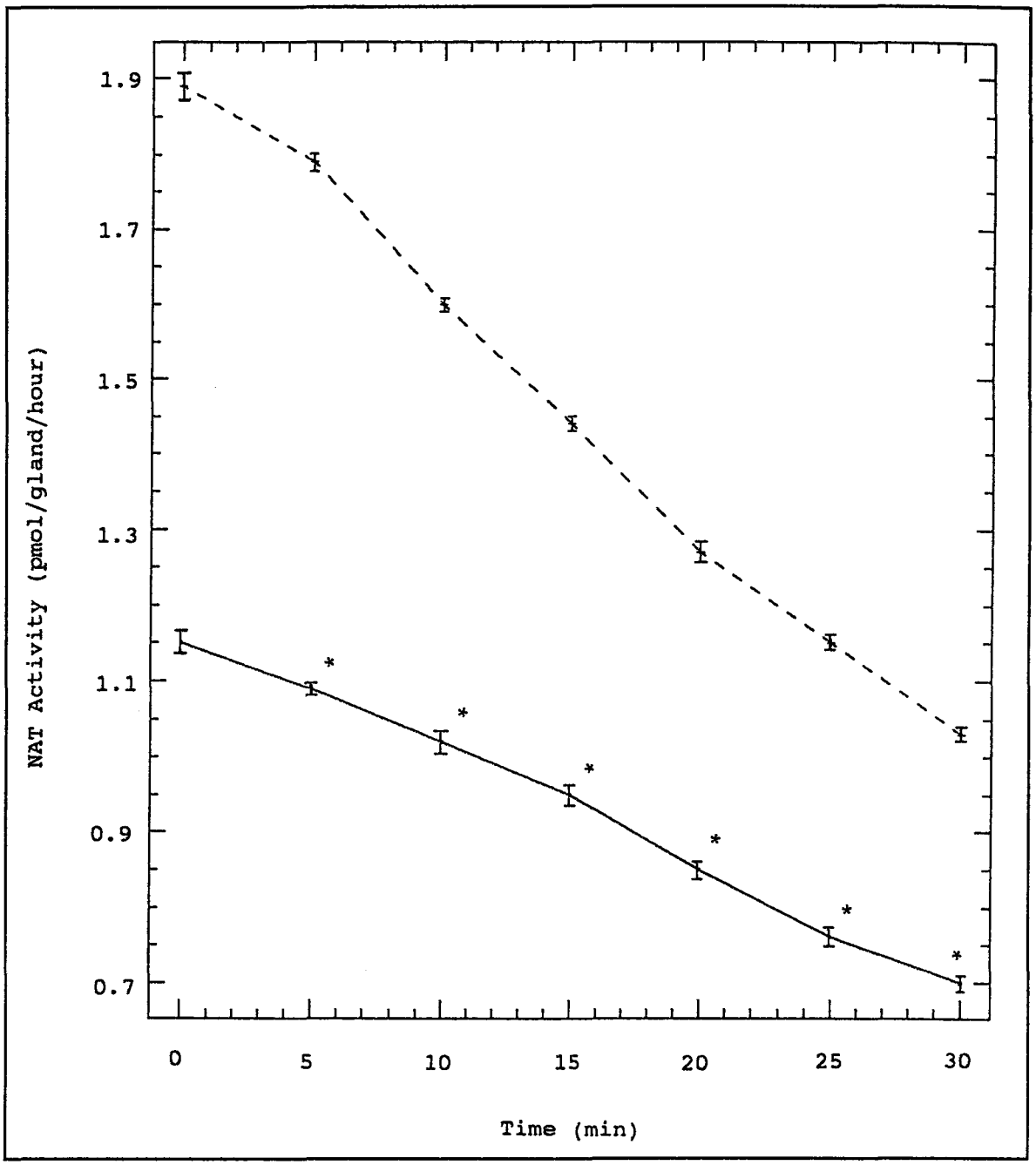

FIGURE 6.2 *Time-course of the loss of isoproterenol-stimulated NAT activity in the presence of (————•————) and absence of (---•---) 10^{-3}M calcium chloride (means ± SEM; n = 5).*

* as compared with the calcium free control, $p < 0.05$, Student's t test.

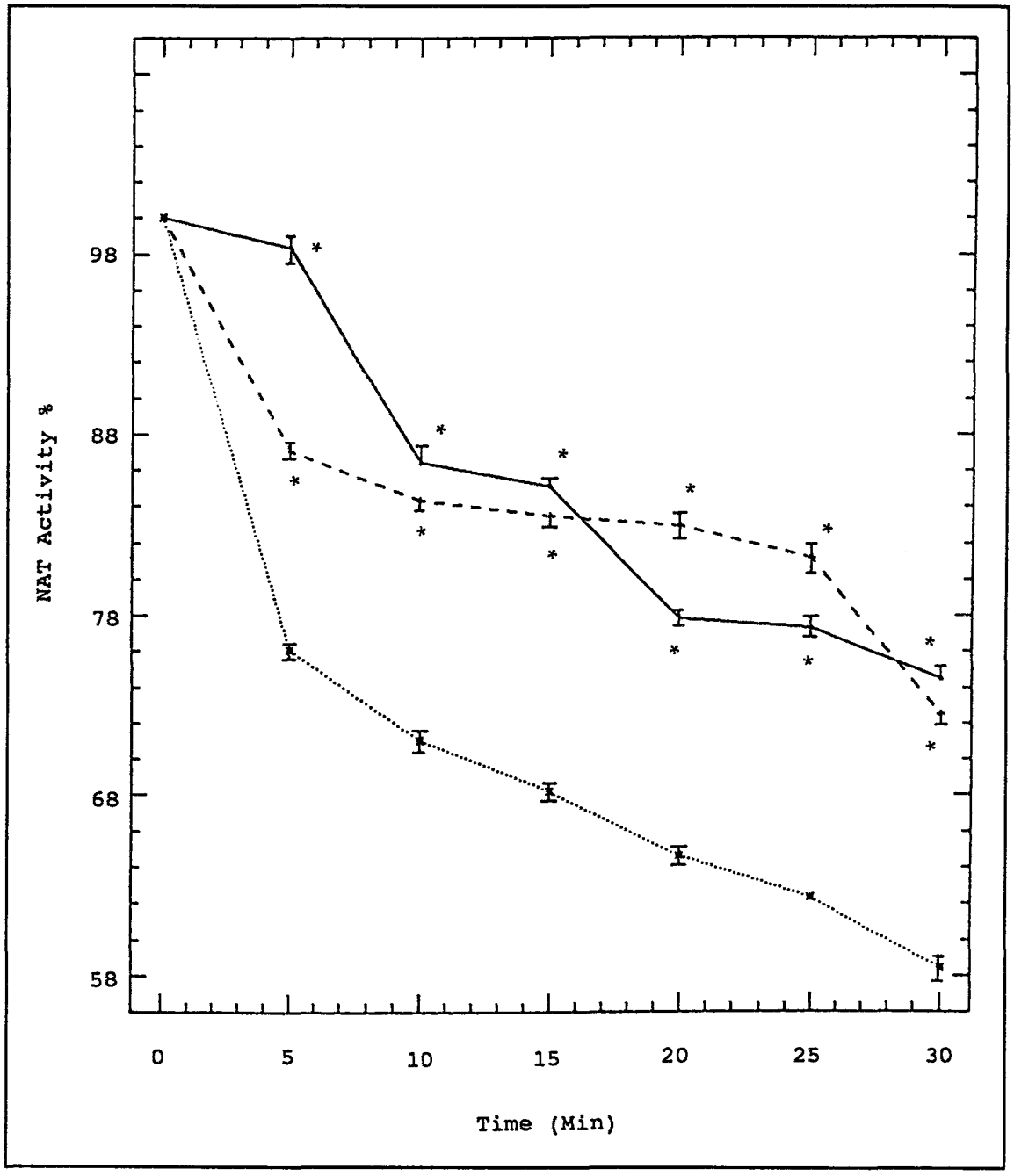

FIGURE 6.3 *Time-course of the loss of isoproterenol-stimulated NAT activity in the presence of 1 mM (————•————), 10 mM (---•---) and absence of EGTA (···■···). The activity measured at zero minutes of pre-incubation was considered as 100% (means ± SEM; n = 5).*

* as compared with the EGTA free control, $p < 0.05$, Student's t test.

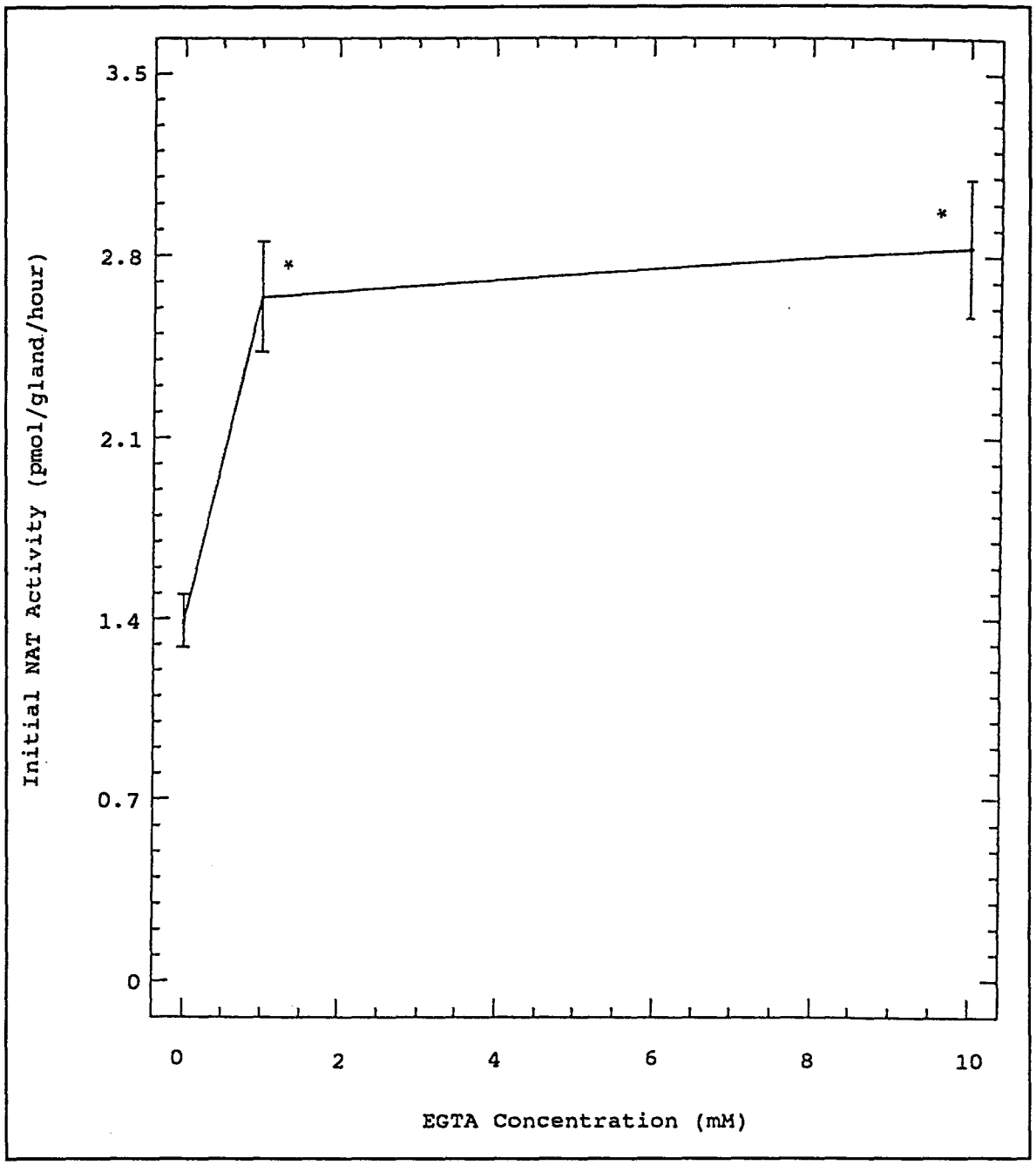

FIGURE 6.4 *Initial NAT activity at different EGTA concentrations (means ± SEM; n = 5).*

* as compared with the EGTA free control, $p < 0.05$, Student's t test.

6.3 Experiment 2: The Effect of the Calcium Ionophore A23187 on Pineal Indoleamine Metabolism.

6.3.1 Introduction

The previous experiment demonstrated the inhibitory effect of calcium on pineal NAT activity in pineal gland homogenates. The opposite has been observed in intact pineal glands, where the chelation of extracellular calcium resulted in a decrease in NAT activity through an indirect effect on the rate of protein synthesis and β-receptor expression (Wilkinson, 1976; Zatz and Romero, 1978). However, long-term exposure of intact pineal glands to elevated calcium levels leads to a reduction in NAT activity (Wilkinson, 1978) through the activation of a NAT inhibitory substance (Chan and Ebadi, 1981; Khoory and Schloot, 1986; Lerchl *et al.*, 1991).

This study investigates the effect of the calcium ionophore A23187 on pineal indoleamine metabolism as a measure of pineal enzyme activity. The advantage of using A23187 is that it is selective for divalent ions (Pressman, 1976). It is therefore capable of stimulating calcium-dependent reactions, without disturbing pre-existing balances of sodium and potassium, by rapidly introducing calcium into cells under mild experimental conditions (Brown, 1992).

6.3.2 Materials and Method

Male Wistar rats weighing between 200 and 250 g were used in this study. The animals were maintained as described in § **2.1** and were sacrificed by neck fracture at 11h00.

Chemicals and Reagents: 5-Hydroxy [side chain-2-^{14}C]-tryptamine creatine sulphate (specific activity 57 mCi/mmol) was purchased from Amersham (England). l-Isoproterenol was purchased from Sigma Chemical Co. (USA). A23187 was purchased from Boehringer Mannheim (West Germany). Dimethyl sulphoxide (DMSO) of analytical grade and all other chemicals and reagents were purchased from local commercial sources.

Assay Procedure: Animals (n = 10) were sacrificed at 11h00, whereafter the pineals were swiftly removed and placed individually into sterile glass culture tubes each containing BGJb culture medium, [^{14}C]-serotonin and 10^{-5}M isoproterenol (final concentration) in the presence of (n = 5) and absence of (n = 5) A23187 at a final concentration of 10 μM. All tubes were incubated at 37°C for 24 hours in a humidified atmosphere of 95% O_2 and 5% CO_2.

After the incubation period, 10 μl aliquots of the culture medium were removed from each tube for analysis. The indoles in the aliquots were isolated by TLC and quantified by liquid scintillometry as described in § **2.2.2.**

A stock solution of A23187 (0.1 M) was made up in DMSO and serially diluted in culture medium to the desired experimental concentration. DMSO (1%) solvent alone showed no effect on indoleamine metabolism.

6.3.3 Results

The calcium ionophore A23187 appeared to inhibit daytime isoproterenol-stimulated indoleamine metabolism (**Table 6.1**). Furthermore, N-acetylserotonin and melatonin levels were significantly reduced in the presence of A23187 (**Figures 6.5** and **6.6**).

6.3.4 Discussion

The introduction of the calcium ionophore A23187 into the organ culture assay method promoted the movement of large quantities of calcium into the pineal. The results of this investigation show that A23187 reduces indoleamine metabolism in daytime isoproterenol-stimulated pineals. These results further support the earlier study (§ **6.2**) which demonstrated that high calcium concentrations retarded NAT activity, while its chelation promoted NAT activity.

The above results are in contrast to reports by Vanecek *et al.* (1986) and Brown (1992) which demonstrated A23187 to potentiate isoproterenol-stimulated cAMP production and increase NAT activity (Brown, 1992).

It is presently unclear whether calcium's action on NAT is as a result of a calcium-dependent inhibitory compound or the direct interaction between NAT and calcium ions.

TABLE 6.1 *The effect of A23187 on daytime isoproterenol-stimulated pineal indoleamine metabolism (means ± SEM; n = 5).*

	DPM recovered as:			
	MEL	**NAS**	**MAOP**	**Total Counts**
Control	4 486[1] ± 361	3 961[1] ± 163	36 094	44 541
A23187	3 468 ± 236	3 089 ± 323	29 693	36 250

MEL = melatonin; NAS = N-acetylserotonin; MAOP = monoamine oxidase products.

[1] as compared with the A23187 group, $p < 0.05$, Student's t test.

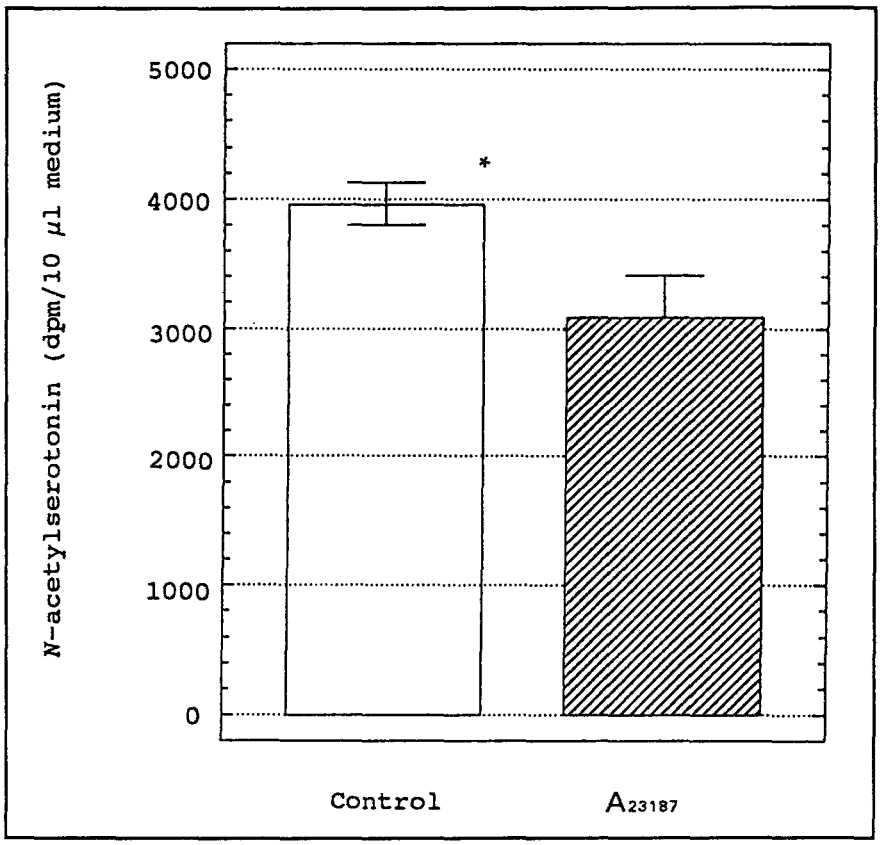

FIGURE 6.5 *The effect of A23187 on daytime isoproterenol-stimulated N-acetylserotonin levels (means ± SEM, n = 5).*

* as compared with the A23187 group, $p < 0.05$, Student's t test.

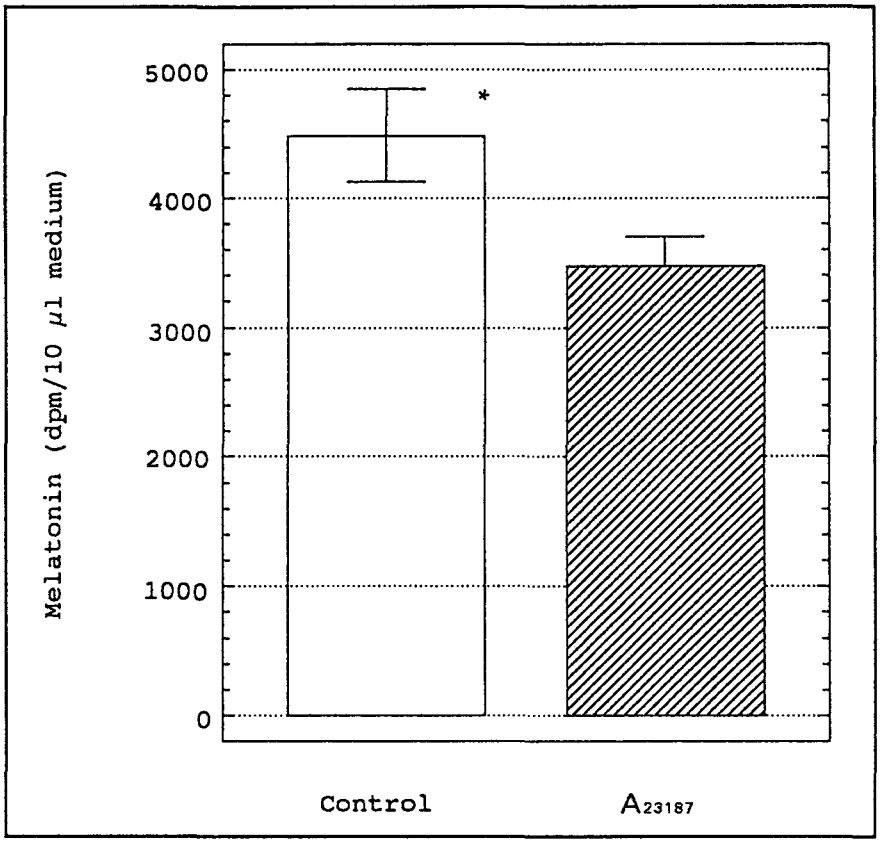

FIGURE 6.6 *The effect of A23187 on daytime isoproterenol-stimulated melatonin levels (means ± SEM, n = 5).*

* as compared with the A23187 group, $p < 0.05$, Student's t test.

6.4 Experiment 3: The Effect of Calmidazolium (R24571) on Pineal Activity.

6.4.1 Introduction

In 1970, Rasmussen afforded calcium "second messenger" status because of its diverse cellular functions. Calcium's intracellular mechanisms have only recently become understood. One such mechanism operates through a calcium binding protein which in turn activates other intracellular enzymes. It has been suggested that calmodulin, the most abundant of these calcium binding proteins, serves an analogous role towards calcium as does protein kinase towards cAMP (Klee and Haiech, 1980; Brown, 1992).

Calmodulin has been found in high concentrations in several mammalian tissues (Hidaka *et al.*, 1979) including the pineal gland (Zhou *et al.*, 1985). In these tissues, calmodulin-stimulated cAMP-phosphodiesterases have also been identified (Beavo *et al.*, 1982; Klee and Vanalan, 1982). The mechanism of the interaction between calmodulin and cAMP-phosphodiesterase has been discussed in **§ 1.10.2.3.**

In the pineal, cAMP-phosphodiesterase is characterised by its two forms, namely a high-Km form and a low-Km form. It is believed that the high-Km form is cytosolic while the low-Km form is membrane-bound (Wells and Hardman, 1977). The membrane-bound enzyme is thought to maintain cAMP levels during steady state conditions, whereas the cytosolic enzyme becomes critical during an upsurge in cAMP levels, for example during stimulated periods.

The importance of calmodulin in translating the "calcium signal" into the pineal cell through its action on adenylate cyclase and cAMP-phosphodiesterase is of paramount importance to pineal function. This experiment investigated the effect of calmidazolium (R24571), a calmodulin inhibitor, on pineal indoleamine metabolism, cAMP levels and cAMP-phosphodiesterase activity.

6.4.2 Materials and Method

Male Wistar rats weighing between 200 and 250 g were used in this study. The animals were maintained as described in § **2.1** and were sacrificed by neck fracture at 11h00.

Chemicals and Reagents: 5-Hydroxy [side chain-2-^{14}C]-tryptamine creatine sulphate (specific activity 57 mCi/mmol) and [8-^{3}H]-adenosine 3′,5′-cyclic phosphate (specific activity 30 Ci/mmol) were purchased from Amersham (England). l-Isoproterenol was purchased from Sigma Chemical Co. (USA). Calmidazolium (R24571), cold cAMP and 5′-AMP were purchased from Boehringer Mannheim (West Germany). Dimethyl sulphoxide (DMSO) of analytical grade and other chemicals and reagents were purchased from local commercial sources.

Experiment A: The following experiment was performed to investigate the effect of R24571 on pineal indoleamine metabolism. Animals (n = 10) were sacrificed, the pineals swiftly removed and individually placed into sterile glass culture tubes each containing BGJb culture medium, [^{14}C]-serotonin and isoproterenol 10^{-5}M (final concentration) in the presence of (n = 5) and absence of (n = 5) R24571 10 μM (final concentration). All tubes were incubated at 37°C for 24 hours in a humidified atmosphere of 95% O_2 and 5% CO_2.

After the incubation period, 10 μl aliquots of the culture medium were removed from each tube for analysis. The indoles present were isolated by TLC and quantified by liquid scintillometry as described in § **2.2.2.**

A stock solution of R24571 (0.1 M) was made up in DMSO and serially diluted in culture medium to the desired experimental concentration. DMSO (1%) solvent alone showed no effect on indoleamine metabolism.

Experiment B: The following experiment was performed to investigate the effect of R24571 on pineal cAMP levels. Animals (n = 10) were sacrificed, the pineal glands swiftly removed and individually placed into sterile glass culture tubes each containing BGJb culture medium in the presence of (n = 5) and absence of (n = 5) R24571 (10 μM). After a 30 minute pre-

incubation at 37°C, isoproterenol (10^{-5}M) was added to all tubes. Thereafter, the tubes were re-incubated at 37°C for three hours in a humidified atmosphere of 95% O_2 and 5% CO_2.

After incubation, the pineals were removed and assayed for cAMP as described in **§ 2.4**, with the exception that cAMP-phosphodiesterase inhibitors were omitted from buffer solutions.

Experiment C: The following experiment was performed to investigate the effect of R24571 on pineal cAMP-phosphodiesterase activity. Animals (n = 10) were sacrificed, the pineal glands swiftly removed and individually placed into sterile glass culture tubes each containing BGJb culture medium in the presence of (n = 5) and absence of (n = 5) R24571 (10 μM). After a 30 minute pre-incubation at 37°C, isoproterenol (10^{-5}M) was added to all tubes. Thereafter, the tubes were re-incubated at 37°C for three hours in a humidified atmosphere of 95% O_2 and 5% CO_2.

After incubation, the pineals were removed and assayed for cAMP-phosphodiesterase activity as described in **§ 2.5**.

6.4.3 Results

Experiment A: The results in **Table 6.2** show that R24571 inhibits pineal indoleamine metabolism. The metabolism of serotonin to N-acetylserotonin and melatonin was significantly reduced in the presence of R24571 (**Figures 6.7** and **6.8**).

Experiment B: Pineal cAMP levels increased significantly in the presence of R24571 (11.0 ± 0.43 pmol/gland) when compared with the R24571 free controls (8.2 ± 0.5 pmol/gland) (**Figure 6.9**). It is important to note that these results were obtained in the absence of cAMP-phosphodiesterase inhibitors.

Experiment C: Pineal cAMP-phosphodiesterase activity was significantly reduced in the presence of R24571 (**Figure 6.10**). The levels were found to decrease from 1.02 ± 0.03 pmol/gland/hour in the control to 0.34 ± 0.01 pmol/gland/hour in the test sample containing R24571.

6.4.4 Discussion

The inhibition of pineal indoleamine metabolism, particularly the reduction in the conversion of serotonin to N-acetylserotonin, suggests that calmidazolium (R24571) affects N-acetyltransferase activity. The results of the cAMP studies show that R24571 enhances pineal cAMP levels with a concomitant decline in cAMP-phosphodiesterase activity. It may thus be inferred that the increase in cAMP is as a result of decreased cAMP-phosphodiesterase activity.

The above results may have been predictable as cAMP-phosphodiesterase is an enzyme reliant on calmodulin for its activation (Haiech *et al.*, 1981). Thus, the blocking of calmodulin's calcium binding sites could result in the absence of active calmodulin. This would result in the absence of an active cAMP-phosphodiesterase, producing an accumulation of cAMP. However, according to Cheung (1982), adenylate cyclase is also believed to be reliant on calmodulin for its activation, but the accumulation of cAMP in the presence of R24571 suggests that cAMP-phosphodiesterase is more reliant on calmodulin than is adenylate cyclase for its activity.

The relevance of the above studies with respect to NAT activity are found at the adenylate cyclase and cAMP-phosphodiesterase level. The activity of these enzymes regulate both cAMP and 5'-AMP levels which, have been shown to be important for NAT synthesis (Klein *et al.*, 1987; Reiter, 1991) and NAT stimulation (**§ 5.2**) respectively. Thus, any factor affecting adenylate cyclase and cAMP-phosphodiesterase activity would indirectly affect NAT activity.

TABLE 6.2 *The effect of R24571 on daytime isoproterenol-stimulated pineal indoleamine metabolism (means ± SEM; n = 5).*

	DPM recovered as:			
	MEL	**NAS**	**MAOP**	**Total Counts**
Control	2 083[1] ± 384	1 644[2] ± 147	36 572	40 299
R24571	1 185 ± 108	860 ± 137	50 095	52 139

MEL = melatonin; NAS = N-acetylserotonin; MAOP = monoamine oxidase products.

[1] as compared with the R24571 group, $p < 0.05$, Student's t test
[2] as compared with the R24571 group, $p < 0.025$, Student's t test.

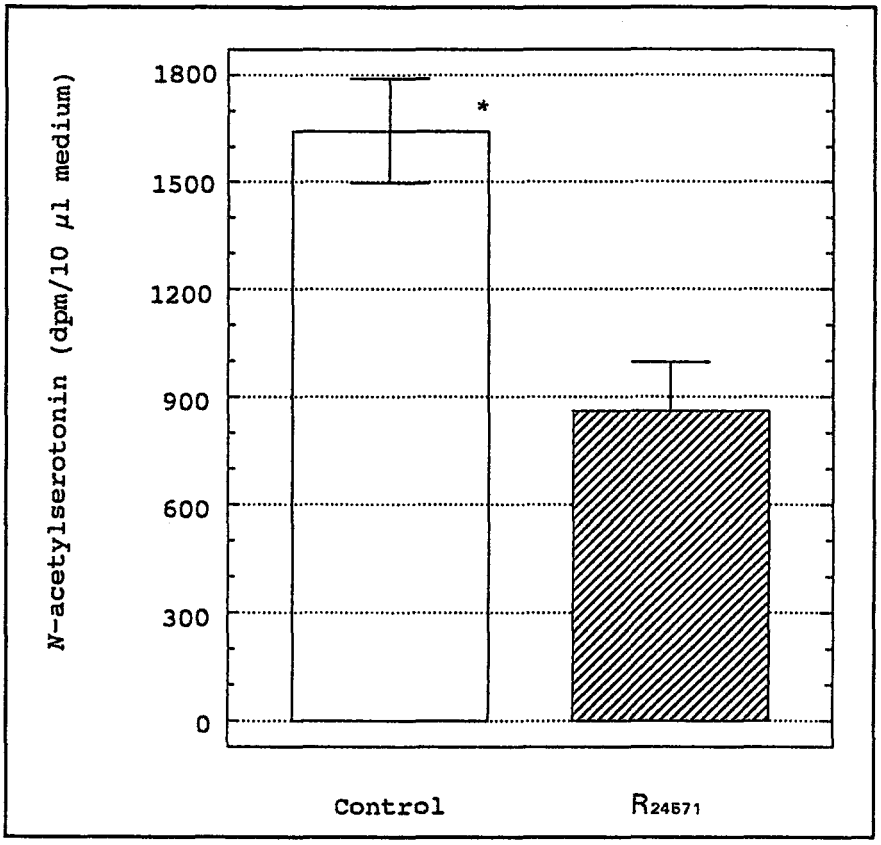

FIGURE 6.7 *The effect of R24571 on daytime isoproterenol-stimulated N-acetylserotonin levels (means ± SEM, n = 5).*

* as compared with the R24571 group, $p < 0.025$, Student's t test.

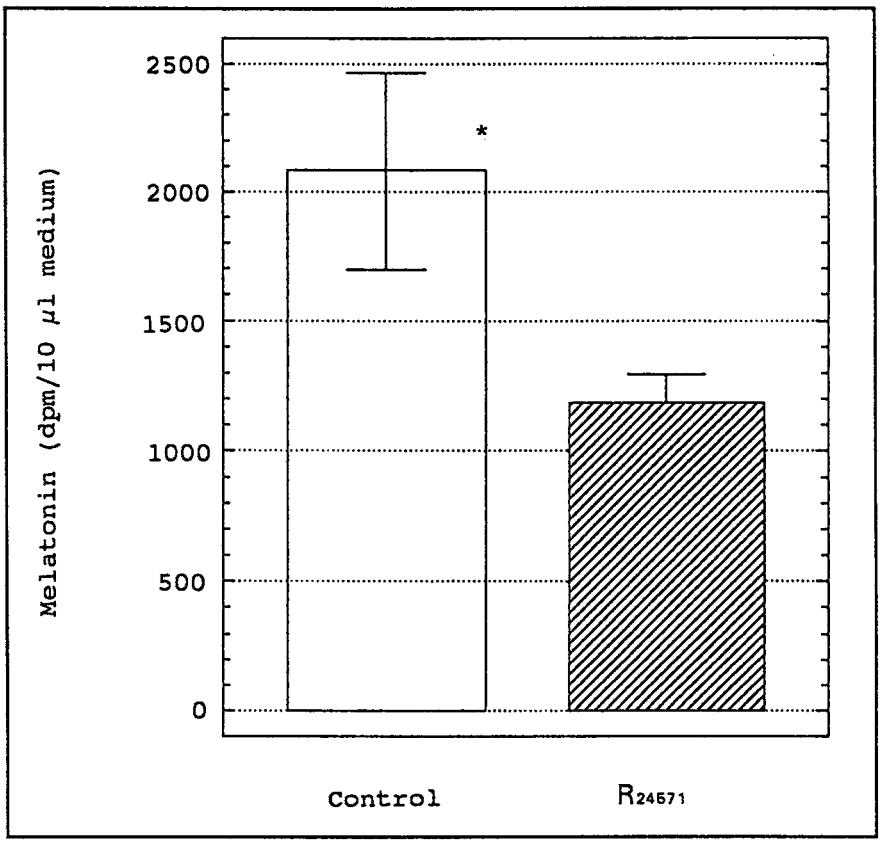

FIGURE 6.8 *The effect of R24571 on daytime isoproterenol-stimulated melatonin levels (means ± SEM, n = 5).*

* as compared with the R24571 group, $p < 0.05$, Student's t test.

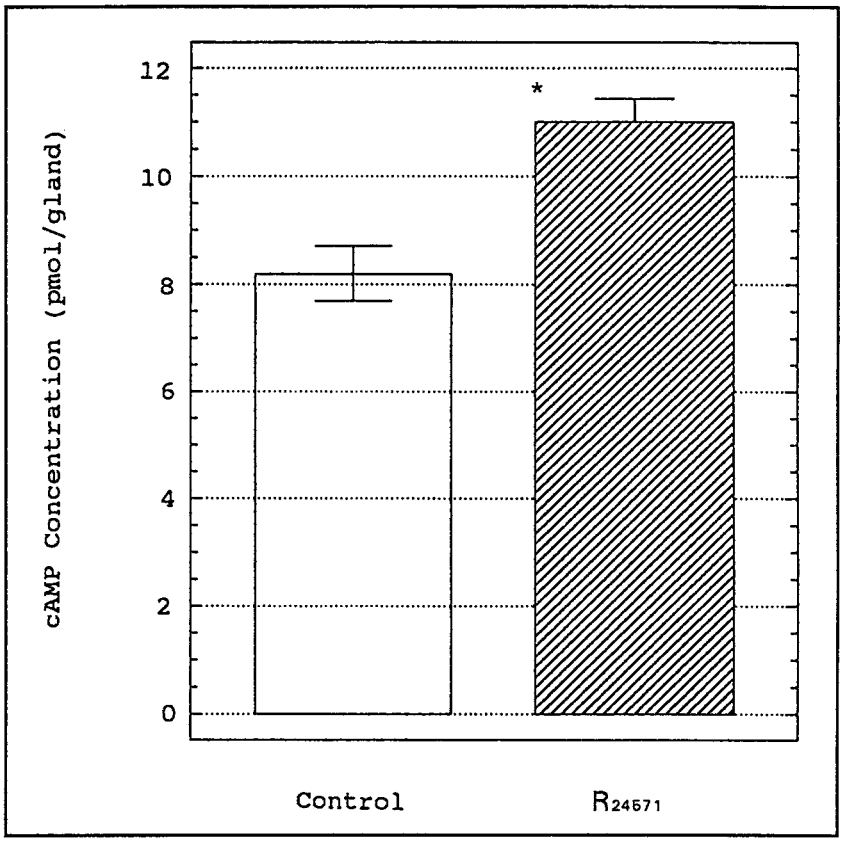

FIGURE 6.9 *The effect of R24571 (10 μM) on daytime isoproterenol-stimulated pineal cAMP levels (means ± SEM, n = 5).*

* as compared with the R24571 free control, $p < 0.05$, Student's t test.

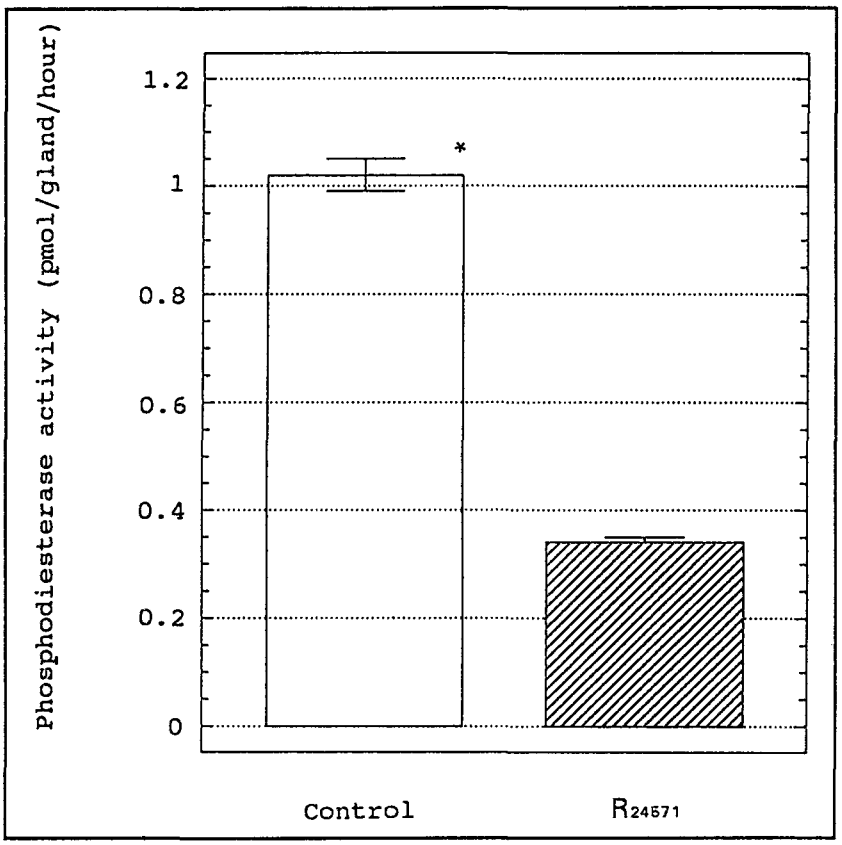

FIGURE 6.10 *The effect of R24571 (10 μM) on daytime isoproterenol-stimulated cAMP-phosphodiesterase activity (means ± SEM, n = 5).*

* as compared with the R24571 group, $p < 0.001$, Student's t test.

6.5 Conclusion

Pineal gland homogenates treated with EGTA showed a marked increase in NAT activity. The opposite was observed in homogenates treated with relatively high concentrations of calcium, suggesting that calcium inhibits NAT activity.

Organ culture studies further support the above in that pineal indoleamine metabolism was markedly reduced in the presence of the calcium ionophore A23187 (**§ 6.3.4**). It can be said that the reduction in indoleamine metabolism is as a result of a reduction in pineal enzyme activity.

The enzymes adenylate cyclase and cAMP-phosphodiesterase are both believed to be reliant on a calcium binding protein, calmodulin, for their activation (Haiech *et al.*, 1981; Cheung, 1982). Organ culture studies in the presence of a calmodulin antagonist, R24571, revealed that it reduces indoleamine metabolism (**§ 6.4.3**). This could be ascribed to a decrease in the availability of the products of adenylate cyclase and cAMP-phosphodiesterase action *i.e.* cAMP and 5′-AMP. This is supported by the findings that in the presence of R24571, cAMP levels increased while cAMP-phosphodiesterase activity was found to decrease. These results support earlier work which suggests that 5′-AMP rather than cAMP is important for sustained pineal enzyme activity and in particular N-acetyltransferase activity (**§ 5.2.4**).

The results presented above pose an interesting problem in that the chelation of calcium from pineal gland homogenates enhances NAT activity while the addition of calcium produces the opposite effect, pointing to an inhibitory function for calcium with respect to NAT. However, it is still unclear whether calcium acts directly on NAT or through a calcium dependent NAT inhibitory compound.

Calcium at specific concentrations, however, is required for the activation of calmodulin-linked enzymes, such as cAMP-phosphodiesterase. The activity of cAMP-phosphodiesterase has been linked through its product of metabolism, 5′-AMP, to NAT activity.

It thus appears that calcium may play a multi-functional role within the pineal, one where it could act directly on NAT through a NAT inhibitory compound, and another where it could

act through the β-adrenergic receptor and associated enzymes *i.e.* adenylate cyclase and cAMP-phosphodiesterase.

CHAPTER VII

SUMMARY

7.1 Chapter I

A general review of the pineal including its discovery, location, anatomy and biochemistry is presented together with a more detailed review of the action, function and regulation of serotonin N-acetyltransferase.

7.2 Chapter II

A detailed explanation of the different investigative techniques used in this study is presented within this chapter.

7.3 Chapter III

This chapter deals with two investigations, namely:

- a 24 hour time profile of tryptophan metabolism by nor-adrenaline stimulated pineal glands in organ culture, and

- the comparison of tryptophan and serotonin metabolism by pineal glands in organ culture.

The results obtained from the tryptophan profile resemble those published by Banoo *et al.* (1987) who used serotonin as the precursor. In both investigations, N-acetylserotonin and melatonin were found to be the major metabolites of metabolism. The differences between the two experiments are found at the levels of NAS and melatonin synthesised. In the tryptophan profile, NAS levels were found to be significantly higher than melatonin levels, whereas in the experiments of Banoo *et al.* (1987) the opposite was found. The characteristic sharp rise in metabolite levels during the first few hours of metabolism found in the serotonin profile of Banoo's *et al.* (1987) was absent in the tryptophan profile.

The comparison of tryptophan and serotonin metabolism in pineal organ culture experiments showed that serotonin is more readily metabolised to melatonin and N-acetylserotonin than tryptophan. This is probably due to serotonin entering the metabolic pathway at a later stage (*i.e.* lower down in the metabolic pathway). Thus serotonin has to undergo fewer enzymatic reactions than tryptophan to reach NAS and melatonin.

These findings further support the use of serotonin as the precursor in rat pineal organ culture studies.

7.4 Chapter IV

The role of S-adenosylmethionine (SAM) within the pineal gland has thus far been restricted to transmethylation reactions. The most important of these reactions is the methylation of N-acetylserotonin to melatonin through the activity of hydroxyindole-O-methyltransferase (HIOMT). A recent report shows that SAM potentiates cAMP levels in stimulated brain slices (Zhong *et al.*, 1990).

The results of similar investigations on the pineal reveal that SAM exerts no significant effect on both indoleamine metabolism and NAT activity (**§ 4.2** and **4.3**). However, cAMP studies reveals that SAM inhibits the isoproterenol-induced rise of these levels (**§ 4.4**). The importance of cAMP for the synthesis and activation of NAT are well documented. Thus, SAM may exert a regulatory role on NAT through cAMP. This speculation, however, needs further investigation.

7.5 Chapter V

Adenosine nucleotide studies revealed that 5′-AMP further induced both dark and isoproterenol-stimulated NAT activity. However, an increase in cAMP levels brought about by the pharmacological inhibition of cAMP-phosphodiesterase resulted in a decline in NAT activity. These results suggest that 5′-AMP could play a role in NAT regulation.

Further studies involving ATP demonstrated that ATP reduced pineal indoleamine metabolism. Whether this reduction in indoleamine metabolism was related to NAT activity

is at present unclear, as NAT studies revealed no statistically significant reduction in NAT activity in the presence of ATP. The interaction between adenosine nucleotides with respect to pineal regulation was also investigated. This investigation showed that pineal activity may be regulated via a nucleotide concentration balance. A shift in the balance could act as a "switch" to "turn on or off" cellular reactions, thereby regulating pineal activity.

7.6 Chapter VI

The calcium signal has emerged as an important component of the intracellular regulation of the pineal gland. Calcification of the gland with increasing age was thought to slow pineal function (Allen *et al.*, 1981). More recently, calcium has been found to play a crucial role in the adrenergic regulation of the gland (Brown *et al.*, 1989).

Recently, studies involving the chelating agent EGTA demonstrated its ability to protect NAT from inactivation in pineal gland homogenates (Rodriguez-Cabello *et al.*, 1990a). The results of the present study support the above and further show that EGTA promotes NAT activity. However, pineal homogenates treated with calcium resulted in a marked decrease in NAT activity. This suggests that calcium actively inhibits NAT activity.

The results of the EGTA and calcium studies are further supported by the studies using A23187, a calcium ionophore. These studies showed that A23187 reduces pineal indoleamine metabolism. This reduction in metabolism is thought to be caused via a reduction in pineal metabolic activity. However, this has not been confirmed.

Studies of the effect of the calmodulin antagonist R24571 on cAMP, cAMP-phosphodiesterase and organ culture experiments produced markedly different results. R24571 was found to increase pineal cAMP levels via a reduction in cAMP-phosphodiesterase activity. Notwithstanding R24571's increase of cAMP levels, pineal indoleamine metabolism is reduced in the presence of R24571. The results further support the view expressed in Chapter V which suggests that 5′-AMP rather than cAMP is important for sustained NAT activity.

CHAPTER VIII

CONCLUSION

The synthesis of the pineal hormone melatonin from serotonin is regulated by the rate-limiting enzyme serotonin N-acetyltransferase (NAT) (Klein *et al.*, 1978). NAT shows a diurnal rhythm in its activity, with highest activity occurring during the night. The exposure of animals to light at night results in the rapid inactivation of NAT (Klein and Weller, 1972; Deguchi and Axelrod, 1972b). The mechanism for this rapid inactivation of NAT remains unclear, due mainly to the inability of the enzyme to be purified without losing its activity.

Numerous studies have shown that NAT is activated via the pineal β-adrenergic receptor (Klein and Weller, 1970; Deguchi and Axelrod, 1972a). The activation is mediated by the intracellular second messenger cAMP. The levels of cAMP are crucial for the induction of NAT. However, cAMP levels return to normal cellular levels within 30 minutes of β-receptor stimulation (Deguchi, 1973), suggesting that cAMP is not involved in the sustained activation of NAT.

Investigators have repeatedly shown pineal cellular components, such as acetyl coenzyme A, nucleotide triphosphates and pyrophosphates, to protect NAT from inactivation during periods of high enzyme activity (Binkley *et al.*, 1976; Namboodiri *et al.*, 1979). The validity of these results is questioned when it is considered that the concentrations of the cellular components tested were far greater than physiological concentrations.

The results of the present study reveal that 5'-AMP activates NAT activity while ATP appears to reduce NAT activity. These results suggest that different nucleotides exert different, and possibly opposing, effects on NAT activity. Furthermore, adenosine nucleotide levels were found to change during different functional states of the gland, further supporting the view that changes in adenosine nucleotide levels result in altered NAT activity.

The role of calcium as a component of intracellular regulation and, more importantly, its role in the activation of adenylate cyclase and cAMP-phosphodiesterase, the enzymes involved in the regulation of nucleotide levels, places it in an important position with respect to NAT.

Calcium appears to exert a two-fold effect on NAT. The first appears to be a direct effect on NAT activity, either through a calcium-dependent NAT inhibitory compound (Chan and Ebadi, 1981; Khoory and Schloot, 1986; Lerchl *et al.*, 1991) or through direct interaction with the enzyme. This was deduced from experiments which showed NAT activity to decrease in the presence of calcium, while the opposite was observed when calcium was chelated by EGTA.

The second effect appears to be an indirect effect through the enzymes adenylate cyclase and cAMP-phosphodiesterase. The activity of these enzymes is regulated via a calcium binding protein, calmodulin (Cheung, 1982). Thus, any factor affecting calcium influx into the pinealocyte would affect the activity of adenylate cyclase and cAMP-phosphodiesterase, thereby affecting nucleotide levels. This in turn would affect NAT activity.

The results presented above lead to speculations on a possible regulatory mechanism for NAT.

During the night, large quantities of nor-adrenaline are released from the sympathetic nerve endings innervating the pineal gland (Brownstein and Axelrod, 1974). The nor-adrenaline binds to the β-adrenergic receptors on the pinealocyte cell surface and induces the activity of both adenylate cyclase and cAMP-phosphodiesterase (Deguchi, 1973). The induction of the above enzymes is also regulated by calmodulin (Cheung, 1982) through the availability of calcium. α_1-Receptor-linked calcium channels have been shown to open in the presence of nor-adrenaline to allow the influx of calcium (Sugden and Klein, 1988).

The activation of adenylate cyclase produces a short-term peak in cAMP levels with a concomitant decrease in ATP levels. The elevated cAMP level induces protein synthesis with the result that *de novo* NAT is synthesised (Morrissey and Lovenberg, 1978; Romero *et al.*, 1978). The metabolism of cAMP to 5′-AMP by cAMP-phosphodiesterase renders large

quantities of 5′-AMP available for the activation of NAT. The removal of 5′-AMP from the system by re-phosphorylation to ADP and ATP could explain the decrease in NAT activity.

Thus, the phenomenon of NAT inactivation when animals are exposed to light at night may be explained as follows:

> light striking the retina of the eye produces an effect on the sympathetic neurons, causing the cessation of nor-adrenaline release at the nerve endings innervating the pineal. The absence of neurotransmitter at the β-receptor results in an altered calcium influx which in turn affects adenylate cyclase and cAMP-phosphodiesterase activity. A decrease in the above enzymes' activities produces a depletion of cAMP and 5′-AMP with a concomitant increase in ATP through phosphorylation of already synthesised 5′-AMP. This results in the inactivation of NAT.

Thus, a "see-saw" between the levels of ATP, ADP, cAMP and 5′-AMP may be responsible for the regulation of NAT, and any external factors affecting the levels of the nucleotides indirectly affect NAT activity.

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