THE EFFECT OF APPETITE SUPPRESSANTS ON PINEAL FUNCTION

THESIS

Submitted in Fulfilment of the Requirements
for the Degree of

MASTER OF SCIENCE

in the Department of Biochemistry and Microbiology
Faculty of Science
Rhodes University

by

BONGANI ISAAC MCHUNU

JANUARY 1994

ABSTRACT

The pineal gland has become the subject of considerable investigation as it provides a productive experimental model for studying circadian rhythms and regulation of end organs. In the rat, the pineal gland provides a convenient model for investigating the noradrenergic receptor system and the effects of various drugs on this system.

The effect of appetite suppressants on the rat pineal gland function is described. Appetite suppressants increase melatonin synthesis in organ cultures of rat pineal glands. This effect appears to be mediated by noradrenaline acting on β-adrenoceptors on the pinealocyte membrane. When β-adrenoceptors are blocked, the appetite suppressant-induced rise in melatonin synthesis is prevented. Depletion of noradrenaline in sympathetic nerve terminals also prevented the appetite suppressant-induced rise in melatonin synthesis. Activation of β-adrenoceptors is followed by a rise in *N*-acetyltransferase activity via a cyclic adenosine monophosphate second messenger system.

The effect of appetite suppressants on the activity of liver tryptophan pyrrolase was also investigated. The activity of this enzyme is an important determinant of tryptophan availability to the brain and consequently of brain serotonin levels. The results show that appetite suppressants inhibit both holoenzyme and total enzyme activities of tryptophan pyrrolase. This finding suggests that appetite suppressants may act by inhibiting tryptophan pyrrolase activity thereby increasing brain serotonin, a phenomenon known to be associated with anorexia. There are two possible mechanisms by which appetite suppressants inhibit tryptophan pyrrolase activity. Firstly, these agents, being drugs of dependence, may increase

liver NADPH concentrations which inhibit pyrrolase activity. Secondly, appetite suppressants may act on the pineal gland to stimulate melatonin synthesis. Melatonin inhibits pyrrolase activity in a dose-dependent manner. This inhibition will elevate plasma tryptophan levels which result in a rise in brain serotonin synthesis.

The present study suggests a possible relationship between the pineal gland and appetite centres in the hypothalamus. Melatonin may have a direct effect on appetite centres since food restriction is associated with an increased melatonin binding in the hypothalamus. If this possible relationship can be extended, melatonin can open new possibilities for the control of food intake and consequently, of pathological obesity.

TABLE OF CONTENTS

CONTENTS PAGE

CONTENTS PAGE

CONTENTS PAGE

CONTENTS PAGE

CONTENTS PAGE

LIST OF ABBREVIATIONS

Ach	Acetylcholine
5-ALA	5-Aminolevulinate
ATP	Adenosine Triphosphate
BZP	Benzodiazepine
cAMP	Cyclic Adenosine Monophosphate
CCK	Cholecystokinin
cGMP	Cyclic Guanosine Monophosphate
CNS	Central Nervous System
cpm	Counts Per Minute
CRF	Corticotrophin-Releasing Factor
DA	Dopamine
DCV	Dense-Core Vesicle
DNA	Deoxyribonucleic Acid
dpm	Disintegration Per Minute
EPO	Evening Primrose Oil
GABA	Gamma Amino Butyric Acid
GER	Granular Endoplasmic Reticulum
GIT	Gastroinestinal Tract
G_s	Stimulatory Guanine Nucleotide-Binding Protein
HCl	Hydrochloride
5-HIAA	5-Hydroxyindole Acetic Acid
HIOMT	Hydroxyindole-O-Methyltransferase
hr	Hour
5-HT	5-Hydroxytryptamine (Serotonin)
5-HTP	5-Hydroxytryptophan
L:D	Light : Dark
MAO	Monoamine Oxidase
MAOP	Monoamine Oxidase Products
MEL	Melatonin
NA	Noradrenaline
NAD	Nicotinamide Adenine Dinucleotide
NADP	Nicotinamide Adenine Dinucleotide Phosphate
NAS	*N*-Acetylserotonin
PFLH	Perifornical Lateral Hypothalamus
PG	Prostaglandin
PVN	Paraventricular Nucleus
QNB	Quinuclidinylbenzilate
SA	South Africa
SAD	Seasonal Affective Disorder
SAM	S-Adenosyl Methionine
SCG	Superior Cervical Ganglion
SCG_x	Superior Cervical Ganglionectomy
SCN	Suprachiasmatic Nucleus
SEM	Standard Error of the Mean
SIDS	Sudden Infant Death Syndrome
T_3	Triiodothyronine

T_4	Thyroxine
THL	Tetrahydrolipstatin
TLC	Thin Layer Chromatography
TRH	Thyroid Releasing Hormone
TRP	Tryptophan
TSH	Thyroid Stimulating Hormone
UK	United Kingdom
USA	United States of America
VMN	Ventromedial Nucleus

LIST OF TABLES

LIST OF FIGURES

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to Professor Santy Daya for his expert supervision, guidance and encouragement throughout this study.

I wish also to thank the following people and organizations for their contribution towards this study :

The German Organization (DAAD) for the financial support which made this study possible.

Rhodes University for additional financial assistance.

Mr. Edward Williams for his expert assistance in maintaining and sacrificing animals.

Dr. Gianfranco Olivieri and Mr. Harold Walsh for their assistance in some of the studies.

Mrs. Joan Miles for her expert assistance with typing.

My family, especially my mother and my only brother, Vuvu, for their support and encouragement, without which this study would not have been possible.

CHAPTER 1

LITERATURE REVIEW

1.1 INTRODUCTION

Obesity is the most common nutritional disorder in developed countries. Life insurance statistics have shown that excessive weight is associated with increased mortality, particularly in young men under 40 years of age (Garrow, 1979). The major factor in this higher relative mortality among obese people is coronary heart disease. In any one year, at least one in ten of the adult population make some attempt to lose weight. Many will attempt to reduce weight on their own using magazine articles or "slimming" foodstuffs or will join a slimming club; sooner or later, about half will eventually seek help from a general practitioner for drug therapy (Ashwell, 1973).

The regulation of feeding behaviour by endogenous substances and by pharmacological agents depends on numerous systems and is mediated by several neurotransmitters, hormones or metabolites, which interact with several recognition sites and at various anatomical areas by complex physiological processes and feedback mechanisms. Research has given considerable evidence that the majority of drugs affecting food intake exert their action by modulating the activity of central monoamine neurons containing serotonin (5-HT), dopamine (DA) and noradrenaline (NA) (Angel, 1990).

For more than a quarter of a century the focus for brain mechanisms controlling feeding has been the hypothalamus. The paraventricular and ventromedial nuclei (PVN and VMN) are known to be involved in controlling energy balance, while the suprachiasmatic nucleus (SCN) determines the circadian patterns of feeding. The ability of animals to alter patterns of protein and carbohydrate intake in relation to need becomes especially apparent during the active period of the diurnal cycle when feeding behaviour is most pronounced. This nocturnal feeding activity in the rat is characterized by peaks of food intake at the beginning and towards the end of the night (Siegel, 1961).

The feeding rhythms are in parallel with the nocturnal rise in the levels of the pineal neurohormone, melatonin, which is also under circadian rhythm. Both of these rhythms are generated in the hypothalamic SCN. Furthermore, specific melatonin binding sites have been demonstrated in this region of the hypothalamus (Vanecek *et al.*, 1987). This relationship between melatonin and appetite centres prompted this investigation on the role of appetite suppressants on the metabolism of pineal indoles, especially melatonin.

The following sections review important aspects of appetite suppressants and the pineal gland, on the other. Clinical data, including the mode of action of appetite suppressants, as well as rhythms involved in neurotransmitter modulation of feeding patterns are briefly discussed. Furthermore, relationships between pineal indoles and feeding behaviour are discussed.

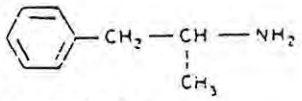
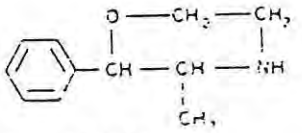
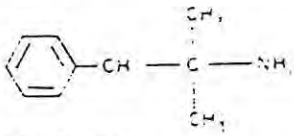
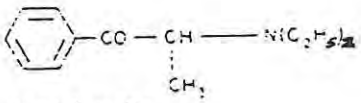
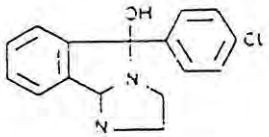
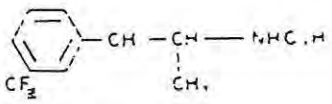
1.2 ANORECTIC COMPOUNDS

The appetite suppressant preparations which are available on prescription are shown in Table 1.1. All but mazindol are chemical derivatives of β-phenylethylamines, the basic structure also shared by the catecholamine neurotransmitters adrenaline, NA and DA.

1.2.1 MODE OF ACTION

The anorectic agents are thought to decrease food intake by interfering with brain mono-amines. Amphetamine, phenmetrazine, phentermine, diethylpropion and mazindol act on catecholaminergic pathways, although it is unclear whether the noradrenergic or dopaminergic system is more important (Garattini *et al.*, 1975a). In contrast, fenfluramine slows the rate of eating and diminishes the size of meal, though not their frequency. Fenfluramine appears to act via serotonergic pathways (Garattini *et al.*, 1975b). This is discussed in further detail on Section 1.2.6.

TABLE 1.1: Antiobesity Drugs Currently Available in the U.K.

Formulation	Structural Formulae	Proprietary Names
Amphetamine* Resin Complex	 Amphetamine	Durophet
Phenmetrazine* (30 mg) Phenbutrazate (20 mg)	 Phenmetrazine	Filon
Phentermine	 Phentermine	Duromine Ionamin
Diethylpropion	 Diethylpropion	Tenuate Apisate
Mazindol	 Mazindol	Teronac
Fenfluramine	 Fenfluramine	Ponderax

*Restricted use under Schedule 2 of the "Misuse of Drugs" Act 1971.

(Adapted from Douglas and Munro, 1982).

1.2.2 CLINICAL PHARMACOLOGY

1.2.2.1 Amphetamine

Amphetamine is a racemic compound comprising equal portions of the *dextro* and *levo* isomers. Both are completely absorbed within 2-3 hours of oral administration, have a plasma half-life of about 5 hours and are excreted in the urine substantially unchanged at the rate closely dependent upon urinary pH (Morselli *et al.*, 1978). Being sympathomimetic amines, their central stimulating effects are marked, the d-isomer being four times more potent than the ℓ-isomer and twice as potent as the racemic compound (Printzmetal and Alles, 1940). With short term administration, amphetamine produces increased alertness, less fatigue, elevation of mood and appetite suppression.

1.2.2.2 Phenmetrazine

Phenmetrazine is well absorbed, rapidly metabolized and excreted by the biliary and renal tracts. Its anorectic and euphoriant properties are similar to those of amphetamine, although it has less central and cardiac stimulating effects (Spillane, 1960).

1.2.2.3 Phentermine and Chlorphentermine

Phentermine is a weak sympathomimetic drug which is well absorbed from the small intestine. It has a plasma half-life of about 24 hours and is excreted in the urine both unchanged and as inactive metabolite. Animal studies have shown inotropic, chronotropic and vasopressor cardiac effects (Yelonsky *et al.*, 1969). Chlorphentermine, like its non-chlorinated analogue, is a weak sympathomimetic drug. It has, however, a longer plasma half-life, more than twice that of phentermine (Beckett and Brookes, 1971).

1.2.2.4 Diethylpropion

The amphetamine congener, diethylpropion, is well absorbed producing peak levels within 2 hours of oral administration. It exhibits first-pass kinetics with rapid hepatic metabolism to active metabolites. It is these and not the parent drug which are responsible for the pharmacological effects. Diethylpropion is therefore twice as potent when administered orally than parenterally (Wright *et al.*, 1975). The central stimulant properties in man are only 10-20% those of amphetamine (Hoekenga *et al.*, 1978).

1.2.2.5 Mazindol

Although mazindol is structurally not a β-phenylethylamine, its neuropharmacological effects are similar to amphetamine. It has cardiac and vasopressor effects and produces central stimulation (Hadler, 1972). Peak drug levels occur two hours after administration and coincide with the onset of the anorectic effect.

1.2.2.6 Fenfluramine

Fenfluramine is rapidly absorbed with an absorption half-life of about 1 hour. It has a plasma half-life of about 20 hours and after multiple dosage, steady state levels are achieved within 5 days. Both fenfluramine and its active metabolite, norfenfluramine, are excreted by the renal tract with an elimination rate which is pH dependent (Campbell, 1971). In man, the weight loss achieved by fenfluramine is frequently associated with a fall in systematic blood pressure (Follows, 1971). Fenfluramine exerts depressant rather than stimulant central effects differing markedly in this respect from amphetamine (Fink *et al.*, 1971).

1.2.3 CLINICAL EFFICACY OF ANORECTIC DRUGS

1.2.3.1 Mode of Administration

Because of the possible development of drug tolerance and the risk of drug abuse, a number of double-blind studies have compared continuous and intermittent therapy with amphetamine, mazindol, diethylpropion and fenfluramine over periods ranging from 12 to 36 weeks (Douglas and Munro, 1982). Intermittent fenfluramine administration was less effective than continuous treatment and was associated with a much higher incidence of adverse side-effects, especially withdrawal depression (Steel and Briggs, 1972). It follows that fenfluramine is best administered continuously. In patients discontinuing treatment, the dose should be reduced stepwise over a period of several weeks.

1.2.3.2 Drug Tolerance

Both the Food and Drug Administration Working Party, and the studies on "refractory obesity" (Douglas and Munro, 1982) have demonstrated an appreciable individual variability in response to anorectic drug therapy. Patients receiving drug treatment have usually been unable to achieve weight loss by dietary means alone. Failure to lose weight following the introduction of an anorectic agent may thus reflect an adaptive fall in resting metabolic rate as a result of persistent calorie deprivation. These subjects may be said to have "**primary drug tolerance**". In others, who are unable to adhere to a calorie reduced diet without the aid of an anorectic agent, effective drug therapy results in a fall in energy consumption and subsequently, the resting metabolic rate may also fall as a result of the metabolic adaptation to weight loss. The rate of weight loss then plateaus. Provided the drug is continued, plateau weight can be maintained and weight regain may be prevented (Hudson, 1977). This suggests that antiobesity agents continue to exert an effect while maintaining plateau weight. In a few subjects, however, weight regain occurs despite continuing drug therapy, *i.e.* **secondary drug tolerance**. This phenomenon probably reflects true drug tolerance as can be readily demonstrated in laboratory animals (Opitz, 1978). If this is the case, a change to

an antiobesity agent acting on a different neurochemical system may help maintain plateau weight and prevent weight regain.

1.2.3.3 Patient Selection

Obese subjects can arbitrarily be divided into three categories :

(i) Those with a "normal" energy expenditure who can curtail their intake sufficiently to create an effective energy gap without recourse to drug therapy.

(ii) Those incapable of making such a change on their own, but in whom the administration of an anorectic drug will produce a negative energy balance, at least while that drug is being taken.

(iii) Those unable, for one reason or another, to achieve weight loss even while receiving an anorectic drug.

Clearly, the centrally acting drugs are unnecessary in the first category of patient and are ineffective in the third. Their use should therefore be restricted to the second category but at present, there is no means of identifying these patients other than by trial and error. Although drug therapy should be withheld until non-pharmacological methods have failed, once "refractory obesity" has developed, the chances of promoting substantial weight loss are poor. However, in one study the combination of phentermine with behavioural therapy produced greater weight losses than that achieved by the drug or behavioural therapy alone (Brightwell and Naylor, 1979).

1.2.3.4 Long Term Therapy

If weight regain following drug withdrawal is to be avoided, the price that may have to be paid is their long term administration. A number of studies have suggested that diethylpropion, phentermine, fenfluramine and mazindol can be given for periods of up to

several years with reasonable safety and without weight regain occurring (Hudson, 1977). Most would consider that the risks of this approach are unacceptable. Some might agree that such treatment would be justified in those patients in whom the initial period of drug treatment had produced substantial weight loss, and, who were at special risk becuase of their obesity. Subjects at special medical risk include the obese diabetic and the obese hypertensive, whereas the combination of obesity with depression may create a vicious cycle of compulsive eating causing further weight gain and aggravating the depression.

1.2.4 ANTIOBESITY DRUGS AND OBESITY RELATED DISORDERS

1.2.4.1 Obesity and Diabetes

Obesity and diabetes mellitus are frequently associated (West and Kalbleisch, 1971), although the nature of this relationship is unclear. Maturity onset diabetes accounts for about 80% of diabetic population and 75% of such patients are overweight. Reduction in weight will improve glucose tolerance and may reduce hyperinsulinemia (Keen *et al.*, 1978). A strong case can therefore be made for the use of antiobesity agents in the management of the overweight diabetic. Both phentermine and diethylpropion given to an obese diabetic for a period of up to 24 weeks will promote weight loss (Campbell *et al.*, 1977a; Gersberg *et al.*, 1977). Mazindol and fenfluramine may have effects on carbohydrate and fat metabolism over and above the benefits attributable to weight reduction (Kirby and Turner, 1977). The additional metabolic effects that have been attributed to fenfluramine include the inhibition of lipogenesis and an insulin-dependent increase in peripheral glucose utilisation (Turner, 1978).

1.2.4.2 Clinical Pharmacology of the Biguanides

Despite nearly 25 years of clinical use, the mode of action of the biguanides remains unclear but is probably multifactorial. They may reduce energy intake by a central anorectic effect (Stowers and Bewsher, 1969), delay gastric emptying (Gomez-Perez *et al.*, 1974), impair the

uptake of nutrients from the small intestine (Czyzyk, 1969), reduce gluconeogenesis and lipogenesis (Sterne, 1964) and enhance peripheral glucose utilisation (Butterfield and Whichelow, 1962). Both phenformin and metformin are well absorbed. Metformin is excreted unchanged in the urine; phenformin is partly metabolized in the liver, with only 50% being excreted unchanged by the kidneys (Douglas and Munro, 1982).

1.2.4.2.1 Side Effects of Biguanides

The most common side effect is lactic acidosis, which carries a high mortality rate and occurs spontaneously, particularly with phenformin (Alberti and Nattrass, 1977). Metformin is now the biguanide of choice. Biguanides produce fibrinolytic effects and caution is necessary during concomitant anticoagulant therapy (Hamblin, 1971).

1.2.4.2.2 Clinical Efficacy of Metformin

Metformin is well established as an effective adjunct to dietary therapy in the obese and non-obese insulin-dependent diabetic. Unlike sulphonylureas, it will improve diabetic control without producing weight gain. It may promote weight loss given alone (Lavieuville and Isnard, 1975; Cairns *et al.*, 1977) or with a sulphonylurea (Patel and Stowers, 1964).

1.2.4.3 Obesity and Hypertension

The evidence linking hypertension to obesity is impressive and the management of the obese hypertensive must always include weight control (Sims, 1981). The sympathomimetic anorectic agents, while promoting weight loss, may potentiate the vasopressor effects of catecholamines and antagonise the adrenergic neuron blockers such as guanethidine, bethanidine and debrisoquine. A number of studies have assessed the effects of appetite suppressants in hypertensive subjects. Mazindol resulted in an additional mean weight loss of 2 kg and a mean reduction in systolic and diastolic blood pressure of 3 mm Hg and 4 mm Hg respectively when compared with placebo (Adams, 1975). In contrast, fenfluramine

appears to have a hypotensive effect in excess of that attributable to weight loss (Hudson, 1977). This is most marked in patients taking methyldopa and reserpine, while those on debrisoquine show minimal change (Waal-Manning and Simpson, 1969). Conversely, weight loss is reduced in patients taking fenfluramine along with hypotensive agents, other than diuretics (Hudson, 1977).

1.2.4.4 Obesity and Depression

Obesity and endogenous depression are both common and often coexist (Rodin, 1981). Fenfluramine and other anorectic drugs, with the possible exception of mazindol, should be avoided in the severely depressed. Unfortunately, tricyclic compounds such as amitriptyline, although of proven value in the treatment of endogenous depression are frequently associated with weight gain. A drug which combines antiobesity and antidepressant properties would be particularly attractive as a long term therapeutic agent.

1.2.5 RECENT PHARMACOLOGICAL APPROACHES

1.2.5.1 Antidepressants

Weight gain commonly occurs during therapy with tricyclic antidepressants (Paykel *et al.*, 1973). This may restrict their value in subjects with coexistent obesity because a rising weight may itself aggravate depressive symptoms. In such conditions, an antidepressant agent with an antiobesity effect would be advantageous. Two such compounds are undergoing clinical evaluation.

1.2.5.1.1 Zimelidine

Zimelidine is a bicyclic antidepressant which is a potent inhibitor of 5-HT uptake. It has antidepressant properties comparable to amitriptyline though may be less likely to produce adverse side effects. In a two week double-blind study in obese depressives, those receiving

amitriptyline all gained weight compared with only half of those taking zimelidine (Coppen *et al.*, 1979).

1.2.5.1.2 Ciclazindol

Ciclazindol is a tetracyclic antidepressant which is structurally related to mazindol. It acts by inhibiting NA uptake and has been reported as being of comparable efficacy to amitriptyline in the management of depression (Ghose *et al.*, 1978). When given to obese, nondepressed subjects it produced a mean weight loss of 3.1 kg in 3 weeks (Gotestam, 1979).

1.2.5.2 Evening Primrose Oil

Evening Primrose Oil (EPO) is a rich, naturally occurring source of essential fatty acids. It contains 72% of linoleic acid and 9% of γ-linoleic acid. The latter is a precursor of prostaglandin E. It has been suggested that prostaglandins or their precursors may influence the development of obesity (Horrobin *et al.*, 1979). It has been shown that the administration of EPO for 6 weeks in obese subjects increases erythrocyte sodium efflux rate; the increase occurring in spite of weight reduction. This observation suggests that EPO exerts an antiobesity effect by altering cellular metabolic activity.

1.2.5.3 Ephedrine

In 1972, a Danish general practitioner made the chance observation that asthmatics taking ephedrine-containing compound lost weight. The preparation also contained caffeine and a barbiturate. The compound, without the barbiturate, has been evaluated by a direct double-blind comparison with diethylpropion in a 12 week study. The mean weight losses achieved with diethylpropion and ephedrine were comparable, both being significantly superior to the placebo (Malchon-Moller *et al.*, 1981). It remains to be established whether the antiobesity action of ephedrine is primarily central or peripheral. It is structurally related to other

centrally acting compounds and also to NA which exerts a significant effect on brown fat metabolic activity.

1.2.5.4 The Use of Thermogenic Drugs

Resting metabolic activity accounts for a large portion of total energy expenditure (Hoffman *et al.*, 1979). Some obese subjects have a reduced metabolic response to food which may become further impaired during dietary restriction (Miller and Parsonage, 1975). It is now recognised that brown adipose tissue plays an important role in energy balance in animals (Rothwell and Stock, 1979). Reduced thermogenesis may be important in some obese subjects (Jung *et al.*, 1979). It follows that such subjects may fail to achieve substantial weight loss in spite of relative caloric restriction and that drugs which promote energy expenditure might prove more effective than those that reduce food intake. The catecholamines increase thermogenesis but unfortunately, they are associated with toxic side-effects in pharmacological dosage. There is therefore considerable interest in the development of nontoxic drugs which can produce weight loss by enhancing thermogenesis. As yet, the only available compounds are the thyroid hormones.

1.2.5.4.1 Thyroid Hormones

In some obese patients, the thyroid stimulating hormone (TSH) response to thyroid releasing hormone (TRH) is exaggerated, although thyroid functioning is unaltered (Douglas and Munro, 1982). Adaptive changes in thyroid hormone metabolism also occur during dietary restriction. Reduction in carbohydrate intake will increase the hepatic conversion of thyroxine (T_4) to reverse triiodothyronine (T_3) which is physiologically inactive (Danforth *et al.*, 1978). The role of thyroid hormones in obesity is therefore of particular interest.

1.2.5.4.1.1 Clinical Pharmacology of Thyroid Hormones

T_3 and T_4 are well absorbed from the small intestine. Thyroid hormones increase the activity

of membrane bound ATPase, mitochondrial glycerol phosphate dehydrogenase and DNA transcriptase, resulting in an increase in resting metabolic rate, decreased lipogenesis and increased protein turnover. The weight loss following thyroid hormone therapy is therefore partly the result of protein catabolism.

1.2.5.4.1.2 Clinical Efficacy of Thyroid Hormones

Both T_3 and T_4, given in pharmacological doses, appear to be well tolerated by the obese. They will promote weight loss during conventional dietary restriction (Hollingsworth *et al.*, 1970; Gonzalez-Barranco *et al.*, 1975) or without dietary advice (Gwinup and Poucher, 1967). They may enhance the weight loss achieved by amphetamine (Kaplan and Jose, 1970). However, mean weight regain usually occurs when treatment is discontinued (Gwinup and Poucher, 1967) and the long term results using T_3 are no better than those achieved by conventional dietary restriction (Goodman, 1969). The use of thyroid hormone in the treatment of obesity is therefore limited. Their use may be justified in obese subjects with elevated serum TSH but without any other clinical or biochemical evidence of thyroid failure.

1.2.5.5 Agents Acting on the Gastrointestinal Tract

Drugs acting primarily on the gastrointestinal tract (GIT) may produce an antiobesity effect either by directly interfering with the digestion and absorption of dietary nutrients or by influencing those humoral and other signals from the GIT which normally are involved in the regulation of appetite and thus, body weight. A number of agents are currently being developed which may prove to be of therapeutic value (Sullivan *et al.*, 1981).

1.2.5.5.1 Agents Reducing Intestinal Absorption of Dietary Lipid

Various drugs in current usage can reduce lipid absorption. These include cholestyramine, hydroxocobalamin and fenfluramine. However, none of these have an effect which is clinically relevant. Pluronic L-101 is a lipophilic nonionic surfactant which is a potent

inhibitor of human pancreatic lipase (Comai and Sullivan, 1980). In rats, it has no effect on food consumption but it produces a dose-dependent reduction in dietary fat absorption and body weight gain due to reduction in carcass weight but not protein (Douglas and Munro, 1982). The new drug, tetrahydrolipstatin (THL) works by blocking the hydrolysis of triglycerides to fatty acids. Without this step, fat cannot be digested. Partially digested fats travel through the gut and are excreted (Saul, 1993). Unfortunately, its safety is already in dispute; it might increase the risk of colonic cancer and heart failure.

1.2.5.5.2 Agents Reducing Intestinal Absorption of Dietary Carbohydrate

The assimilation of dietary carbohydrate may be influenced by the biguanides and the "bulking agents". Inhibitors of the enzymes, α-glucosides, α-amylase and sucrase will reduce the rate of absorption of glucose. The drug, Bay-g-5421, has been shown to have such properties and has a dose-dependent influence on weight gain in genetically obese rats (Puls *et al.*, 1977). It may prove particularly valuable in the management of the obese diabetic (Sachse and Willms, 1979).

1.2.5.5.3 Anorectic Drugs Which Function Primarily in the GIT

The monosubstituted citric acid analogs, *threo*-chlorocitric acid, *threo*-epoxyaconitic acid and *threo*-hydroxycitric acid appear to exert a profound anorectic activity. The exact mode of action is unclear but probably relates, at least in part, to their effects on delaying the rate of gastric emptying. The resultant weight loss can be attributed to a selective reduction in body fat. *Threo*-chlorocitric acid is the most potent of the three; it has no CNS stimulant effects, tolerance does not develop nor does it produce conditioned aversion behaviour (Sullivan *et al.*, 1981).

Clearly, these various anorectic drugs will require considerable development before their introduction into clinical usage can be justified. However, if further evaluation confirms their potential, they may offer a more effective and acceptable pharmacological approach to

the long term management of obesity.

1.2.6 ANORECTIC DRUGS AND BRAIN NEUROTRANSMITTERS

1.2.6.1 Noradrenergic System

On the basis of biochemical studies, it has been suggested that amphetamine acts on brain NA mainly by releasing it from presynaptic terminals and by inhibiting its uptake into the neurons (Glowinski, 1970). It has also been suggested that amphetamine can exert an inhibitory effect on monoamine oxidase (MAO), the enzyme responsible for the intraneural metabolism of NA (Glowinski *et al.*, 1966). These effects of amphetamine would lead to an increased availability of NA at the receptors and consequently would increase the activity of the NA system in the brain. In addition, it appears that amphetamine can possess some direct stimulatory action on postsynaptic NA receptors (Van Rossum *et al.*, 1962; Smith, 1965).

It has been reported that mazindol and diethylpropion can also produce a decrease in brain NA concentrations, whereas phentermine appeared to be ineffective (Garattini *et al.*, 1975a). It has been further suggested that mazindol may act mainly by blocking the neuronal uptake of NA with little or no effect on the release and synthesis of this amine (Engstrom *et al.*, 1975). The perifornical lateral hypothalamus (PFLH) is involved in suppression of feeding mediated by NA, adrenaline and DA, and via β-adrenoceptor (Leibowitz and Rossakis, 1978). The anorectic effects of phenylethylamines and mazindol in this area are mediated by β-adrenergic and dopaminergic receptors (Heffner *et al.*, 1977). Using the selective β_2 adrenergic agonist salbutamol and the antagonist propranolol (Garattini and Samanin, 1984; Bendotti *et al.*, 1986), it was further demonstrated that the anorectic activity in this site is specifically mediated by β_2 adrenoceptor.

1.2.6.2 Dopaminergic System

Amphetamine and related phenylethylamines are both DA releasers and uptake inhibitors. These compounds are known to be potent anorectic drugs in animals (Cox and Maickel, 1972; Leibowitz, 1975) as well as in humans (Kyriakides and Silverstone, 1979; Douglas and Munro, 1982), although orexigenic and body-weight gain were also reported (Holtzman, 1974). Most effects mediated by these drugs are not selective for DA, since they similarly affect NA-mediated system. The anorectic activity of amphetamine was antagonised by the non-selective DA antagonists haloperidol, chlorpromazine and pimozide (Clineschmidt *et al.*, 1974; Heffner *et al.*, 1977; Liebowitz and Rossakis, 1978) or by the selective D_1 antagonist SCH-23390, but not by the D_2 selective antagonist sulpiride (Gilbert and Cooper, 1985), thus indicating an involvement of D_1 dopaminergic receptors in the anorectic activity of amphetamine. Nevertheless, a large number of studies indicate the involvement of adrenergic receptors, particularly β-adrenoceptors, in the anorectic activity of amphetamine (Stein and Wise, 1969; Leibowitz and Rossakis, 1978; Ross, 1979).

1.2.6.3 Serotonergic System

Serotonin and drugs that affect serotonergic transmission have been systematically shown to affect food intake and body weight essentially by anorectic and satiety-promoting activities (Blundell, 1984; Leibowitz and Shor-Posner, 1986; Sugrue, 1987). Drugs that release 5-HT from presynaptic stores, such as *p*-chloroamphetamine, fenfluramine, or norfenfluramine (Trulson and Jacobs, 1976) are known to be powerful anorectic drugs in both experimental animals and humans (Cox and Maickel, 1972; Douglas and Munro, 1982). Agonists at 5-HT_{1B}, 5-HT_{1C}, or 5-HT_2 receptors are inhibitors of feeding behaviour (Bendotti and Samanin, 1987; Kennett *et al.*, 1987). Direct application of the selective 5-HT_{1B} agonist, RU 24969, into the PVN causes hypophagia without motor-stimulant activity (Hutson *et al.*, 1988). Because this site is enriched in 5-HT_{1B} receptors in the rat, it was suggested that similar to endogenous 5-HT which is active at the PVN, 5-HT_{1B} receptors at this site may directly relay the observed anorectic activity (Angel, 1990).

A summary of the effect of anorectic drugs on brain monoamines is shown in Table 1.2.

1.2.7 ANORECTIC DRUG RECOGNITION SITES

1.2.7.1 [^{3}H](+) Amphetamine Binding

A saturable and stereospecific binding site for [^{3}H](+) amphetamine was first described by Paul *et al.*, (1982) in a membrane preparation from rat hypothalamus. Using high sodium concentrations (0.5 M), two sites were found. Using the lower affinity site, which represents 70 - 80% of specific binding sites, it was further found that the relative affinities of a series of anorectic phenylethylamine derivatives for [^{3}H](+) amphetamine binding is highly correlated to their potencies as anorectic agents. No significant correlation with the stimulant activity of these drugs was observed. The [^{3}H](+) amphetamine binding site was of micromolar affinity and of relatively high capacity (Hauger *et al.*, 1984).

1.2.7.2 [^{3}H](+) Mazindol Binding

Angel *et al.*, (1987) found that [^{3}H](+) mazindol binding was quite similar, if not identical to, [^{3}H](+) amphetamine binding. This site is essentially centrally located and particularly enriched in the hypothalamus and brainstem. Recent preliminary autoradiographic studies further confirm these observations and expand the localization of high levels of the [^{3}H](+) mazindol binding site also to the dorsal raphe, median eminence, arcuate nucleus of the hypothalamus and locus ceruleus, olfactory tubercule, and pineal gland (Vincent and Levin, 1989).

TABLE 1.2: Effect of Anorectics on Brain Monoamines and Some Metabolites

DRUG (mg/kg, i.p.)		5-HT	5-HIAA	NA	DA
Saline		0.43 ± 0.01	0.34 ± 0.01	0.40 ± 0.01	8.2 ± 0.5
d, amphetamine sulphate	15	0.40 ± 0.01	0.34 ± 0.03	0.24 ± 0.02*	7.5 ± 0.3
d, l, fenfluramine HCl	15	0.16 ± 0.01*	0.20 ± 0.03*	0.38 ± 0.01	7.2 ± 0.2
Mazindol	15	0.50 ± 0.08	0.34 ± 0.02	0.32 ± 0.03*	-
Phentermine HCl	15	0.47 ± 0.01	0.37 ± 0.03	0.40 ± 0.01	-
Diethylpropion HCl	15	0.40 ± 0.01	0.28 ± 0.01	0.30 ± 0.01*	-

Each figure represents the mean (μg/g ± SEM) of 6 animals

* p 0.01 versus saline group (Dunnett's test)

5-HT	Serotonin
5-HIAA	5-Hydroxyindoleacetic acid
NA	Noradrenaline
DA	Dopamine

(Adapted from Garattini and Samanin, 1975).

1.2.7.3 [^{3}H](+) *p*-Chloroamphetamine Binding

p-Chloroamphetamine is a selective 5-HT releaser and uptake blocker, with anorectic activity on acute treatment, and is among the most potent of the phenylethylamine anorectic drugs (Cox and Maickel, 1972). Saturable, low-affinity and high capacity binding of [^{3}H](+) *p*-chloro-amphetamine has been demonstrated in rat hypothalamic membranes (Angel *et al.*, 1985). The pharmacological characteristics of this site are very similar, if not identical, to the hypothalamic [^{3}H](+) amphetamine and [^{3}H](+) mazindol binding sites, and positive and significant intercorrelations between the effects of a series of phenylethylamine derivatives and the three different sites were obtained (Angel *et al.*, 1985).

1.2.7.4 [^{3}H] Fenfluramine Binding

A site of relatively high affinity and low capacity was described in rat brain membranes (Garattini *et al.*, 1987). The regional distribution of [^{3}H] fenfluramine binding site differs from previous ones, because the cortex and cerebellum had the highest levels of binding and lower levels were found in the hypothalamus. The regional distribution of this site did not parallel the endogenous concentrations of 5-HT and had no obvious relationship to feeding centres. The site was stereoselective for the *d*-enantiomer of fenfluramine and for its respective metabolite. Amphetamine and similar phenylethylamines did not interact with this site, but 5-HT uptake inhibitors such as imipramine, fluoxetine, or indalpine show the highest affinity for this site (Garattini *et al.*, 1987).

1.3 THE ROLE OF GUT-BRAIN PEPTIDES IN ANOREXIA

The number of centrally injected peptides that inhibit food intake is rapidly growing and includes cholecystokinin (CCK), bombesin, neurotensin, adrenocorticotrophin, anorexigenic peptide, corticotrophin-releasing factor (CRF), vasopressin, insulin, calcitonin, somatostatin, TRH, substance P, satietin, and glucagon (Leibowitz, 1985). In as much as in most studies these peptides were injected intraventricularly and tested at quite high doses, it is difficult

to draw any specific conclusion concerning the site(s) of action of these peptides and, thus, to identify the mechanism of action or their potential physiological function.

In as much as brain peptides and monoamines are known to coexist in a single neuron and may affect each other's pattern of release and receptor activity, it has been proposed that these substances may interact closely in their control of appetite for food (Leibowitz, 1985; Morley *et al.*, 1985). This suggestion has been made for neurotensin, calcitonin, CCK, and CRF in relation to NA function in the PVN, and for CCK, neurotensin and the opioids in relation to catecholamine activity in the PFLH. The specific nature and physiological significance of these peptide-monoamine interactions need some elaboration. Hypothetically, the gut-brain peptides may be expected to act through a variety of hypothalamic areas, inhibiting monoamine function where it normally stimulates feeding and potentiating its function where it normally induces satiety.

1.4 DIURNAL RHYTHMS IN NEUROTRANSMITTER MODULATION OF FEEDING PATTERNS

The ability of animals to alter patterns of protein and energy intake in relation to need becomes especially high during the active period of the diurnal cycle when feeding behaviour is more pronounced (Armstrong, 1980). This nocturnal feeding activity in the rat is characterised by peaks of food intake at the beginning and towards the end of the night with a relatively stable trough in the middle of the cycle (Siegel, 1961). In freely feeding rats maintained on pure macronutrient diets (Tempel *et al.*, 1987), the animals during the first 1 - 2 hours of the night exhibit an initial preference for large carbohydrate meals. This pattern is shortly followed by a shift towards increased protein intake. Superimposed on this pattern of alternating carbohydrate and protein meals appears to be an additional rhythm in which carbohydrate ingestion is favoured in the early hours of the dark cycle whereas protein ingestion is favoured in the late hours of the dark and exhibited particularly during the last meal of the cycle (Tempel *et al.*, 1987).

With regard to serotonergic function, a diurnal rhythm of hypothalamic content of this indoleamine, with a peak during the early hours of the dark cycle, has been indicated (King *et al.*, 1982; Angren *et al.*, 1986). Investigations involving peripheral injection of dexfenfluramine (Tempel *et al.*, 1987) revealed, in the early dark tests, a strong and relatively selective suppression of carbohydrate intake, accompanied by no change in protein and fat intake. This pattern of relative selectivity for carbohydrate, which can also be seen with injection of 5-HT directly into the PVN (Walsh *et al.*, 1987), is not observed later in the dark period when non-selective suppression of intake occurs with all three diets. These findings are of particular interest in light of other findings that have demonstrated that PVN noradrenaline similarly produces its strongest stimulatory effect on food intake during this period (Bhakthavatsalam and Leibowitz, 1985), when a peak is known to occur in PVN α_2-receptor density and a simultaneous peak in circulating corticosterone (Krieger and Hauser, 1978).

1.5 OVERVIEW

The indications for the pharmacological treatment of obesity remains poorly defined. A number of new approaches are being evaluated, and the future may lie in the development of drugs which enhance thermogenesis (Section 1.2.5.4) or primarily act upon the GIT (Section 1.2.5.5). Various studies suggest that anorectic agents exert an inhibitory effect on food intake by interacting with the monoaminergic systems in the brain. Although the separate role of NA and DA in the anorectic activity remains to be elucidated, the evidence suggests that catecholamines are involved in the effect of amphetamine and other anorectics such as phentermine, chorphentermine, mazindol and diethylpropion (Section 1.2.6.1). Fenfluramine appears to exert its anorectic effect through an action on brain 5-HT (Section 1.2.6.3). This fact is of great interest, since it can suggest an involvement of brain 5-HT in the regulation of food intake and, therefore, can open new possibilities for the chemical control of food intake and, consequently, of pathological obesity.

Furthermore, other findings suggest that NA and 5-HT, possibly within the PVN, may become activated specifically at the beginning of the dark (active) cycle, to interact antagonistically and directly in the control of carbohydrate intake in relation to protein intake (Section 1.4). It is likely that activation of these systems at the onset of darkness enables an animal to modulate the ratio of nutrient intake and thus maintain a dietary balance at this time of most active feeding.

1.6 THE PINEAL GLAND

1.6.1 INTRODUCTION

Although the pineal gland was considered a functional component by many just two decades ago, research in the last few years has clearly established it as an integral and important component of the neuroendocrine system. The bulk of the investigative effort was initially directed at clarifying the endocrine role of the pineal organ in non-human mammals. Although the data suggested a niche for the pineal gland in the control of the hypothalamic-pituitary-gonadal axis (Reiter, 1981; Goldman, 1983), its effects are by no means restricted to the reproductive system. On the contrary, the pineal gland and its indole products have been functionally related to virtually every endocrine gland in the organism (Mathews and Seamark, 1981; Reiter, 1982).

A great deal of effort has been directed at the mechanisms concerned with the conversion of serotonin (5-HT) to *N*-acetyl-5-methoxytryptamine or melatonin (MEL). Research on this hormone has overshadowed investigative efforts on other indoleamines that may also be secretory products of the pineal. The mammalian pineal gland is considered to be an active neuroendocrine transducer (Wurtman and Axelrod, 1965); it converts a neural input, *viz.* neurotransmitter, to a hormonal output, i.e. MEL and pineal polypeptides.

In humans, melatonin may affect circadian rhythms as evidenced by studies on seasonal affective disorder (SAD) (Rosenthal *et al.*, 1986), depression (Arendt, 1989), jet lag (Arendt *et al.*, 1988), puberty (Lang, 1986), and sleep and wakefulness (Arendt, *et al.*, 1988). Thus, it becomes clear that the human pineal gland is highly active, light : dark-dependent and capable of a variety of endocrine effects.

This subject tries to correlate between melatonin production and the feeding behaviour of animals which both seem to be under circadian rhythm. Therefore, topics on food intake rhythms and the effects of feeding on pineal indoleamines are also reviewed.

1.6.2 ANATOMY

1.6.2.1 Location

In the rat, the pineal gland rests between the two cerebral hemispheres in front of the cerebellum where it is covered by the confluence of the superior saggital and transverse sinuses (Figure 1.1 and 1.2). The pineal recess lies between the two laminae that form the stalk. The pineal stalk is generally divided into 3 distinct sections : a distal part, midpart and a proximal part. The proximal part is connected to the brain between the caudal and habenular commissures. The midpart is extremely thin and connects the proximal and distal parts (Wurtman *et al.*, 1968).

1.6.2.2 Blood Supply

The pineal gland is supplied by 2 - 4 branches from the posterior cerebral arteries. These branches do not give off any side branches except to part of the choroid plexus. Venous blood is collected by 12 - 16 superficial veins which drain into the great cerebral vein and via the superior sagittal sinus into the systemic venous circulation (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; Reiter, 1981).

1.6.2.3 Innervation

The mammalian pineal gland, unlike other endocrine organs, relies heavily on its innervation for its endocrine activity. Sensory information perceived by the eyes is essential in determining the production of pineal MEL. In general, light suppresses MEL production, whereas darkness is associated with a rise in the biosynthesis of several indoleamines (Reiter, 1984). Information about ambient lighting conditions is transferred from the retinas to the pineal gland via a complex series of neurons that are located in both the central and

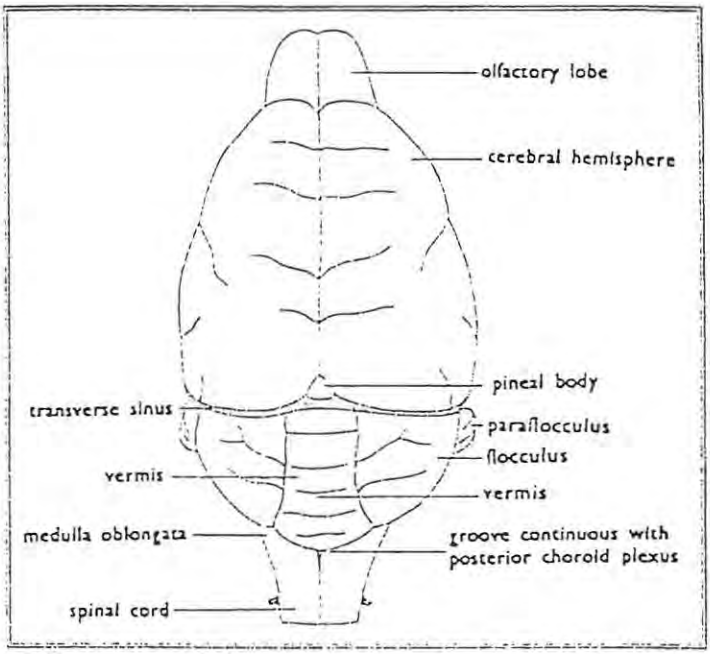


FIGURE 1.1: **Dorsal View of the Rat Brain** (Daya, 1982).

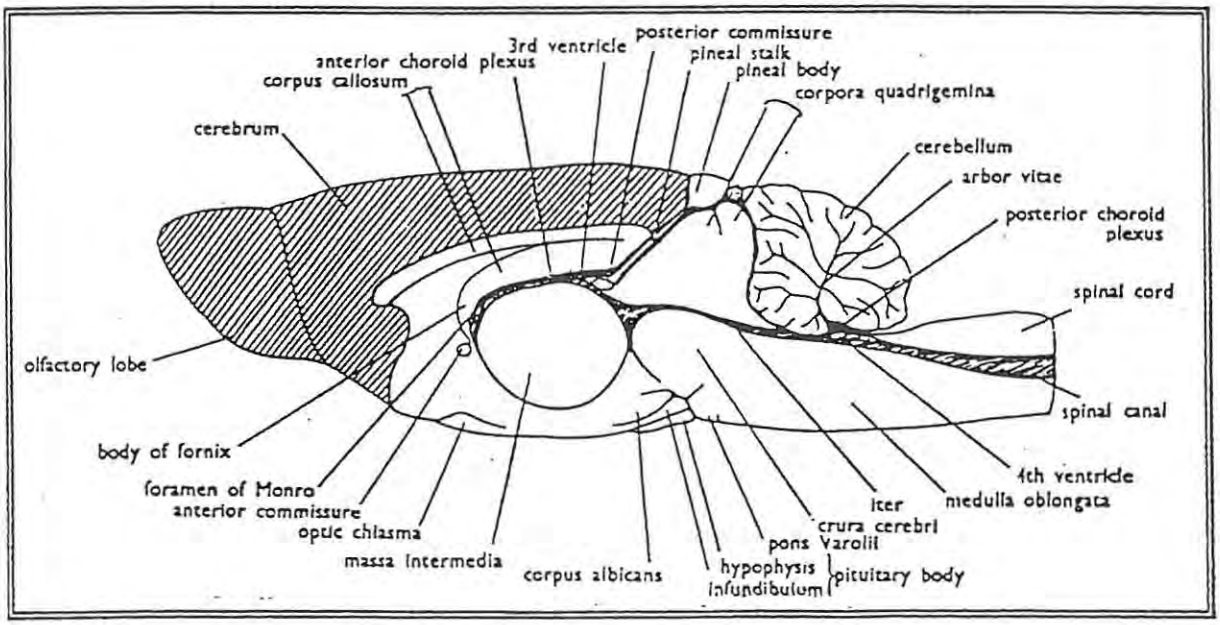


FIGURE 1.2: **Medial Saggital View of the Rat Brain** (Daya, 1982).

peripheral nervous systems (Kappers, 1965). After transduction into a neural signal in the photoreceptors of the retinas, the message is sent to the hypothalamus via ganglion cell axons, which form the retinohypothalamic tract (Moore, 1978). At the level of the optic chiasma these fibres diverge from this region and terminate in the suprachiasmatic nuclei (SCN) of the anterior hypothalamus (Sonofriew and Weindl, 1982). After a synapse at this location, fibres project possibly to the PVN of the hypothalamus (Bittman, 1984). Long descending axons then supposedly carry the neural message to the intermediolateral cell column of the upper thoracic spinal cord (Swanson and Sawchenki, 1983). The axons of these preganglionic perikarya leave the spinal cord and pass up the sympathetic trunk to synapse on postganglionic cell bodies in the superior cervical ganglia (SCG); the postganglionic fibres enter the skull and, among other regions, terminate within the pineal gland (See Figure 1.3) (Kappers, 1965).

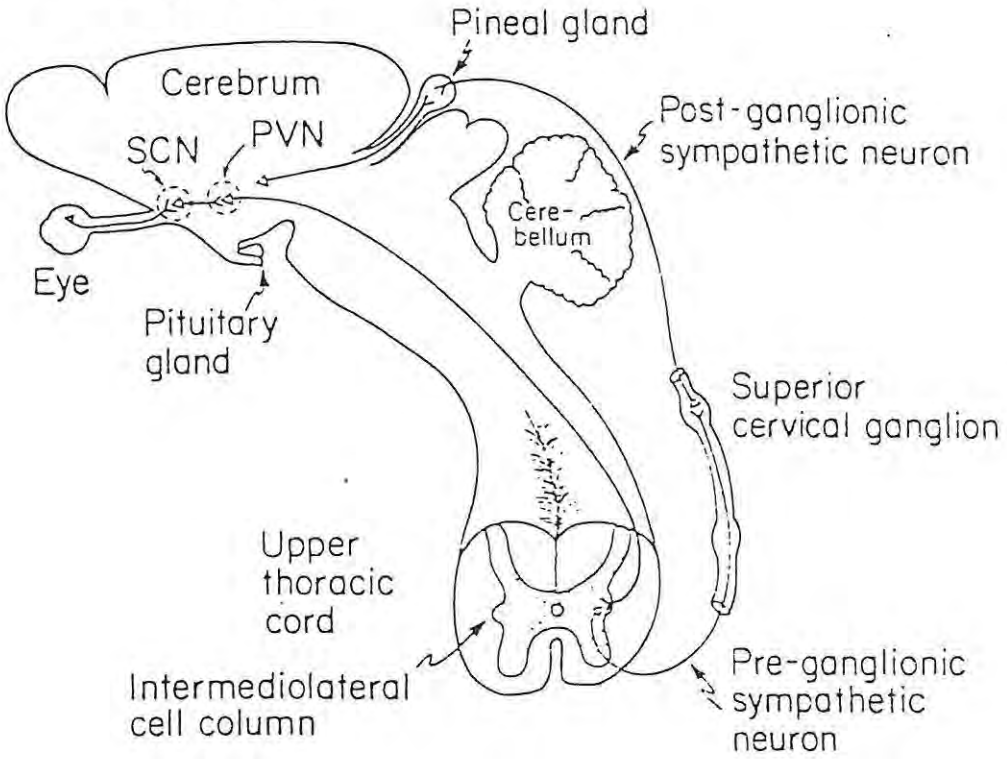


FIGURE 1.3: **Proposed Neural Connections Between the Eyes and the Pineal Gland in Mammals, Including Man.** PVN = Paraventricular Nuclei; SCN = Suprachiasmatic Nuclei (Reiter, 1989).

The sympathetic neural connections between the SCN and the pineal gland must remain intact in order for the pineal to function in its normal capacity. Interruption of the neural connections caudal to the SCN in either the central or peripheral nervous systems interferes with the cyclic production of MEL as well as with the endocrine capability of the pineal gland (Kneisley *et al.*, 1978; Klein and Moore, 1979; Reiter, 1981).

1.6.3 CYTOLOGY

Pinealocytes are predominant cells in the mammalian pineal gland. Although of neuroectodermal origin and secretory, the pinealocyte is not a neurosecretory cell and not a neuron. The mammalian pinealocyte belongs to the APUD cell type. In addition to pinealocytes, glial cells are present in varying numbers and, in some species, fibroblasts, mast cells, plasma cells, pigment-containing cells and nerve cells are occasionally present (Vollrath, 1981). Pinealocytes are generally irregularly shaped containing varying numbers of cytoplasmic processes emerging from cell bodies.

The **nucleus** is usually oval or irregular in shape with varying numbers of indentations, and is characterised by electron-dense nucleoplasm containing condensed chromatin.

The **Golgi apparatus** in most pinealocytes is well developed and consists of a system of sacs which are associated with numerous vesicles of varying diameter. Most of these vesicles are clear, although dense-core vesicles (DCV) are also present.

A moderate amount of **granular endoplasmic reticulum** (GER) is present within the pinealocyte, and GER generally consists of isolated cisternae or vesicles. However, in some pinealocytes complexes of parallel oriented cisternae are also present. Smooth endoplasmic reticulum is consistently present in the mammalian pinealocyte, though in varying amounts.

A common feature of the mammalian pinealocyte is a relatively large number of **mitochondria**, which show many variations in shape and size. Typically, round, oval or

elongated mitochondria are observed.

Lysosomes are another cell organelle typically present in the mammalian pinealocyte, although their number shows great variations among species.

"Synaptic" ribbons, structures consisting of an electron-dense rod surrounded by a single layer of electron-lucent vesicles, have been reported to be present in pinealocytes of every mammalian species. In addition to "synaptic" ribbons, other similar structures, *viz.* "synaptic" spherules, are present in the mammalian pinealocyte.

Other cell components of the mammalian pinealocyte include : lipid droplets, multivesicular bodies, subsurface cisternae, centrioles, cilia, multitubular sheaves, microtubules, microfilaments, annulate lamellae and related structures, concentric lamellae, pigment granules, glycogen particles, membrane-bound bodies and inclusion bodies.

Although no particular structure of the mammalian pinealocyte has yet been proved to be the morphological marker of the pineal secretory substance, there is substantial ultrastructural evidence which allows the delineation of few putative secretory processes (Pévet, 1979; Karasek, 1983).

The first of such processes (**neurosecretory-like**) is characterized by the formation of DCV by the Golgi apparatus. The second type of secretory process (**ependymal-like**) consists of the formation of vacuoles containing flocculent material of moderate electron density by the cisternae of the GER. The possibility of yet other modes of pineal secretion cannot be excluded (Karasek, 1983).

1.6.4 MELATONIN BIOSYNTHESIS

The production of MEL within the pinealocyte requires the uptake of the amino acid tryptophan (TRP) from the circulation, which is believed to involve an active uptake

mechanism (Sugden, 1979). An active uptake mechanism for TRP is supported by the observation that pineal levels of the amino acid are conserved (over brain TRP concentrations) in rats treated with the heme precursor 5-aminolevulinate (5-ALA) (Daya *et al.*, 1989); 5-ALA saturates hepatic TRP pyrrolase enzyme thereby enhancing TRP catabolism and lowering circulating levels of the amino acid. Once taken up into the pinealocyte, TRP is oxidised to form 5-hydroxytryptophan (5-HTP) (Ebadi, 1984).

Once formed, 5-HTP is converted to serotonin (5-HT) in the presence of the enzyme aromatic L-amino acid decarboxylase. Serotonin concentrations in the pineal gland are very high and exceeds those of any other organ in the body (Quay, 1974). Serotonin can be acetylated to *N*-acetyl-serotonin (NAS), oxidized to hydroxyindole-3-acetic acid or metabolized to 5-hydroxytryptophol. The 5-hydroxyindoles and *N*-acetylserotonin can be methylated by hydroxyindole-O-methyltransferase (HIOMT) (Axelrod and Weissbach, 1960; Cardinali and Wurtman, 1972). From the methylated products, MEL and 5 methoxy-tryptophol seem to be physiologically the most important substances (Lerner and Case, 1960; McIsaac *et al.*, 1965). Once melatonin is formed in the pineal gland, it is immediately released; as a consequence there is usually a strong correlation between pineal MEL production and plasma levels of this hormone (Wilkinson *et al.*, 1977). Schematic representation of pineal indole metabolism is shown in Figure 1.4.

1.6.5 PHARMACOLOGY

1.6.5.1 Pineal β-Adrenoceptor-mediated Activation

The rat pineal gland represents an extremely rich source of β-adrenergic receptors. The numbers of these receptors have been shown to have a circadian rhythm, being decreased at night (Romero *et al.*, 1975). The pharmacological characteristics of the β-adrenoceptors indicated a β_1-subclass nature in the rat pineal gland (Dickinson *et al.*, 1986). Beta adrenoceptors have a high affinity for NA and adrenaline. Beta adrenergic stimulation of the pinealocyte results in the activation of adenylate cyclase enzyme via a stimulatory guanine

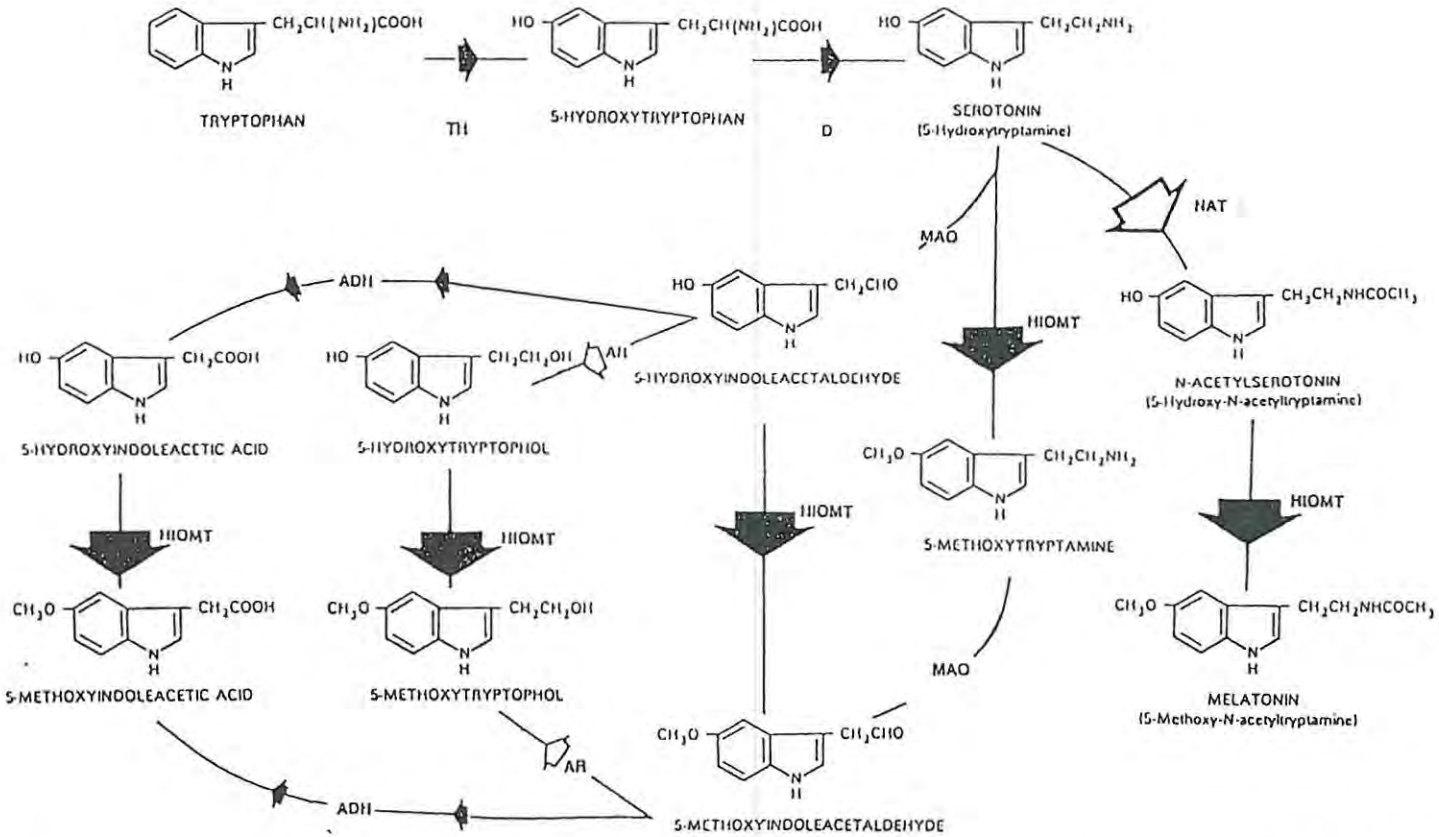


FIGURE 1.4: **Schematic Representation of Pineal Indole Metabolism.** TH = Tryptophan Hydroxylase; D = L-Aromatic Amino Acid Decarboxylase; MAO = Monoamine Oxidase; AR = Aldehyde Reductase; ADH = Aldehyde Dehydrogenase; NAT = *N*-acetyltransferase; and HIOMT = Hydroxyindole-O-methyltransferase. (Adapted from Smith and Hauser, 1981).

nucleotide-binding regulatory protein (G_s) (Spiegel, 1989); this activation results in a rapid, large increase of intracellular cAMP. Cyclic AMP serves as a second messenger in the nocturnal elevation of MEL biosynthesis by activating a cAMP-dependent protein kinase, transcription of mRNA, and an eventual rise in serotonin NAT, the presumed rate-limiting enzyme in MEL production (Figure 1.5) (Klein, 1985). Whether new mRNA induces the de novo synthesis of NAT molecules or merely stimulates a NAT activator protein remains unknown, although recent studies suggest that the former possibility is more likely.

1.6.5.2 Pineal α-Adrenoceptor-mediated Activation

The information originating in α-adrenergic receptor flows from the cell surface into the cell interior through two routes, Ca^{2+} mobilisation and protein kinase activation (Exton, 1985). Alpha-adrenergic receptor stimulation of the rat pineal gland causes an increase in Ca^{2+} influx (Cardinali *et al.*, 1986; Sugden and Sugden, 1986), phosphatidylinositol turnover (Smith *et al.*, 1979) and prostaglandin (PG) synthesis (Ritta and Cardinali, 1982). Data suggesting the possible role of α-adrenoceptor-induced PGE_2 synthesis in MEL regulation include :

(i) the impairment by PG synthesis inhibitors of the nocturnal rise of NAT and MEL content *in vitro* and of the NA-stimulated increase of MEL release *in vitro*;

(ii) the stimulation by PGE_2 of cAMP synthesis, NAT and MEL synthesis;

(iii) the occurrence of specific PG receptor sites in pineal membranes (Cardinali and Ritta, 1983).

Sugden *et al.*, (1985b) demonstrated that the activation of protein kinase C by Ca^{2+} potentiates β-adrenergic cAMP accumulation in rat pinealocytes. Zatz (1985) reported that stimulation of protein kinase C mimics the potentiation of rat pineal NAT produced by β-adrenergic agonism. These data suggest an active interaction between α- and β-adrenoceptors in the mammalian pineal gland (See Figure 1.5).

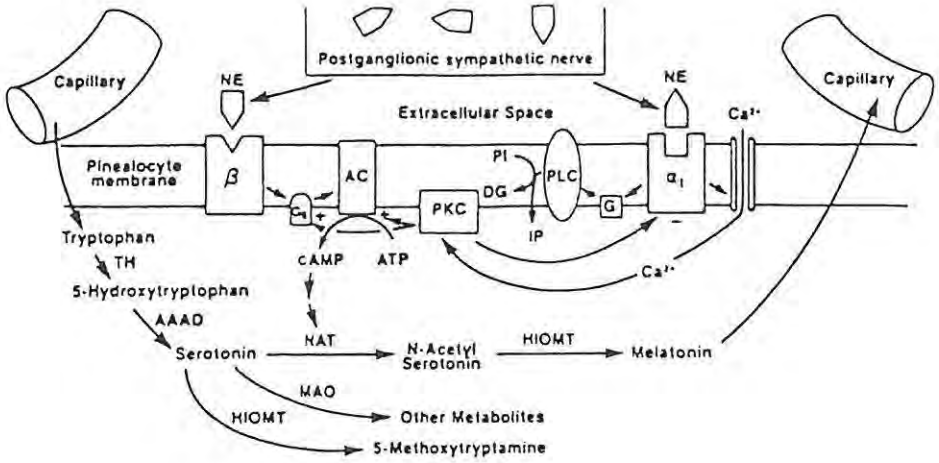


FIGURE 1.5: **Diagrammatic Representation of the Presumed Signal Transduction Mechanisms Between Postganglionic Sympathetic Neurons and the Pinealocyte.** AAAD = L-Aromatic Amino Acid Decarboxylase; AC = Adenylate Cyclase; ATP = Adenosine Triphosphate; DG = Diacylglycerol; G = Guanine Nucleotide-binding Protein; G_s = Stimulatory G; IP = Inositol Phosphate; MAO = Monoamine Oxidase, PI = Phosphatidylinositol; PKC = Protein Kinase C; PLC = Phospholipase C; TH = Tryptophan Hydroxylase; HIOMT = Hydroxyindole-O-methyltransferase; NAT = *N*-acetyltransferase; NE = Norepinephrine; α = α-adrenergic receptor; β = β-adrenergic receptor. (Adapted from Reiter, 1991b).

1.6.5.3 The Role of Other Transmitters

Active synthesis and metabolism of gamma amino butyric acid (GABA) occur in the pineal gland. A Na^+-dependent GABA uptake compatible with that reported in other regions of the brain was detectable in bovine pineals (Ebadi and Chan, 1980). When rat pineal cultures are incubated in the presence of GABA, a significant depression of MEL release was found (Cardinali *et al.*, 1987). This finding is in agreement with the depression of cAMP levels

produced by the exposure of rat pineals to GABA. Additionally, BZP binding sites have been reported in the pineal gland (Lowenstein *et al.*, 1984). After superior cervical ganglionectomy (SCGx) rat pineal BZP binding decreased by about 30 - 60% (Mathew *et al.*, 1984; Quirion, 1984). These results suggest that BZP site is partially located at or is under the trophic influence of pineal sympathetic nerves. Administration of the mixed "central-peripheral" BZP agonist, diazepam, blocked the nocturnal rise of NAT (Zatz and Brownstein, 1979).

Based on direct and indirect evidences implicating DA in the modulation of the synthesis or action of MEL in the pineal gland, Govitrapong *et al.* (1984) characterized two DA binding sites in the bovine pineal. DA may have a synergistic effect with that of NA in stimulating the activity of pineal NAT in the rat (Axelrod *et al.*, 1969; Deguchi and Axelrod, 1972; Lynch *et al.*, 1973; Altar *et al.*, 1981).

Using [^{3}H]-quinuclidinylbenzilate ([^{3}H]-QNB), Taylor *et al.* (1980) detected muscarinic receptors in rat and sheep pineal glands. Bilateral SCGx did not decrease the numbers of pineal [^{3}H]-QNB binding sites.

Indirect evidence suggested that 5-HT binding sites may exist in the pineal gland (King *et al.*, 1982; Juillard *et al.*, 1983). 5-HT may regulate the synthesis of MEL (Sugden *et al.*, 1985a), and this effect may occur independently of and be unrelated to the activity of NAT.

The concentration of glutamic acid is considerably higher than GABA in the bovine pineal gland (Ebadi *et al.*, 1986). It has been shown that glutamic acid, like acetylcholine, is an excitatory transmitter (Fonnum, 1984), depicting a rapid onset of action and a short duration of action. Also, using the frog pineal gland, Meissl and George (1984) reported that glutamate and aspartate exerted stimulatory actions which were dose-dependent and reversible and could be partly blocked by aspartate antagonist, aminoadipate.

Other transmitters and neuropeptides which have been reported in the pineal gland include

neuropeptide Y (Shiotani *et al.*, 1986); vasoactive intestinal peptide (Moller and Mikkelsen, 1989); substance P (Govitrapong and Ebadi, 1986); peptide histidine isoleucine (Moller and Mikkelsen, 1989) and opioid peptides (Fraschini *et al.*, 1989).

1.6.6 THE PINEAL MELATONIN RHYTHMS IN MAMMALS

Normally, during darkness, MEL synthesis is stimulated by action potentials arising in the gland via the sympathetic nervous system; these action potentials originate in the SCN. Because light suppresses the neural activity of the SCN during the day, pineal MEL synthesis remains low. Thus, the rhythm in the MEL synthesis is generated by the SCN and synchronised by the prevailing light-dark environment (Reiter, 1988).

1.6.6.1 Generation of the Pineal Melatonin Rhythm

Melatonin production in the pineal gland increases at night because action potentials in the postganglionic sympathetic fibres cause the increased synthesis and release of NA (Reiter, 1989). The production of NA is controlled by the enzyme tyrosine hydroxylase; the activity of this enzyme in the intrapineal nerve endings rises at night (Craft *et al.*, 1984), as does the release of the neurotransmitter into the postsynaptic cleft. The action of NA on pinealocyte β-adrenergic receptors seem also to be assisted by the night-time increase in the number of receptors in the pinealocyte membrane (Gonzalez-Brito *et al.*, 1988). Melatonin, once synthesized, is rapidly released from the pineal gland, primarily, if not exclusively, into the vascular system. As a result, blood MEL levels fluctuate in parallel with the production of the indole in the pineal (Reiter, 1986).

1.6.6.2 Nature of the Pineal Melatonin Rhythm

The 24 hour patterns of MEL production in mammals have been arbitrarily classified according to the nature of the nocturnal increase (Reiter, 1983). This classification is based on mammals kept under laboratory conditions with either long or short daily photoperiods.

Refer to Figure 1.6.

In some species, darkness onset is not associated with an immediate rise in MEL production; rather, values remain low for several hours in the dark phase. Thereafter, pineal MEL production and secretion rise rapidly to reach a short-term peak in the latter half of the dark cycle; this peak is followed by a rapid reduction in pineal MEL levels so that at the time of light onset, values have returned to near basal levels. This sequence is classified as a **type A pattern** of MEL production. Animals that have a **type B pattern** exhibit a gradual rise in pineal and plasma MEL levels, beginning with light offset; in these species, maximal values are reached near the middle of the dark period, with the latter half of the night being associated with a gradual decrease in the MEL synthetic ability. In animals with a **type C pattern** of MEL production, the quantities of the hormone in the pineal and blood increase rapidly after darkness onset and maintain a high plateau during the bulk of the night; the levels again drop rapidly to the basal values before light onset in the morning.

The drop in MEL production near the end of the dark phase may be related to the down-regulation of the β-adrenergic receptors in the pinealocyte membrane, which attenuates the ability of released NA to promote the intracellular events required to maintain MEL production at high levels (Reiter, 1988).

1.6.6.3 Physiological Significance of the Melatonin Rhythms

Which aspect of the MEL rhythm actually conveys the endocrine message to a specific organ system remains debated. Several testable hypothesis have been proposed (Reiter, 1987). The most widely accepted theory to explain MEL action is what is referred to as the **duration theory**. According to this theory, seasonal fluctuations in day length alter the duration of elevated MEL and adjust organismal physiology, e.g. reproduction, on an annual basis (Carter and Goldman, 1983). A second theory emphasises the possibility of two separate rhythms that must be synchronized before MEL has physiological consequences. These are

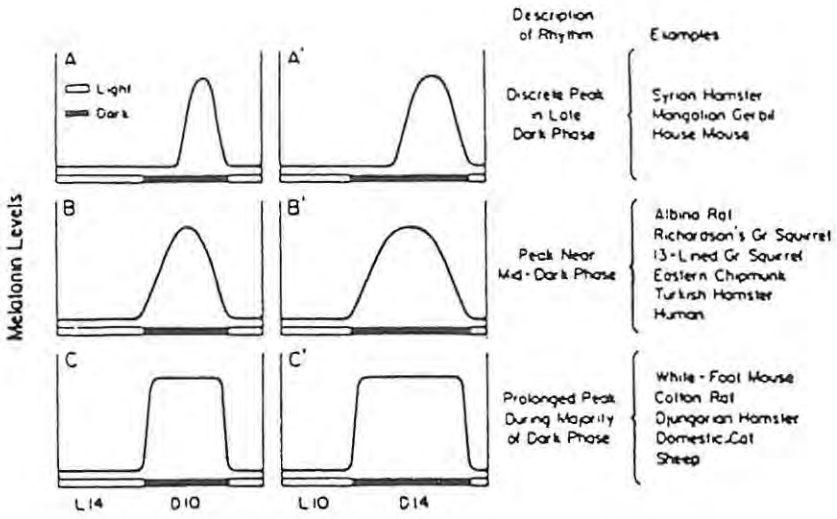


FIGURE 1.6: **Provisional Classification (A, B and C) of the Melatonin Rhythm in Various Mammalian Species.** Regardless of the type of rhythm a particular species displays, if the daily dark period is increased the duration of elevated melatonin is likewise prolonged. (Reiter, 1987).

referred to as coincidence models, i.e. **internal and external coincidence** (Reiter, 1987). Accordingly, only when nightly elevated MEL levels overlap an increased sensitivity of the MEL receptor ("Window of sensitivity") is a physiological response forthcoming. The final theory, the **amplitude hypothesis**, states that it is the amplitude of the nocturnal rise that is the critical feature in determining melatonin's functional significance (Berga *et al.*, 1988).

1.6.7 MELATONIN BINDING SITES AND THEIR RELEVANCE WITHIN THE CNS

The actual or presumptive MEL receptors have been localised in a large number of neural areas and extraneural tissues (Dubocovich, 1988; Morgan and Williams, 1989; Stankov and Reiter, 1990). Using *in vitro* autoradiography in combination with the high affinity radioligand (2-[^{125}I]-MEL), MEL binding sites have been reliably identified in the SCN, striatum, hippocampus, medulla pons and in the cells of the pars tuberalis of the adenohypophysis (Williams and Morgan, 1988; Zisapel *et al.*, 1988). In the rabbit retina and hamster hypothalamus MEL receptors have been classified into two types (ML-1 and ML-2)

based on their kinetic and pharmacological properties (Dubocovich, 1988).

The rat hypothalamus exhibits circadian changes in both receptor density and affinity (English and Arendt, 1988). Autoradiographically, a conspicuous diurnal rhythm in MEL binding density was demonstrated in the SCN of the rat with a nadir late in the light phase (Laitinen *et al.*, 1989). These diurnal variations in the density of MEL binding sites in the hypothalamus may, however, underlie the physiological response of the neuroendocrine system to MEL.

Although the identification of MEL binding sites assists in an understanding of the molecular basis of melatonin's action, the subcellular and biochemical mechanisms affected by MEL in the brain are not yet clearly characterized. Various relevant brain functions and components are affected by treatment with MEL. These include protein synthesis; 5-HT and GABA brain concentrations; neurotransmitter synthesis, uptake, reuptake and release; axonal transport; tubulin levels; and prostaglandin release (Review - Cardinali, 1981).

1.6.8 FOOD INTAKE RHYTHMS

1.6.8.1 Circadian Variation

Most feeding occurs in the part of the 24 hours when the organism sleeps least. This is true of many species, including the nocturnal rat and the diurnal human being. One model shows that "visceral activation" during the behaviourally active night phase is sufficient to explain the circadian variation of the food intake pattern, metabolic rate and lipoflow in the rat (Le Magnen and Devos, 1970); "visceral activation" means the acceleration of the gastric clearance rate for given contents of the GIT, with the consequent facilitation of exocrine secretions.

In the rat, there are two mechanisms which account for the fact that food is less satiating during the night (Le Magnen and Devos, 1970; Panksepp, 1973) :

(i) The fast gut clearance function produces more lipogenesis for a given size of meal;

(ii) Greater motor activity by night causes more heat loss.

Both lipogenesis and heat production direct energy from appetite satiety receptor systems, so attenuating satiation. In a prototype model of the control of human feeding by internal energy flows (Mather and Booth, 1977), the difference accounts for three meals a day and yet no hunger at night. Rats on a 2 hour lighting cycle eat more in the dark than in the light, although the proportion is attenuated (Borbely and Huston, 1974). A weakened circadian rhythm of feeding and activity also persists. Another model predicts the same pattern (Booth and Toates, 1974), because any lighting cycle which permits entrainment of gut clearance should produce more intake in the dark, and yet this rhythm should be lower in amplitude than that on circadian lighting and a weak circadian feeding rhythm should persist, because the endogenous circadian activity rhythm diverts more energy from satiation in the active period.

1.6.8.2 Oestrous Cycle Variation

By a close analysis of feeding patterns in mice, Petersen (1975) has identified two major factors in the variation in food intake around the oestrous cycle and the body weight differences between the sexes. Food intake is reduced during sexual receptivity by an increased distractibility. This presumably might be represented as effects of motivational time-sharing on feeding rate and the timing of meals, i.e. increased randomness in meal onset time will tend to produce the observed lower meal size by raising the average starting gut contents. Secondly, Petersen identified an oestrogenic failure to compensate intake in metoestrus which was attributed to a changed body weight "set point". If this is mediated by the VMH, like the effect of lighting on oestrus (Booth, 1980), that would explain the similarity of hyperphagia in males and females after VMH lesions (Gold, 1970), attributable to loss of daytime slowing of the stomach.

1.6.9 THE EFFECT OF FEEDING ON PINEAL INDOLEAMINES

1.6.9.1 Effects of Scheduled Feeding

1.6.9.1.1 Feeding Schedule as a Time Cue

Cycles of eating and fasting can act as zeitgebers. Richter (1922) established that a single meal daily would synchronise the rest-activity pattern of a rat in constant darkness. It has been shown that when food availability is restricted to specific periods in animals who are concurrently on fixed L:D cycles, various circadian rhythms are altered (Krieger, 1974). These effects did not require an intact SCN (Krieger *et al.*, 1977). Food shifted rhythms of corticosteroids and body temperature persisted in animals with SCN lesions. Moreover, animals who had previously been made arrhythmic by SCN lesions demonstrated rhythmicity of corticosteroids and body temperature when placed on scheduled feeding (Krieger *et al.*, 1977). Thus, there is evidence that in mammals food availability may be an important zeitgeber.

1.6.9.1.2 Scheduled Feeding and Pineal Indoleamines

Periodic availability of food in rats on a fixed L:D cycle has been shown to have no effect on the rhythm of pineal NAT (Ho *et al.*, 1985a), pineal NAS (Ho *et al.*, 1985a) pineal MEL (Ho *et al.*, 1985a) and serum MEL (Ho *et al.*, 1985a). Thus, unlike the other rhythms described above, pineal rhythms are predominantly controlled by the L:D cycle as compared to the feeding cycle.

Serum 5-HT showed a 24 hour rhythm; lowest levels preceded food intake and gradually rises with peak levels observed 10 - 13 hours afterwards. This alteration occurred whether food was presented during the dark or light period and was cued by food presentation. With-holding food at the expected time of feeding had no effect on serum 5-HT (Ho *et al.*, 1985b). A likely source of the peak in serum 5-HT is altered synthesis in the gut.

1.6.9.2 Effects of Food Restriction on Endocrine Function

Restriction of food intake has multiple effects on endocrine function including changes in the hypothalamic-pituitary-gonadal axis, in sympathetic nervous activity, thyroid function, adrenal function and altered growth hormone and prolactin regulation (Campbell *et al.*, 1977b; Landsberg and Young, 1982; Garfinkel, 1984). Particularly noteworthy are changes in the hypothalamic-pituitary-gonadal axis.

In the rat, oestrous cyclicity is lost, and there is suppression of the levels of circulating sex hormones and of the pituitary gonadotropins (Campbell *et al.*, 1977b). With restoration of food intake these changes are reversed. Suppression of the hypothalamic-pituitary-gonadal axis is believed to be due to a reduced output of gonadotropin releasing hormone since the pituitary gland will respond to gonadotropin releasing hormone (Piacsek and Mietes, 1967).

In weight loss associated amenorrhea in humans there is low and non-cyclic ovarian sex steroid secretion which is secondary to hypogonadotropism (Vigersky, 1984). With restoration of weight, abnormalities in gonadotropin regulation are reversed. How weight loss induces these abnormalities is unclear. Factors implicated have included the amount of body fat and changes in particular constituents of the diet (Vigersky, 1984; Dubey *et al.*, 1986).

Studies on pineal function in relation to these reproductive changes are of interest as there is convincing evidence in the rat that MEL can influence reproductive function (Lang *et al.*, 1983), and that underfeeding is a factor which is known to sensitise the reproductive axis to such pineal effects (Sorrentino *et al.*, 1971; Blask *et al.*, 1980). Thus, increased secretion of MEL could be a factor in the reproductive changes produced by underfeeding.

1.6.9.3 Effect of Food Restriction on Serum and Pineal Melatonin

Reduced levels of gonadotropins are found in underfed rats, an effect which is reversed by

constant illumination (Piacsek and Meites, 1967). The nocturnal rise in MEL is suppressed by constant illumination suggesting that MEL may mediate these effects. Also, underfeeding will potentiate an inhibitory effect of MEL administration on reproductive axis organ weight in the prepubertal rat (Blask *et al.*, 1980).

There is evidence of altered pineal activity in rats undergoing chronic food restriction. Walker *et al.* (1978) reported increased pineal activity as assessed by O_2 consumption and morphological changes in food restricted adult rats, while Herbert and Reiter (1981) reported a reduction in daytime and nighttime pineal MEL content in food restricted prepubertal rats. Consideration of these findings suggests that in food restricted animals there might be a higher turnover rate of pineal MEL, so that pineal MEL content drops. However, the decreased pineal MEL content might also indicate a reduced synthesis. Thus, it becomes important to assess the effect of food restriction on serum levels of circulating MEL. Chik (1986) studying the effect of food restriction in the young adult rat found no effects on the 24 hour pattern of serum or pineal MEL after 1 week of food restriction. However, when food restriction was prolonged to 3 weeks, changes in pineal and serum MEL patterns were observed. Both pineal and serum demonstrated an earlier nocturnal rise in MEL. The nighttime rise in serum MEL was increased while the peak pineal content of MEL was reduced. The reason for the increased nocturnal rise in serum MEL concomittant with a reduced pineal content remains to be established. The increase may reflect an increased rate of synthesis, a decreased volume of distribution, a decreased clearance rate, or perhaps a combination of these factors.

In parallel studies, Chik (1986) has examined the pineal NAT, pineal and serum MEL response to isoproterenol to determine whether alteration in the sympathetic control of the pineal, secondary to food restriction, might be responsible for the alterations in MEL in underfed animals. The findings indicated that pineal β-adrenergic responsiveness to isoproterenol was altered in food restricted animals. In a dose response study, peak responses occurred at a lower dose of isoproterenol in underfed animals. Peak levels of pineal NAT and MEL were of reduced magnitude, but serum MEL levels were not reduced.

This discrepancy between peak levels is similar to the discrepancy seen for the peak nocturnal rise in underfed animals and may reflect a specific effect of underfeeding.

Changes in β-adrenergic responsivity in the underfed rat do not fit simply with a super or subsensitivity of the β-adrenoceptors. This is not unexpected as underfeeding produces numerous effects in addition to possible effects on sympathetic outflow.

1.6.10 OVERVIEW

Armstrong *et al.* (1982) stated that "considering the multitude of biochemical, physiological, endocrinological and behavioural changes attributable to the pineal, it is unlikely to have so many specific functions" and these authors suggested that the function of the pineal gland must be at a higher level of integration. This suggestion, coupled with others, such as the proposals that the pineal gland acts as a "regulator of regulators" [(Reiter and Hester, 1966) cited in Armstrong *et al.* (1982)] and that MEL acts as a "central inhibitory modulator", "neuroendocrine transducer" or "biological clock" (Datta and King, 1980), supported the hypothesis proposed by Armstrong *et al.* (1982), that the pineal is a "synchroniser of regulators", with environmental lighting acting as the main **ZEITGEBER** (synchroniser).

Regarding its clinical applications, MEL has been implicated in controlling reproduction in a number of non-human mammals. Also, what are considered to be abnormal or unusual MEL rhythms have been observed in females with hypothalamic amenorrhea (Berga *et al.*, 1988), in patients with reproductive dysfunction associated with anorexia nervosa (Tortosa *et al.*, 1989), and in males with oligospermia or aspermia (Reiter, 1991b).

Other conditions in which MEL either may be involved or may have therapeutic utility include SAD, jet lag, hormone-responsive tumour growth, immunocompetence, sleep and sudden infant death syndrome (SIDS).

Melatonin's actions are not exclusive to reproductive system and, in fact, the reproductive effects may be secondary to its more important role of adjusting the entire physiology of the organism on a seasonal basis. Possible sites of action of MEL are shown in Figure 1.7.

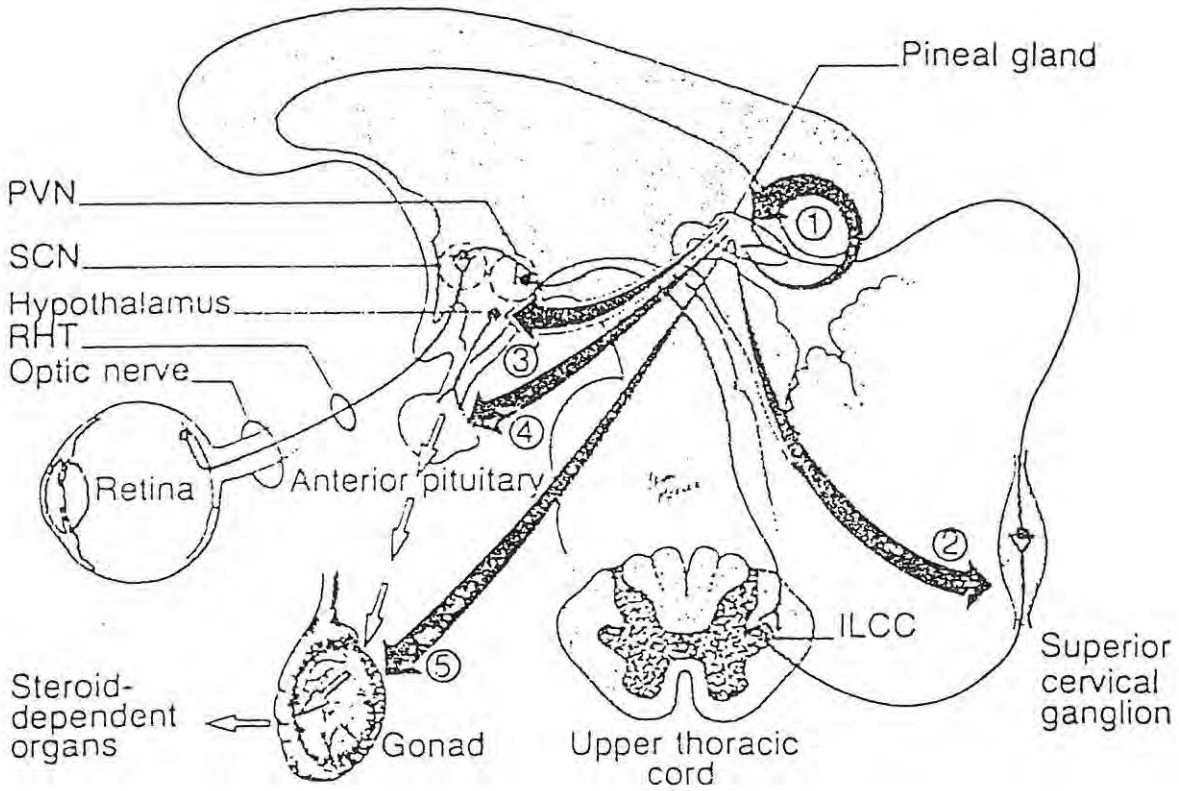


FIGURE 1.7: **Summary of the Relations of the Visual System to the Pineal and Possible Sites of Action of Pineal Hormone(s)**

1. The pineal itself;
2. The superior cervical ganglia;
3. The hypothalamus and the brain stem;
4. The anterior pituitary gland;
5. The gonads.

ILCC = Intermediolateral Cell Columns; PVN = Paraventricular Nuclei; RHT = Retinohypothalamic Tract; SCN = Suprachiasmatic Nuclei (Reiter, 1989).

CHAPTER 2

EXPERIMENTAL PROCEDURES

2.1 ANIMALS

Adult male rats of the Wistar strain weighing between 200 and 250 g were used throughout this study. All animals were maintained under an automatically regulated lighting cycle of light : dark (12:12); lights on at 06h00. The light intensity was approximately 300 μWatts/cm^2 provided by cool white fluorescent tubes. The animal room was maintained at a constant temperature between 20 and 24°C and an extractor fan ensured the constant removal of stale air. The rats were housed in groups of 5 in opaque, white plastic cages with food and water *ad libitum*.

Rats were sacrificed swiftly by cervical fracture followed by decapitation. An incision was made through the bone from the foramen magnum to near the orbit using a pair of scissors. The top of the skull was removed using clean forceps and the pineal gland quickly removed. To remove the liver, a mid-ventral incision was made through the abdominal musculature from the pelvic region to the posterior edge of the sternum. A transverse cut was made anteriorly to expose the liver which was rapidly removed and dissected free of adhering tissues. When assays were not conducted immediately, livers were frozen in liquid nitrogen and stored at -70°C until further use.

2.2 INDOLE METABOLISM BY PINEAL ORGAN CULTURES

2.2.1 INTRODUCTION

The pineal gland of certain species such as rat and chicken, being small and readily accessible, makes them ideal for intact organ culture studies, and numerous methods have been developed and used with comparative success (Trowell, 1959; Klein and Notides, 1969; Chan and Ebadi, 1981; Daya and Potgieter, 1982; Morton, 1987). The pharmacological manipulations can be studied in organ cultures, free from hormonal, neuronal and other

complicating influences of the *in vivo* milieu.

The pineal gland in organ culture is able to utilize exogenous radioactive serotonin to produce various indoles including melatonin. It has been shown that approximately 95% of the synthesized radioactive indoles are secreted into the culture medium during the incubation period (Klein and Rowe, 1970; Skene, 1985). The radioactive indoles thus synthesized are then separated using bi-dimensional thin layer chromatography (TLC) (Klein and Notides, 1969; Daya and Fata, 1982) and assayed quantitatively by liquid scintillation spectrometry.

2.2.2 MATERIALS AND METHODS

Animals: Male Wistar rats weighing between 200 and 250 g were used and maintained as previously described (Section 2.1). All animals were sacrificed between 11h00 and 12h00 in these experiments.

Chemicals and Reagents: 5-Hydroxy (side chain-2-^{14}C) tryptamine creatinine sulphate (specific activity 55 mCi/mmol) was purchased from Amersham (England); the synthetic pineal indoles from Sigma Chemical Co. (USA); aluminium TLC plates coated with silica gel 60 F_{254} (0.2 mm) from Merck (West Germany) and BGJb culture medium (Fitton-Jackson Modification) from Gibco (Europe). All other chemicals and reagents were obtained from local commercial sources.

Culture Medium: The sterile BGJb culture medium was aseptically supplemented with benzyl penicillin sodium (0.06 mg/ml), streptomycin sulphate (0.1 mg/ml) and amphotericin B (2.5 μg/ml) to prevent the growth of contaminating micro-organisms during the culture period. The composition of this medium is shown in Table 2.1.

Pineal Gland Culture: Rat pineal glands were aseptically removed with minimum delay and dissected free from adhering tissue. The pineal glands were then individually placed in sterile glass culture tubes containing 52 μl of culture medium; 8 μl of [^{14}C]-5-HT was then

added. The tubes were then aerated with carbogen (95% O_2 : 5% CO_2), sealed and incubated in the dark at 37°C for 24 hours. Following incubation, the glands were removed and the culture medium analyzed as described below.

TLC Analysis of Radioactive Indoles: The technique employed was a modification of the method used by Klein and Notides (1969). A 10 μl aliquot of the culture medium was spotted on a 10 cm x 10 cm TLC plate. Following this, a solution (10 μl) containing synthetic unlabelled standards (0.2 mg/ml) of all pineal indoles to be measured was spotted on top of the culture medium spot. The indole standard solution was prepared as follows : 1.0 mg of each standard (listed in Figure 2.1) was dissolved together in 2.5 ml of 95% ethanol. To this, 2.5 ml of 1% ascorbic acid in 0.1 NHCl was added to prevent oxidation of indoles. The solution was stored in darkness at -20°C until further use. In all instances the TLC plates were spotted in subdued light and a gentle stream of nitrogen was used to dry the spots. The plates were then placed in a TLC tank and developed twice in the same direction in a solvent containing chloroform : methanol : glacial acetic acid (93:7:1). The total solvent front was allowed to move 8 cm up the plate and dried under nitrogen between each development. Following this, the plate was rotated 90° and developed once in a solvent containing ethyl acetate. The solvent was allowed to reach a height of 6 cm. The plates were removed, dried with nitrogen and sprayed with Van Urk's reagent (1.0 g 4-dimethylaminobenzaldehyde in 50 ml of 25% HCl and 50 ml of 95% ethanol).

The plates were then heated at 60°C in an oven to visualize the various metabolites. The spots were then cut out and individually placed into plastic scintillation vials containing 1 ml 95% ethanol and allowed to stand for 5 minutes to extract radioactivity. After extraction, 3 ml of emulsifier scintillator 299™ (Packard, Netherlands) was added to each vial, shaken for 20 minutes and the radioactivity quantified using liquid scintillometry.

2.2.3 RESULTS

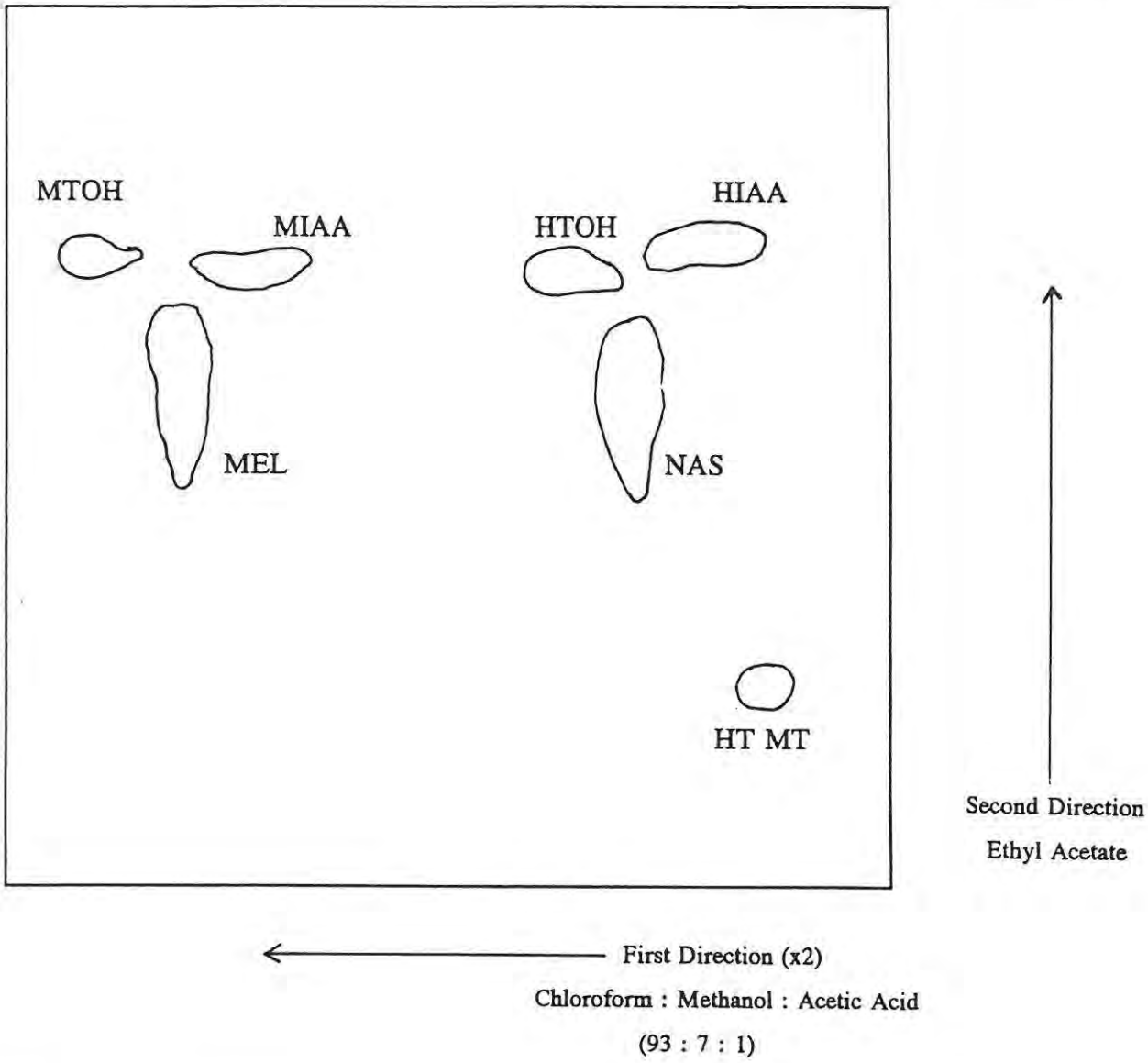
A typical bi-dimensional thin layer chromatogram of [^{14}C]-labelled indole metabolites is presented in Figure 2.1. Adequate separation of the pineal indoles was achieved except for 5-methoxytryptamine and 5-HT which remained at the original spot.

2.2.4 DISCUSSION

Using organ culture technique and TLC separation, pineal indoles were adequately separated. This was a confirmation of the work done by Daya and Fata (1986). The procedure described was therefore employed for all subsequent studies.

TABLE 2.1: The Composition of BGJb Culture Medium (Fillton-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	0.04
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



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

FIGURE 2.1: **A Typical Bi-Dimensional Thin Layer Chromatogram of Pineal Indoles.**

2.3 PINEAL cAMP ASSAY

2.3.1 INTRODUCTION

Extracellular signals such as hormones, neurotransmitters and growth factors control their physiological effects on target cells by binding to specific membrane receptors and thus activating a signalling cascade within the cell.

The physiological responses to such extracellular signals are mediated through "second messengers". This is the term described by Sutherland for molecules which are able to transmit intracellularly, the biological effects of compounds not able to enter the target cells themselves (Sutherland *et al.*, 1968).

Cyclic AMP, since its discovery more than 30 years ago, has played a decisive role in the understanding of the part played by "second messengers" in transmembrane signalling systems (Cook *et al.*, 1957; Lipkin *et al.*, 1959). There remains considerable interest in the measurement of intracellular cAMP in tissues, and this may help to provide an understanding of the physiology and pathology of many diseased states.

Brown *et al.* (1971) developed a saturation assay for cAMP using purified bovine adrenal protein as the specific binding protein. This assay was considered simpler and relatively inexpensive, and was chosen to measure pineal cAMP for the purpose of this study. The assay is based on the competition between unlabelled cAMP and a fixed quantity of the tritium labelled cAMP for binding to a bovine protein which has a high affinity and specificity for cAMP. The amount of labelled protein-cAMP complex formed is inversely related to the amount of unlabelled cAMP present in the assay mixture. Separation of protein bound cAMP from the unbound nucleotide is achieved by adsorption of the free nucleotide on the coated charcoal, followed by centrifugation. An aliquot of the supernatant is then removed for liquid scintillometry. The concentration of unlabelled cAMP in the sample is then determined from a linear standard curve.

2.3.2 MATERIALS AND METHODS

Animals: Male Wistar rats weighing between 200 and 250 g were used in these experiments and were maintained as previously described (Section 2.1).

Chemicals and Reagents: (8 - ^{3}H) Adenosine 3′, 5′-cyclic phosphate, ammonium salt (specific activity 30 Ci/mmol) was purchased from Amersham (England); theophylline and bovine serum albumin (BSA) from Sigma Chemical Co. (USA); activated charcoal from Merck (West Germany). All other chemicals and reagents were obtained from local commercial sources.

Preparation of cAMP Binding Protein: Bovine adrenals, collected from the local abbatoir as soon as possible after slaughter, were transported on ice to the laboratory. The cortices were separated, chopped and homogenized in 1.5 vol of ice-cold medium comprising 0.25M sucrose; 50 mM Tris-HCl buffer (pH 7.4); 25 mM potassium chloride; and 5 mM magnesium chloride (homogenizing buffer). The supernatant, after centrifugation of the homogenate at 2 000 g for 10 minutes, was respun at 5 000 g for 15 minutes at 4°C. The resulting supernatant was stored in 1.0 ml portions at -20°C. When used, this preparation was thawed and diluted with 50 mM Tris-HCl buffer (pH 7.4), containing 8 mM theophylline and 6 mM 2-mercaptoethanol. This protein diluting buffer was used for all subsequent procedures.

Preparation of Pineal Tissue and Incubation Procedures: Pineal glands (groups of 5) were rapidly removed from animals and dissected free of adhering tissue. Glands were placed in sterile dishes with 2.0 ml of BGJb culture medium in the presence or absence of a drug. The pineals were then incubated at 37°C for 30 minutes in the atmosphere of 95% O_2 : 5% CO_2. Following incubation, the pineals were removed and individually sonicated with 100 μl of homogenizing buffer using a Vibra Cell™ Sonicator (Sonics and Materials Inc., USA). A 50 μl aliquot of homogenate was removed and used for the cAMP assay.

Cyclic AMP Assay Procedure: The scheme for the assay is presented in Table 2.2. The assay was done in duplicate. The reaction mixture contained diluted amounts of standard cAMP to give the concentrations of 0 - 8 pmol cAMP/tube, and 50 μl of the unknown sample; 50 μl of [^{3}H] cAMP (8 n Ci); 100 μl of diluted binding protein (1:3) and the protein diluting buffer to a final volume of 350 μl. The tubes were gently mixed and incubated at 4°C for 100 minutes. Following incubation, 100 μl of a 10% w/v suspension of activated charcoal in buffer containing 2% w/v BSA was added to each reaction tube and the tubes were vortexed for 15 seconds and centrifuged at 1 200 g for 15 minutes at 4°C. A 100 μl aliquot of the supernatant was removed and added into plastic scintillation vials containing 3 ml of emulsifier scintillator 299™ (Packard, Netherlands). The vials were shaken for 10 minutes and radioactivity quantified using liquid scintillometry (Beckman LS 2800).

2.3.3 RESULTS

Calculations: To determine the blank counts per minute (cpm), cpm values for the blank were averaged. Then, cpm values for the zero dose were averaged and the blank values subtracted; this result is the cpm bound in the absence of unlabelled cAMP (Co). The cpm values of each pair of duplicates were then averaged and the blank value subtracted from each result to give cpm bound in the presence of standard or unknown unlabelled cAMP (Cx).

The Co/Cx ratio for each level of cAMP was calculated and plotted against the concentration of cAMP (pmol/tube). A straight line was obtained between 0 and 8 pmol of cAMP (Figure 2.2, Linear Regression Analysis $r^2 = 0.997$).

TABLE 2.2: Scheme for cAMP Assay

Reagents (μl)	Blank	Cyclic AMP Standards (pmoles)							Sample
		0	0.25	0.5	1.0	2.0	4.0	8.0	
Buffer	250	150	150	150	150	150	150	150	150
Standard: - 20 nM - 200 nM	 --- ---	 --- ---	 12.5 ---	 25 ---	 50 ---	 --- 10	 --- 20	 --- 40	 --- ---
Sample	---	---	---	---	---	---	---	---	50
Water	50	50	37.5	25	---	40	30	10	---
[^{3}H] cAMP	50	50	50	50	50	50	50	50	50
Mix Gently									
Protein	---	100	100	100	100	100	100	100	100
Incubate at 4°C for 100 minutes									
Charcoal	100	100	100	100	100	100	100	100	100
Vortex for 15 seconds Centrifuge at 1 200 x g for 15 minutes at 4°C Add 100 μl of supernatant to 3 ml scintillation cocktail Shake for 10 minutes and count radioactivity									

[Adapted from Van Wyk, 1993].

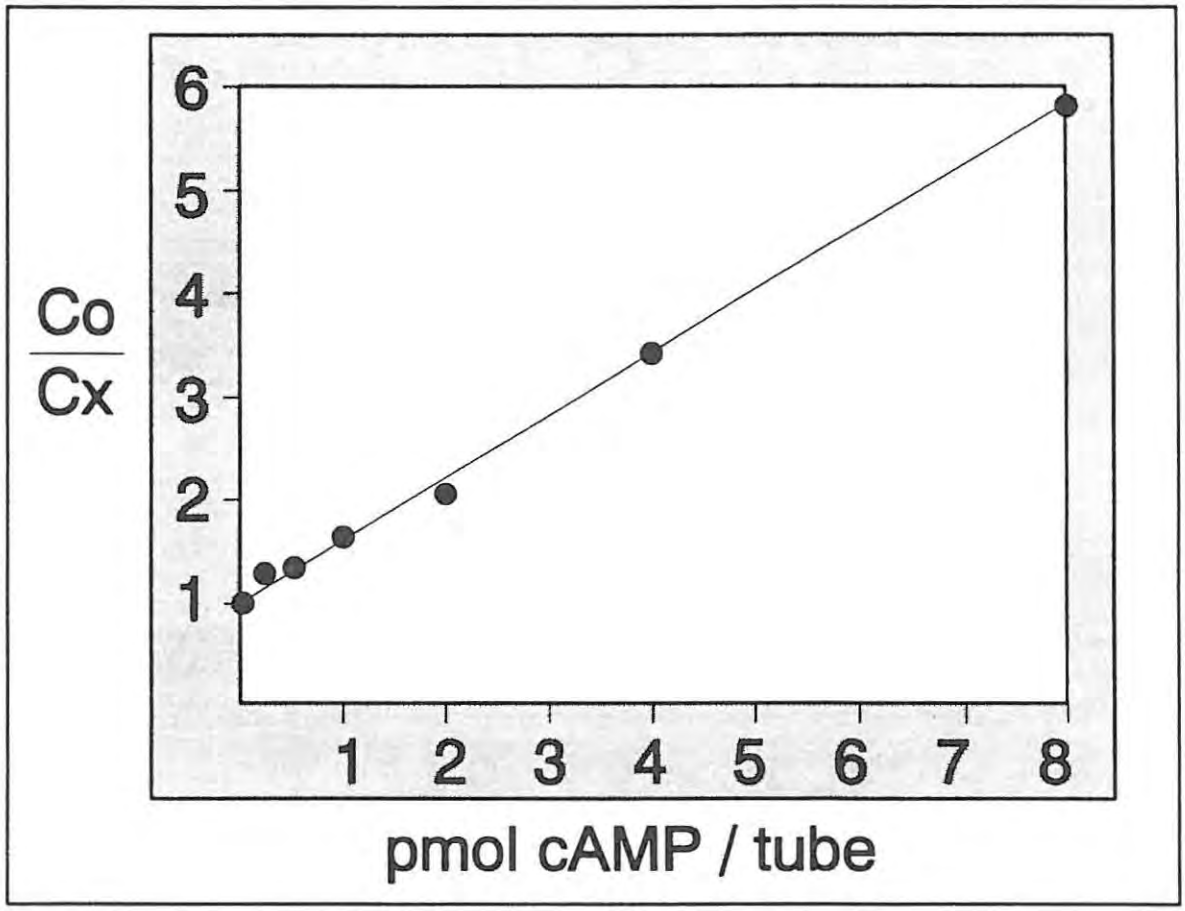


FIGURE 2.2: Typical cAMP Standard Curve
[Each point represents the mean of duplicate determinations].

2.4 THE DETERMINATION OF SEROTONIN *N*-ACETYLTRANSFERASE ACTIVITY

2.4.1 INTRODUCTION

Serotonin *N*-acetyltransferase (NAT) is the enzyme responsible for the conversion of 5-HT to NAS, the precursor of MEL. NAT is known to be a rate-limiting enzyme in the biosynthesis of MEL (Klein *et al.*, 1981; Reiter, 1991b).

The activity of the pineal NAT is currently used as a probe in many studies concerning pineal activation and deactivation. *In vivo*, NAT activity decreases dramatically in chickens or rats with a half-life of 3 - 10 minutes when the animals are exposed to light during the dark phase (Hamm *et al.*, 1983). The same half-life value has been found *in vitro* when pulses of light are applied to cultured chicken pineal glands kept in the dark (Hamm *et al.*, 1983) and when both rat and chicken pineals are homogenized (Binkley *et al.*, 1976). The most commonly used assay for this enzyme was developed by Deguchi and Axelrod (1972) and is used in these experiments with some slight modifications.

The principle involves the *N*-acetylation by endogenous pineal NAT of a substrate, tryptamine hydrochloride, using [^{3}H] acetyl coenzyme A as the acetyl donor. The radio-labelled product, *N*-acetyltryptamine, is extracted into an organic solvent and the radioactivity evaluated by liquid scintillometry.

2.4.2 MATERIALS AND METHODS

Animals: Male Wistar rats weighing between 200 and 250 g were used in these experiments. The animals were maintained as previously described (Section 2.1). The animals were sacrificed between 11h00 and 12h00 for the purpose of these experiments.

Chemicals and Reagents: [^{3}H] Acetyl coenzyme A (specific activity 3.3 Ci/mmol) was

purchased from Amersham (England); tryptamine hydrochloride and ℓ-isoproterenol from Sigma Chemical Co. (USA) and unlabelled acetyl coenzyme A from Boehringer Mannheim (West Germany). All other chemicals and reagents were obtained from local commercial sources.

Working Solution: The working solution for NAT assay is shown in Table 2.3. This was prepared immediately before the assay and kept on ice.

TABLE 2.3: Working Solution for NAT Assay

Reagents		Volume (μℓ/sample)
Tryptamine HCl	(5.6 mM)	5
Acetyl CoA	(800 μM)	2
[^{3}H]-Acetyl CoA	(0.04 μCi)	2
Phosphate Buffer	(0.05 M, pH 6.8)	1
Total Volume		10

NAT Assay Procedure: Pineal glands were rapidly removed from animals. Each pineal was individually sonicated in 100 μl of ice-cold 50 mM phosphate buffer (pH 6.8) for 5 seconds. The assay was carried out in Eppendorf microfuge tubes. 20 μl aliquots of homogenate from each gland were transferred to the bottom of the microfuge tubes. A 10 μl aliquot of the working solution was transferred to the side of the tube.

The tubes were then tapped and vortexed to ensure thorough mixing of the two solutions. This was followed by incubation at 37°C in a shaking water bath for 30 minutes. A tube containing no homogenate was also included in the incubation (blank tube). Following incubation, the reaction was terminated by the addition of 100 μl of ice-cold borate buffer (200 mM, pH 10) to each tube. A 1.0 ml solution of toluene : isoamyl alcohol (97:3) was

then added to each tube. The tubes were shaken for 5 minutes and centrifuged at 2 000 g for 30 seconds. The tubes were opened and washed with 100 μl of ice-cold borate buffer, shaken and centrifuged as before. A 0.5 ml aliquot of organic solvent was transferred to plastic scintillation vials containing 3 ml of scintillation cocktail (emulsifier scintillator 299™, Packard, Netherlands) and radioactivity quantified by liquid scintillometry.

***In Vivo* Studies:** Groups of 5 rats were injected with a test drug (e.g. isoproterenol HCl in 0.9% NaCl, 5 mg/kg) intraperitoneally (i.p.) at 11h00 and sacrificed 3 hours later. Pineal glands were removed, sonicated and NAT activity determined as described above.

***In Vitro* Studies:** Groups of 5 pineal glands were incubated in sterile glass culture tubes containing 500 μl of BGJb culture medium. Drugs were dissolved in distilled water and added to the culture medium. The tubes were then incubated at 37°C in an atmosphere of 95% O_2 : 5% CO_2 for 4 hours. Following incubation, the glands were removed, sonicated and NAT activity determined as described above.

2.4.3 RESULTS

The cpm values obtained were converted to dpm by correcting the counting efficiency of the scintillation counter. The NAT activity was then calculated according to the following equation :

$$\frac{(DPM_{sample} - DPM_{blank}) \times a \times b \times 2 \times 4.5 \times 5}{DPM_{total}} = \text{pmol NAT/pineal x hour}$$

where
- a = pineal fraction assayed (1 for whole pineal; 2 for half pineal, etc).
- b = pmol of acetyl coenzyme A per sample.
- 2 = For counting only half toluene : isoamyl alcohol
- 4.5 = For incubating 30 minutes.
- 5 = For taking 20 μl homogenate from 100 μl.
- DPM_{total} = Total radioactivity per sample.

[Deguchi and Axelrod, 1972].

2.5 THE DETERMINATION OF PINEAL HYDROXYINDOLE-O-METHYLTRANSFERASE ACTIVITY

2.5.1 INTRODUCTION

Pineal *N*-acetylserotonin (NAS) is converted to melatonin by the pineal cytosolic enzyme, hydroxyindole-O-methyltransferase (HIOMT). HIOMT catalyzes the O-methylation of 5-hydroxyindoles by the methyl donor S-adenosyl methionine (SAM). This enzyme is found in high concentration in the pineal gland (Jackson and Lovenberg, 1971), which presumably accounts for the relatively high concentration of melatonin in this gland.

HIOMT activity, when first described in rat pineal gland, was reported to exhibit a nocturnal increase (Axelrod *et al.*, 1965); however, when saturating concentrations of the substrate were employed in the assay a rhythm could no longer be verified (Sugden *et al.*, 1987). The preferred substrate for HIOMT is generally considered to be NAS and, as a consequence, during the night when pineal NAS concentrations are elevated, it is rapidly converted to melatonin (Axelrod and Weissbach, 1961). The assay used for this enzyme is a modification by Axelrod and Weissbach (1961).

The principle for the assay involves the O-methylation by endogenous pineal HIOMT of a substrate, NAS, using [^{14}C]-SAM as the methyl donor. The radiolabelled product, N-acetyl-5-methoxytryptamine (MEL), is extracted into an organic solvent and the radioactivity quantified by the liquid scintillometry.

2.5.2 MATERIALS AND METHODS

Animals: Male Wistar rats weighing between 200 and 250 g were used in these experiments and maintained as previously described (Section 2.1).

Chemicals and Reagents: S-Adenosyl-L-[methyl-^{14}C]methionine (specific activity 56

mCi/mmol) was purchased from Amersham (England); *N*-acetylserotonin from Sigma Chemical Co. (USA) and unlabelled methionine from Boehringer Mannheim (West Germany). All other chemicals and reagents were obtained from local commercial sources.

Working Solution: The working solution for HIOMT assay is presented in Table 2.4. This solution was prepared immediately before the assay and kept on ice.

TABLE 2.4: Working Solution for HIOMT Assay

Reagents		Volume (μl/sample)
N-acetylserotonin	(3 mM)	3
Methionine	(400 μM)	3
[^{14}C]-SAM		1
Phosphate Buffer	(50 mM, pH 7.9)	2
Deionized Water		1
Total Volume		10

HIOMT Assay Procedure: Pineal glands were rapidly removed from rats as previously described (Section 2.1). Each pineal was individually sonicated in 500 μl of ice-cold 50 mM phosphate buffer (pH 7.9) for 5 seconds. The assay was carried out in eppendorf microfuge tubes. A 20 μl aliquot of homogenate from each gland was transferred to the bottom of the microfuge tube. A 10 μl aliquot of working solution was transferred to the side of the tube.

The tubes were then tapped and vortexed to ensure uniform mixing of two solutions. The tubes were then incubated at 37°C for 30 minutes in a shaking water bath. A blank tube, containing no homogenate, was included in the incubation. Following incubation, the reaction was terminated by the addition of 100 μl of ice-cold borate buffer (200 mM, pH 10)

to each tube. A 1.0 ml solution of toluene : isoamyl alcohol (97:3) was then added to each tube, which was followed by shaking for 5 minutes and centrifugation at 2 000 g for 30 seconds. The tubes were opened and the contents washed with 100 μl of ice-cold borate buffer (pH 10), shaken and centrifuged as before. A 0.5 ml aliquot of organic solvent was transferred to plastic scintillation vials containing 3 ml of scintillation cocktail (emulsifier scintillator 299™, Packard, Netherlands). The vials were shaken for 10 minutes and the radioactivity quantified by liquid scintillometry (Beckman LS 2800).

For *in vivo* and *in vitro* studies, refer to Section 2.4.2.

2.5.3 RESULTS

The dpm values for the blank were substrated from the sample values as indicated in Section 2.4.3. The results were expressed in pmol *N*-acetyl-5-[^{14}C]-methoxytryptamine formed per pineal per hour.

2.6 THE MEASUREMENT OF LIVER TRYPTOPHAN PYRROLASE ACTIVITY

2.6.1 INTRODUCTION

Tryptophan pyrrolase (L-tryptophan-oxygen 2,3-oxidoreductase, EC 1.13.11.11) is the haem-dependent liver cytosolic enzyme that catalyzes the oxidative cleavage of the pyrrole ring of L-tryptophan to produce *N'*-formylkynurenine during the first and rate-limiting step of the kynurenine-nicotinic acid pathway of tryptophan degradation in the rat (Badawy and Evans, 1976). There is considerable evidence that pyrrolase activity is a major peripheral determinant of tryptophan availability to the brain (Badawy *et al.*, 1981) and that the cerebral concentration of this amino acid is, in turn, an important factor in 5-HT synthesis (Curzon, 1979).

In the liver of man, rat and other animal species (Badawy and Evans, 1976), the enzyme exists in two forms. The already active reduced holoenzyme which does not require the addition of haematin for the demonstration of its activity *in vitro* (Feigelson and Greengard, 1961), whereas the haem-free predominant form, or apoenzyme, does.

Measurement of both apoenzyme and holoenzyme activities is important, not only to find out which form is inhibited or activated by a particular agent, but also to examine the presence or absence of the enzyme. By performing such simultaneous measurements, it has been shown (Badawy and Evans, 1976) that, although both forms are present in the liver of the chicken, mouse, pig, rat, turkey and possibly man, the apoenzyme is absent from the cat, frog, gerbil, guinea pig, hamster, ox, rabbit and sheep. It has also been shown that species lacking the apoenzyme are sensitive to tryptophan toxicity because they metabolize the amino acid largely via the indoleamine routes (Badawy, 1979).

Tryptophan pyrrolase activity was determined by the method of Badawy and Evans (1975) by measuring the formation of kynurenine from ℓ-tryptophan either in the absence (holoenzyme activity) or in the presence (total enzyme activity) of added haematin. The

apoenzyme activity was calculated by the difference.

2.6.2 MATERIALS AND METHODS

Animals: Male Wistar rats weighing between 200 and 250 g were used and maintained as previously described (Section 2.1). The animals were sacrificed between 11h00 and 12h00.

Chemicals and Reagents: L-Tryptophan and haematin hydrochloride were purchased from Sigma Chemical Co. (USA); trichloroacetic acid from Riedel-DeHaën (Europe). All other chemicals and reagents were obtained from local commercial sources.

Preparation of Liver Homogenates: The liver was rapidly removed and homogenized in 100 ml of 140 mM KCl - 2.5 mM NaOH at O°C (ice bath) with a Waring blender for 1 minute at 1100 rev/min. This suspension was further homogenized in a glass homogenizer with a loose-fitting teflon pestle at O°C. The homogenates were used within 10 minutes of preparation. Another liver tissue was frozen in liquid nitrogen and kept at -70°C for 24 hours prior to the enzyme assay.

Determination of Tryptophan Pyrrolase Activity: Liver homogenate samples (15 ml) were added to a mixture containing 5 ml of 0.03 M L-tryptophan, 15 ml of 0.2 M sodium phosphate buffer (pH 7.0) and 25 ml of deionized water at O° C. The assay was performed in duplicate. On one set of tubes, 0.1 ml of 1.2 mM of haematin hydrochloride was added in the overall mixture to give a final concentration of 2 μM solution. Control tubes (without tryptophan) were included to assess the formation of kynurenine from tryptophan.

Samples (3 ml) of the mixture were incubated with shaking (120 oscillations per minute) in stoppered boiling tubes in an atmosphere of 95% O_2 : 5% CO_2 at 37°C for the following times : 0, 15, 30, 45, 60 and 75 minutes. The reaction was terminated by the addition of 2 ml of 0.9 M trichloroacetic acid; the tubes were shaken for further 2 minutes and then

filtered using Whatman No. 1 filter paper. To 2.5 ml of the filtrate, 1.5 ml of 0.6 M NaOH was added. The kynurenine present was determined by measuring the absorbance at 365 nm in a Unicam SP 1001 spectrophotometer.

***In Vivo* Studies:** Rats (groups of 3) were injected with drug (5 mg/kg) intraperitoneally at 09h00 and sacrificed 6 hours later. Livers were removed, homogenized and pyrrolase activity determined as described above.

2.6.3 RESULTS

The concentration of the kynurenine formed was determined by using the Beer-Lambert's law (ϵ = 4540 litre/mol/cm). The results were expressed as μmol kynurenine formed per mg protein. The pyrrolase activity was calculated from the increase in absorbance at 365 nm with time during the linear phase. With the holoenzyme, the linear phase was preceded by a lag phase that persisted for the first 15 minutes. There was no lag phase in the total enzyme activity; the activity was linear with all incubation times (Figure 2.3).

2.6.4 DISCUSSION

The results were consistent with those obtained by previous investigators (Jago *et al.*, 1964; Badawy and Evans, 1975). The effect of freezing liver tissue at -70°C did not change the pyrrolase activity.

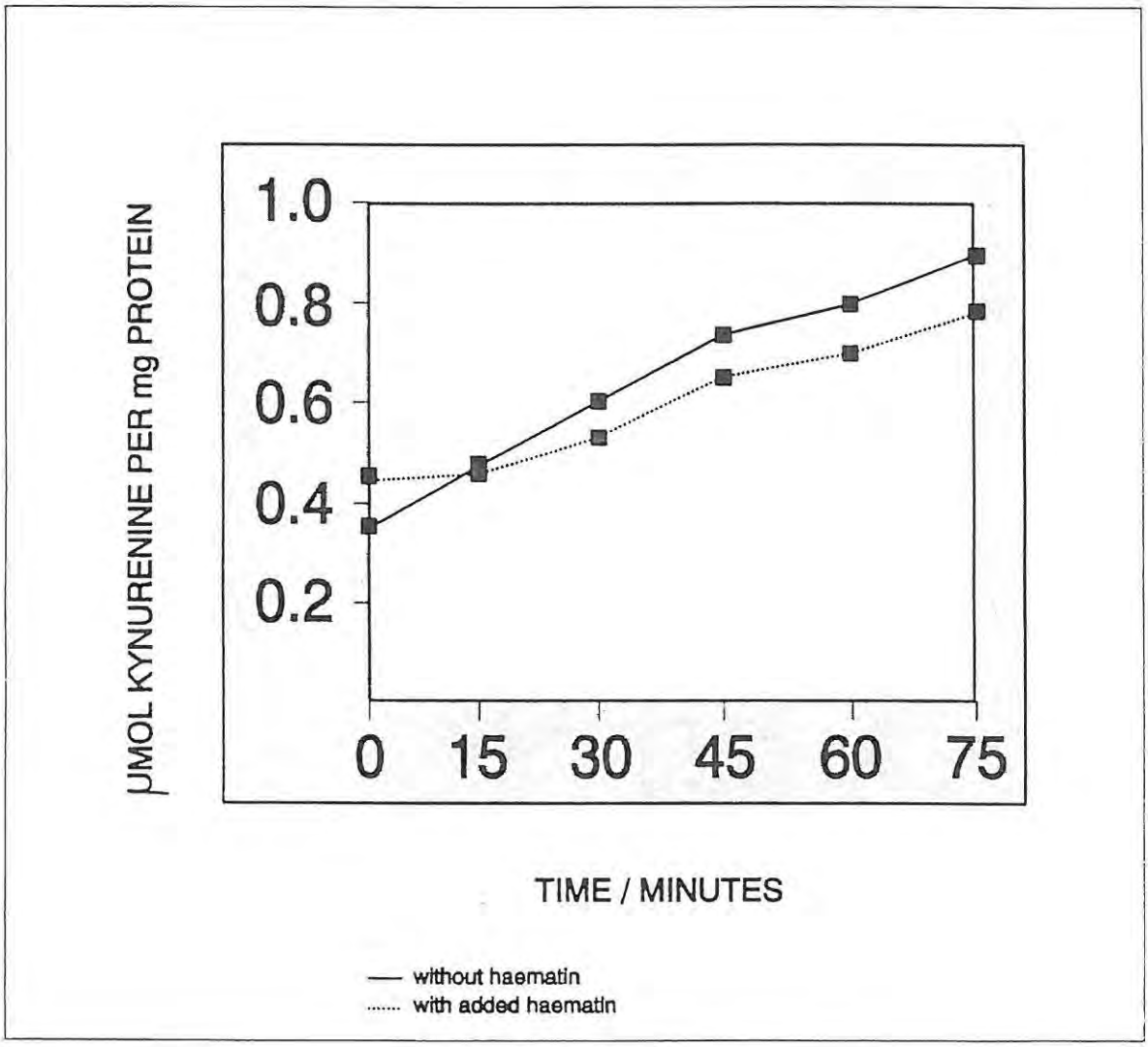


FIGURE 2.3: Time Course of the Tryptophan Pyrrolase Reaction in Rat Liver Homogenate
[Each point represents the mean of triplicate determinations].

CHAPTER 3

PINEAL INDOLEAMINE METABOLISM IN ORGAN CULTURE

3.1 INTRODUCTION

The sympathetic nerves in the pineal gland contain both noradrenaline and serotonin, and it was not certain whether these compounds released from the nerve terminals exert their effects on pineal indoleamines. If, as appeared to be, the neurotransmitter noradrenaline stimulates the pinealocyte, does it act on an α- or β-adrenergic receptor? Also, does it exert its effects directly on a receptor or are other mechanisms involved?

The rat pineal in organ culture proved to be a useful experimental tool. In such a system, the effects of biogenic amines, adrenergic blocking agents and catecholamine depletors on pineal function could be examined directly. The studies on pineal organ culture were initiated by Klein *et al.* (1970). These authors added [^{14}C] serotonin to the culture medium and the formation of radioactive indoles was measured.

The addition of ℓ-noradrenaline to the pineal culture caused a marked increase in the formation of radioactive melatonin from [^{14}C] serotonin following 24-hour incubation (Axelrod *et al.*, 1969). The stimulation of melatonin synthesis in the pineal organ culture by noradrenaline was prevented by the addition of ℓ-propranolol, a β-adrenergic receptor blocker. Alpha-adrenergic receptor blockers had no effect on the increased formation of melatonin in the presence of ℓ-noradrenaline. Reserpine, a drug that depletes neurons of both catecholamines and serotonin, also prevented the elevated formation of melatonin. These observations indicate the involvement of ℓ-noradrenaline and the β-adrenoceptor in the elevation of pineal melatonin biosynthesis.

This section investigates the effect of appetite suppressants on pineal indoleamine metabolism and an eventual secretion of the pineal neurohormone, melatonin. The effect of these agents following ℓ-propranolol and reserpine treatment will also be examined.

3.2 EXPERIMENT 1: THE EFFECT OF APPETITE SUPPRESSANTS ON THE METABOLISM OF PINEAL INDOLEAMINE IN ORGAN CULTURE.

3.2.1 Introduction

Most of the appetite suppressant compounds are chemical derivatives of β-phenylethylamine, the basic structure also shared by the catecholamine neurotransmitter noradrenaline. From this structural background, the following experiments were performed to investigate the effects of these compounds on the metabolism of pineal indoleamines (with particular emphasis on acetylated indoles) in organ culture.

As previously discussed (Section 2.2.1), the pineal gland in organ culture is able to utilize exogenous radioactive serotonin to produce various indoles including melatonin which are then secreted into the medium. The radioactive indoles thus synthesized are separated using two-dimensional TLC and assayed quantitatively by liquid scintillometry.

3.2.2 Materials and Methods

Animals: Male Wistar rats weighing between 200 and 250 g were used in these experiments. The animals were maintained as previously described (Section 2.1). The animals were sacrificed between 11h00 and 12h00 when performing these experiments.

Chemicals and Reagents: 5-Hydroxy (side chain-2-^{14}C) tryptamine creatinine sulphate (specific activity 55 mCi/mmol) was purchased from Amersham (England); chlorphentermine hydrochloride, ephedrine chloride and synthetic indoles from Sigma Chemical Co. (USA); fenfluramine hydrochloride was a gift from Reckitt and Coleman (SA). All other chemicals and reagents were obtained from local commercial sources.

Pineal Organ Culture: Pineal glands were aseptically removed and individually cultured

in 52 μl of BGJb culture medium and 0.4 μCi of [^{14}C] serotonin in the presence of chlorphentermine hydrochloride (n = 5), fenfluramine hydrochloride (n = 5) and ephedrine chloride (n = 5) all at a final concentration of 10^{-4}M. A set of control pineal glands (n = 5) were cultured in the absence of drugs. The culture tubes were then incubated at 37°C in an atmosphere of 95% O_2 : 5% O_2 for 24 hours. Following incubation, a 10 μl aliquot of culture medium was analysed for radioactive metabolites using TLC and liquid scintillometry as previously described (Section 2.2.2).

Data Analysis: Statistical comparisons were determined using Student's t-test and the data was expressed as DPM/10 μl medium/pineal. Values represent the mean $\pm$ SEM (n = 5).

3.2.3 Results

The effect of appetite suppressant compounds (chlorphentermine HCl, fenfluramine HCl and ephedrine chloride) on pineal acetylated indoles and monoamine oxidase products (MAOP) is shown on Tables 3.1, 3.2 and 3.3. The appetite suppressant compounds stimulated the synthesis of *N*-acetylserotonin and melatonin as compared to the controls ($p < 0.05$). The mean DPM values of *N*-acetylserotonin were higher than those of melatonin ($p < 0.001$). There was no significant change in MAOP levels after the addition of appetite suppressant compounds.

3.2.4 Discussion

The results show that the appetite suppressants stimulated the synthesis of melatonin in organ culture. This increase mimics the elevation of melatonin synthesis induced by addition of ℓ-noradrenaline to pineal cultures. It has been shown that the rise in cAMP, an increase in *N*-acetyltransferase activity and an eventual synthesis of melatonin in rat pinealocytes is regulated by noradrenaline acting through synergistic dual receptor mechanisms involving α_1 and β-adrenoceptors (Klein *et al.*, 1983). Considering this information, it became necessary

to establish the site(s) of action of appetite suppressants which result in stimulation of melatonin synthesis. Does the increase in melatonin synthesis due to the interaction of appetite suppressants with α_1- or β-adrenoceptor, or an interaction with another pinealocytic site?

The *N*-acetylserotonin values were higher than those of melatonin. The possible explanation for this may be that these compounds might enhance the activity on *N*-acetyltransferase without changing the activity of hydroxyindole-O-methyltransferase.

TABLE 3.1: The Effect of Chlorphentermine on Pineal Indoleamine Metabolism in Organ Culture.

Indoleamine	DPM/10 μl Medium/Pineal	
	Control	Chlorphentermine
NAS	1442 ± 82	3085* ± 105
MEL	887 ± 121	2065* ± 155
MAOP	19809 ± 2430	18346 ± 2687

NAS = *N*-Acetylserotonin; MEL = Melatonin; MAOP = Monoamine Oxidase Products

* $p < 0.01$ vs control (Student's t-test)

Values represent the mean ± SEM (n = 5).

TABLE 3.2: The Effect of Fenfluramine on Pineal Indoleamine Metabolism in Organ Culture.

Indoleamine	DPM/10 μl Medium/Pineal	
	Control	Fenfluramine
NAS	934 ± 147	2714* ± 195
MEL	760 ± 112	2114* ± 161
MAOP	8338 ± 1480	7887 ± 1615

NAS = *N*-Acetylserotonin; MEL = Melatonin; MAOP = Monoamine Oxidase Products

* $p < 0.05$ vs control (Student's t-test)

Values represent the mean ± SEM (n = 5).

TABLE 3.3: The Effect of Ephedrine on Pineal Indoleamine Metabolism in Organ Culture.

Indoleamine	DPM/10 μl Medium/Pineal	
	Control	Ephedrine
NAS	1541 ± 175	2765* ± 163
MEL	1085 ± 152	2234* ± 174
MAOP	4527 ± 1501	7947 ± 2602

NAS = *N*-Acetylserotonin; MEL = Melatonin; MAOP = Monoamine Oxidase Products

* $p < 0.05$ vs control (Student's t-test)

Values represent the mean ± SEM (n = 5).

3.3 EXPERIMENT 2: THE EFFECT OF THE β-ADRENOCEPTOR BLOCKER, PROPRANOLOL ON THE APPETITE SUPPRESSANTS - INDUCED INCREASE IN PINEAL *N*-ACETYLATED INDOLES.

3.3.1 Introduction

The release of noradrenaline (NA) from the post-ganglionic sympathetic neurons in the pineal gland is followed by interaction of the catecholamine with adrenergic receptors in the pinealocyte membrane. That released NA interacts with β-adrenoceptor to augment melatonin synthesis in the pineal gland is shown by the impaired methoxyindole synthesis observed after adding the β-adrenoceptor blocker, propranolol to rat pineal cultures (Cardinali *et al.*, 1987).

On the basis of these considerations, this study was designed to aid in identifying the site of action of appetite suppressants to induce an increase of pineal *N*-acetylated indoles. Therefore, the effect of propranolol on [^{14}C] serotonin metabolism in the presence of appetite suppressants was determined.

3.3.2 Materials and Methods

Animals: Male Wistar rats weighing between 200 and 250 g were used in this study. The animals were maintained as previously described (Section 2.1). The animals were sacrificed between 11h00 and 12h00 for the purpose of this study.

Chemicals and Reagents: 5-Hydroxy (side chain-2-^{14}C) tryptamine creatinine sulphate (specific activity 55 mCi/mmol) was purchased from Amersham (England); ℓ-propranolol from Sigma Chemical Co. (USA). All other chemicals and reagents were obtained from local commercial sources.

Pineal Organ Cultures: Organ culture studies and analysis of [^{14}C]-labelled metabolites were performed as outlined in Section 2.2.2. In summary, pineal glands were aseptically removed and individually cultured in the presence of chlorphentermine HCl (n = 5), fenfluramine HCl (n = 5) and ephedrine chloride (n = 5); also included in these culture tubes was ℓ-propranolol at a final concentration of 10^{-4}M. The tubes were then incubated at 37°C in an atmosphere of 95% O_2 : 5% CO_2 for 24 hours. Following incubation, a 10 μl aliquot of culture medium was analysed for radioactive metabolites using TLC and liquid scintillometry as previously described (Section 2.2.2).

Data Analysis: Statistical comparisons were determined using Student's t-test and the data expressed as DPM/10 μl medium/pineal. Values represent the mean $\pm$ SEM (n = 5).

3.3.3 Results

The β-adrenoceptor blocker, propranolol prevented the increase in pineal *N*-acetylated indoles induced by appetite suppressants. These results are shown in Tables 3.4, 3.5 and 3.6. Propranolol significantly suppressed fenfluramine-induced increase in pineal melatonin with $p < 0.001$ (Figure 3.3 and 3.4). The effect of propranolol on chlorphentermine-induced and ephedrine-induced increase of *N*-acetylated pineal indoles is graphically presented on Figures 3.1, 3.2 and Figures 3.5, 3.6, respectively. There was no significant decrease on monoamine oxidase product (MAOP) levels after the addition of propranolol.

3.3.4 Discussion

It has been clearly established in rats that the stimulation of pineal melatonin synthesis induced by noradrenaline or isoproterenol (direct β-adrenoceptor agonist) can be blocked by the addition of ℓ-propranolol to pineal organ cultures (Strada *et al.*, 1972). The results show that the increase in *N*-acetylated indole synthesis induced by appetite suppressants is prevented in pineal glands pretreated with propranolol.

This observation indicates the possible involvement of β-adrenoceptors in the action of appetite suppressants in the pineal gland. This finding made it necessary to determine whether these compounds were acting directly or indirectly on pineal β-adrenoceptors.

TABLE 3.4: The Effect of Propranolol on Chlorphentermine-Induced Increase of Pineal Indoles in Organ Culture.

Indole	DPM/10 μl Medium/Pineal		
	Control	Chlorphentermine	Chlorphentermine + Propranolol
NAS	1442 ± 82	3080 ± 105	1284* ± 214
MEL	887 ± 121	2065 ± 155	898** ± 187
MAOP	19809 ± 2430	18346 ± 2678	15189 ± 1082

NAS = *N*-Acetylserotonin; MEL = Melatonin; MAOP = Monoamine Oxidase Products

* $p < 0.01$ vs chlorphentermine

** $p < 0.05$ vs chlorphentermine (Student's t-test).

TABLE 3.5: The Effect of Propranolol on Fenfluramine-Induced Increase of Pineal Indoles in Organ Culture.

Indole	DPM/10 µl Medium/Pineal		
	Control	Fenfluramine	Fenfluramine + Propranolol
NAS	934 ± 147	2304 ± 188	480* ± 55
MEL	760 ± 112	2114 ± 161	218* ± 21
MAOP	8570 ± 1591	7887 ± 1615	4806 ± 813

NAS = *N*-Acetylserotonin; MEL = Melatonin; MAOP = Monoamine Oxidase Products

* $p < 0.001$ vs fenfluramine (Student's t-test)

TABLE 3.6: The Effect of Propranolol on Ephedrine-Induced Increase of Pineal Indoles in Organ Culture.

Indole	DPM/10 µl Medium/Pineal		
	Control	Ephedrine	Ephedrine + Propranolol
NAS	1541 ± 175	2658 ± 343	2287 ± 455
MEL	1085 ± 152	2234 ± 376	1042* ± 267
MAOP	4527 ± 1501	7067 ± 2142	5183 ± 2276

NAS = *N*-Acetylserotonin; MEL = Melatonin; MAOP = Monoamine Oxidase Products

* $p < 0.05$ vs ephedrine (Student's t-test).

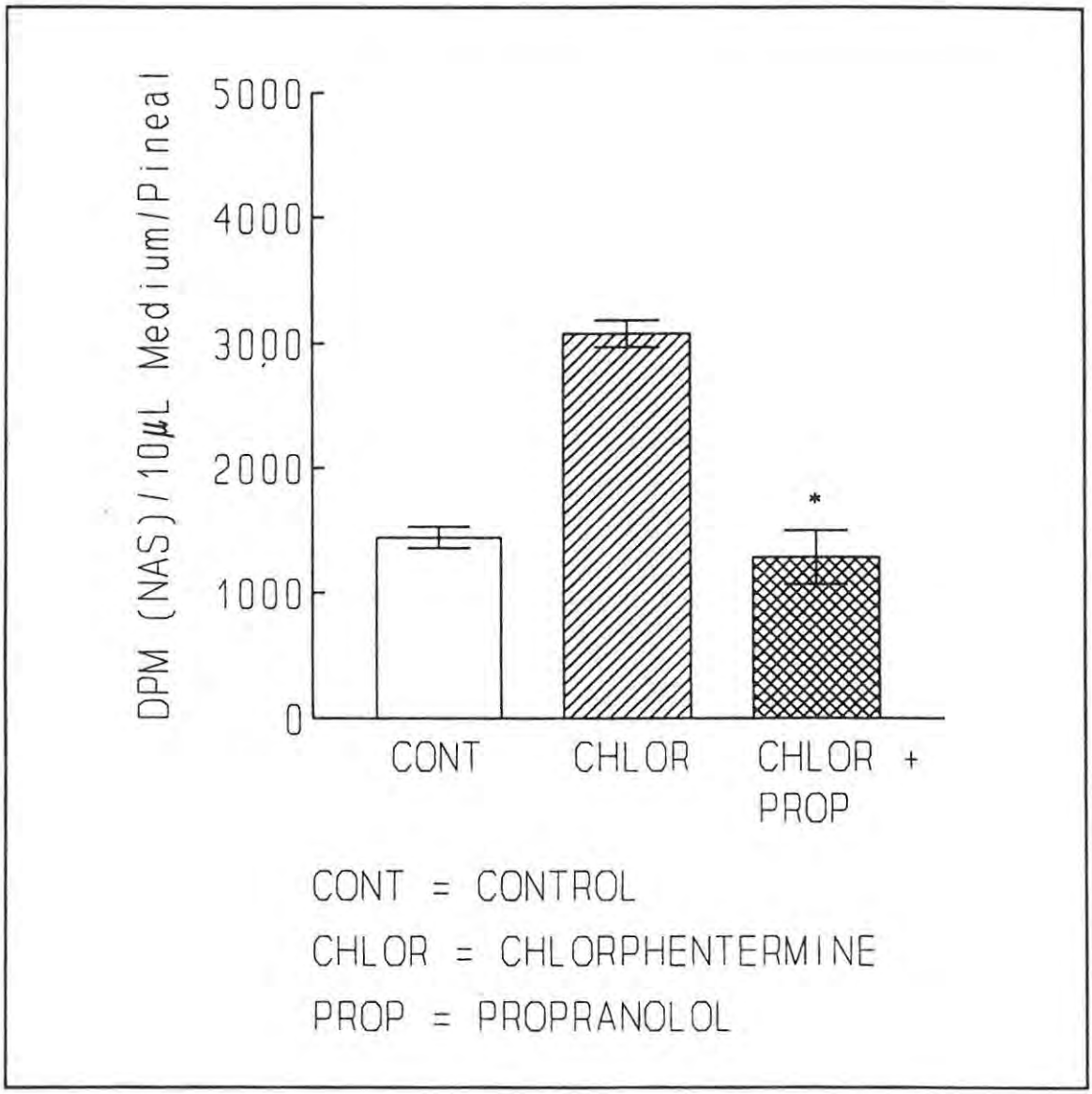


FIGURE 3.1: **The Effect of Propranolol on Chlorphentermine-Induced Increase of Pineal NAS in Organ Culture.**

Values represent the mean ± SEM (n = 5)

* $p < 0.01$ vs Chlorphentermine.

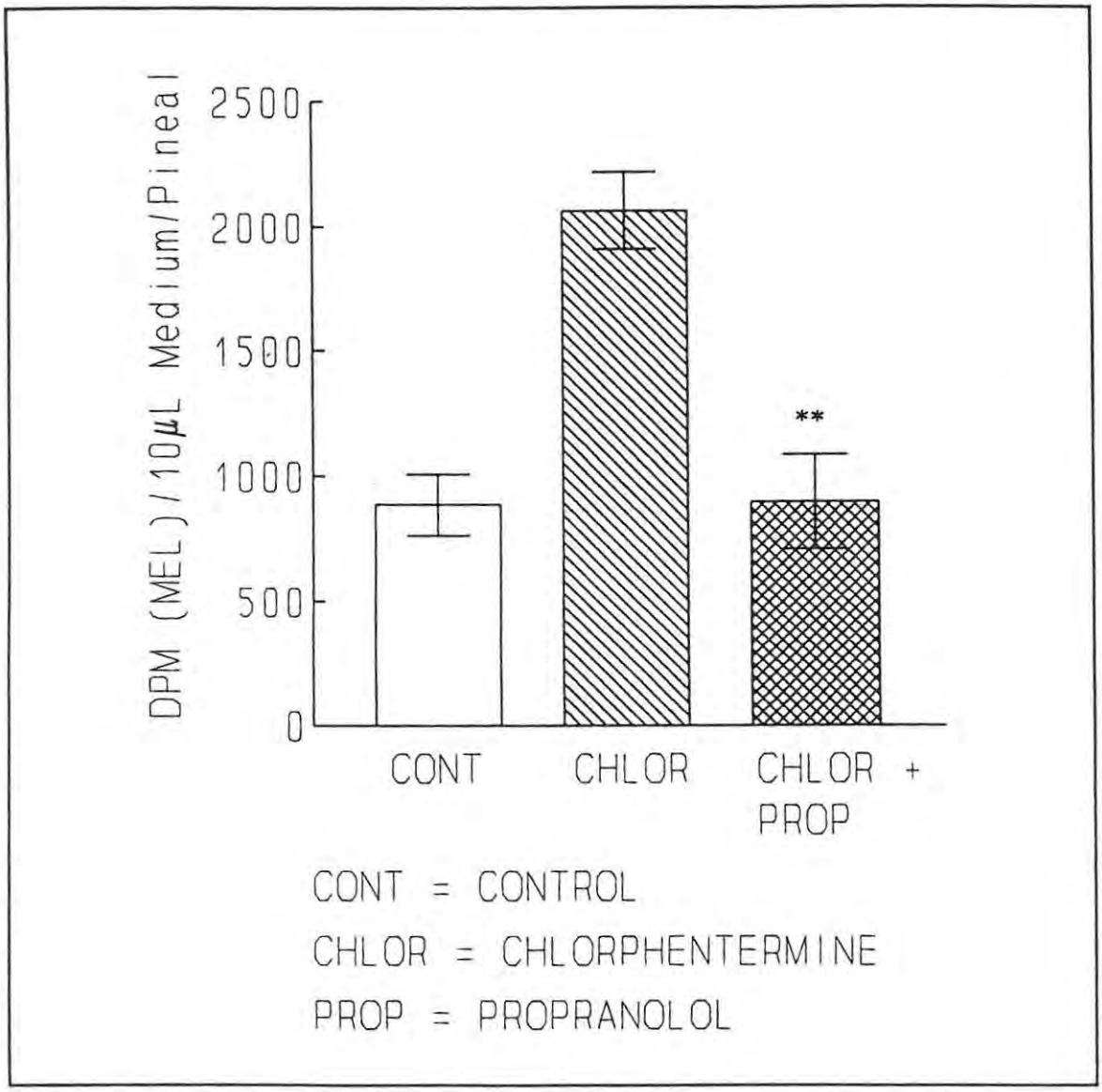


FIGURE 3.2: **The Effect of Propranolol on Chlorphentermine-Induced Increase of Pineal Melatonin in Organ Culture.**

Values represent the mean ± SEM (n = 5)

** $p < 0.05$ vs Chlorphentermine.

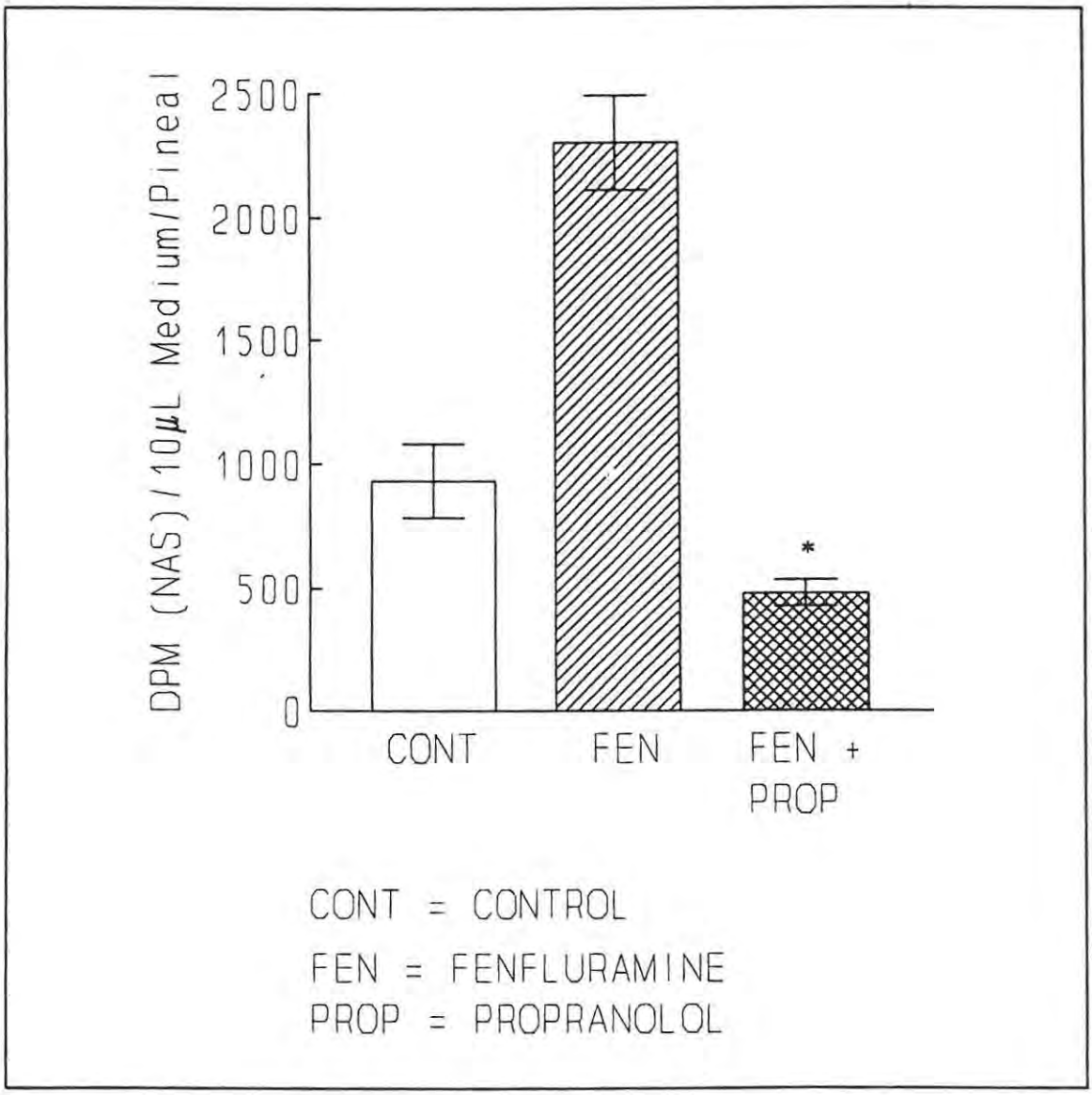


FIGURE 3.3: **The Effect of Propranolol on Fenfluramine-Induced Increase of Pineal NAS in Organ Culture.**

Values represent the mean ± SEM (n = 5)

* $p < 0.05$ vs Fenfluramine.

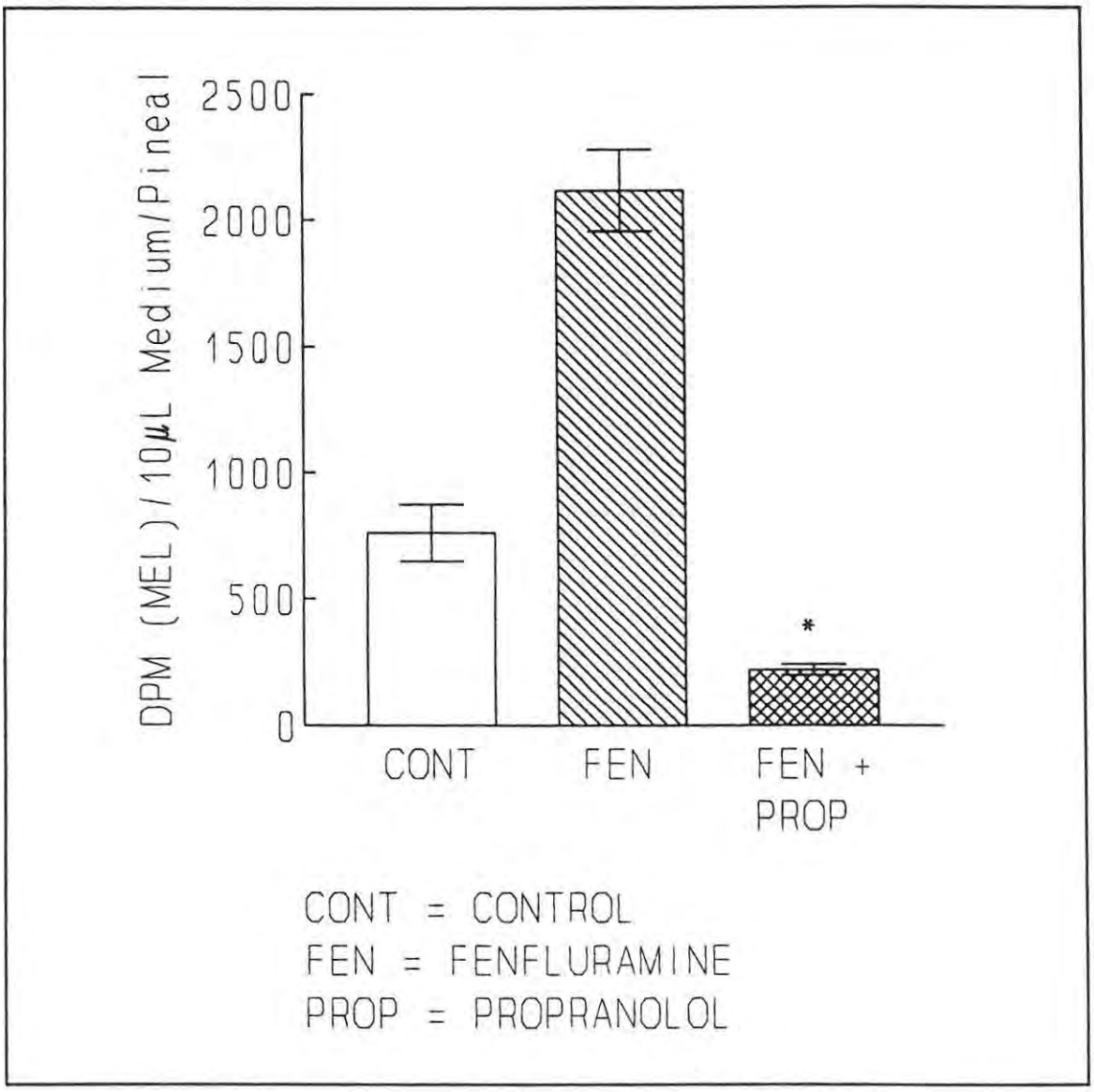


FIGURE 3.4: **The Effect of Propranolol on Fenfluramine-Induced Increase of Pineal Melatonin in Organ Culture.**

Values represent the mean ± SEM (n = 5)

* $p < 0.05$ vs Fenfluramine.

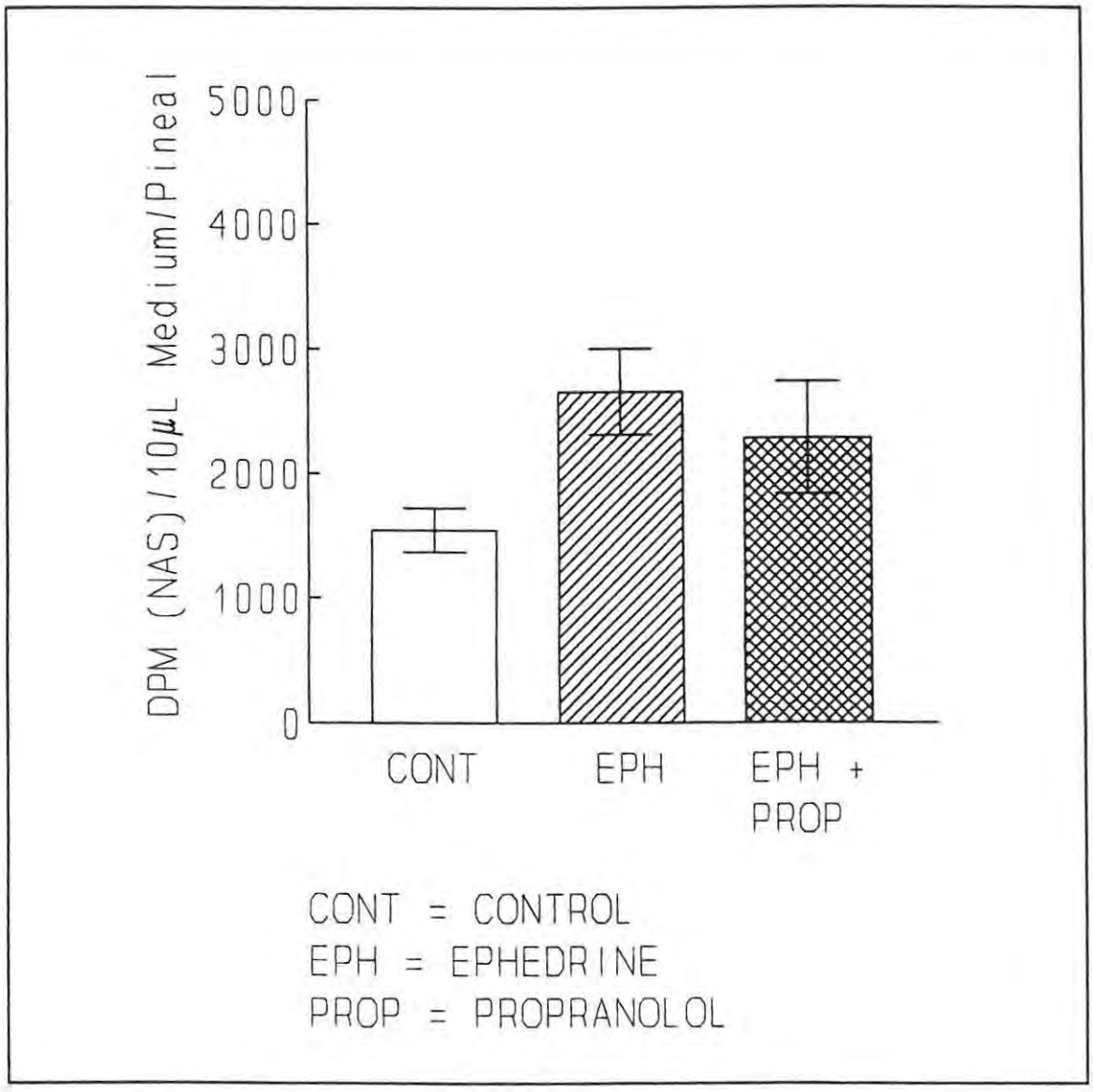


FIGURE 3.5: **The Effect of Propranolol on Ephedrine-Induced Increase of Pineal NAS in Organ Culture.**

Values represents the mean ± SEM (n = 5).

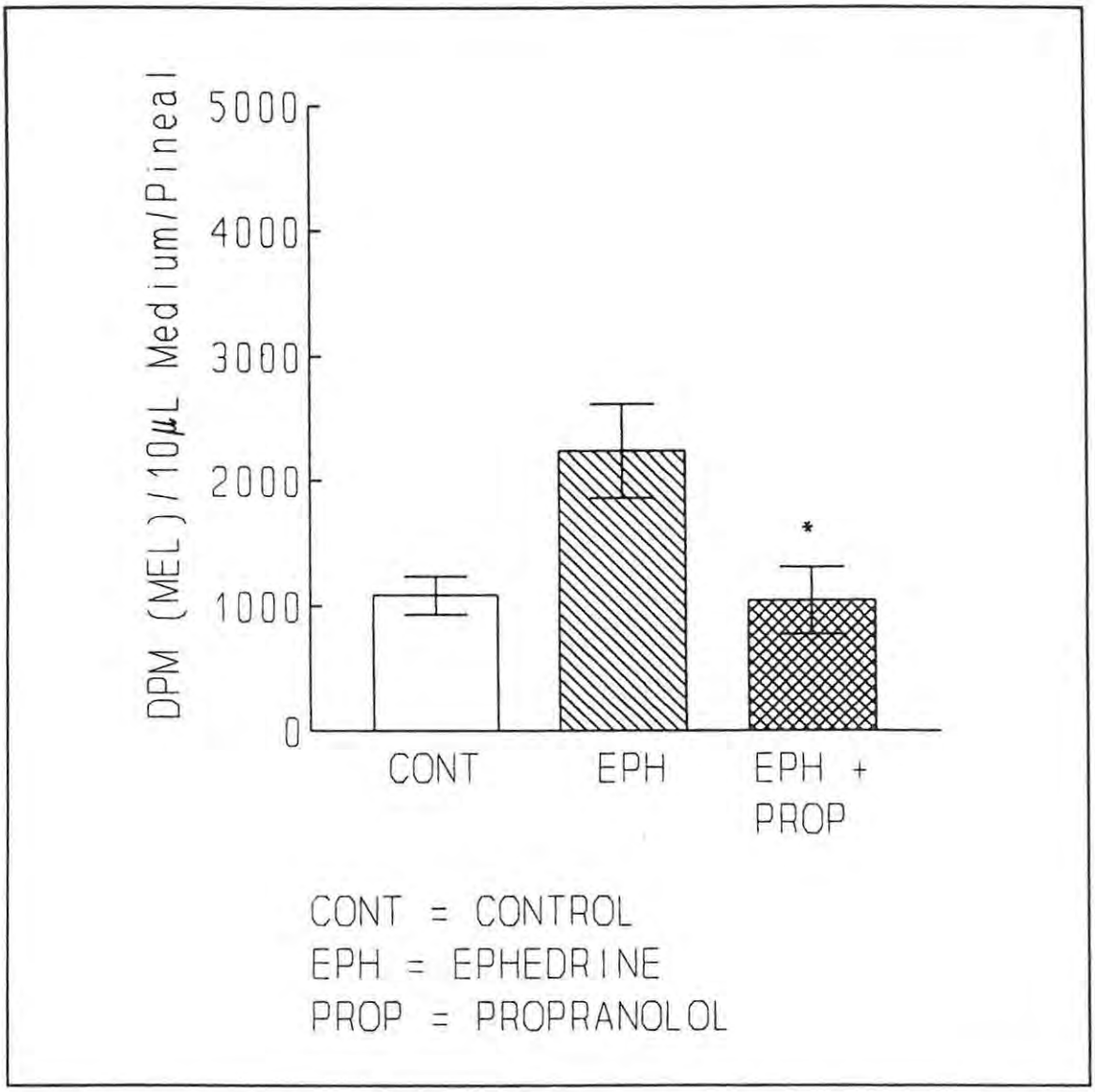


FIGURE 3.6: **The Effect of Propranolol on Ephedrine-Induced Increase of Pineal Melatonin in Organ Culture.**

Values represent the mean ± SEM

* $p < 0.05$ vs Ephedrine.

3.4 EXPERIMENT 3: THE EFFECT OF RESERPINE ON APPETITE SUPPRESSANT-INDUCED INCREASE IN PINEAL *N*-ACETYLATED INDOLES.

3.4.1 Introduction

The concentrations of melatonin and serotonin in the rat pineal gland are influenced by signals transmitted to the organ by way of its sympathetic innervation. The nerve endings of these neurons contain both noradrenaline and serotonin; however, studies *in vitro* (Axelrod *et al.*, 1969) have provided evidence that noradrenaline, and not serotonin, is the neurotransmitter which controls the pineal indole content.

This study was designed to investigate the effect of reserpine, a drug that depletes neurons of both catecholamines and serotonin, on appetite suppressant-induced increase in pineal *N*-acetylated metabolites.

3.4.2 Materials and Methods

Animals: Male Wistar rats weighing between 200 and 250 g were used in this study. The animals were maintained as previously described (Section 2.1). The animals were sacrificed between 11h00 and 12h00 for the purpose of this study.

Chemicals and Reagents: 5-Hydroxy (side chain-2-^{14}C) tryptamine creatinine sulphate (specific activity 55 mCi/mmol) was purchased from Amersham (England); reserpine hydrochloride, from Sigma Chemical Co. (USA). All other chemicals and reagents were obtained from local commercial sources.

Drug Administration: Reserpine hydrochloride was administered as a solution in a vehicle consisting of the mixture of 2% benzyl alcohol, 10% polysorbate and deionized water. Rats (groups of 5) were injected intraperitoneally with reserpine (5 mg/kg) and

sacrificed 3 hours later.

Pineal Organ Cultures: Refer to Section 2.2.2. In summary, pineal glands were aseptically removed and individually cultured in BGJb culture medium in the presence of chlorphentermine hydrochloride (n = 5), fenfluramine hydrochloride (n = 5) and ephedrine chloride (n = 5). The final concentration of drugs in culture tubes was 10^{-4}M. The tubes were then incubated at 37°C in an atmosphere of 95% O_2 : 5% CO_2 for 24 hours. Following incubation, a 10 μl aliquot of culture medium was removed and analysed for radioactive metabolites using TLC and liquid scintillometry as previously described (Section 2.2.2).

Data Analysis: Statistical comparisons were performed using Student's t-test and the data expressed as DPM/10 μl medium/pineal. Values represent the mean $\pm$ SEM (n = 5).

3.4.3 Results

The effect of reserpine treatment on appetite suppressant-induced increase in *N*-acetylated pineal indoles is shown in Tables 3.7, 3.8 and 3.9, and graphically presented in Figures 3.7 to 3.12. Reserpine depressed the levels of chlorphentermine-induced (Figure 3.7 and 3.8) and ephedrine-induced (Figure 3.11 and 3.12) increase of pineal *N*-acetylated indoles. Reserpine did not have any effect on the fenfluramine-induced increase in pineal *N*-acetylated indoles (Table 3.8 and Figure 3.9). MAOP levels seemed to increase after reserpine treatment as compared to levels of appetite suppressants (Tables 3.7, 3.8 and 3.9).

3.4.4 Discussion

Reserpine, a drug that depletes neurons of both catecholamines and serotonin, depressed the increased levels of pineal *N*-acetylated indoles induced by appetite suppressants, with the exception of fenfluramine. There is evidence that noradrenaline, and not serotonin, is the neurotransmitter which controls the pineal indole content (Axelrod *et al.*, 1969). Furthermore, it has been shown that the interaction of noradrenaline with β-adrenoceptor

elevates cAMP levels and increases *N*-acetyltransferase activity with a consequent rise in the synthesis of melatonin in rat pineals (Strada *et al.*, 1972).

It therefore appears that appetite suppressants act indirectly by displacing noradrenaline from nerve endings. It is therefore likely that it is the action of noradrenaline, and not these agents, which results in the stimulation of pineal *N*-acetylated indole synthesis.

The effect of fenfluramine on pineal *N*-acetylated indoles was unaltered by reserpine treatment (Figures 3.9 and 3.10). These results are in agreement with the observations on the rat brain that fenfluramine releases noradrenaline from a reserpine-insensitive pool (Garattini *et al.*, 1986); these effects, however, depend on isomers of fenfluramine, the *ℓ*-isomer being stronger than the d-isomer. On the other hand, the effect of fenfluramine may indicate its direct action on *β*-adrenoceptor in the pinealocyte membrane in inducing melatonin synthesis. Further studies are required to clarify this fenfluramine effect.

TABLE 3.7: The Effect of Reserpine on Chlorphentermine-Induced Increase of Pineal Indoles in Organ Culture.

Indole	DPM/10 μl Medium/Pineal		
	Control	Chlorphentermine	Chlorphentermine + Reserpine
NAS	1442 ± 82	3080 ± 105	1757* ± 455
MEL	887 ± 121	1624 ± 164	921* ± 186
MAOP	19809 ± 2430	9099 ± 1565	9914 ± 3009

NAS = *N*-Acetylserotonin; MEL = Melatonin; MAOP = Monoamine Oxidase Products

* $p < 0.05$ vs Chlorphentermine.

TABLE 3.8: The Effect of Reserpine on Fenfluramine-Induced Increase of Pineal Indoles in Organ Culture.

Indole	DPM/10 μl Medium/Pineal		
	Control	Fenfluramine	Fenfluramine + Reserpine
NAS	934 ± 147	2304 ± 188	2228 ± 146
MEL	760 ± 112	1000 ± 198	805 ± 210
MAOP	8338 ± 1480	8569 ± 1591	11937 ± 3117

NAS = *N*-Acetylserotonin; MEL = Melatonin; MAOP = Monoamine Oxidase Products.

TABLE 3.9: The Effect of Reserpine on Ephedrine-Induced Increase of Pineal Indoles in Organ Culture.

Indole	DPM/10 μl Medium/Pineal		
	Control	Ephedrine	Ephedrine + Reserpine
NAS	1541 ± 175	2357 ± 315	526* ± 60
MEL	1085 ± 152	2232 ± 376	735 ± 173
MAOP	4527 ± 1501	7755 ± 2149	8219 ± 2531

NAS = *N*-Acetylserotonin; MEL = Melatonin; MAOP = Monoamine Oxidase Products

* $p < 0.05$ vs Ephedrine.

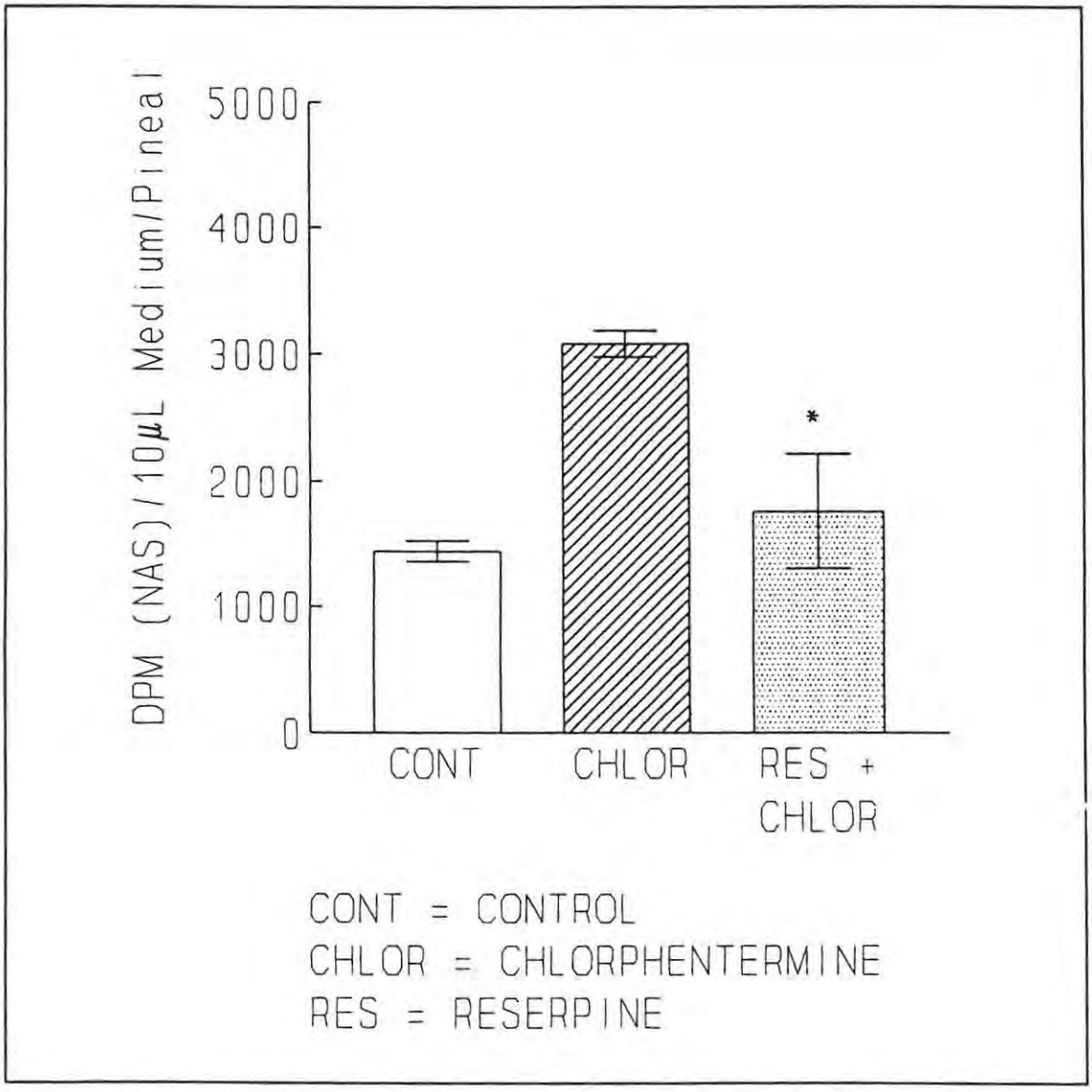


FIGURE 3.7: **The Effect of Reserpine on Chlorphentermine-Induced Increase of Pineal NAS in Organ Culture.**

Values represent the mean ± SEM (n = 5)

* $p < 0.05$ vs Chlorphentermine.

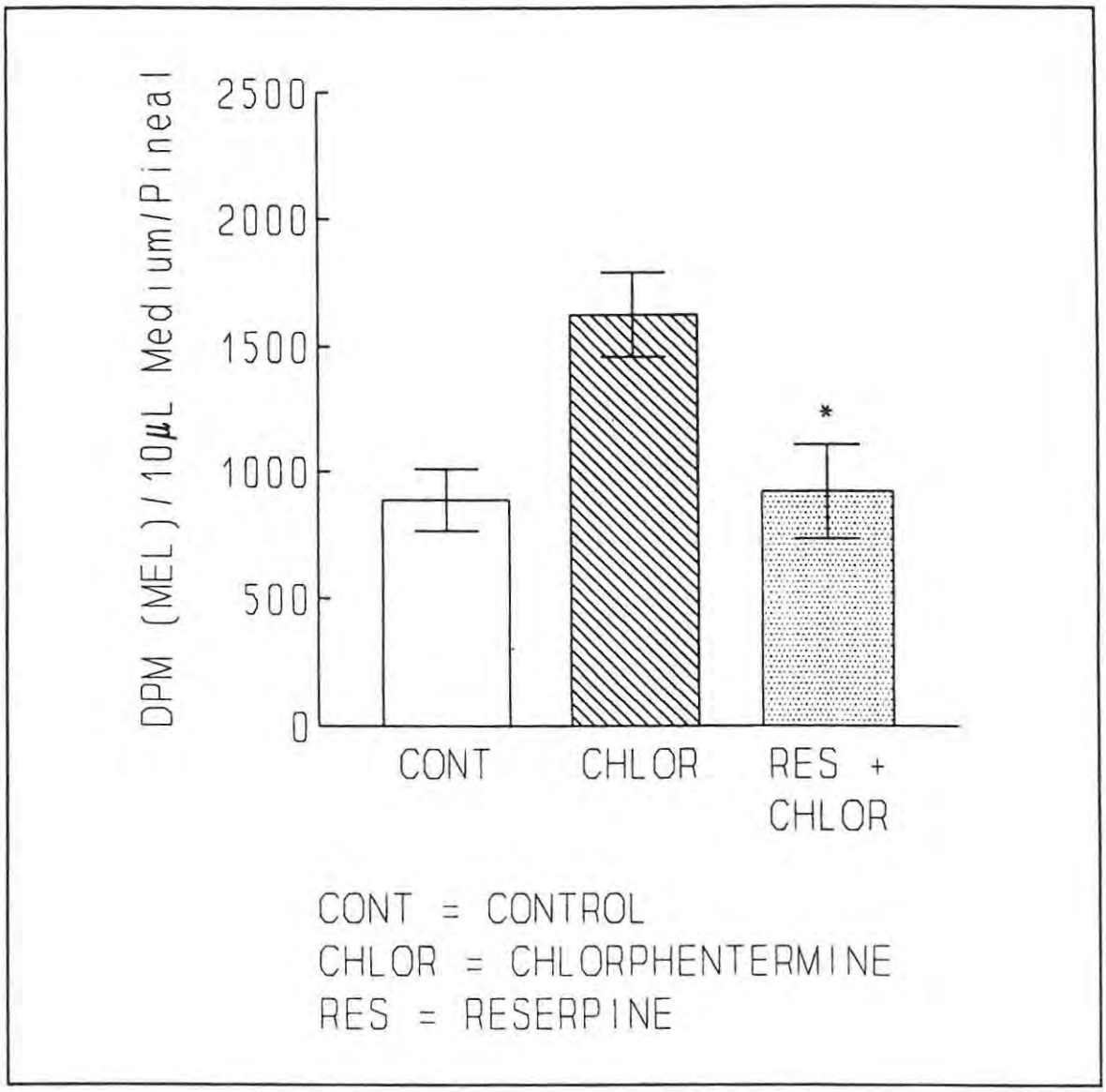


FIGURE 3.8: **The Effect of Reserpine on Chlorphentermine-Induced Increase of Pineal Melatonin in Organ Culture.**

Values represent the mean SEM (n = 5)

* $p < 0.05$ vs Chlorphentermine.

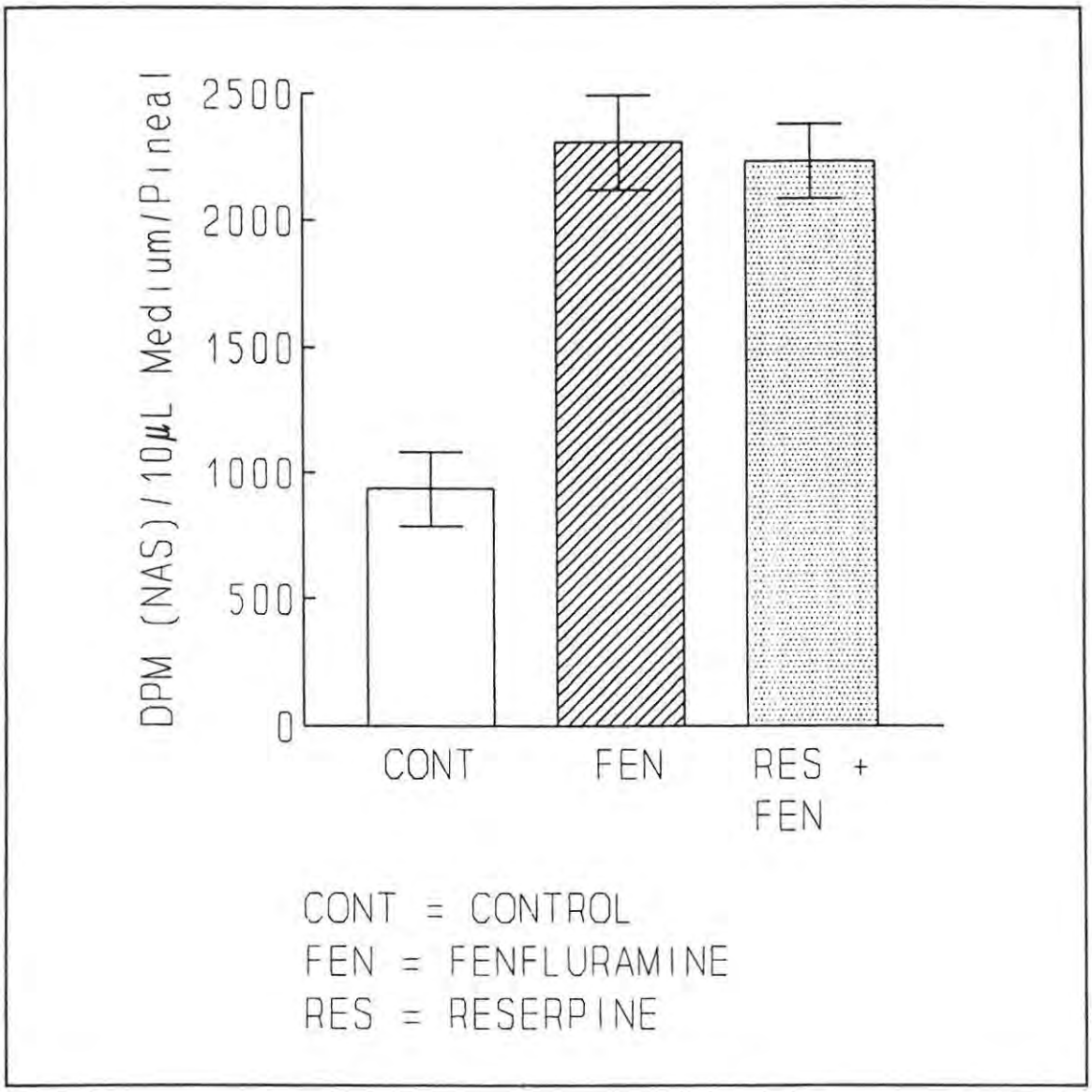


FIGURE 3.9: **The Effect of Reserpine on Fenfluramine-Induced Increase of Pineal NAS in Organ Culture.**

Values represent the mean ± SEM (n = 5).

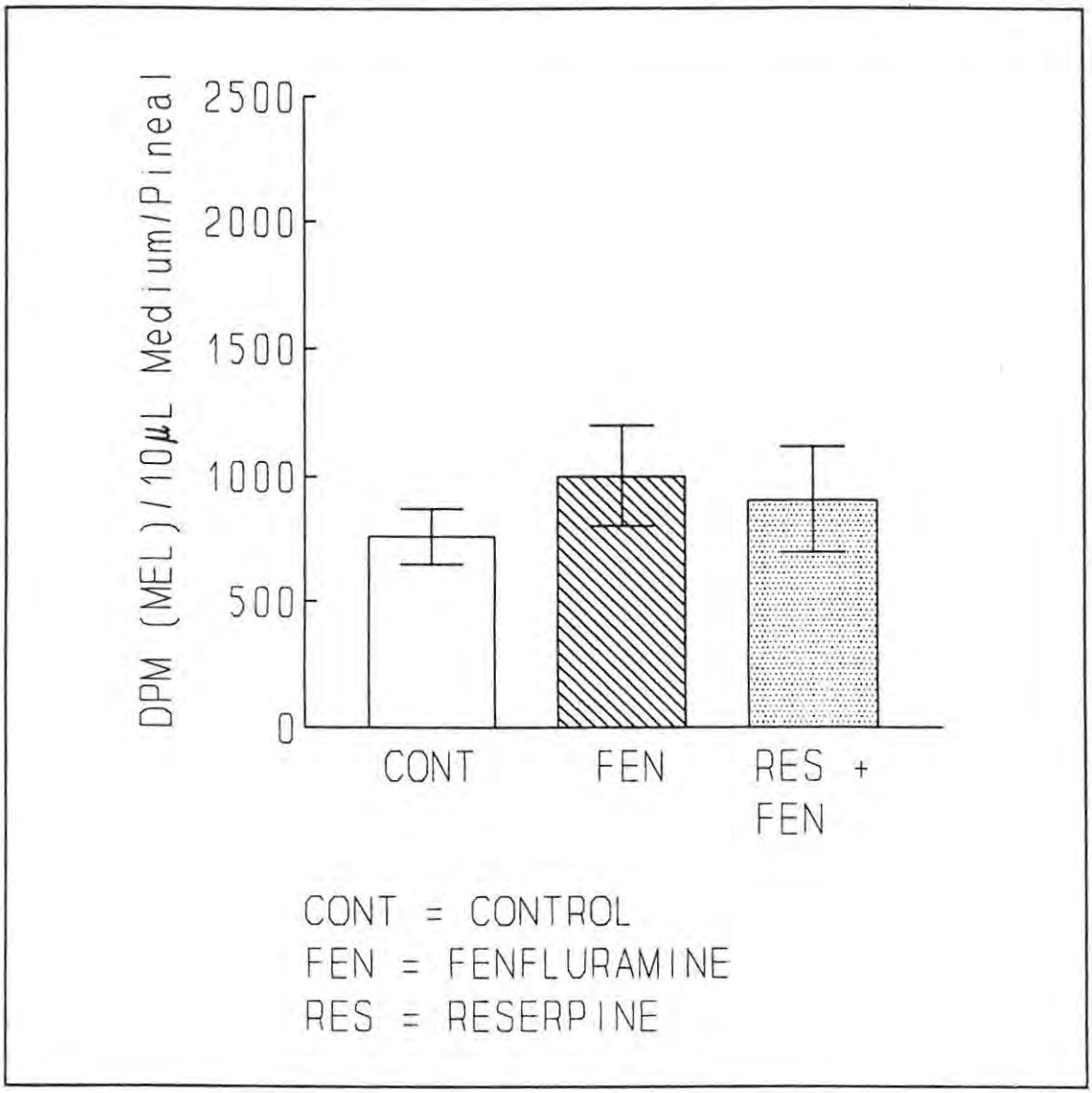


FIGURE 3.10: **The Effect of Reserpine on Fenfluramine-Induced Increase of Pineal Melatonin in Organ Culture.**

Values represent the mean ± SEM (n = 5).

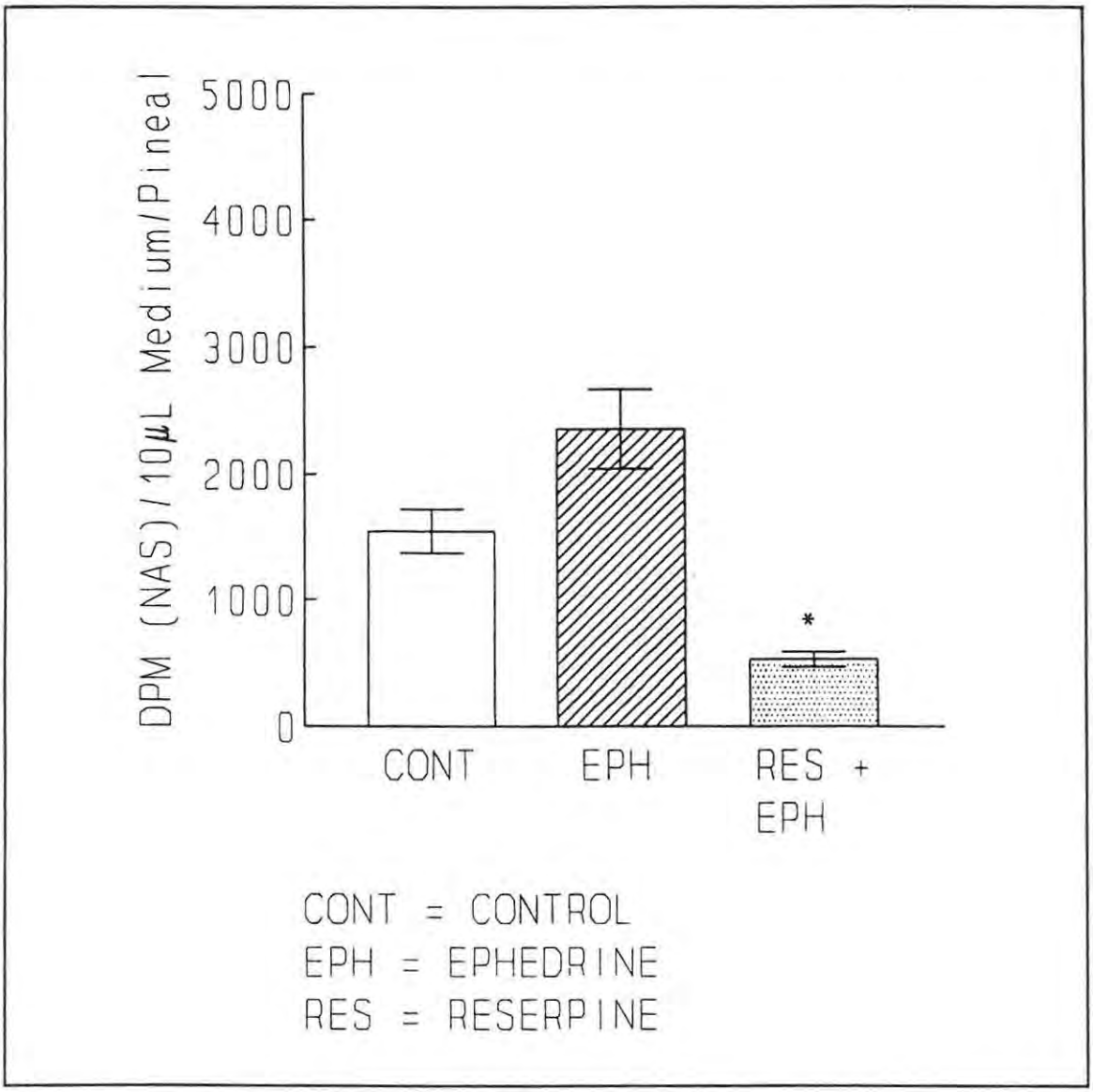


FIGURE 3.11: **The Effect of Reserpine on Ephedrine-Induced Increase of Pineal NAS in Organ Culture.**

Values represent the mean ± SEM (n = 5)

* $p < 0.05$ vs Ephedrine.

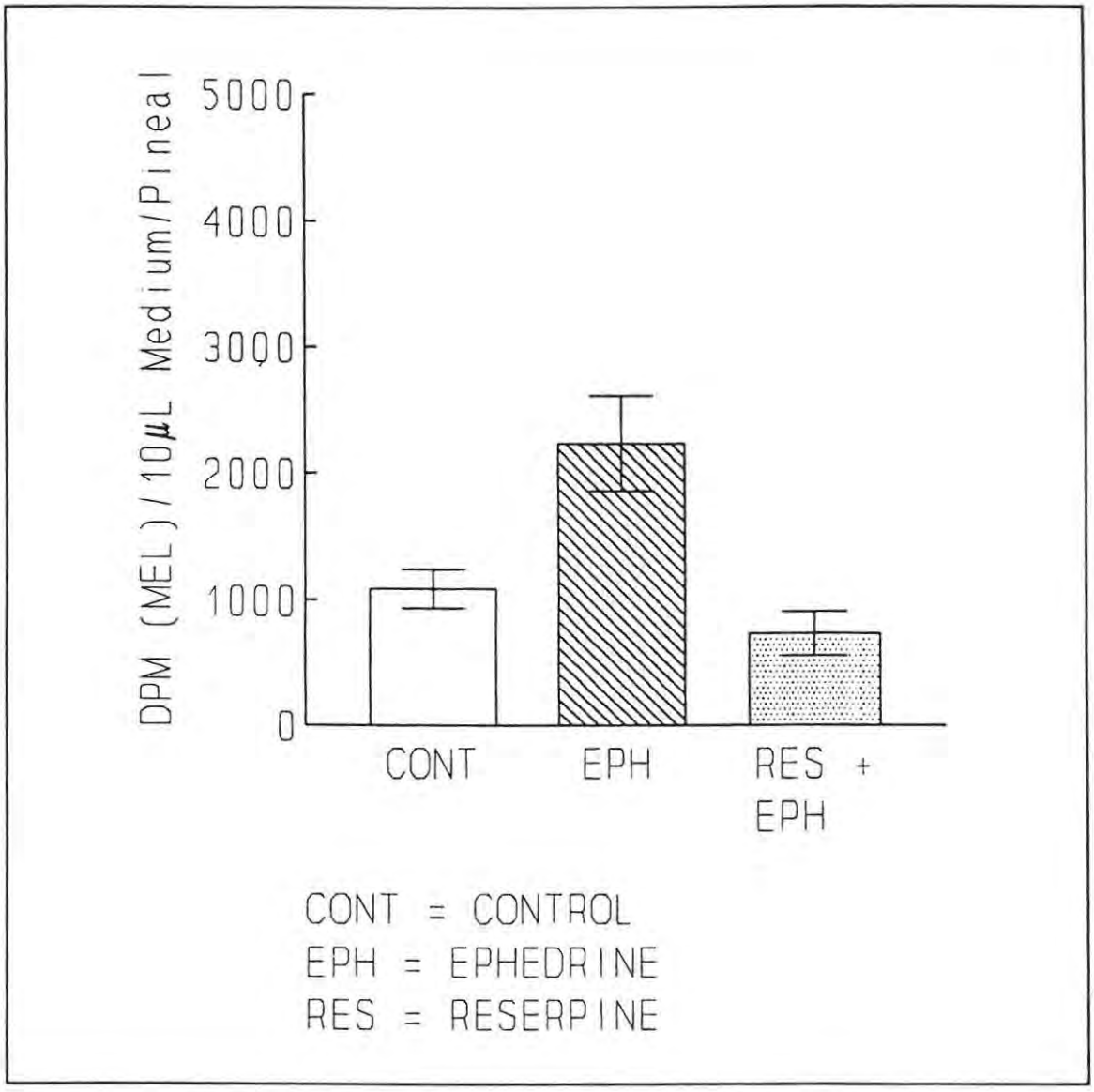


FIGURE 3.12: **The Effect of Reserpine on Ephedrine-Induced Increase of Pineal Melatonin in Organ Culture.**

Values represent the mean ± SEM (n = 5).

3.5 CONCLUSION

The investigation on the effect of appetite suppressants on the metabolism of pineal indoleamine in organ culture revealed that these drugs stimulate synthesis of *N*-acetylated indoles (*N*-acetylserotonin and melatonin). The degree of stimulation varies on the drug used with chlorphentermine being superior.

The addition of ℓ-propranolol, a β-adrenoceptor blocker, prevented the increase of pineal *N*-acetylated indoles indicating that these drugs probably act on β-adrenoceptors. Furthermore, reserpine treatment depresses the rise in pineal *N*-acetylated indoles induced by appetite suppressants.

Therefore, the results of this study indicate that appetite suppressants act by displacing noradrenaline from pineal nerve terminals resulting in the interaction of the catecholamine with β-adrenoceptors to elevate the pineal *N*-acetylated indole synthesis.

CHAPTER 4

THE EFFECT OF APPETITE SUPPRESSANTS ON PINEAL BIOCHEMISTRY

4.1 INTRODUCTION

The production of melatonin within the pinealocyte requires the interaction of noradrenaline with the β-adrenoceptor on the pinealocyte membrane. Beta-adrenergic stimulation activates the adenylate cyclase enzyme via a stimulatory guanine nucleotide-binding regulatory protein (G_s) (Spiegel, 1989). Adenylate cyclase is responsible for the conversion of adenosine triphosphate (ATP) to cAMP. Activation of adenylate cyclase results in a rapid, large increase of intracellular cAMP. Cyclic AMP serves as a second messenger in the nocturnal elevation of melatonin biosynthesis by activating a cAMP-dependent protein kinase and an eventual rise in *N*-acetyltransferase (NAT) activity, the presumed rate-limiting enzyme in melatonin production (Klein, 1985).

The mechanism involved in the regulation of pinealocyte cAMP and cGMP by adrenoceptors (α_1 and β) remains unclear. Several observations indicate that calcium may play a role (Sugden *et al.*, 1987; Ho *et al.*, 1988). Firstly, α_1-adrenergic potentiation of β-adrenergic cAMP accumulation has been shown to involve the activation of calcium-dependent enzyme protein kinase C (Sugden *et al.*, 1985b); secondly, removal of calcium markedly reduces the cAMP and cGMP response to noradrenaline in cultured pineal glands (Vanecek *et al.*, 1986); and thirdly, preliminary observations indicate that the calcium ionophore A 23187 potentiates the β-adrenergic stimulation of cAMP and cGMP (Vanecek *et al.*, 1986).

When the elevation of cAMP is blocked by prior treatment of rat pineal glands with a β-adrenoceptor blocker, NAT activity cannot be stimulated suggesting a causal relationship between cAMP and the synthesis of NAT (Deguchi, 1973). Maintenance of the high level of NAT activity seems to require continuous stimulation of the β-adrenoceptor on the pinealocyte membrane. Once the receptor has been blocked regardless of the levels of

cAMP, NAT activity disappears immediately (Deguchi, 1973). The rapid disappearance of NAT activity could be due either to rapid conversion of active to an inactive form of the enzyme or to disaggregation of the enzyme molecule to its subunits (Deguchi and Axelrod, 1972b).

Hydroxyindole-O-methyltransferase (HIOMT) is the final enzyme in the biosynthesis of melatonin from tryptophan. The enzyme is largely restricted to the pineal gland (Axelrod and Weissbach, 1961), yet its activity has also been found in some extrapineal tissues, such as the retina and the Harderian gland (Quay, 1965; Reiter, 1984). Although *N*-acetylserotonin appears to be the preferred physiological substrate for HIOMT in the pineal gland of many mammalian species, other 5-hydroxyindoles present in the gland have been shown to undergo the process of O-methylation to varying degrees (Axelrod and Weissbach, 1961; Morton and Forbes, 1989). Klein and Berg (1970) examined the effect of ℓ-noradrenaline stimulation of β-adrenoceptor on HIOMT activity. Noradrenaline induced a small rise in HIOMT activity, not enough to account for the large increase in melatonin synthesis.

Appetite suppressants have been found to increase the levels of pineal melatonin (Chapter 3). Therefore, this section deals with the effect of these agents on pineal cyclic nucleotide (cAMP) levels and on the activity of enzymes involved in melatonin biosynthesis, namely NAT and HIOMT.

4.2 EXPERIMENT 1: THE EFFECT OF APPETITE SUPPRESSANTS ON PINEAL CYCLIC AMP LEVELS

4.2.1 Introduction

The production of cAMP and cGMP in rat pinealocytes is regulated by noradrenaline acting through synergistic dual receptor mechanisms involving both α_1- and β-adrenoceptors (Klein *et al.*, 1983). Activation of the β-adrenoceptor by itself produces a 7 - 10 fold increase in the accumulation of cAMP. Selective activation of the α_1-adrenoceptor potentiates the β-adrenergically stimulated cyclic nucleotide responses. Stimulation by both the β- and α_1-adrenoceptors results in 100 fold increases in both cAMP and cGMP accumulation (Ho *et al.*, 1991).

Since appetite suppressants seem to act on the adrenergic system, their effect on pineal cAMP levels was investigated.

4.2.2 Materials and Methods

Animals: Male Wistar rats weighing between 200 and 250 g were used. The animals were maintained as previously described (Section 2.1). The animals were sacrificed between 9h00 and 10h00.

Chemicals and Reagents: (8 - ^{3}H) Adenosine 3′,5′-cyclic phosphate, ammonium salt (specific activity 30 Ci/mmol) was purchased from Amersham (England); theophylline and bovine serum albumin from Sigma Chemical Co. (USA); activated charcoal from Merck (West Germany). All other chemicals and reagents were obtained from local commercial sources.

Cyclic AMP Assay: Preparation of cAMP binding protein, pineal tissue and incubation procedures were carried out as previously described (Section 2.3.2).

Animals were sacrificed and the pineal glands rapidly removed. Glands were placed into sterile glass tubes containing 2 ml of culture medium in the absence (n = 5) and presence of chlorphentermine HCl (n = 5), fenfluramine HCl (n = 5) and ephedrine chloride (n = 5) at a final concentration of 10^{-4}M. The tubes were incubated at 37°C for 20 minutes in an atmosphere of 95% O_2 : 5% CO_2. Following incubation, glands were removed and individually sonicated in 100 μl of homogenizing buffer (Section 2.3.2). A 50 μl aliquot was removed for cAMP assay. The assay was carried out as previously outlined (Section 2.3.2 and Table 2.2).

Data Analysis: Statistical comparisons were determined using Student's t-test. The cAMP concentration was expressed as pmol cAMP per pineal, i.e. pmol per tube x2 (50 μl of homogenate was used from a total of 100 μl).

4.2.3 Results

Chlorphentermine significantly increased the levels of pineal cAMP ($p < 0.05$). Fenfluramine and ephedrine increased the cAMP levels but not significantly. The results are shown on Table 4.1 and graphically presented in Figure 4.1.

4.2.4 Discussion

Klein *et al.* (1983) showed that the production of cAMP in rat pinealocytes is regulated by the stimulation of β-adrenoceptors. Activation of the β-adrenoceptor produces a 7 - 10 fold increase in the accumulation of cAMP within 10 - 15 minutes. The results of this study show that chlorphentermine produces about a 5-fold increase in the levels of cAMP after 20 minutes of incubation. Fenfluramine and ephedrine also stimulate the increase in cAMP levels although not profoundly.

The results show that the appetite suppressants, particularly chlorphentermine, has part of its action on the adrenergic system in that these agents have the ability to induce a marked increase in pineal cAMP levels.

TABLE 4.1: The Effect of Appetite Suppressants on Pineal cAMP Levels.

Treatment	pmol cAMP per pineal
Control	8.11 ± 3.24
Ephedrine	17.20 ± 4.43
Fenfluramine	28.62 ± 5.42
Chlorphentermine	37.56 ± 4.08*

* $p < 0.05$ vs control

Values represent the mean ± SEM.

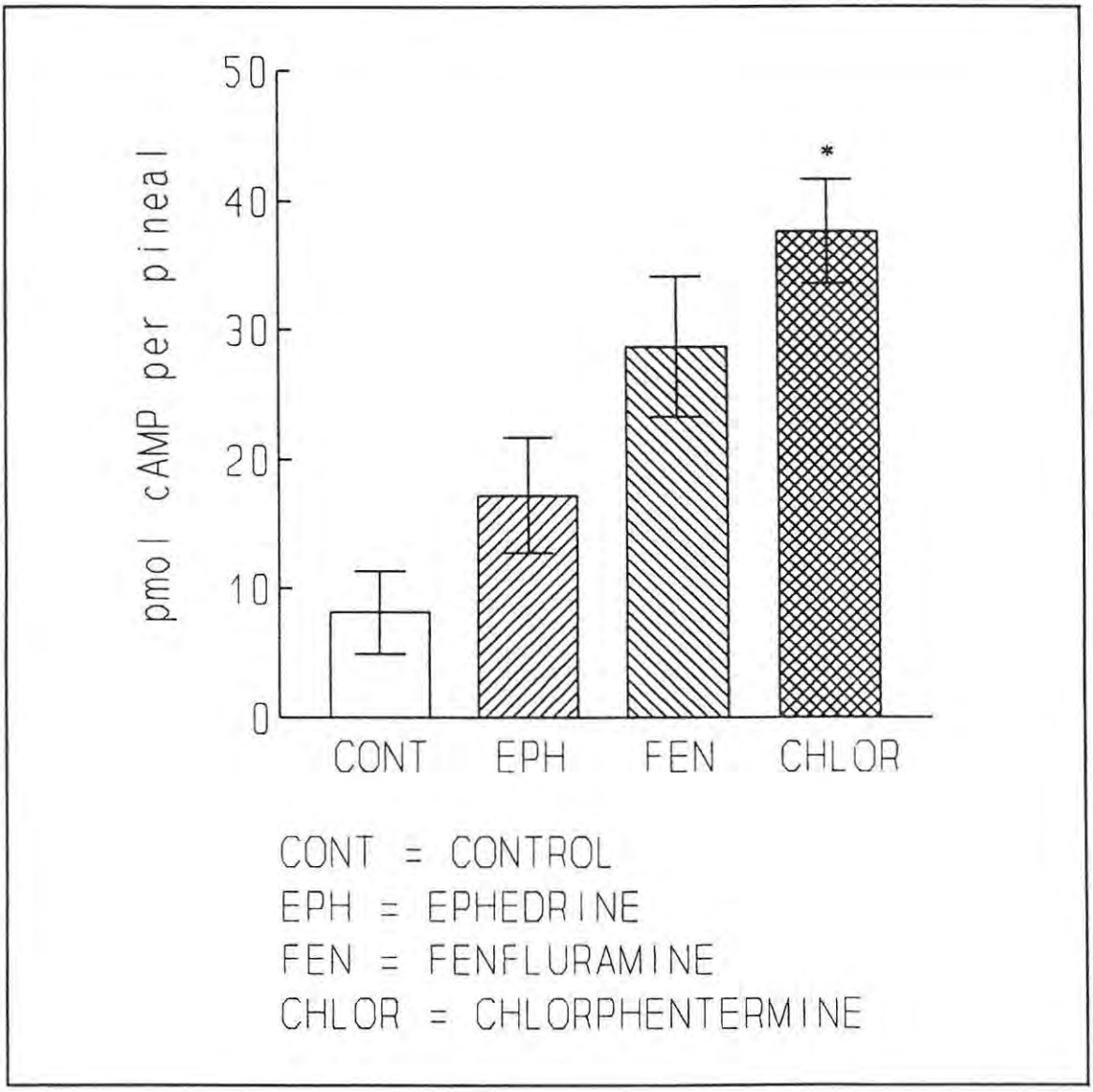


FIGURE 4.1: **The Effect of Appetite Suppressants on Pineal cAMP Levels.**

* $p < 0.05$ vs control

Values represent the mean ± SEM (n = 5).

4.3 EXPERIMENT 2: THE EFFECT OF APPETITE SUPPRESSANTS ON PINEAL *N*-ACETYLTRANSFERASE ACTIVITY

4.3.1 Introduction

The activity of rat pineal *N*-acetyltransferase (NAT) at night reaches values that are 30 - 70 times higher than the daytime values (Klein and Weller, 1970). When rats are exposed to light during the dark period, a rapid decrease in enzyme activity occurs (Klein and Weller, 1972). The pineal gland NAT activity can also be stimulated during the day by treatment with noradrenaline and/or isoproterenol *in vivo* or in organ culture (Klein and Weller, 1972).

Since appetite suppressants were found to increase pineal cAMP levels (Chapter 4 - Experiment 1), this study therefore investigates the effect of these agents on pineal NAT activity.

4.3.2 Materials and Methods

Animals: Male Wistar rats weighing between 200 and 250 g were used. The animals were maintained as previously described (Section 2.1). The animals were sacrificed between 11h00 and 12h00 for *in vitro* studies and between 14h00 and 15h00 for *in vivo* studies.

Chemicals and Reagents: [^{3}H] Acetyl coenzyme A (specific activity 3.3 Ci/mmol) was purchased from Amersham (England); tryptamine hydrochloride and ℓ-isoproterenol from Sigma Chemical Co. (USA) and unlabelled acetyl coenzyme A from Boehringer Mannheim (West Germany). All other chemicals and reagents were obtained from local commercial sources.

NAT Assay Procedure

***In Vivo* Studies:** Animals were injected at 11h00 with isoproterenol (n = 5),

chlorphentermine HCl (n = 5), fenfluramine HCl (n = 5) and ephedrine chloride (n = 5) intraperitoneally at a dose of 5 mg/kg. Control animals (n = 5) were injected with 0.9% NaCl solution. Animals were sacrificed 3 hours after treatment. Pineal glands were removed, sonicated and NAT activity determined as previously described (Section 2.4.2).

***In Vitro* Studies:** Animals were sacrificed at 11h00 and pineal glands rapidly removed. Pineals were individually placed in sterile glass culture tubes containing 500 μl of culture medium in the absence (n = 5), and presence of chlorphentermine HCl (n = 5), fenfluramine HCl (n = 5) and ephedrine chloride (n = 5) at a final concentration of 10^{-4}M. The tubes were incubated at 37°C for 4 hours in an atmosphere of 95% O_2 : 5% CO_2. Following incubation, the pineals were removed, sonicated and NAT activity determined as previously described (Section 2.4.2).

4.3.3 Results

***In Vivo* Studies:** Fenfluramine and chlorphentermine caused a significant increase ($p < 0.001$) in NAT activity. Isoproterenol produced 8 - 9 fold increase in NAT activity as compared to the control ($p < 0.05$). There was no significant increase on NAT activity following ephedrine treatment. These results are shown in Table 4.2 and graphically presented in Figure 4.2.

***In Vitro* Studies:** Isoproterenol significantly stimulated the increase in NAT activity ($p < 0.05$). Chlorphentermine produced about 5-fold increase in NAT activity as compared to the controls ($p < 0.05$). There was a 2-fold increase in NAT activity after fenfluramine treatment. Ephedrine did not significantly increase NAT activity.

4.3.4 Discussion

It has been established that the maintenance of the high level of NAT activity seems to require continuous stimulation of the β-adrenoceptor on the pinealocyte membrane (Deguchi,

1973). Once the receptor has been blocked regardless of the levels of cAMP, NAT activity disappears immediately.

The results of the present study show that isoproterenol stimulates NAT activity both *in vivo* and *in vitro*. These results also confirm the findings of Deguchi (1973) that isoproterenol elevates the cAMP levels and increases NAT activity in the pineal gland at a dose of 0.5 mg/kg. Appetite suppressants differentially stimulate NAT activity. Chlorphentermine is the most effective drug in stimulating NAT activity both *in vivo* and *in vitro*.

These results further confirm the involvement of appetite suppressants with the β-adrenergic system in stimulating the synthesis of pineal melatonin.

TABLE 4.2: The Effect of *In Vivo* Appetite Suppressant Treatment on Pineal NAT Activity.

Treatment	pmol *N*-Acetyltryptamine formed per pineal per hour
Control	0.351 ± 0.041
Ephedrine	0.619 ± 0.065
Fenfluramine	1.337 ± 0.147*
Chlophentermine	1.785 ± 0.182*
Isoproterenol	3.30 ± 1.24**

* $p < 0.001$ vs control

** $p < 0.05$ vs control

Values represent the mean ± SEM.

TABLE 4.3: The Effect of Appetite Suppressants on Pineal NAT Activity *In Vitro*.

Treatment	pmol *N*-Acetyltryptamine formed per pineal per hour
Control	0.203 ± 0.035
Ephedrine	0.368 ± 0.084
Fenfluramine	0.749 ± 0.168*
Chlophentermine	0.937 ± 0.237*
Isoproterenol	2.94 ± 1.02*

* $p < 0.05$ vs control

Values represent the mean ± SEM (n = 5).

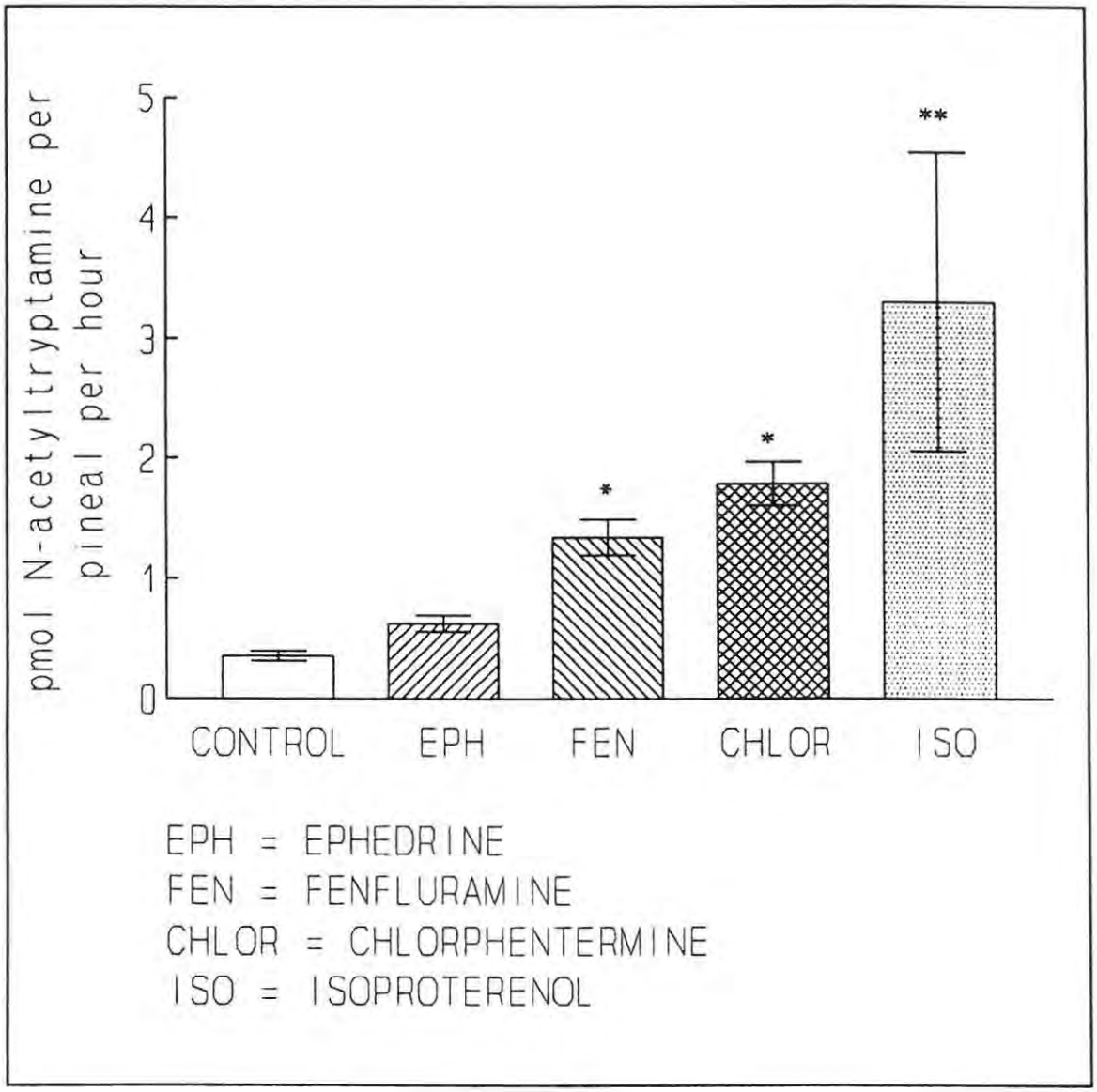


FIGURE 4.2: **The Effect of Appetite Suppressants on Pineal NAT Activity *In Vivo*.**

* $p < 0.001$ vs control

** $p < 0.05$ vs control

Values represent the mean $\pm$ SEM ($n = 5$).

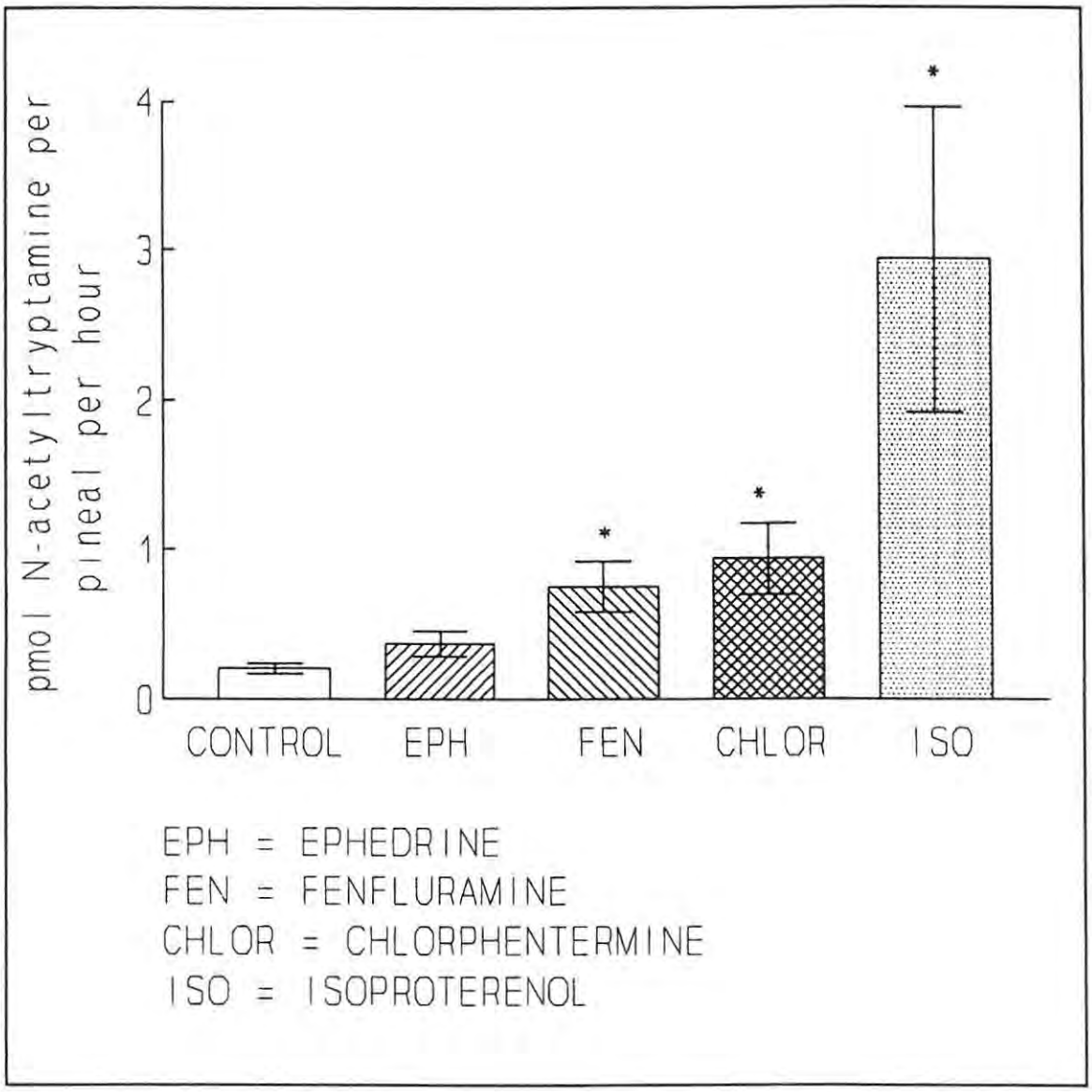


FIGURE 4.3: **The Effect of Appetite Suppressants on Pineal NAT Activity *In Vitro*.**

* $p < 0.05$ vs control

Values represent the mean ± SEM (n = 5).

4.4 EXPERIMENT 3: THE EFFECT OF APPETITE SUPPRESSANTS ON PINEAL HYDROXYINDOLE-O-METHYLTRANSFERASE ACTIVITY

4.4.1 Introduction

Pineal *N*-acetylserotonin is converted to melatonin by the pineal cytosolic enzyme, hydroxyindole-O-methyltransferase (HIOMT). This enzyme is found in high concentration in the pineal gland, which accounts for the relatively high concentration of melatonin in this gland (Axelrod and Weissbach, 1961).

Having demonstrated that appetite suppressants stimulate NAT activity, the effect of appetite suppressants on HIOMT activity was investigated.

4.4.2 Materials and Methods

Animals: Male Wistar rats weighing between 200 and 250 g were used. The animals were maintained as previously described (Section 2.1). The animals were sacrificed between 11h00 and 12h00.

Chemicals and Reagents: S-Adenosyl-L-(methyl-^{14}C)methionine (specific activity 56 mCi/mmol) was purchased from Amersham (England); *N*-acetylserotonin from Sigma Chemical Co. (USA). All other chemicals and reagents were obtained from local commercial sources.

HIOMT Assay Procedure: Animals were sacrificed and the pineals were rapidly removed. The pineals were individually placed in sterile glass culture tubes containing 500 μl of culture medium in the absence (n = 5) and presence of chlorphentermine HCl (n = 5), fenfluramine HCl (n = 5) and ephedrine chloride (n = 5) at a final concentration of 10^{-4}M. The tubes were incubated at 37°C for 4 hours in an atmosphere of 95% O_2 : 5% CO_2. Following

incubation, the glands were removed, sonicated and HIOMT activity determined as previously described (Section 2.5.2).

Data Analysis: Statistical comparisons were determined using Student's t-test. The data was expressed as pmol melatonin formed per pineal per hour. Values represent the mean $\pm$ SEM, n = 5.

4.4.3 Results

Appetite suppressants did not significantly affect pineal HIOMT activity *in vitro* with the exception of chlorphentermine. Chlorphentermine significantly inhibited the activity of HIOMT ($p < 0.05$). These results are shown on Table 4.4 and graphically presented in Figure 4.4.

4.4.4 Discussion

Klein and Berg (1970) examined the effect of ℓ-noradrenaline stimulation of HIOMT and found a small increase in HIOMT activity. Reiter (1991b) found that HIOMT activity diminishes in chronically sympathetically denervated glands, suggesting that noradrenaline may be involved in the maintenance of basal HIOMT levels. Furthermore, the enzyme appears to be very stable and does not exhibit rapid or extreme changes in activity.

Since appetite suppressants possibly act through the β-adrenergic system, it is not unexpected that these agents do not have an effect on HIOMT activity. Surprisingly, chlorphentermine inhibits the activity of the enzyme. Chlorphentermine, however, was shown to stimulate the conversion of radiolabelled serotonin to melatonin in organ culture studies (Chapter 3). These conflicting results need further investigation.

TABLE 4.4: The Effect of Appetite Suppressants on Pineal Hydroxyindole-O-Methyltransferase Activity.

Treatment	pmol melatonin formed per pineal per hour
Control	49.2 ± 11.2
Ephedrine	47.4 ± 9.8
Fenfluramine	48.9 ± 15.5
Chlorphentermine	14.8 ± 5.7*

* $p < 0.05$ vs control

Values represent the mean ± SEM (n = 5).

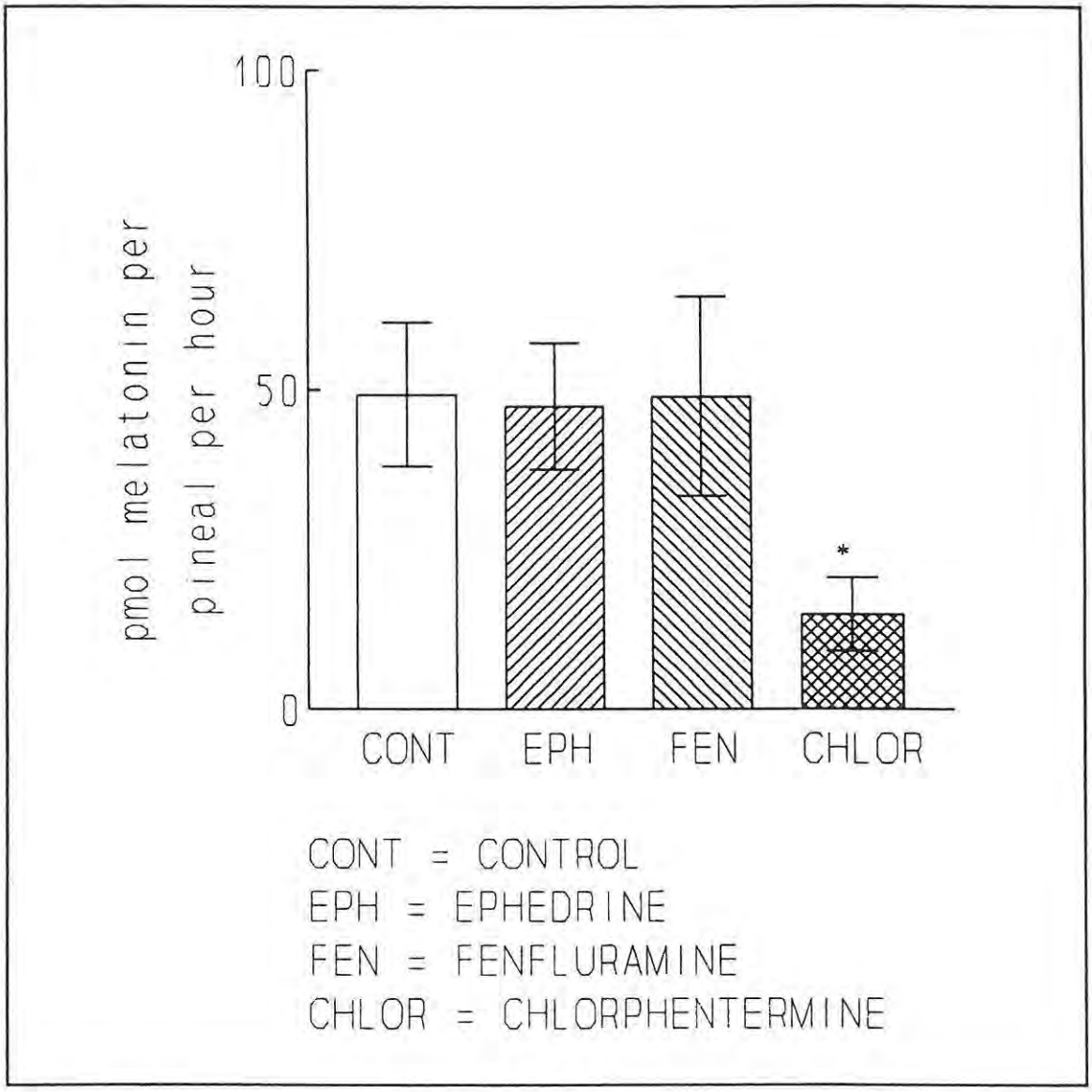


FIGURE 4.4: **The Effect of Appetite Suppressants on Pineal HIOMT Activity.**

* $p < 0.05$ vs control

Values represent the mean $\pm$ SEM ($n = 5$).

4.5 CONCLUSION

Appetite suppressants were shown to increase the synthesis of pineal melatonin (Chapter 3). In this present section, the effect of these agents on pineal cAMP and on the activity of *N*-acetyltransferase (NAT) and hydroxyindole-O-methyltransferase (HIOMT) was investigated.

Appetite suppressants stimulated the increase in pineal cAMP levels at a final concentration of 10^{-4}M. Chlorphentermine produced a 5-fold rise in the levels of cAMP. The effect of fenfluramine and ephedrine was lower than that of chlorphentermine. These findings further suggest the involvement of these agents with adrenergic system, since Klein *et al.* (1983) showed that the production of cAMP in rat pinealocytes is regulated by the stimulation of β-adrenoceptors.

Isoproterenol stimulated NAT activity both *in vivo* and *in vitro*. These findings confirm the work of Deguchi (1973) which showed that isoproterenol elevates the cAMP levels and increases NAT activity in cultured rat pineals. Appetite suppressants stimulated the increase in NAT activity although the magnitude of the increase differed with the agent used. Chlorphentermine produced a 5-fold increase in NAT activity both *in vivo* and *in vitro*. It has been demonstrated that a sudden elevation of cAMP levels is followed by a delayed, slow increase in NAT activity (Deguchi, 1973). Thus, the results of this study appear to support this finding.

Appetite suppressants did not have an effect on pineal HIOMT activity with the exception of chlorphentermine which inhibited the activity. This is expected however, since Reiter (1991b) suggested that noradrenaline may be involved in the maintenance of basal HIOMT levels. Also, the enzyme appears to be stable and does not exhibit extreme changes in activity. The inhibition of HIOMT activity by chlorphentermine will result in reduced pineal melatonin production. On the contrary, chlorphentermine was shown to increase the synthesis of pineal melatonin through its action on adrenergic system (Chapter 3).

The possible explanation for the conflicting results of these experiments may be that chlorphentermine exercises the dual kind of action. Firstly, this agent may have part of its action on the adrenergic system thereby facilitating the interaction of noradrenaline with the β-adrenoceptor. Secondly, chlorphentermine could directly affect HIOMT activity. Further investigation on the effect of this agent on purified enzyme is required.

A conclusion can therefore be drawn on the possible mechanism of action of appetite suppressants in elevating pineal melatonin synthesis. Appetite suppressants act through the β-adrenoceptor system by displacing noradrenaline from sympathetic nerve terminals such that released noradrenaline interacts with β-adrenoceptors. This is followed by the elevation of cAMP levels and an increase in NAT activity.

CHAPTER 5

STUDIES WITH APPETITE SUPPRESSANTS ON TRYPTOPHAN METABOLISM

5.1 INTRODUCTION

Tryptophan enters the systematic circulation from two main sources : as the overflow from the portal circulation following protein ingestion and as the efflux from the bound and free tryptophan pools in the tissues (Fernstrom and Wurtman, 1971). The ingestion of dietary protein elevates plasma tryptophan concentrations. The magnitude of this elevation apparently depends on the time of the day that the protein is consumed, perhaps reflecting variations in the activity of the main enzyme that catabolizes tryptophan, hepatic tryptophan pyrrolase (Young *et al.*, 1969).

Tryptophan pyrrolase is the haem-dependent liver cytosolic enzyme that catalyzes the conversion of L-tryptophan into formylkynurenine. The rat liver enzyme is regulated by four known mechanisms : hormone induction by glucocorticoids, substrate activation and stabilization by tryptophan, cofactor activation by haem and feedback inhibition by NAD(P)H (Badawy, 1979). Activation of this enzyme results in enhanced catabolism of tryptophan, thus reducing the availability of circulating tryptophan for uptake by the brain (Badawy *et al.*, 1987).

Some psychotropic drugs have been shown to influence the concentration of tryptophan in tissues (Leonard and Shallice, 1971). Treatment with amphetamine and some of its derivatives elevates the concentrations of tryptophan in rat and mouse brain (Schubert *et al.*, 1970). Since tryptophan is not synthesized in mammalian tissues, the increase in tryptophan levels in the brain could be due to interference with the uptake or transport of tryptophan, and/or the blockade of metabolic pathways for the amino acid. The finding by Schubert and Sedvall (1972) that the levels of tryptophan in the brain of amphetamine-treated rats were higher than controls during the first hour after injection, could indicate a block of tryptophan

degradation by amphetamine.

The kynurenine pathway is the major route for tryptophan catabolism involving tryptophan pyrrolase in the rat liver. Since pyrrolase is the rate-limiting enzyme in this pathway (Sourkes *et al.*, 1969), the blockade of this metabolic step also results to the increase in brain tryptophan levels. Since tryptophan is the precursor of 5-hydroxytryptamine (5-HT; serotonin), the changes of the amino acid concentration in tissues have been shown to influence 5-HT concentration in the brain (Fernstrom and Wurtman, 1971). Thus, the effects of amphetamine on tryptophan concentrations influence 5-HT synthesis and the turnover in the brain and other tissues, including the pineal gland.

Serotonin and drugs that affect serotonergic transmission have been demonstrated to affect food intake by anorectic and satiety-promoting activities (Leibowitz and Shor-Posner, 1986; Sugrue, 1987). Furthermore, neurons that contain 5-HT in the brain have been implicated in the control of sleep, body temperature, feeding and other rhythmic physiological processes (Beckman and Eisenman, 1970). Fenfluramine, its metabolite norfenfluramine and chloroamphetamine are both 5-HT releasers and uptake inhibitors. The effects of these agents would result to an increased availability of 5-HT at the 5-HT receptors, and consequently would increase the activity of serotonergic system in the brain.

The present study, therefore, investigates the effects of fenfluramine and other appetite suppressants, namely chlorphentermine and ephedrine, on tryptophan pyrrolase activity in the rat liver. Fenfluramine could exert part of its action by inhibiting pyrrolase activity to elevate 5-HT availability in the brain.

5.2 EXPERIMENT 1: THE EFFECT OF APPETITE SUPPRESSANTS ON RAT LIVER TRYPTOPHAN PYRROLASE ACTIVITY

5.2.1 Materials and Methods

Animals: Male Wistar rats weighing between 200 - 250 g were used. The animals were maintained as previously described (Section 2.1). The animals were sacrificed between 15h00 and 16h00.

Chemicals and Reagents: L-Tryptophan and haematin hydrochloride were purchased from Sigma Chemical Co. (USA); trichloracetic acid from Riedel-de Haën (Europe). All other chemicals and reagents were obtained from local commercial sources.

Determination of Tryptophan Activity: Animals were injected at 9h00 with chlorphentermine HCl (n = 5), fenfluramine HCl (n = 5) and ephedrine chloride (n = 5) intraperitoneally at a dose of 5mg/kg. Control animals (n = 5) were injected with 0.9% NaCl solution. The animals were sacrificed 6 hours later. Livers were removed, homogenized and pyrrolase activity determined as previously described (Section 2.6.2).

Data Analysis: Statistical analysis was determined using Student's t-test. The data was expressed as μmol kynurenine formed per hour per mg protein. Values represent the mean $\pm$ SEM, n = 5.

5.2.2 Results

The concentration of the kynurenine formed was determined using the Beer-Lambert's Law (ϵ = 4540 litre/mol/cm). The activity of the enzyme was determined in either the absence (holoenzyme activity) or presence (total enzyme activity) of added haematin. The apoenzyme activity was calculated by a difference.

Appetite suppressants inhibited the activity of tryptophan pyrrolase. Ephedrine did not have an effect on the holoenzyme activity. The results are shown on Tables 5.1, 5.2 and 5.3 and graphically presented in Figures 5.1, 5.2 and 5.3. Fenfluramine inhibited both holoenzyme and total enzyme activities (Figure 5.2). Chlorphentermine, like fenfluramine, significantly inhibited the total enzyme activity ($p < 0.05$) (Figures 5.2 and 5.3).

The time course effect of appetite suppressants on pyrrolase activity is shown on Figures 5.4 - 5.9. The inhibition of total enzyme activity by ephedrine was evident after 1 hour of incubation (Figure 5.4). The inhibition produced by fenfluramine and chlorphentermine was evident from the first 15 minutes. The pyrrolase activity reached the plateau after 1 hour of incubation. This is most obvious with chlorphentermine on the total enzyme activity (Figure 5.9).

TABLE 5.1 The Effect of Ephedrine on Tryptophan Pyrrolase Activity.

Treatment	μmol kynurenine formed per hour per mg protein		
	Holoenzyme	Total Enzyme	Apoenzyme
Control	0.33 ± 0.051	0.54 ± 0.065	0.21 ± 0.014
Ephedrine	0.32 ± 0.054	0.44 ± 0.076	0.12 ± 0.022

Values represent the mean ± SEM (n = 5).

TABLE 5.2: The Effect of Fenfluramine on Tryptophan Pyrrolase Activity.

Treatment	μmol kynurenine formed per hour per mg protein		
	Holoenzyme	Total Enzyme	Apoenzyme
Control	0.51 ± 0.061	0.68 ± 0.051	0.03 ± 0.003
Fenfluramine	0.40 ± 0.051	0.48 ± 0.042*	0.04 ± 0.005

* $p < 0.05$ vs control

Values represent the mean ± SEM (n = 5).

TABLE 5.3: The Effect of Chlorphentermine on Tryptophan Pyrrolase Activity.

Treatment	μmol kynurenine formed per hour per mg protein		
	Holoenzyme	**Total Enzyme**	**Apoenzyme**
Control	0.37 ± 0.065	0.56 ± 0.045	0.27 ± 0.012
Chlorphentermine	0.28 ± 0.051	0.41 ± 0.035*	0.13 ± 0.0061

* $p < 0.05$ vs control

Values represent the mean ± SEM (n = 5).

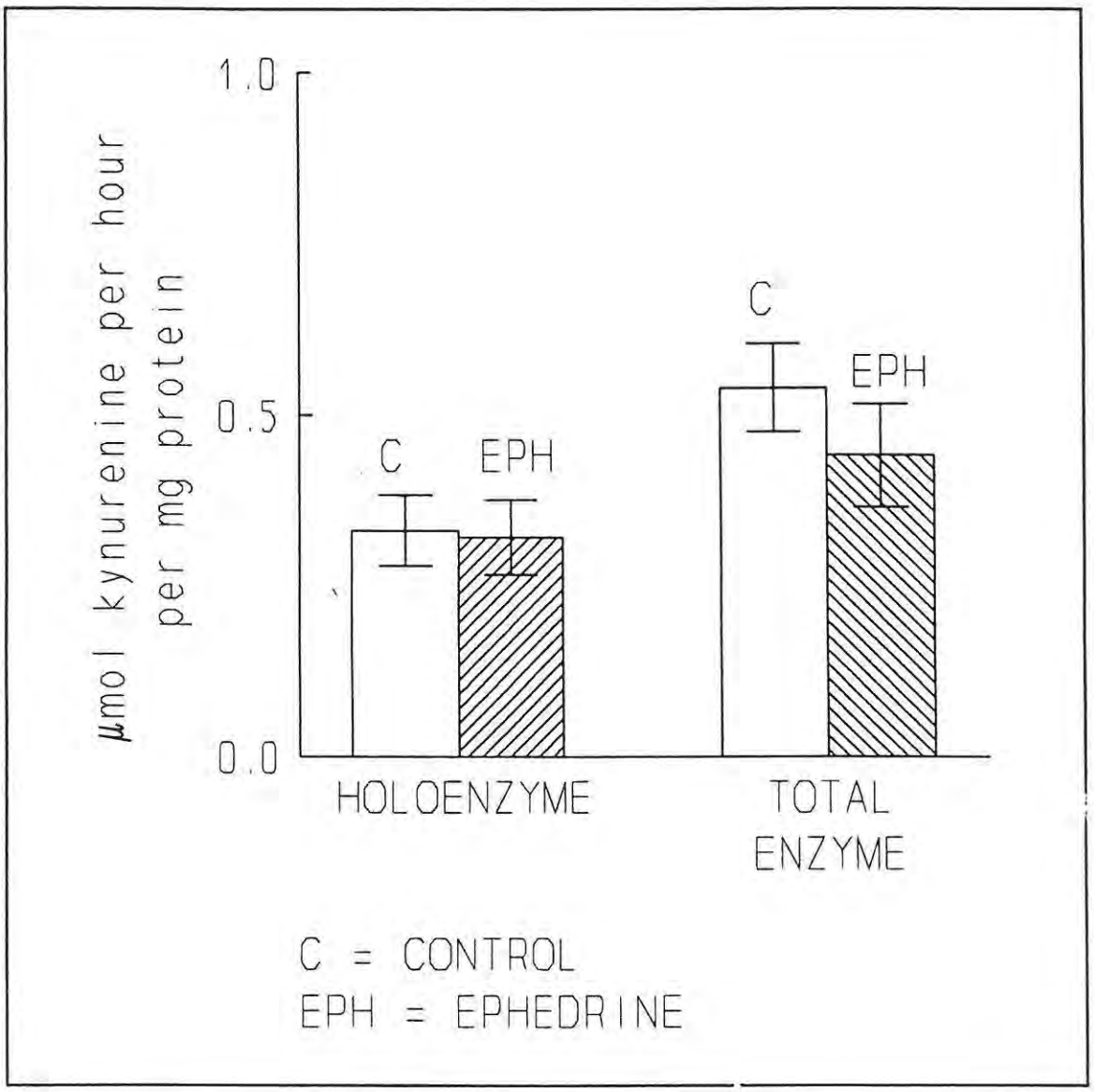


FIGURE 5.1: **The Effect of Ephedrine on Tryptophan Pyrrolase Activity.**

Values represent the mean ± SEM (n = 5).

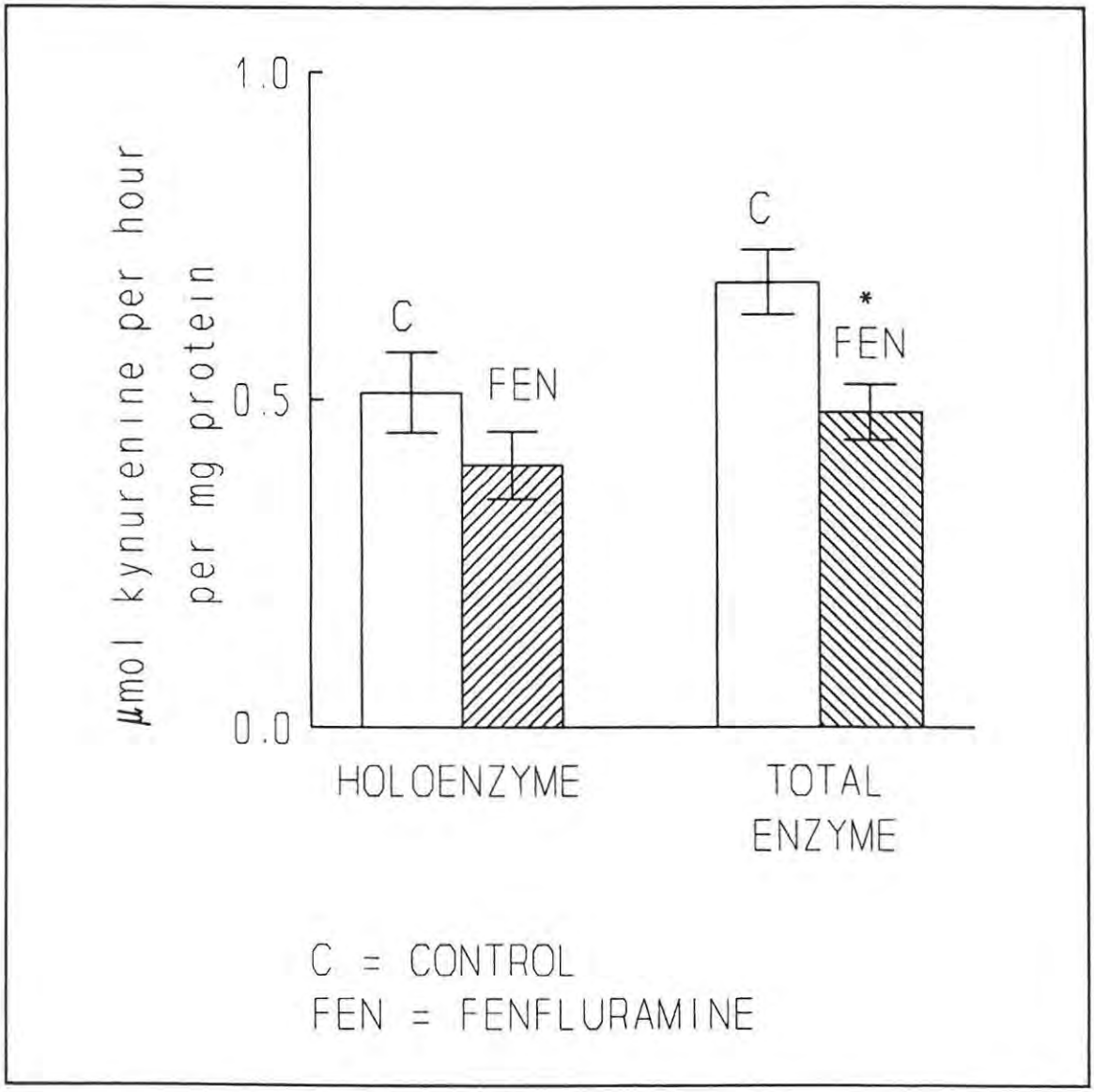


FIGURE 5.2: **The Effect of Fenfluramine on Tryptophan Pyrrolase Activity.**

* $p < 0.05$ vs control

Values represent the mean ± SEM (n = 5).

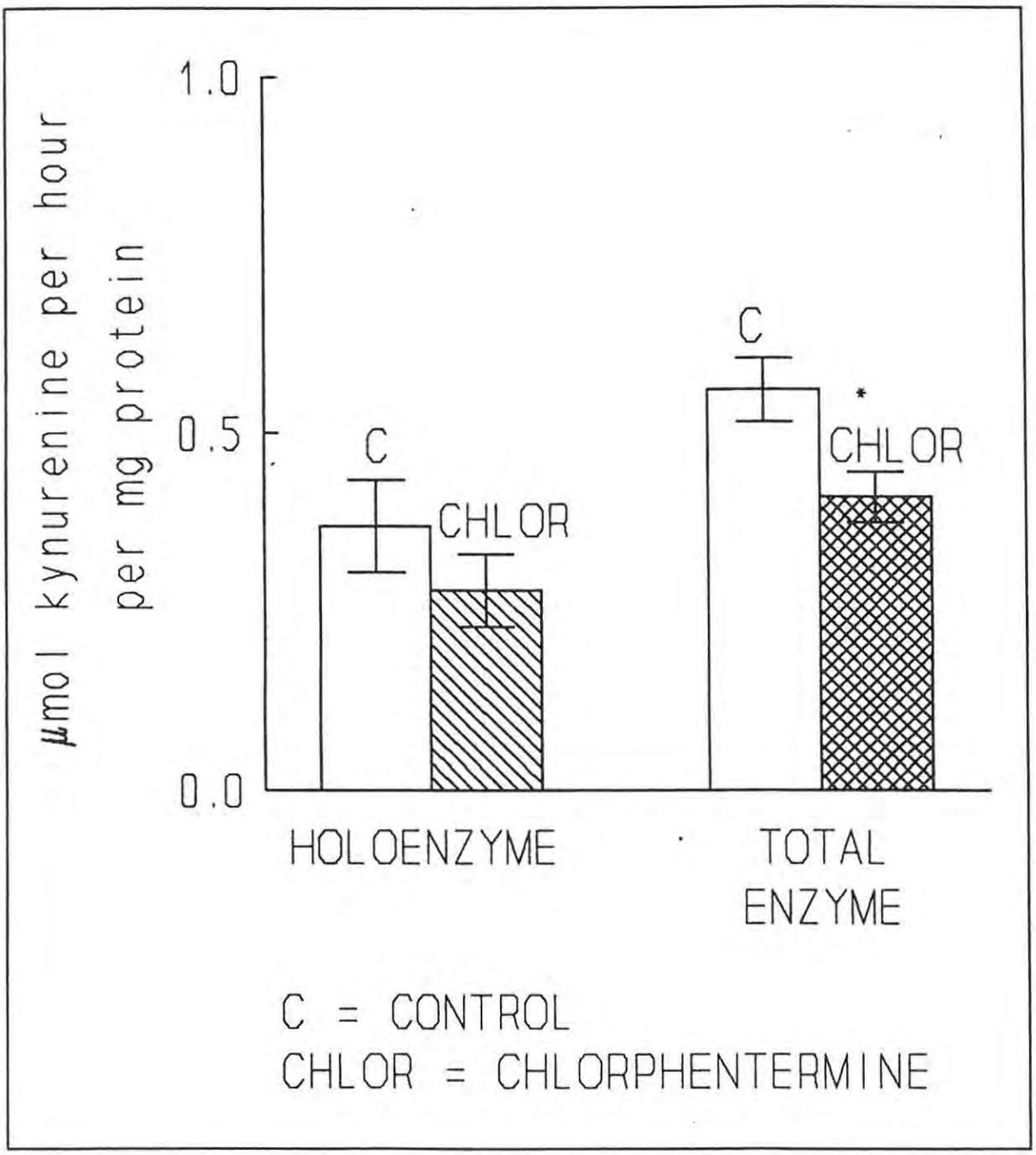


FIGURE 5.3: **The Effect of Chlorphentermine on Tryptophan Activity.**

* $p < 0.05$ vs control

Values represent the mean ± SEM (n = 5).

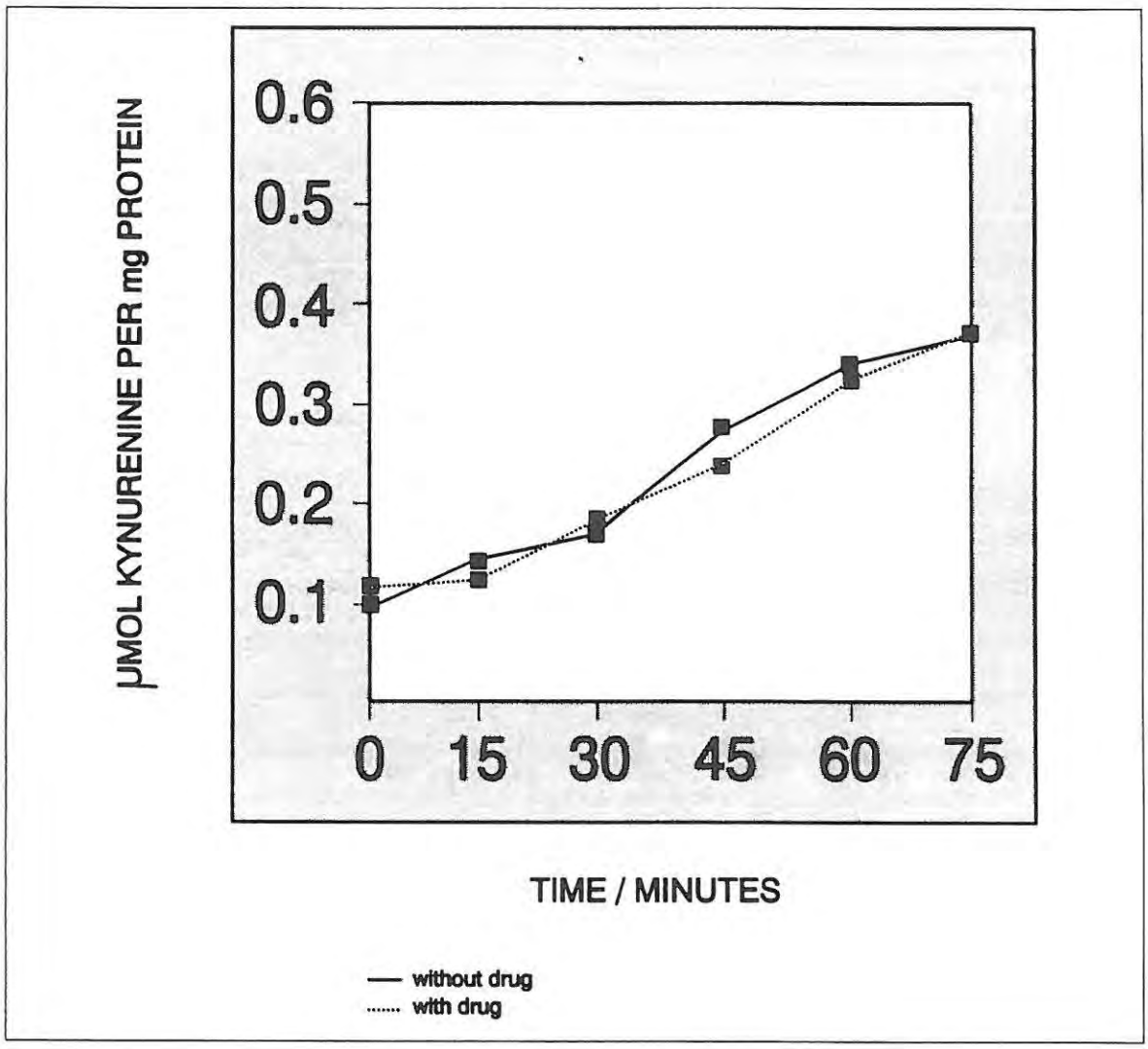


FIGURE 5.4: Time Course Effect of Ephedrine on the Holoenzyme Activity.

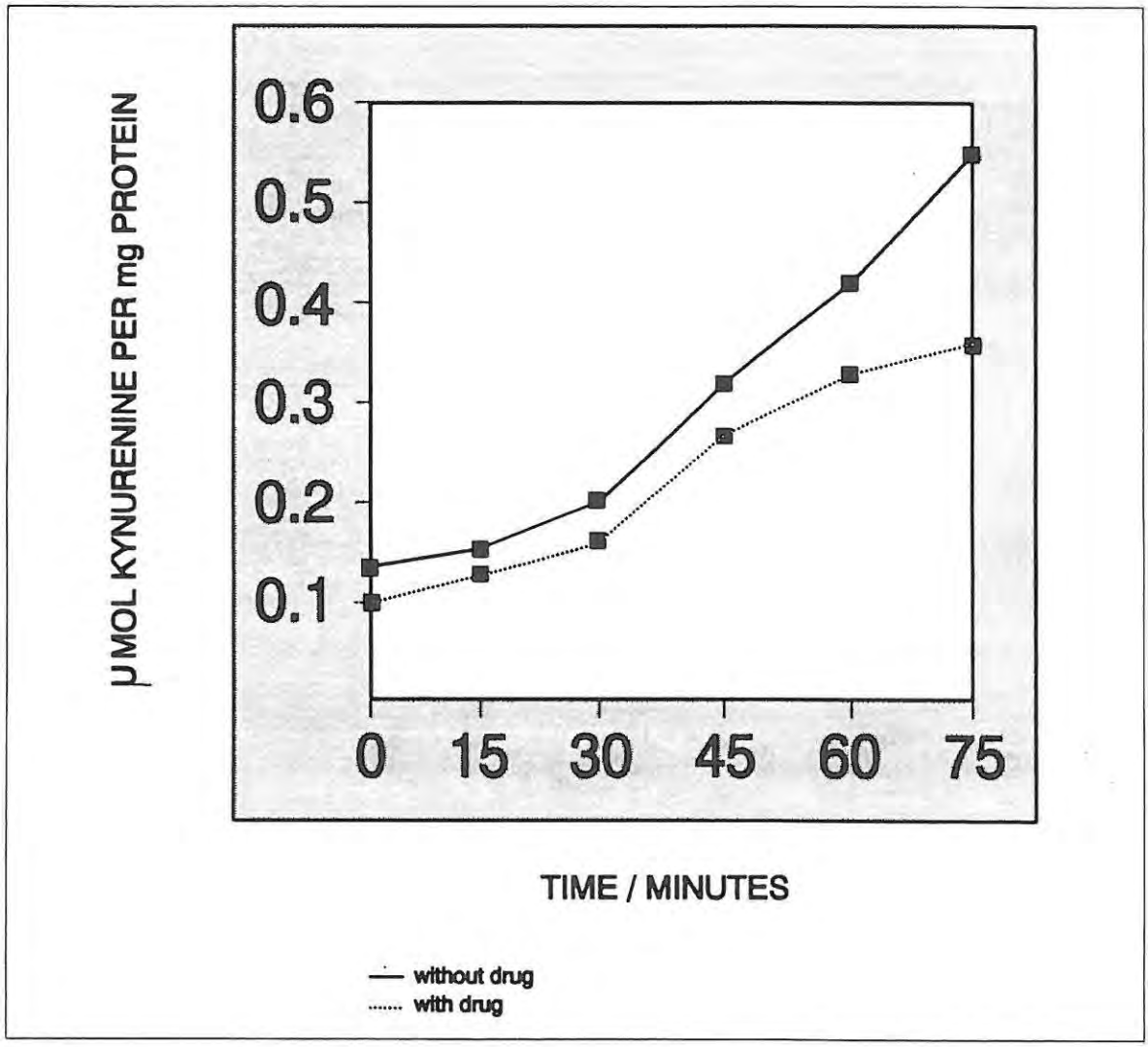


FIGURE 5.5: Time Course Effect of Ephedrine on the Total Enzyme Activity.

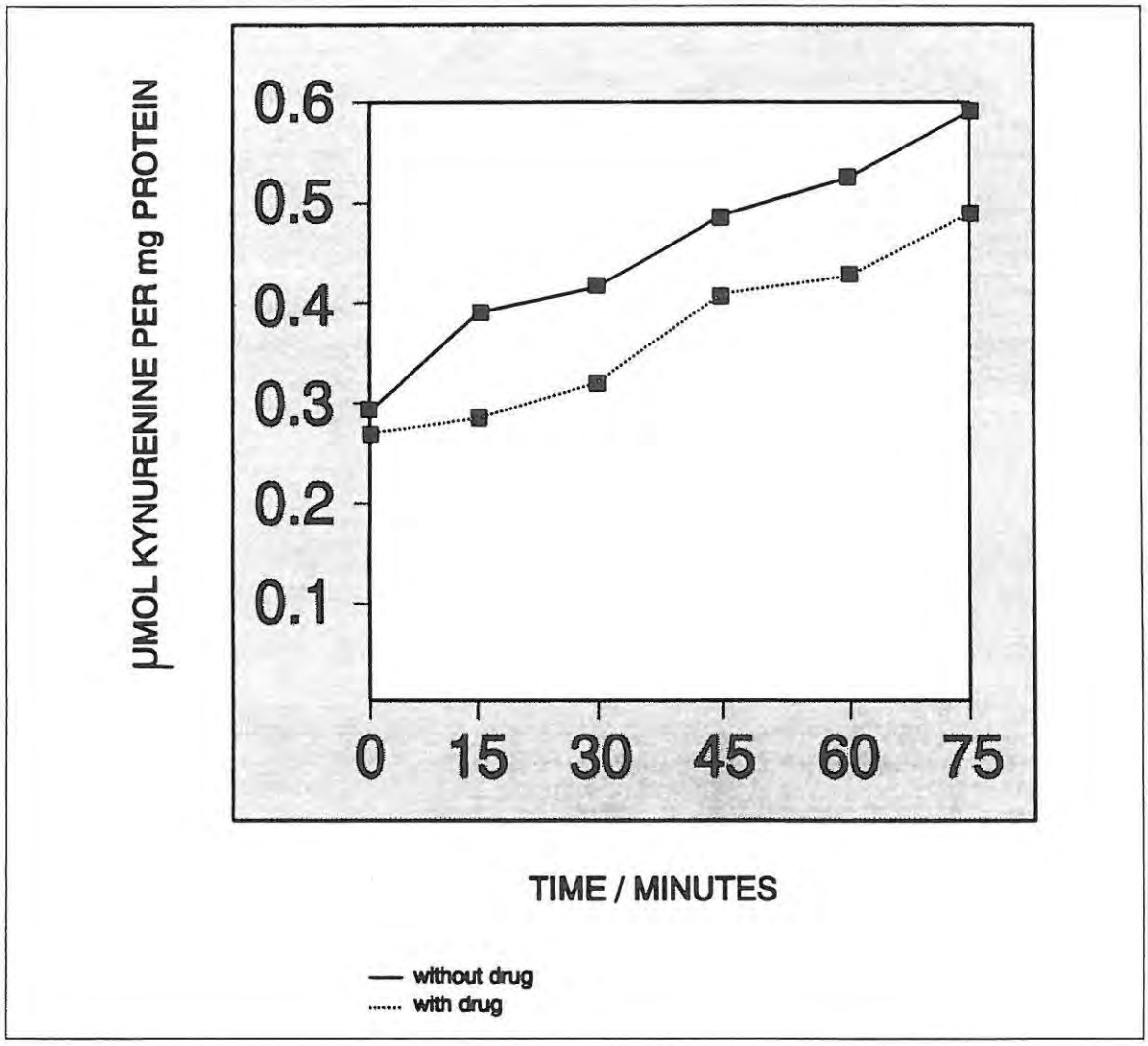


FIGURE 5.6: Time Course Effect of Fenfluramine on the Holoenzyme Activity.

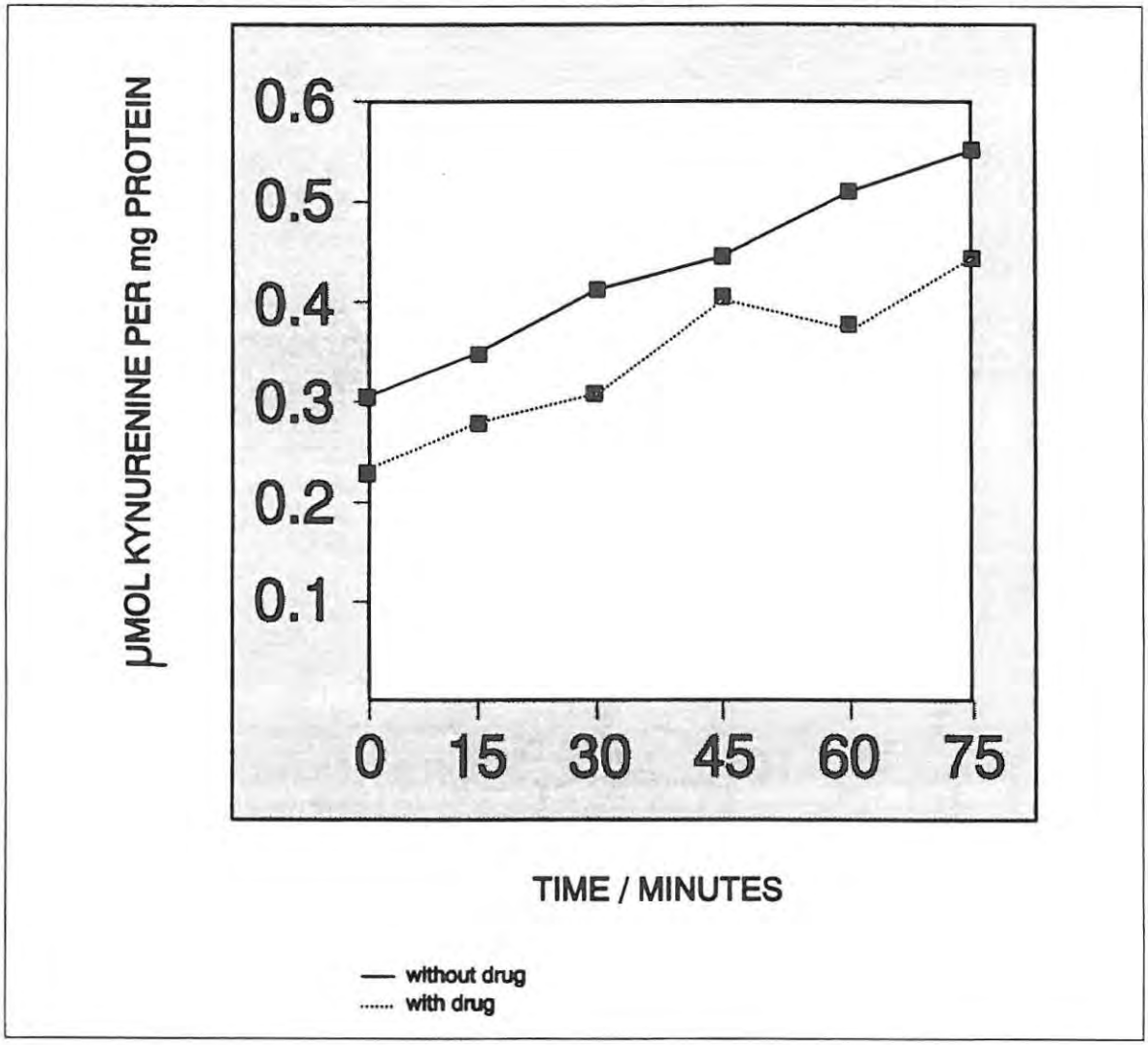


FIGURE 5.7: Time Course Effect of Fenfluramine on the Total Enzyme Activity.

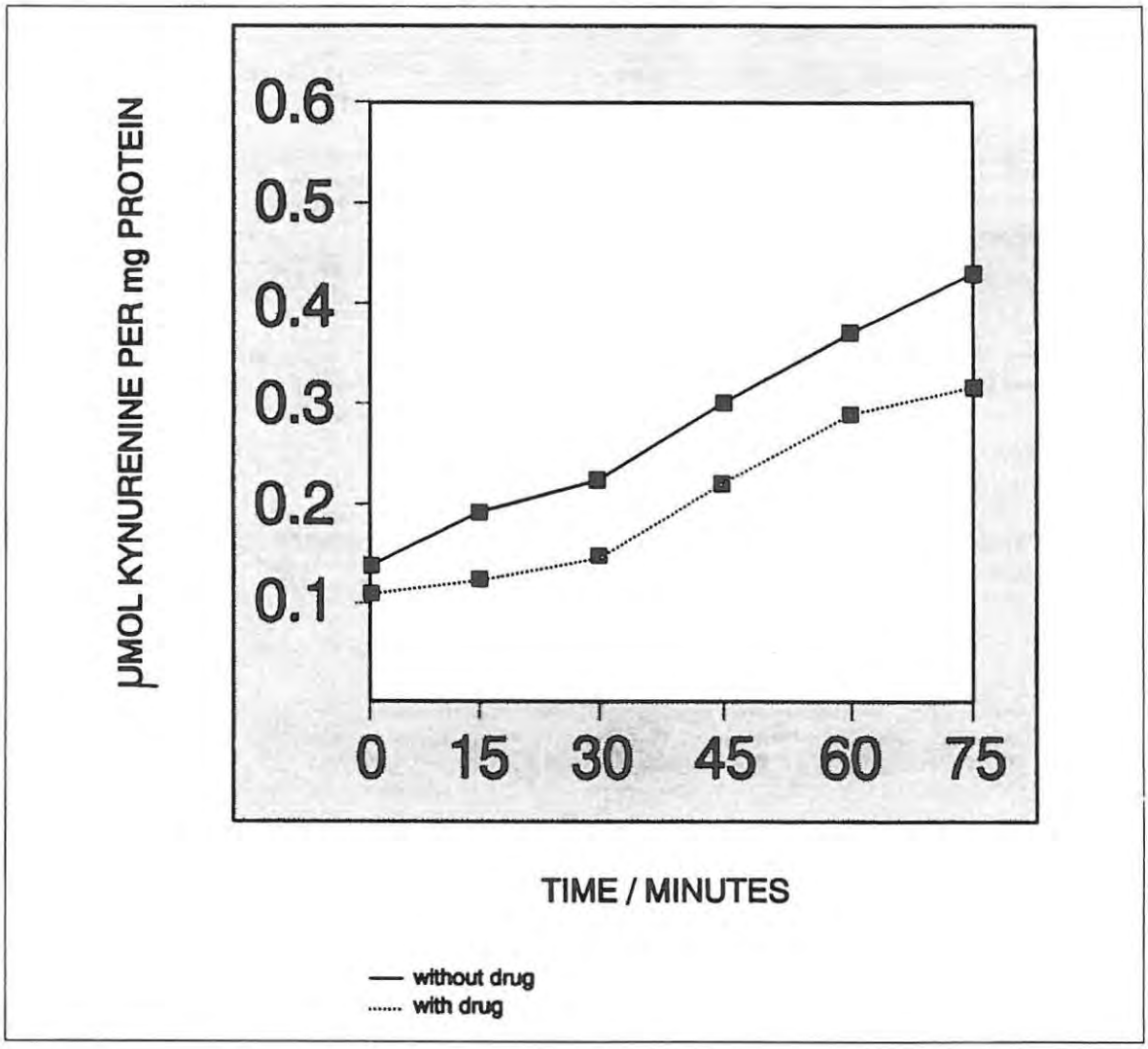


FIGURE 5.8: Time Course Effect of Chlorphentermine on the Holoenzyme Activity.

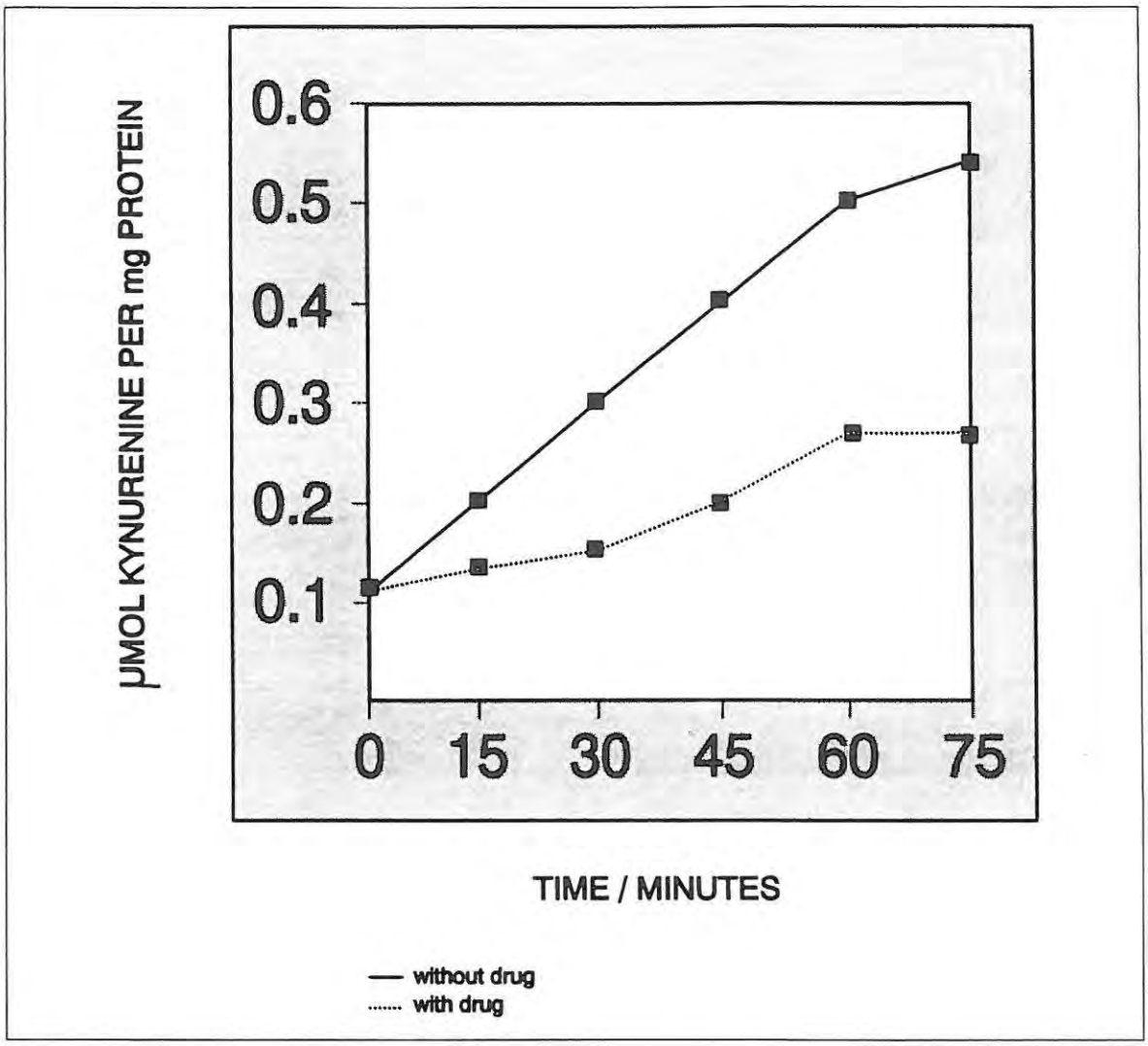


FIGURE 5.9: Time Course Effect of Chlorphentermine on the Total Enzyme Activity.

5.2.3 Discussion

The addition of haematin increased tryptophan pyrrolase activity (total enzyme activity) as compared to the holoenzyme activity (without haematin).

Appetite suppressants inhibit both holoenzyme and total enzyme activities with the exception of ephedrine. Ephedrine did not affect haloenzyme activity. Further studies are required to determine the site of inhibition on the enzyme. Possibly, these agents might interfere with the conjugation of haem with the apoenzyme.

The inhibition of liver tryptophan pyrrolase activity will increase the plasma tryptophan concentrations. It has been demonstrated that physiological changes in plasma tryptophan concentrations cause parallel changes in brain serotonin synthesis (Fernstrom and Wurtman, 1971). Therefore, elevated plasma tryptophan concentrations will result in a rise in brain serotonin synthesis. The results of the present study suggests that appetite suppressants might enhance brain serotonin synthesis by increasing tryptophan availability to the brain. These effects are secondary to the inhibition of liver pyrrolase activity and an associated decrease in hepatic tryptophan catabolism. Further studies are required to investigate the effect of appetite suppressant administration on brain tryptophan and serotonin concentrations. Schubert and Sedvall (1972) demonstrated that intraperitoneal administration of amphetamine, another appetite suppressant, at a dose of 1 mg/kg significantly increases the brain tryptophan concentrations in rats.

5.3 CONCLUSION

The results of the present study show that appetite suppressants inhibit rat liver tryptophan pyrrolase activity. Appetite suppressants are the chemical derivatives of β-phenyl-ethylamines. Since this class of compounds induce a marked central stimulant effect, they are sometimes referred to as "drugs of dependence". It has been shown that the drugs of dependence of different chemical classes share, other than stimulation or depression of the

CNS and the production of physical dependence, the single property of influencing brain serotonin synthesis by acting on the liver tryptophan pyrrolase (Badawy *et al.*, 1981). Chronic administration of drugs of dependence inhibits liver pyrrolase activity by a mechanism not involving a defective apoenzyme synthesis, but associated with an increased liver NADPH concentrations (Badawy and Evans, 1975).

It is, therefore, assumed that appetite suppressants, being drugs of dependence, might also act by increasing liver NADPH concentrations thereby inhibiting pyrrolase activity. That NADH is the cause of the inhibition is suggested by the reversal of inhibition by the addition *in vitro* of $NAD(P)^+$ or by administration *in vivo* of the regenerator of oxidized dinucleotides (Badawy and Evans, 1975).

On the other hand, appetite suppressants might act on the pineal gland by releasing melatonin. Appetite suppressants were demonstrated to induce the synthesis of pineal melatonin (Chapter 3). It has been shown that the intraperitoneal injection of melatonin is followed by a rapid rise in the concentration of brain serotonin (Anton-Tay *et al.*, 1968). Melatonin might elevate brain serotonin concentration by inhibiting the release of the amine, enhancing its reuptake or inhibiting its intraneural or extraneural metabolism. The other possible mechanism might be that melatonin inhibits tryptophan pyrrolase activity such that brain tryptophan concentrations rise with the consequent increase in brain serotonin synthesis. The latter mechanism is more likely to take place, since Walsh *et al.* (1991) have shown that melatonin significantly inhibits tryptophan pyrrolase activity at concentrations above 50 μM.

CHAPTER 6

SUMMARY

The important aspects of appetite suppressants and the pineal gland were reviewed (**Chapter 1**). Clinical data, including the mode of action of appetite suppressants, was presented. The rhythms involved in neurotransmitter modulation of feeding patterns as well as relationships between pineal indoles and feeding were discussed.

From the review of appetite suppressants, various studies suggest that appetite suppressants exert an inhibitory effect on food intake by interaction with monoaminergic systems in the brain. Although the separate role of noradrenaline and dopamine in the anorectic action remains to be elucidated, the evidence suggests that these catecholamines are involved in the effect of amphetamine and its analogues. Fenfluramine appears to exert its anorectic effect through brain serotonin.

A general review of the pineal gland including anatomy, pharmacology and function was presented. Although initial investigations suggested a possible reproductive regulatory function for the pineal gland, its effects appear not to be restricted to the reproductive system. The pineal gland has been found to interact with virtually every endocrine gland in the organism.

The ability of animals to alter patterns of protein and carbohydrate intake in relation to need becomes apparent during the active period of the diurnal cycle. This nocturnal feeding activity in the rat is characterized by peaks of food intake at the beginning and towards the end of the night. The rat feeding behaviour is in parallel with the nocturnal rise in the levels of pineal neurohormone, melatonin. Both the pineal and feeding rhythms are generated in the hypothalamic suprachiasmatic nuclei. The primary objective of this study was therefore to investigate the possible effect of appetite suppressants on pineal function.

The effect of appetite suppressants on the metabolism of pineal indoleamines in organ culture was investigated (**Chapter 3**). Appetite suppressants were found to induce the synthesis [^{14}C]

N-acetylated pineal indoles from [^{14}C] serotonin. Blocking the β-adrenoceptors with propranolol prevented the increase in pineal [^{14}C] *N*-acetylated indoles induced by appetite suppressants. These results suggest the possible involvement of appetite suppressants with the β-adrenergic system. Furthermore, prior-treatment of animals with reserpine lowered the appetite suppressant-induced increase in pineal *N*-acetylated indoles. Reserpine studies suggest that appetite suppressants may act by releasing noradrenaline from the postganglionic nerve terminals. The released noradrenaline then interacts with the β-adrenoceptor to augment melatonin synthesis.

The β-adrenergic stimulation induces changes on pineal biochemistry. Therefore, an investigation on the effect of appetite suppressants on pineal cAMP and on the activity of the enzymes in the melatonin pathway, i.e. *N*-acetyltransferase (NAT) and hydroxyindole-O-methyltransferase (HIOMT), was undertaken (**Chapter 4**). Appetite suppressants induced a marked rise in pineal cAMP levels. This rise in cAMP levels was comparable to that induced by activation of the β-adrenoceptor. Appetite suppressants stimulated the increase in NAT activity though not significantly. Pineal β-adrenergic stimulation is followed by the elevation of cAMP levels and an increase in NAT activity. These results further support the involvement of appetite suppressants with the pineal β-adrenergic system. These agents were ineffective in changing HIOMT activity, with the exception of chlorphentermine. The finding that chlorphentermine inhibits HIOMT activity is confusing because this agent was initially shown to induce an increase of [^{14}C] melatonin in organ culture studies. The possible explanation for these conflicting results may be that chlorphentermine exercises the dual kind of action. Firstly, it may have part of its action on the adrenergic system in elevating melatonin synthesis. Secondly, chlorphentermine could directly affect HIOMT activity. Further studies on the effect of this agent on the purified form of enzyme are required.

Fenfluramine, as an appetite suppressant, acts on the brain serotonin system. The activity of liver tryptophan pyrrolase is an important peripheral determinant of tryptophan availability to the brain, and subsequently of brain serotonin levels. Therefore, the effect of appetite

suppressants on the activity of liver tryptophan pyrrolase was investigated (**Chapter 5**). The results show that appetite suppressants inhibit both holoenzyme and total enzyme activities. There are two possible mechanisms by which appetite suppressants inhibit pyrrolase activity. Firstly, these agents, being drugs of dependence, might increase liver NADPH concentrations thereby inhibiting pyrrolase activity. Secondly, appetite suppressants might act on the pineal gland by releasing melatonin. Melatonin inhibits the pyrrolase activity in a dose-dependent manner. Whichever mechanism is involved, the inhibition of tryptophan pyrrolase results in an increased plasma tryptophan concentrations. Physiological changes in plasma tryptophan concentrations cause parallel changes in brain serotonin synthesis. Elevated plasma tryptophan levels will result in a rise in brain serotonin synthesis. Therefore, appetite suppressants used in this study, with particular emphasis to fenfluramine, might also act by inhibiting pyrrolase activity thereby increasing brain serotonin concentrations which induce anorectic activity.

The present study suggests a possible relationship between the pineal gland and the appetite centres in the hypothalamus. This study shows that appetite suppressants can alter pineal function by increasing the synthesis of pineal neurohormone, melatonin. Melatonin might have a direct effect on appetite centres since food restriction is associated with an increased melatonin binding in the hypothalamus. Further investigations are required on the relationship of melatonin binding sites with the appetite centres in the hypothalamus. On the other hand, melatonin might exercise its inhibitory effect on liver tryptophan pyrrolase activity. This effect will elevate brain serotonin synthesis and increase the activity of brain serotonergic system which results in anorexia.

If further studies can support and extend the relationship between appetite centres and the pineal, melatonin can open new possibilities for the control of food intake and consequently, of pathological obesity.

REFERENCES

Adams, V.J. (1975). In: *Recent Advances in Obesity Research*, **1**, pp. 393-395. Newman, London.

Alberti, K.G.M.M. and Nattrass, M. (1977). *Lancet*, **II**: 25-29.

Altar, A., Goldwag, D., Terry, R.L. and Lytle, L.D. (1981). *Proc. West Pharmacol. Soc.*, **24**: 101-104.

Angel, I. (1990). *Clin. Neuropharmacol.*, **13**: 361-391.

Angel, I., Luu, M.D., Hauger, R. and Paul, S.M. (1985). *Soc. Neurosci. Abstr.*, **11**: 670.

Angel, I., Luu, M.D. and Paul, S.M. (1987). *J. Neurochem.*, **48**: 491-497.

Angren, H., Koulu, M., Saaredra, J.M., *et al.* (1986). *Brain Res.*, **397**: 353-358.

Anton-Tay, F., Chou, C., Anton, S. and Wurtman, R.J. (1968). *Science*, **112**: 277-278.

Arendt, J. (1989). *Br. J. Psychiatry*, **155**: 585-590.

Arendt, J., Aldhous, M. and Marks, V. (1986). *Ergonomics*, **30**: 1379-1393.

Arendt, J. and Broadway, J. (1987). *Chronobiol. Int.*, **4**: 271-282.

Arendt, J., Aldhous, M. and Wright, J. (1988). *Lancet*, **2**: 772-773.

Armstrong, S. (1980). *Neurosci. Biobehav. Rev.*, **4**: 27-53.

Armstrong, S., Ng, K.T. and Coleman, G.J. (1982). In: *The Pineal Gland*, Vol. **III**: Extra-Reproductive Effects (Reiter, R.J., ed.), pp. 81-106. CRC Press, Bocca Raton.

Ashwell, M.A. (1973). *Practitioner*, **211**: 653-658.

Axelrod, J. and Weissbach, H. (1960). *Science*, **131**: 1312.

Axelrod, J. and Weissbach, H. (1961). *J. Biol. Chem.*, **236**: 211-213.

Axelrod, J., Wurtman, R.J. and Synder, S.H. (1965). *J. Biol. Chem.*, **240**: 1949.

Axelrod, J., Shein, H.M. and Wurtman, R.J. (1969). *Proc. Natl. Acad. Sci., USA* **62**: 544-549.

Badawy, A.A.B. (1979). *Biochem. Soc. Trans.*, **7**: 575-583.

Badawy, A.A.B. and Evans, M. (1975). *Biochem. J.*, **150**: 511-520.

Badawy, A.A.B. and Evans, M. (1976). *Biochem. J.*, **158**: 79-88.

Badawy, A.A.B., Punjani, N.F. and Evans, M. (1981). *Biochem. J.*, **196**: 161-170.

Badawy, A.A.B., Morgan, C.J. and Davis, N.R. (1987). *Biochem. J.*, **248**: 293-295.

Becket, A.H. and Brookes, L.G. (1971). *J. Pharmacol.*, **23**: 288-294.

Beckman, A.L. and Eisenman, J.S. (1970). *Science*, **170**: 334.

Bendotti, C., Villa, M. and Samanin, R. (1986). *Life Sci.*, **28**: 259-266.

Bendotti, C. and Samanin, R. (1987). *Life Sci.*, **41**: 635-642.

Berga, S.L., Mortola, J.F. and Yen, S.S.C. (1988). *Experientia*, **45**: 939.

Bhakthavatsalam, P. and Leibowitz, S.F. (1985). *Am. J. Physiol.*, **250**: R83-R88.

Binkley, S., Klein, D.C. and Weller, J.L. (1976). *J. Neurochem.*, **26**: 51-55.

Bittman, E.L. (1984). In: *The Pineal Gland* (Reiter, R.J., ed.), pp. 155-192. Raven Press, New York.

Blask, D.E., Nodelman, J.L., Leadmen, C.A. and Richardson, B.A. (1980). *Biol. Reprod.*, **22**: 507-512.

Blundell, J.E. (1984). *Neuropharmacology*, **23**: 1537-1551.

Booth, D.H. (1980). In: *Obesity* (Stunkard, A.J., ed.), pp. 101-143. W.B. Saunders Co., Philadelphia.

Booth, D.A. and Toates, F.M. (1974). Mark 1. *Bull. Psychon. Soc.*, **3**: 442-444.

Borbely, A.A. and Hutson, J.P. (1974). *Physiol. Behav.*, **13**: 795-802.

Brightwell, D.R. and Naylor, C.S. (1979). *Int. J. Obesity*, **3**: 141-145.

Brown, B.L., Albans, J.D.M., Ekins, R.P., *et al.* (1971). *Biochem. J.*, **121**: 561-562.

Butterfield, W.J.H. and Whichelow, M.J. (1962). *Diabetes*, **II**: 281-286.

Cairns, A., Shalet, S., Marshall, A.J. and Hartog, M. (1977). *Diabete Metab.*, **3**: 183-188.

Campbell, D.B. (1971). *Br. J. Pharmacol.*, **43**: 465-466.

Campbell, C.J., Bhalla, I.P., Steel, J.M. and Duncan, L.J.P. (1977a). *Practitioner*, **218**: 851-855.

Campbell, G.A., Rurcz, M., Marshall, S. and Meites, J. (1977b). *Endocrinology*, **100**: 580-587.

Cardinali, D.P. (1981). *Endocrin. Reviews*, **2**: 327-346.

Cardinali, D.P. and Wurtman, R.J. (1972). *Endocrinology*, **91**: 247-252.

Cardinali, D.P. and Ritta, M.N. (1983). *Neuroendocrinology*, **36**: 152-160.

Cardinali, D.P., Vacas, M.I., Rosenstein, R., Lavenstein, P.R., Gonzalez Solreyra, C., Romeo, H.E. and Keller Sarmiento, M.I. (1986). In: *Advances in Pineal Research*, Vol. **1** (Reiter, R.J. and Karasek, M., eds.), pp. 127-138. John Libbey, London.

Cardinali, D.P., Vacas, M.I., Rosenstein, R.E., Etchegoyen, G.S., Keller Sarmiento, M.I., Gonzalez Solreyra, C. and Pereyra, E.N. (1987). In: *Advances in Pineal Research*, Vol. **2** (Reiter, R.J. and Fraschini, F., eds.), pp. 51-66. John Libbey, London.

Carter, D.S. and Goldman, B.D. (1983). *Endocrinology*, **113**: 1261-1267.

Chan, A. and Ebadi, M. (1981). *Endocr. Res. Commun.*, **8**: 25-44.

Chik, C. (1986). *Effects of Food Restriction on Serum and Pineal Indoles*. PhD Thesis.

Clineschmidt, B.V., McGuffin, J.C. and Werner, A.B. (1974). *Eur. J. Pharmacol.*, **27**: 313-323.

Comai, K. and Sullivan, A.C. (1980). *Int. J. Obesity*, **4**: 33-42.

Cook, W.H., Lipkins, D. and Markham, R. (1957). *J. Am. Chem. Soc.*, **81**: 6198-6203.

Coppen, A., Rama Rao, V.A., Swade, C. and Wood, K. (1979). *Psychopharmacology*, **63**: 199-208.

Cox, R.H. Jr. and Maickel, R.P. (1972). *J. Pharmacol. Exp. Ther.*, **181** 1-9.

Craft, C.M., Morgan, W.W. and Reiter, R.J. (1984). *Neuroendocrinology*, **38**: 193-198.

Curzon, G. (1979). *J. Neural Transm. Suppl.*, **15**: 81-92.

Czyzyk, A. (1969). *Acta Diab. Lat.*, **62** (Suppl. 1): 636-643.

Danforth, E., Burger, A.G., Goldman, R.F. and Sims, E.A.H. (1978). In: *Recent Advances in Obesity Research* II, pp. 229. Newman, London.

Datta, P.C. and King, M.G. (1980). *Neuroscience and Biobehavioural Rev.*, **4**: 451-458.

Daya, S. (1982). MSc Thesis : Rhodes University, South Africa.

Daya, S. and Potgieter, B. (1982). *S. Afr. J. Sci.*, **78**: 174.

Daya, S. and Fata, M. (1986). *IRCS Med. Sci.*, **14**: 1153-1154.

Daya, S., Nonaka, K.O., Buzzell, G.R. and Reiter, R.J. (1989). *J. Neurosci. Res.*, **23**: 304-309.

Deguchi, T. (1973). *Mol. Pharmac.*, **9**: 184-190.

Deguchi, T. and Axelrod, J. (1972a). *Anal. Biochem.*, **50**: 174-179.

Deguchi, T. and Axelrod, J. (1972b). *Proc. Natl. Acad. Sci.*, USA, **69**: 2208-11.

Dickinson, K.E.J., Leeb-Lundberg, L.M.F., Strasser, R.H., Caron, M.G. and Lefkowitz, R.J. (1986). *J. Neurochem.*, **46**: 1153-1160.

Douglas, J.G. and Munro, J.F. (1982). *Pharmac. Ther.*, **18**: 351-373.

Dubey, A.K., Cameron, J.L., Steiner, R.A. and Plant, T.M. (1986). *Endocrinology*, **118**: 518-525.

Dubocovich, M.L. (1988). *FASEB J.*, **2**: 2765.

Ebadi, M. (1984). In: *The Pineal Gland* (Reiter, R.J. ed.), pp. 1-38. Raven Press, New York.

Ebadi, M. and Chan, A. (1980). *Brain Res. Bull.*, **5** (Suppl. 2): 179-187.

Ebadi, M., Hama, Y., Govitrapong, P. and Awad, A. (1986). In: *Gabaergic Mechanisms in the Mammalian Periphery* (Bowers, N.G. and Eroo, S.L. eds.). Raven Press, New York.

English, J. and Arendt, A. (1988). *J. Reprod. Fertil. Abstr. Series* No. 1, Abstr. 6.

Engstrom, R.G., Kelly, L.A. and Goberty, J.H. (1975). *Arch. Int. Pharmacodyn Ther.*, **214**: 308-321.

Exton, J.H. (1985). *J. Clin. Invest.*, **75**: 1753-1757.

Feigelson, P. and Greengard, O. (1961). *J. Biol. Chem.*, **236**: 153-157.

Fernstrom, J.D. and Wurtman, R.J. (1971). *Science*, **173**: 149-152.

Fink, M., Shapiro, D.M. and Itil, T.N. (1971). *Psychopharmacologia*, **22**: 369-383.

Follows, O.J. (1971). *Br. J. Clin. Pract.*, **25**: 236-238.

Fonnum, F. (1984). *J. Neurochem.*, **42**: 1-11.

Fraschini, F., Esposti, D., Lucini, V., *et al.* (1989). In: *Advances in Pineal Research*, Vol. III (Reiter, R.J. ed.), pp. 127-132. John Libbey, London.

Garattini, S. and Samanin, R. (1975). In: *Appetite and Food Intake* (Silverstone, T., ed.), pp. 83-108. Dahlem Konferenzen, Berlin.

Garattini, S., Bizza, A., de Geatano, G., *et al.* (1975a). In: *Recent Advances in Obesity Research*, Vol. **1**, pp. 354-367. Newman, London.

Garattini, S., Buczke, W., Jori, A. and Samanin, R. (1975b). *Postgrad. Med. J.*, **51** (Suppl. 1): 27-35.

Garattini, S. and Samanin, R. (1984). *Int. J. Obesity*, **8** (Suppl.): 151-157.

Garattini, S., Mennini, T., Bendotti, C., *et al.* (1986). *Appetite*, **7**: (Suppl.), 15-38.

Garattini, S., Mennini, T. and Samanin, R. (1987). In: *Recent Advances in Obesity Research*, Vol. **5** (Berry, E.M., Blondheim, S.H., Eliahou, E.A., *et al.*, eds.), pp. 272-284. John Libbey, London.

Garfinkel, P.E. (1984). In: *Neuroendocrinology and Psychiatric Disorder* (Brown G. M., Koslow S.A. and Reichlin S., eds.), pp. 301-314. Raven Press, New York.

Garrow, J.S. (1979). *Br. Med. J.*, **2**: 1171-1172.

Gersberg, H., Kare, R., Hulse, M. and Phenggen, E. (1977). *Curr. Ther. Res.*, **22**: 814-820.

Ghose, K., Rama Rao, U.A., Bailey, J. and Coppen, A. (1978). *Psychopharmacologia*, **57**: 109-114.

Gilbert, D.B. and Cooper, S.J. (1985). *Brain Res. Bull.*, **15**: 385-389.

Glowinski, J. (1970). In: *Amphetamine and Related Compounds* (Costa, E. and Garattini, S., eds.), pp. 301-316. Raven Press, New York.

Glowinski, J., Axelrod, J. and Iversen, L.L. (1966). *J. Pharmacol. Exp. Ther.*, **153**: 30-41.

Gold, R.M. (1970). *Physiol. Psychol.*, **71**: 347-356.

Goldman, B.D. (1983). In: *Pineal Research Reviews*, Vol. 3 (Reiter, R.J., ed.), pp. 145-182. Alan R. Liss, New York.

Gomez-Perez, F.J., Ryan, J.R. and Staub, B. (1974). *J. Clin. Pharmac.*, **14**: 261.

Gonzalez-Barranco, J., Schulte, J., Rull, J.A. and Lozano-Castaned, O. (1975). In: *Recent Advances in Obesity Research*, **1**, pp. 386. Newman, London.

Gonzalez-Brito, A., Jones, D.J., Ademe, R.M. and Reiter, R.J. (1988). *Brain Res.*, **438**: 128-114.

Goodman, N.G. (1969). *Med. Ann. Distr. Columbia*, **38**: 658-662.

Gotestam, K.G. (1979). *Curr. Med. Res. Opinion*, **6** (Suppl. 1): 255-259.

Govitrapong, P., Murrin, L.C. and Ebadi, M. (1984). *Pineal Res.*, **1**: 215-226.

Govitrapong, P. and Ebadi, M. (1986). *Endocrinol. Res.*, **12**: 293-324.

Gwinup, G. and Pucher, R. (1967). *Am. J. Med. Sci.*, **254**: 416-420.

Hadler, A.J. (1972). *J. Clin. Pharmacol.*, **12**: 453-458.

Hamblin, T.J. (1971). *Lancet*, **II**: 1323.

Hamm, H.E., Takahashi, J.S. and Menaker, M. (1983). *Brain Res.*, **266**: 287-293.

Hauger, R.L., Hulihan-Giblin, B., Skolnick, P. and Paul, S.M. (1984). *Life Sci.*, **34**: 771-782.

Heffner, T.G., Zigmond, M.J. and Stricker, E.M. (1977). *J. Pharmacol. Exp. Ther.*, **201**: 386-399.

Herbert, D.C. and Reiter, R.J. (1981). *Proc. Soc. Exp. Biol. Med.*, **164**: 257-261.

Ho, A.K., Burns, T.G., Grota, L.J. and Brown, G.M. (1985a). *Endocrinol.*, **116**: 1858-1862.

Ho, A.K., Chik, C.L. and Brown, G.M. (1985b). *Life Sci.*, **37**: 1619-1626.

Ho, A.K., Thomas, T.P., Chik, C.L., Anderson, W.B. and Klein, D.C. (1988). *J. Biol. Chem.*, **263**: 9292-9297.

Ho, A.K., Young, I. and Chik, C.L. (1991). *Biochem. Pharmac.*, **41**: 897-903.

Hodde, K.C. (1979). *Prog. Brain. Res.*, **52**: 39-44.

Hoekenga, M.J., Dillon, R.H. and Leyland, H.M. (1978). In: *Central Mechanisms of Anorectic Drugs*, pp. 391. Raven Press, New York.

Hoffman, M., Pfeiffer, W.A., Gundlach, B.L. Nikrake, H.G.M., Oude Ophius, A.J.M. and Hantvast, J.G.A.J. (1979). *Int. J. Obesity*, **3**: 111-118.

Hollingsworth, D.R., Amatruda, T.T. and Scheig, R. (1970). *Metabolism*, **19**: 934-939.

Holtzman, S.G. (1974). *J. Pharmacol. Exp. Ther.*, **189**: 51-60.

Horrobin, D.R., Oka, M. and Manky, M.S. (1979). *Med. Hypoth.*, **5**: 849-858.

Hudson, K.D. (1977). *J.R. Coll. Gen. Pract.*, **27**: 497-501.

Hutson, P.H., Donhoe, T.P. and Curzon, G. (1988). *Psychopharmacology*, **95**: 550-552.

Jackson, R.L. and Lovenberg, W. (1971). *J. Biol. Chem.*, **246**: 4280.

Jago, M.V., Newson, J.F. and Rose, S. (1964). *Biochim. Biophys. Acta*, **92**: 44-51.

Juillard, M.T., Balemans, M.G.M., Collin, J.P., Legerstee, W.C. and Van Benthem, J. (1983). *Biol. Cell.*, **47**: 365-379.

Jung, R.T., Shetty, P.S., James, W.P.T., Barrand, M.A. and Callingham, B.A. (1979). *Nature*, **279**: 322-323.

Kaplan, N.W. and Jose, A. (1970). *Am. J. Med. Sci.*, **260**: 105-111.

Kappers, J.A. (1965). *Prog. Brain Res.*, **10**: 87-153.

Karasek, M. (1983). *Pineal Res. Rev.*, **1**: 1-48.

Keen, H., Thomas, B.J., Jarret, R.J. and Fuller, J.F. (1978). In: *Diet of Man : Needs and Wants*, pp. 89-96. Applied Science, London.

Keller Sarmiento, M.I., Gonzalez Solveyra, C. and Pereyra, E.N. (1987). In: *Advances in Pineal Research*, **2** (Reiter, R.J. and Fraschini, F., eds.), pp. 51-66. John Libbey and Co., London.

Kennett, G.A., Dourish, C.T. and Curzon, G. (1987). *Eur. J. Pharmacol.*, **141**: 429-435.

King, T.S., Steinlechner, S., Steger, R.W., Richardson, B.A. and Reiter, R.J. (1982). *Trans. Soc. Neurosci.*, **10**: 763.

King, T.S., Steinlechner, S. and Steger, R.W. (1985). *Experientia*, **41**: 417-419.

Kirby, M.J. and Turner, P. (1977). *Br. J. Clin. Pharmac.*, **4**: 459-461.

Klein, D.C. (1985). In: *Photoperiodism, Melatonin and the Pineal* (Ciba Foundation Symposium 117), pp. 51. Pitman, London.

Klein, D.C. and Notides, A. (1969). *Anal. Biochem.*, **31**: 480-483.

Klein, D.C. and Berg, G.R. (1970). *Adv. Biochem. Psychopharmacol.*, **3**: 241-263.

Klein, D.C. and Rowe, J. (1970). *Mol. Pharmacol.*, **6**: 164-171.

Klein, D.C. and Weller, J.L. (1970). *Science*, **169**: 1093-1095.

Klein, D.C., Berg, G.R. and Weller, J. (1970). *Science*, **168**: 979.

Klein, D.C. and Weller, J.L. (1972). *Science*, **177**: 532-533.

Klein, D.C. and Moore, R.Y. (1979). *Brain Res.*, **174**: 245-262.

Klein, D.C., Ayerback, D. and Weller, J.L. (1981). *Proc. Natl. Acad. Sci.*, USA, **78**: 4625-4628.

Klein, D.C., Sugden, D. and Weller, J.L. (1983). *Proc. Natl. Acad. Sci.*, USA, **80**: 599-603.

Kneisley, L.W., Moskowitz, M.A. and Lynch, H.J. (1978). In: *The Pineal Gland* (Nir, I., Reiter, R.J. and Wurtman, R.J., eds.), pp. 311-323. Springer, Vienna.

Krieger, D.T. (1974). *Endocrinol.*, **95**: 1195-1201.

Krieger, D.T., Hanser, H. and Krey, L.C. (1977). *Science*, **197**: 398-399.

Krieger, D.T. and Hauser, H. (1978). *Proc. Natl. Acad. Sci.*, USA, **75**: 1577-1581.

Kyriakides, and Silverstone, T. (1979). *Curr. Med. Res. Opin.*, **6** (Suppl.): 180-187.

Laitinen, J.T., Castre, E., Vakkuri, O. and Saaredra, J.M. (1989). *Endocrinology*, **124**: 1585.

Landsberg, L. and Young, J.B. (1982). *Am. J. Clin. Nutr.*, **35**: 1234-1240.

Lang, V. (1986). *Pineal Res. Rev.*, **4**: 199-244.

Lang, U., Aubert, M.L., Conne, B.S., *et al.*, (1983). *Endocrinology*, **112**: 1578-1584.

Lavieyville, M. and Isnard, F. (1975). *J. Diab. Hotel Diey.*, **16**: 341-348.

Le Magnen, J. and Devos, M. (1970). *Physiol. Behav.*, **5**: 805-814.

Leibowitz, S.F. (1975). *Brain Res.*, **84**: 160-167.

Leibowitz, S.F. (1985). *Psychopharmacol. Bull.*, **21**: 412-418.

Leibowitz, S.F. and Rossakis, C. (1978). *Cur. J. Pharmacol.*, **53**: 69-81.

Leibowitz, S.F. and Shor-Posner, G. (1986). *Appetite*, **7** (Suppl.): 1-14.

Leanard, B.E. and Shallice, S.A. (1971). *Br. J. Pharmac.*, **41**: 198-212.

Lerner, A.B. and Case, J.D. (1960). *Fed. Proc.*, **19**: 590-593.

Lipkin, D., Cook, W.H. and Markham, R. (1959). *J. Am. Chem. Soc.*, **81**: 6198-6203.

Lowensein, P.R., Gonzalez Solveyra, C. and Cardinali, D.P. (1984). *J. Pineal Res.*, **1**: 207-213.

Lynch, H.J., Wang, P. and Wurtman, R.J. (1973). *Life Sci.*, **12**: 145-151.

Malchon-Moller, A., Larsen, S., Hey, H., *et al.* (1981). *Int. J. Obesity*, **5**: 183-187.

Mather, P. and Booth, D.A. (1977). In: *Hunger Models : Computable Theory of Feeding Control* (Booth, D.A., ed.). Academic Press, London.

Mathews, C.S. and Seamark, R.F. (1981). *Pineal Function.* Elsevier/North Holland, Amsterdam.

Mathew, Parfitt, A.G., Sugden, D., Engelhardt, D.L., Zimmerman, E.A. and Klein, D.C. (1984). *J. Pharmacol. Exp. Ther.*, **228**: 434-438.

McIsaac, W.M., Farrell, G., Taborsky, R.G. and Taylor, A.N. (1965). *Science*, **148**: 102-103.

Meissl, H. and George, S.R. (1984). *Opthalmic Res.*, **16**: 114-118.

Miller, D.S. and Parsonage, S. (1975). *Lancet*, **1**: 773-775.

Moller, M. and Mikkelsen, J.D. (1989). In: *Advances in Pineal Research*, Vol. **3**, pp. 1-10. John Libbey, London.

Moore, R.Y. (1978). In: *The Pineal and Reproduction*, Vol. **4** (Reiter, R.J., ed.), pp. 1-29. Basel, Karger.

Morgan, P.J. and Williams, L.M. (1989). *Experientia*, **45**: 955.

Morley, J.E., Levine, A.S., Gosnell, B.A. and Krahn, D.D. (1985). *Brain Res. Bull.*, **14**: 511-519.

Morselli, P.L., Maggini, C., Placidi, G.F., Gemeni, R. and Tegneni, G. (1978). In: *Central Mechanisms of Anorectic Drugs*, pp. 243. Raven Press, New York.

Morton, D.J. (1987). *S. Afr. J. Sci.*, **83**: 167-168.

Morton, D.J. and Forbes, H.J. (1989). *J. Neural Transm.*, **75**: 65-71.

Olivieri, G. (1992). PhD Thesis : Rhodes University, South Africa.

Opitz, K. (1978). *Int. J. Obesity*, **2**: 59-65.

Panksepp, J. (1973). *J. Comp. Physiol. Psychol.*, **82**: 78-94.

Patel, D.P. and Stowers, J.M. (1964). *Lancet*, I: 282-284.

Paul, S.M., Hulihan-Giblin, B. and Skolnick, P. (1982). *Science*, **218**: 487-489.

Paykel, E.S., Mueller, P.S. and De La Vergne, P.M. (1973). *Br. J. Psychiat.*, **123**: 501-507.

Peterson, S.A. (1975). *Gonadal Hormones and Feeding Behaviour in the Mouse*. PhD Thesis, University of Edinburgh.

Pévet, P. (1979). *Progr. Brain Res.*, **52**: 149-192.

Piacsek, B.E. and Meites, J. (1967). *Endocrinology*, **81**: 535-546.

Printzmetal, M. and Alles, G.A. (1940). *Am. J. Med. Sci.*, **200**: 665-673.

Puls, W., Keup, U., Krause, H.P., *et al.* (1977). *Naturwissarschaffer*, **64**: 537.

Quay, W.B. (1965). *Life Sci.*, **4**: 983-991.

Quay, W.B. (1972). *Physiologist.*, **15**: 241-248.

Quay, S.B. (1974). In: *Pineal Chemistry* (Thomas, C.C., ed.), Springfield IL.

Quirion, R. (1984). *Eur. J. Pharm.*, **102**: 559-560.

Reiter, R.J. (1981). *Am. J. Anat.*, **162**: 287-313.

Reiter, R.J. (1981). In: *The Pineal Gland*, Vol. **1** : *Anatomy and Biochemistry*, CRC Press, Florida.

Reiter, R.J. (1981). In: *The Pineal Gland*, Vol. **II**., CRC Press, Boca Raton.

Reiter, R.J. (1982). In: *The Pineal Gland*, Vol. **III**, CRC Press, Boca Raton.

Reiter, R.J. (1983). In: *The Pineal Gland and its Endocrine Role* (Axelrod, J., Fraschini, F. and Volo, G.P., eds.), pp. 227-241. Plenum, New York.

Reiter, R.J. (1984). In: *Neuroendocrin. Perspectives*, Vol. **III**, pp. 345-377. Elsevier/North Holland, Amsterdam.

Reiter, R.J. (1986). *Neural Transm.*, **21** (Suppl): 35-54.

Reiter, R.J. (1987). *Life Sci.*, **60**: 2119-2131.

Reiter, R.J. (1988). *ISI Atlas of Science : Plants and Animals*, Vol. 1. pp. 111-116.

Reiter, R.J. (1989). *De Groots Endocrinology*, Vol. 1, 2nd Edition (De Groot, L.J. ed.), pp. 240-253. Saunders, Philadelphia.

Reiter, R.J. (1991a). *Mol. and Cell. Endocrinol.*, **79**: 153-158.

Reiter, R.J. (1991b). *Trends in Endocrinol. and Metab.*, **2**: 13-19.

Richter, C.P. (1922). *Comp. Psych. Monographs*, **1**: 1-55.

Ritta, M.N. and Cardinali, D.P. (1982). *Neurosci. Lett.*, **31**: 307-311.

Rodin, J. (1981). In: *Recent Advances in Obesity Research* III, pp. 106-123. Newman, London.

Romero, J.A., Zatz, M., Kebabian, J.W. and Axelrod, J. (1975). *Nature*, **258**: 435-436.

Rosenthal, N.E., Sack, D.A., Jacobsen, F.M., *et al.* (1986). *J. Neural Transm.*, **21** (Suppl.): 257-267.

Ross, S.B. (1979). *Life Sci.*, **24**: 159-168.

Rothwell, N.J. and Stock, M.J. (1979). *Nature*, **281**: 31-36.

Sachse, G. and Willms, B. (1979). *Diabetologia.*, **17**: 287-290.

Saul, H. (1993). *New Scientist*, July : 28-31.

Schubert, J., Fyrö, B., Nyback, H. and Sedrall, G. (1970). *J. Pharm. Pharmac.*, **22**: 136-139.

Schubert, J. and Sedvall, G. (1972). *J. Pharm. Pharmac.*, **24**: 53-62.

Shiotani, Y., Yamano, M., Shiosaka, S., *et al.*, (1986). *Neurosci. Lett.*, **70**: 187-192.

Siegel, P.S. (1961). *J. Comp. Physiol. Psychol.*, **54**: 294-301.

Sims, E.A.H. (1981). *Int. J. Obesity*, **5** (Suppl.): 9-18.

Skene, D.J. (1985). PhD Thesis : Medunsa, South Africa.

Smith, C.B. (1965). *J. Pharmacol. Exp. Ther.*, **147**: 96-102.

Smith, T.L., Eichberg, J. and Hauser, G. (1979). *Life Sci.*, **24**: 2179-2184.

Smith, T.L. and Hauser, G. (1981). *J. Neurochem.*, **37**: 427-435.

Sonofrien, M.V. and Weindl, A. (1982). In: *Vertebrate Circadian Systems : Structure and Physiology* (Aschoff, J., Daan, S. and Gross, G.A., eds.), pp. 75-86. Springer, Berlin.

Sorrentino, S., Reiter, R.J. and Schalch, D.S. (1971). *Neuroendocrinol.*, **7**: 105-115.

Sourkes, T.L., Missala, K. and Madras, B.K. (1969). *J. Pharmac. Exp. Ther.*, **165**: 289-293.

Spiegel, A.M. (1989). *Trends Endocrinol. Metab.*, **1**: 72.

Spillane, J.P. (1960). *Practitioner*, **185**: 102-106.

Stankov, B. and Reiter, R.J. (1990). *Life Sci.*, **46**: 971.

Steel, J.M. and Briggs, W. (1972). *Br. Med. J.*, **3**: 26-27.

Stein, L. and Wise, C.D. (1969). *J. Comp. Psychol.*, **67**: 189-198.

Sterne, J. (1964). *Metabolism*, **13**: 791-798.

Stowers, J.M. and Bewsher, P.D. (1969). *Postgrad. Med. J.*, **45** (Suppl.): 13-16.

Strada, S.J., Klein, D.C., Weller, J. and Weiss, B. (1972). *Endocrinology*, **90**: 1470-1475.

Sugden, D. (1979). *J. Neurochem.*, **33**: 811.

Sugden, A.L. and Sugden, D. (1986). *68th Annual Meeting : The Endocrine Society*, Abs. 283.

Sugden, D., Namboodiri, M.A.A., Klein, D.C., Grndy, R.K. Jr. and Mefford, I.N. (1985a). *J. Neurochem.*, **44**: 769-772.

Sugden, D., Vanecek, J., Klein, D.C., Thomas, T.P. and Anderson, W.B. (1985b). *Nature*, **314**: 359-361.

Sugden, D., Gena, V. and Klein, D.C. (1987). *Methods Enzymol.*, **142**: 590.

Sugden, L.A., Sugden, D. and Klein, D.C. (1987). *J. Biol. Chem.*, **262**: 741-745.

Sugrue, M.F. (1987). *Pharmacol. Ther.*, **32**: 145-182.

Sullivan, A.C., Comai, K. and Triacari, J. (1981). In: *Recent Advances in Obesity Research* **III**, pp. 199-207. John Libbey, London.

Sutherland, E.W., Robinson, G.A. and Butcher, R.W. (1968). *Circulation*, **37**: 279-306.

Swanson, L.W. and Sawchenki, P.E. (1983). *Annu. Rev. Physiol.*, **6**: 269-324.

Taylor, R.L., Albuquerque, M.L.C. and Burt, D.R. (1980). *Life Sci.*, **26**: 2195-2200.

Tempel, D., Smith, D. and Leibowitz, S.F. (1987). *Proc. East. Psychol. Assoc.*, **12**: 592.

Tortosa, F., Puig-Domingo, M., Peinado, M.A., *et al.*, (1989). *Acta Endocrinol.*, (Copenh), **120**: 574.

Trowel, O.H. (1959). *Exp. Cell Res.*, **16**: 118.

Trulson, M.E. and Jacobs, B.L. (1976). *Eur. J. Pharmacol.*, **36**: 149-154.

Turner, P. (1978). *Int. J. Obesity*, **2**: 343-348.

Van Rossum, J.M., Van Der Schoot, J.B. and Hurksmans, J.A. (1962). *Experientia*, **18**: 229-231.

Van Wyk, E.J. (1993). MSc Thesis : Rhodes University, South Africa.

Vanecek, J., Sugden, D., Weller, J.L. and Klein, D.C. (1986). *J. Neurochem.*, **47**: 678-686.

Vanecek, J., Paulik, A. and Illnerova, H. (1987). *Brain Res.*, **435**: 359-362.

Vigersky, R.A. (1984). In: *Neuroendocrinology and Psychiatric Disorder* (Brown, G.M., Koslow, S.H. and Reichlia, S., eds.), pp. 291-300. Raven Press, New York.

Vincent, G.P. and Levin, B.E. (1989). *Soc. Neurosci. Abstr.*, **15**: 1131.

Vollrath, L. (1981). *The Pineal Organ.* Springer Verlag, Berlin.

Waal-Manning, H.J. and Simpson, F.O. (1969). *Lancet*, **II**: 1392-1395.

Walker, R.F., McMahon, K.M. and Pirorum, E.B. (1978). *Exp. Gerontol.*, **13**: 91-99.

Walsh, V.A., Weiss, G.F., Long, E.A. and Leibowitz, S.F. (1987). *Proc. East. Psychol. Assoc.*, **58**: 32.

Walsh, H., Daya, S. and Whiteley, C.G. (1991). *Med. Sci. Res.*, **19**: 849-850.

West, K.M. and Kalbleisch, J.M. (1971). *Diabetes*, **20**: 99-108.

Wilkinson, M., Arendt, J., Bradtke, J. and De Ziegler, D. (1977). *J. Endocrinol.*, **72**: 243-244.

Williams, L.M. and Morgan, P.J. (1988). *J. Endocrinol.*, **119**: R1.

Wright, G.J., Lang, J.F., Lemieux, R.E. and Goodfriend, M.J. (1975). *Drug Metab. Rev.*, **4**: 267.

Wurtman, R.J. and Axelrod, J. (1965). *Sci. Am.*, **213**: 50-60.

Wurtman, R.J., Axelrod, J. and Kelly, B.E. (1968). *The Pineal.* Academic Press, New York.

Yelonsky, J.O., Panasevich, R.E., Borreli, A.R. and Lawlor, R.B. (1969). *Archs. Int. Pharmacodyn. Ther.*, **178**: 62-76.

Young, V.R., Hussein, M.A., Murray, E. and Scimshaw, N.S. (1969). *Am. J. Clin. Nutr.*, **22**: 1563.

Zatz, M. (1985). *J. Neurochem.*, **45**: 637-642.

Zatz, M. and Brownstein, M.J. (1979). *Brain Res.*, **160**: 381-385.

Zisapel, N., Nir, I. and Laudon, M. (1988). *FEBS*, **232**: 172-176.