SYNTHESIS AND SECRETION OF RAT PINEAL PROTEINS IN-VITRO

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"In the absence of a God-granted Solomonic Wisdom to be visionary, we sought refuge in the presumption that starting to solve a puzzle without finding the solution may not be in vain, that planting a small weakling tree may be as joyful as seeing it bear fruit and it, may be more exciting to travel than to arrive."

Ebadi et al., 1989

This thesis is dedicated to my dear wife Sue and to my four wonderful children John, Athanasia, Katherine and Anastasi who gave me the courage to keep on going and who endured with me the frustrations, anxieties and pleasures of a PhD student's life.

FORWORD

After two millenia of speculation vested with philosophical thoughts, fantasies, myths and superstitions about its origins and physiological functional significance, the pineal has been properly identified as an endocrine gland with a distinctive role in translating photic and other enviromental information into hormonal messages.

The majority of pineal research in the last 35 years has focused on its indoleamine secretions especially melatonin. However, recent advances in molecular technology and better scientific interdisciplinary cooperation, has generated renewed interest in the plethora of polypeptide secretions of the pineal gland.

The forthcoming era of pineal research will pave the way for an even better understanding of pineal function and hopefully ascertain its role as a "regulator of regulators."

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PREFACE

Disillusioned by the commercial world, I decided to call Seamark and return to the study of the pineal gland which I had left for nearly ten years. It was for the love of science and the fascination of the pineal which brought me back and I am glad I did.

Those that choose to study the pineal gland should do so with a prior knowledge that rewards will come, if and only if, they accept at the onset that the pineal is a gland of great interspecies variation displaying circadian, circalunar and circannual rhythmical changes. Lack of understanding of these factors may have been a major contributing factor as to why the exact function(s) of the pineal gland remains unresolved.

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1992

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Abstract

This thesis reassesses pineal proteins. The highly sensitive technique of two dimensional gel electrophoresis [2D-SDS/PAGE] was established, evaluated and applied to answer questions about the nature and patterns of pineal proteins synthesized and secreted in-vitro and factors influencing their production. In conjunction with radiolabelling, 2D-SDS/PAGE demonstrated a large number of proteins synthesized and secreted from individual rat pineal glands in-vitro. Major factors found to influence these protein secretions were the photoperiod and melatonin. The photoperiodic influences indicated two peaks of incorporation of ^{35}S-methionine into secreted proteins during the 24 hour diurnal cycle - a major peak at the midlight phase [ML] and a second less intense but significant peak at the middark phase [MD]. The diurnal fluctuations in total protein content of the pineal glands followed a similar bimodal distribution. Protein patterns as determined by 1and 2D-SDS/PAGE were significantly different [$p<0.01$] at these two times in all samples examined. Melatonin at physiological doses was able to inhibit protein synthesis and secretion and at a dose of 10^{-7}M mimicked the MD pattern very closely. Further assessment of the possible mechanisms of action of melatonin on pineal protein synthesis demonstrated that it's mode of action is via transcription. Melatonin inhibition of protein synthesis was reversible and dose related between 10^{-7}M and 10^{-4}M.

These results were the highlight of the current study and although raising further questions, clearly add to our understanding and reassessment of the importance of pineal bioactive secretions [both indoles and proteins]. In particular, the data further emphasizes the need to review the potential autocrine and/or paracrine mechanisms of action of melatonin. It is possible that the photoperiodic influences on pineal protein synthesis and secretion are mediated via melatonin. The techniques established in these studies provide a valuable tool for providing insights into a hitherto neglected arm of the functions of this very important gland.

Declaration

I hereby declare that the experiments reported in this manuscript were carried out by myself and that no part of this work has been submitted to any other University or institution for consideration for any degrees or diplomas.

I further grant my consent to the University of Adelaide to make this thesis available for photocopying and loan once accepted for the degree of Doctor of Philosophy.

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Acknowledgements

To my supervisor Dr. Robert F. Seamark for giving me the opportunity to return and complete my studies, for his advice and encouragement during the course of these studies and for instilling in me the true meaning of scientific excellence - I extend my sincere gratitude. Thanks Bob!

It was a pleasure working in the Department of Obstetrics and Gynaecology under the Chairmanship of Professor Jeffrey Robinson. I sincerely thank him and his staff for their cheerful support and encouragement throughout my studies.

I acknowledge the assitance and support extended to me by Dr. David J. Kennaway, his student Rehema White [donation of Bush Rat pineals] and his staff Pauline Blake and Richard Mason [occassional assistance with killing of the animals].

I further acknowledge the assistance of Drs Andrew and Gillian Borthwick with computing and general advice, Mr. Don Bigham and Frank Carbone for their prompt attention to materials and equipment supplies and repairs, and Mrs.Glenys King for her cheerful secretarial support.

Finally I owe a profound thanks to my parents, who from my early school years always highlighted and instilled in me the values of learning. Thankyou Mum and Dad.

Publications

1. Kotaras, P.J. and Seamark, R.F. (1990). Circadian protein synthesis and secretion by rat pineal glands in-vitro. Australian Society for Reprod. Biol. Proceedings of the 22nd Annual Meeting Perth Aust. Sept 24-26.

2. Kotaras, P.J. and Seamark, R.F. (1990). Protein synthesis by the rat pineal gland. The Aust. Soc. For Med. Research. Proceedings of the Annual Meeting Adelaide, Aug 17.

3. Kotaras, P.J. and Seamark, R.F. (1991). The effects of photoperiod and melatonin on protein synthesis and secretion by rat pineal glands in-vitro. Advances in Pineal Research 6:123-126, Foldes, A and Reiter, R.J. eds John Libbey & Co .

Part One

Literature Review

Chapter One

Historical Considerations

(1). Superstitions and Philosophical Notions

The pineal organ was first described by Herophilus and Erasistratus in the period 300 BC. At that time the organ was identified with mental functions, and seen as a valve controlling memory and thoughts. This primitive notion of the pineal gland was superceeded by Galen (A.D. 130-200) who regarded the pineal as a supportive structure filling the gap between cerebral veins. Descartes (A.D. 1640) introduced the notion that the pineal was the seat of the soul. Whilst this new notion was largely rejected by medical scientists of the time this Cartesian notion did persist and its influence was sufficient to prevent publications and circulation of views which would have placed the pineal on a true scientific path of understanding. Bonet in 1700 published his "Sepulchretum" in which he related pineal calcification to mental illnesses. Bonet was influential and his view consequently widely accepted, and it was not until 1830 that his data was challenged by more critical investigations meanwhile allowing the pineal to once again relinguish into obscurity as an organ of no important function.

(2). Preliminary Pineal Research

This second historical period began with the initiation of experimental studies involving ablation of the organ. As the pineal proved difficult to

remove observations were mostly made on small numbers of subjects and critical influences such as age, sex and time of removal of the pineal were not recognized rendering much information as either inconclusive or irreproducible. Hence whilst this important era did lay the foundation for our understanding of pineal function the interim conclusions drawn by reviewers such as Kitay and Altschule 1954, that the pineal was a gland of functional significance with influence on reproduction, photoperiodism, pigmentation and possibly behaviour, were all tentative.

(3). Studies of Internal Pineal Function

The third period of research saw the discovery of the pineal indoleamine melatonin by Lerner and coworkers in 1958 and establishment of the pineal as an endocrine gland [reviews by Wurtman, Axelrod and Kelly 1968] and is generally regarded as initiating the modern period of pineal research.

(4). Studies of Overall Biological Role

The final and current period of research is focussed on determining the extent of the pineal's role in the overall biological functioning of the organism. This period of research is regarded as still being very much in its infancy and is preoccupied with identifying the bioactive pineal principles.

Development

1. Introduction

The pineal gland or epiphysis cerebri, develops as an integral part of the brain and has been demonstrated in all mammalian species examined to date. The potential role relationship of the pineal as a mediator in the adaptation of an organism to its enviroment, is evident early in its evolutionary phase. Here sensitive photoneuroendocrine cells found in pineal tissues act as "timing devices" in biological events. These photosensitive cells are absent in mammals, but photic inputs continue to play a central role in pineal regulation.

Evolution has resulted in significant variation at both the ultrastructural and molecular level of pineal tissues [see Ralph, 1970; Quay, 1974; Dodt, 1973; Collin, 1981; and Oksche and Pevet, 1981 for recent exhaustive reviews]. The capacity of the pineal to actively synthesize and secrete several biologically active compounds was evident at an early evolutionary stage [Oksche, 1983], but both sensory and secretory organelles and presumably the secretory products of the pineal gland have undergone significant evolutionary adaptations. Fig. (1) summarizes the developmental origins of mammalian pineal cells.

(2) Anatomy

Mammalian pineals are compact organs which display interspecies variations in their exact anatomical position and intracranial relations. Generally the pineal is found between the habenular and posterior commissures Fig. (2). In rodents, the pineal is separate from the roof of

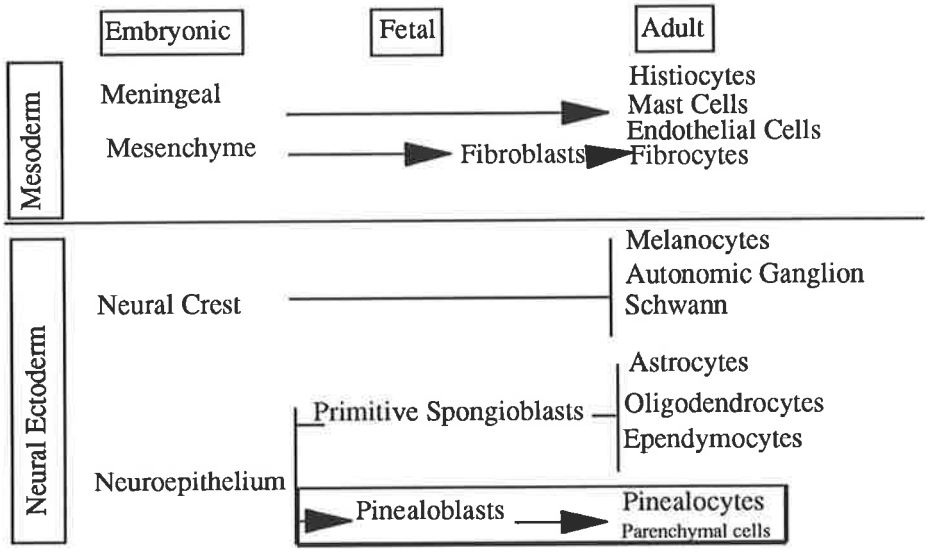

Figure 1. Pineal Histiogenesis

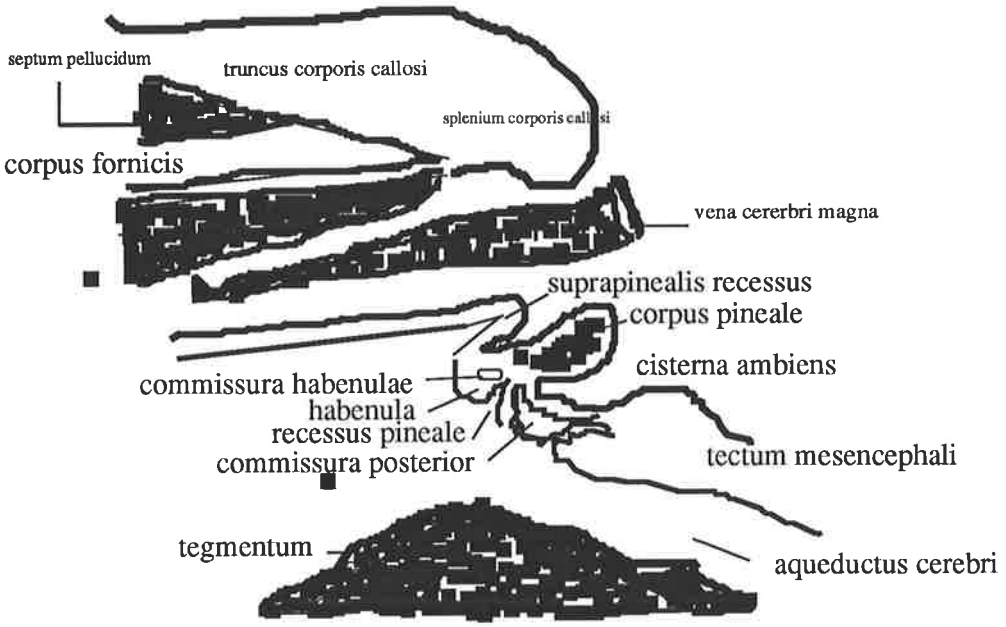

Figure 2. Anatomical relationships of the pineal [adapted from Quay, 1974]

the diencephalon and attached by a few nerve fibres from the habenular and posterior commissures [Kappers, 1965]. This is useful experimentally as it facilitates surgical ablation, however, in other species the gland lies basally making it difficutlt to remove the entire organ [Quay, 1965]. Cytological evidence suggests that the distal and basal parts of the pineal may differ in composition and function. Quay, 1965 for example has shown that the distal pineal cells are more responsive to photoperiodic changes in some species. The pineal gland has been shown to have a close anatomical relationship to the third ventrical [Quay, 1970] and in the human and other mammals lies in the subarachnoid space and is bathed in cerebrospinal fluid.

Pineal innervation in mammals has been studied extensively and has been shown to be entirely autonomic [Kappers, 1965; 1969]. Fig. (3) illustrates the anatomical relations and sources of pineal nerve fibres found in mammals. There is now ample evidence to implicate both sympathetic and parasympathetic innervation of the mammalian pineal contrary to a earlier view that the innervation was entirely sympathetic [Moller, 1991]. Many of the nerve fibre terminals supplying the pineal have been shown to contain clear as well as dense-cored or granulated vesicles.

The blood supply to the pineal is via small arterioles, branches from the posterior choroid arteries. The arterial and venule anatomical relations to the mammalian pineal are shown in Fig.(4a & 4b). There is vascular variability both within and between species [Kaplan and Ford, 1966]. Cannulation of the pineal blood vessels although difficult to perform has been attempted to provide information about pineal metabolism and the effects of various factors on pineal function. Rat pineal blood flow has been shown to be as high as that of the pituitary

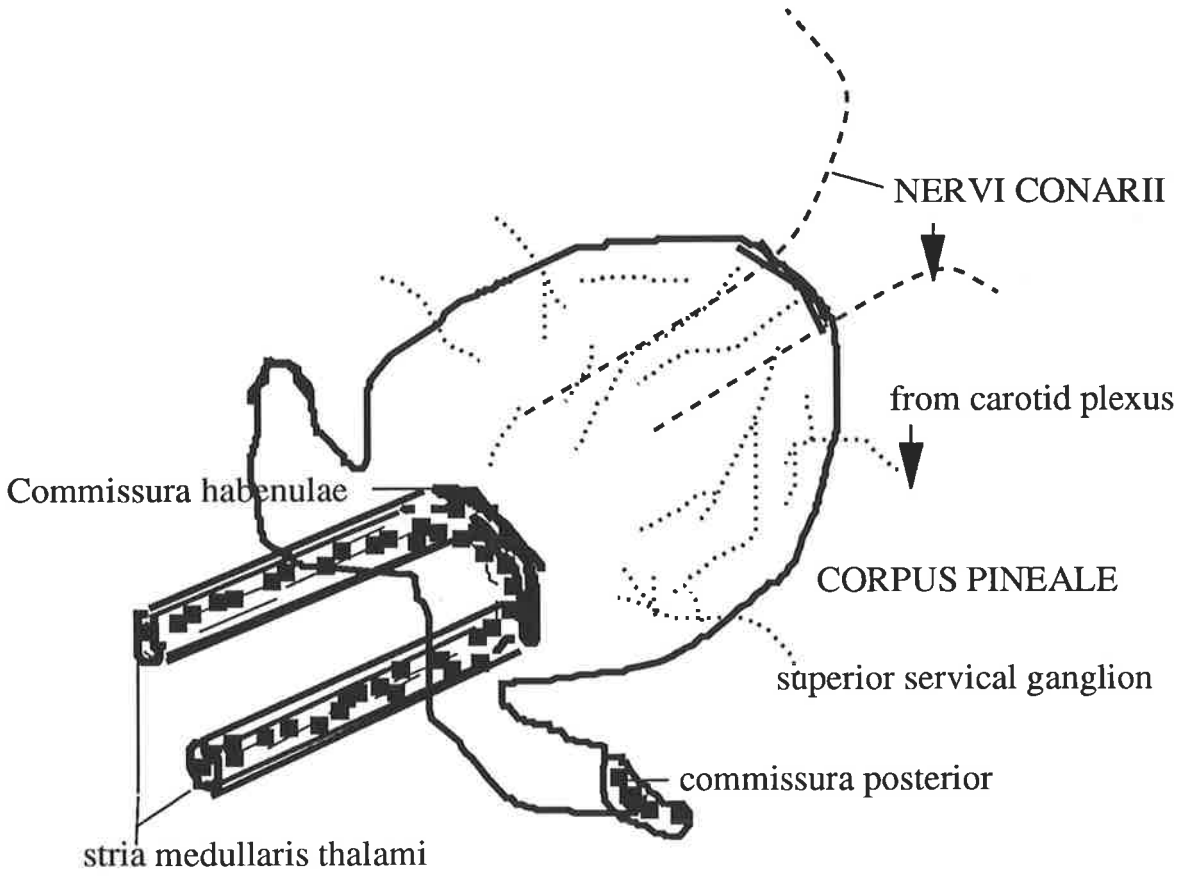

Figure 3. Pineal innervation [adapted from Quay, 1974]

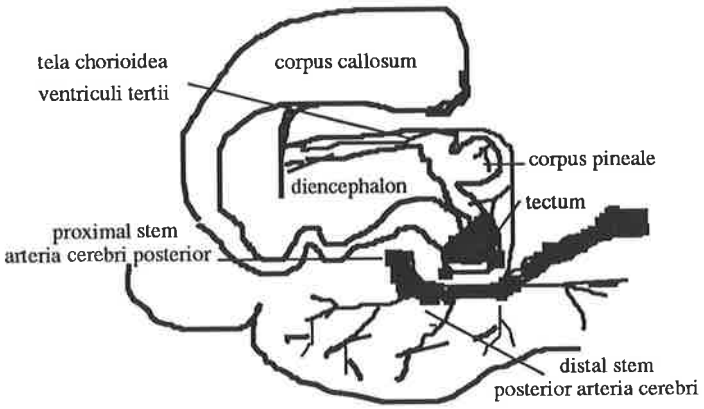

Figure 4[a]. Arterial blood supply to the pineal

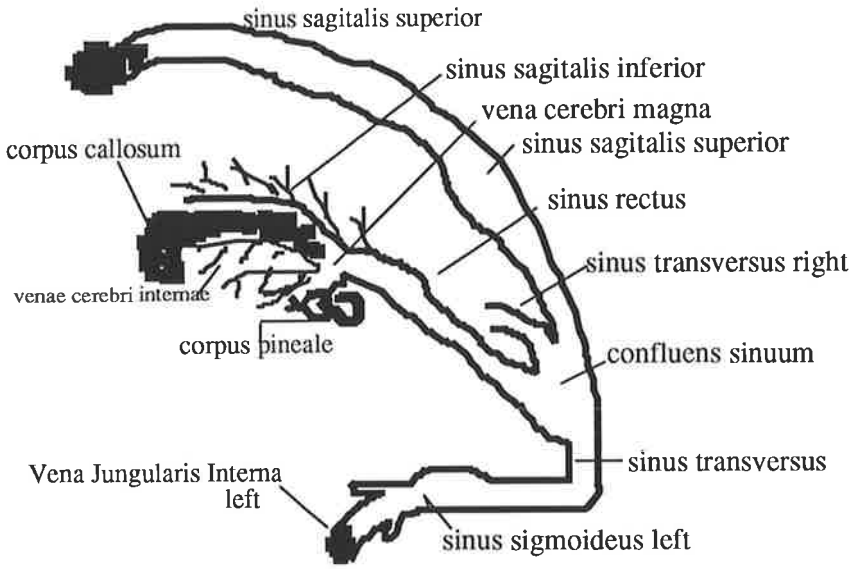

Figure 4[b]. Cerebral veins and the pineal gland

gland and is exceeded only by that of the kidney [Goldman and Wurtman 1964]. No specific arteries or arterioles have been identified for the pineal and any arterioles found on the connective tissue capsule do not extend far within the organ. Blood within the pineal is contained by the capillaries and venules which drain into larger veins or dural venous sinuses. Eventually pineal blood drains into the cerebral vein via the sinus rectus, confluens sinuum, sinus transversus, sinus sigmoidens to the internal jugular vein. Angiographic studies have been valuable in demonstrating pineal vascular patterns in both normal and diseased states [Johanson, 1954; Lofgren, 1958]. In the Wistar rat, several (12-16) peripheral pineal veins were shown to drain into the great cerebral vein. In the central region only one major vein was observed [Hodde, 1979, 1981].

Pronounced vasoconstriction has been observed at the onset of light [Quay, 1972] and removal of the sympathetic innervation to the pineal is known to reduce pineal blood flow [Goldman, 1967]. Pineal blood flow may serve as a vehicle for the pineal secretory products although the cerebral spinal fluid has also been implicated.

(3) Histology and Cytology

The major cell type of the pineal parenchyma proper is the pinealocyte also termed pineocyte. In the rat these cell types make up 82% of the total cell content of the pineal [Wallace et al., 1969]. Typically, the pinealocyte of mammals consists of a cell body and cell processes which vary in length, width and number. Ultramicroscopically, the terminal processes of the pinealocytes are difficult to distinguish from nerve endings especially when they contain

granulated vesicles . In some mammals, the presence of striated muscle fibres are observed, their origin or functional significance being unknown. Pineal connective tissue in most mammalian pineals is absent or scarce. Where present, the connective tissue separate the organ into follicles and are endowed with blood vessels and small nerve fibres. Other pineal cells shown are the mast cells, pigment containing melanophores,the glial or interstitial cells [Wallace et al., 1969; Wartenberg, 1968] and astrocytes [Moller et al., 1978; Sheridan and Reiter, 1973]. There are both species and individual differences in the relative distribution of these pineal cells and their functional significance still to be fully determined. Attainment of full differentiation and activity is characterized by pinealocyte cytoplasmic, nuclear and nucleolar hypertrophy combined with nuclear creasing, folding and pleomorphisms. These changes are evident in the postnatal pineals of humans and laboratory species studied thus far. Acquisition of these characteristics corresponds in time to the acquisition of full metabolic and biochemical activity within the pineals. However, extensive quantitative and comprehensive comparative studies of pinealocyte cytology, differentiation and metabolism in mammals are still lacking.

Electron microscopical studies have enhanced the view that pineal cells in particular the pinealocytes resemble neurons capable of endocrine secretory activity. Fig.(5) shows a diagramatic representation of a mammalian pinealocyte. Cytoplasmic organelles capable of protein synthesis are particularly apparent and of specific interest. For extensive reviews on pineal ultrastructure see Wartenberg, 1968 and Wolfe, 1965. In more recent ultrastructural studies [Pevet, 1977, 1978] different types of pinealocytes have been distinguished. Some attribute the distinction to different functional states of the same cell type

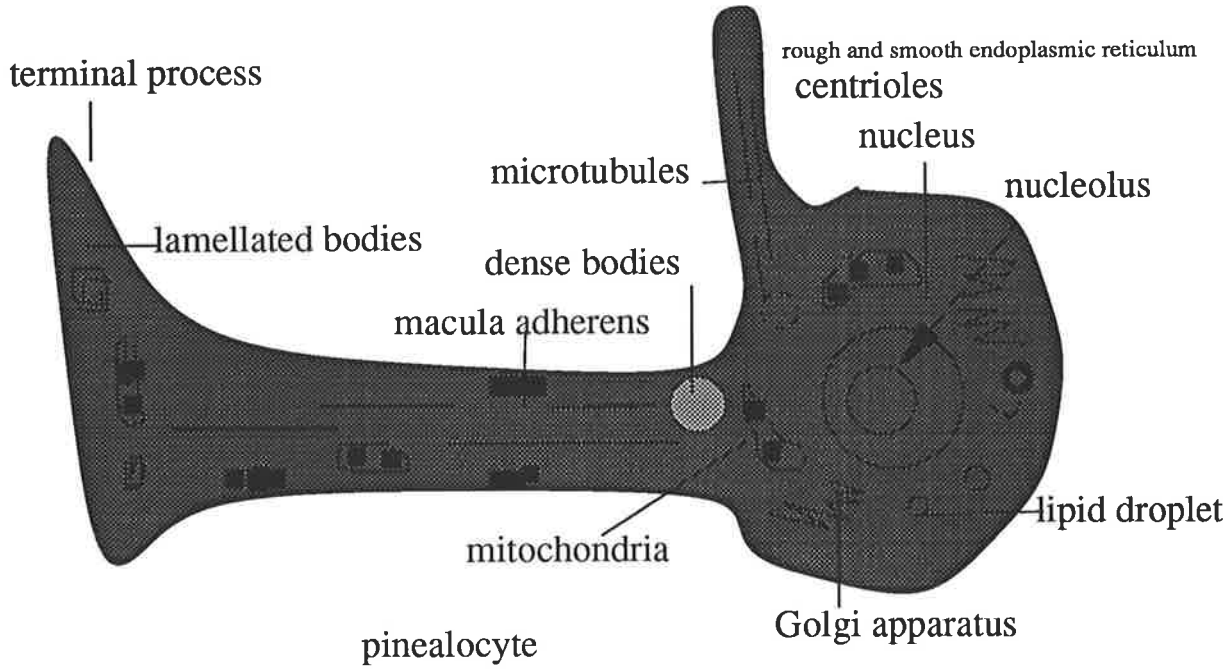

Figure 5. Pinealocyte schematic representation

[Vollrath, 1981]. There is some evidence to suggest that the different pinealocytes differ in their ability to synthesize and secrete proteinaceous compounds [Pevet, 1979].

Chapter Two

Pineal Proteins and Peptides as Bioactive Secretions

1.Introduction

Once the morphologists had established that the pineal gland contained structures capable of active synthesis and secretion of biochemical substances, most pineal workers concentrated in defining pineal specific bioactive compounds synthesized and secreted by the pineal in an attempt to affirm the glands endocrine status. Excellent reviews on pineal secretions common to other neuronal tissues such as the neuropeptides and neurotransmitters are provided by Ebadi et al., 1989. This review focuses at two major classes of pineal secretions: pineal proteins and peptides and pineal indoleamines as specific pineal hormones. In this chapter, literature pertaining to the pineal proteins and peptides will be reviewed.

Although, the study of pineal proteins and peptides dates back to the early 1920's, their significance in pineal function remains overshadowed by the discovery of the indolamine melatonin in 1958 by Lerner and coworkers.

As discussed in several authoratative reviews [Benson et al., 1983, Benson and Ebels, 1978; Collin, 1979,1981; Pevet, 1983, 1986; Vaughan, 1984 Noteborn, 1986; Reiter and Karasek, 1986; Reiter and

Fraschini, 1987; Trentini et al., 1987 and Gupta et al., 1988] evidence to date both supports and opposes the functional significance of pineal proteins and peptides. There is now compelling experimental evidence identifying the pineal as a site of active protein synthesis and secretion, but the main bioactive products remain to be identified. Table (2) presents a summary of bioactive proteins and peptides reported as being present in pineal tissue. As is readily appreciated the proteins listed are by no means pineal specific.

TABLE (1) BIOACTIVE PINEAL PROTEINS AND PEPTIDES

1. Pineal Antigonadotrophin
2. Vasotocin
3. Vasopressin
4. Oxytocin
5. Neurophysin I & II
6. Renin
7. Angiotensin
8. Corticotrophin
9. Endorphin
10. Melanocyte Inhibiting Factor
11. Melanocyte Stimulating Hormone
12. Thyrotrophin releasing Hormone
13. Threonylserinyllysine
14. Substance P
15. Vasoactive Intestinal Peptide
16. Retinol Binding Protein

17. Rhodopsin
18. S-Antigen
19. Zinc-Binding Protein
20. Gonadotrophin Releasing Factors
21. Prolactin-releasing and inhibiting factors.

(1) PINEAL ANTIGONADOTROPHINS

Kitay and Altschule, 1954 in their review of the pineal concluded that pineal extracts exhibited antigonadotrophic activities. In 1971, Thieblot and Menigot isolated five putative peptides which affected the neuroendocrine reproductive axis with molecular weights ranging from 1000 to 3000 daltons. In 1971 Moszkowska et al., 1965 had demonstrated that similar pineal peptides derived from sheep and mouse pineals, altered hypothalamic gonadotrophin releasing factors in-vitro, with both inhibitory and stimulatory effects. Benson et al., 1971 isolated a pineal factor capable of altering the sensitivity of the hypothalamic-hypophyseal axis to the feedback effects of gonadal steroids. This factor was called pineal antigonadotrophin (PAG). Other observed actions of PAG were the inhibition of the postcastration rise in serum luteinizing hormone (LH), the delayed opening of the vagina an inhibition of compensatory ovarian hypertrophy (COH), a reduction in circulating levels of prolactin and a significant increase in newly synthesized dopamine in the hypothalamus. Benson and coworkers 1972 concluded PAG was probably acting through inhibition of gonadotrophin secretion in particular LH. Benson and Ebels (1981) reviewed the evidence for the small molecular weight presumptive peptides isolated from ovine and bovine pineal glands which showed

both inhibitory and stimulatory affects on reproduction in the models tested. In immature mice, some of these peptides reduced ventral prostate, gonadal and accessory gonadal weights. Two active putative peptides that were characterized had molecular weights of 1000 and 500 daltons and trypsinization resulted in the loss of their antigonadotrophic activities. Orts et al., 1989, 1979. extracted two distinct antigonadotrophic fractions from bovine pineals and demonstrated to inhibit COH and block pregnancy. One of these peptides was characterized as a small tripeptide threonylserinyllysine (TSL). Neascu, 1972 isolated and characterized a peptide with antigonadotrophic activities from bovine pineal glands. This peptide- E_5 behaved like oxytocin and reduced uterine weight, reduced the incorporation of radioactive phosphorus in LH primed prepubertal female mice, inhibited spermatogenesis in frogs and significantly reduced plasma prolactin levels Vaughan, 1982. E_5 was characterized as ending with the same carboxyterminal tripeptide group as arginine vasotocin and arginine vasopressin but further analysis showed that E_5 was of higher molecular weight to AVT and AVP and differed immunologically from these peptides. The exact role of E_5 has yet to be resolved.

(2) ARGININE VASOTOCIN AND OXYTOCIN

The identification of Arginine Vasotocin (AVT) in the bovine pineal gland as an antigonadotrophic factor prompted one of the most controversial debates on pineal peptides [Milcu and co-workers 1963], which was partially resolved in 1983 when Prechel and co-workers found that AVT fluctuates seasonaly with a maxima in August and a

minima in January. Similar seasonal fluctuations were shown by Vivien-Roels et al., 1979 in the levels of AVT in the tortoise pineal.
The extensive affects of the neurohypohyseal hormones on the reproductive axis have been reviewed by several authors [Vaughan, 1981] and it is was widely accepted that pineal AVT is an important pineal antigonadotrophin with inhibitory effects on the neuroendocrine - reproductive axis [summarized by Vaughan, 1984; Johnson et al., 1980; and Cheeseman et al., 1983].

AVT and AVP have been demonstrated to have both stimulatory and inhibitory effects on corticotrophin levels. Spinedi and Negro-Vilar, 1983 have demonstrated that at the pituitary level AVT and AVP are stimulatory and their effects can be be enhanced by serotonin. Pavel et al., 1977 have shown that at high doses AVT and AVP stimulate adrenocorticotrophin (ACTH) secretion both in-vitro and in-vivo and at lower doses inhibitory and the mechanism of action involving the serotonergic system.

Pineal AVP and AVT have been examined in terms of their effects on sleep and behaviour (memory,learning) [Van Ree et al., 1978; and Walter et al., 1978] AVT has been demonstrated to effect sleep patterns in humans and is postulated as acting via the serotonergic pathway [Coculescu et al., 1979; Pavel et al., 1980 and Popoviciu et al ., 1982].

(3) NEUROPHYSINS

In the posterior pituitary, AVT and AVP are secreted with cysteine rich proteins identified by Reinharz et al., 1974 as neurophysins. Subsequently, immunoreactive neurophysins have been isolated from human [Legros et al., 1976] and rat [Reinharz et al., 1978] pineal

glands. However, the occurence of neurophysins in the pineal is not interpreted as products of the gland but rather it is postulated that they originate in hypothalamic nuclei and comigrate to the pineal with AVT and AVP.

(4) PROGONADOTROPHINS
HYPOTHALAMIC RELEASING-LIKE PEPTIDES

There are numerous reports indicating the presence of immunoreactive and/or bioactive luteinizing hormone-releasing like peptides (LHRH-like) in rat, bovine,porcine and ovine pineal glands [Redding and Schally, 1973; Dupont et al., 1974; Benson et al., 1983; White et al., 1974; Millar et al., 1981; and Kotaras et al., 1980]. The pineal LHRH-like peptides have been shown by some workers to differ from the hypothalamic decapeptides on account of molecular size and seasonal variations [Kotaras et al., 1980; King and Millar, 1981]. The presence of thyrotropin-releasing like peptides (TRH) in the pineal of some mammals has also been demonstrated both immunologically and biologically [Brammer et al., 1979; Wilber et al., 1976; Youngblood et al., 1979 and White et al., 1974]. These latter results on TRH-like peptides must be treated with caution since Kellokumpu et al., 1980; and Youngblood et al., 1979 revealed that no authentic TRH is present in the TRH-like complexes found in pineal extracts. Seasonal and circadian fluctuations were demonstrated by Brammer and co-workers 1979 in the levels of TRH-like pineal peptides, however the exact nature and mechanism of action of these putatative pineal hypothlamic like releasing peptides are not known.

(5) STUDIES ON OTHER PINEAL ACTIVITIES

Identitified by deAgostini et al., 1982, Angiotensin converting enzymes responsible for the conversion of Angiotensin I to Angiotensin II were found to display circadian rhythms with a peak at the end of the light phase of the 24 hour photoperiod [Nahmod et al., 1982]. Proposed function of renin-angiotensin peptides in the pineal includes regulaton of vasoconstriction and thus blood flow through the pineal Quay, 1958 and Rollag et al., 1978. Another proposal is that at least Angiotensin II may regulate the neurotransmitter (norepinephrine) release from the pinealocyte endings [Peach, 1977]. The pineal has also been linked to the secretion of mineralcorticoids [Johnson, 1981].

(6) CORTICOTROPHIN-ENDORPHIN DERIVED PINEAL PEPTIDES

The evidence for pineal alpha-melanocyte-stimulating hormone (MSH), ACTH, beta-endorphins, enkephalins and melanocyte-inhibiting factor (MIF) is reviewed by Vaughan 1984. All these peptides are derived from a common glycoprotein precursor found in the adenohypophysis [Fisher and Fernstrom, 1981]. Alpha-MSH displays a diurnal rhythm with its peak during the dark phase and is postulated as a modulator of indoleamine synthesis [O'Donohue et al., 1980]. through interaction with the noradrenergic stimulation of cyclic AMP formation [Sakai et al., 1976]. MIF has been identified in the rat pineal gland which displays circadian variations but with large individual variations and inconsistencies [Kastin et al., 1980]. .

(7) MISCELLANEOUS PINEAL PEPTIDES: S-ANTIGEN, RHODOPSIN, VASOACTIVE INTESTINAL POLYPEPTIDE, NEUROPEPTIDE-Y, OPIOID PEPTIDE, SUBSTANCE-P.

This group of pineal peptides are extensively reviewed by Ebadi et al., 1989. In their review, they provide data indicating that the peptides are also localized in the retina of several mammalian species. The pineal and retina have developed from the diencephalon and share a number of morphological, biochemical, biological and neurochemical properties. A summary of the common pineal and retinal neurochemical properties can be found in recent reviews by Ebadi et al., 1989; Gupta et al., 1988; Awad et al., 1988 and Takahashi et al., 1988. The reviews, discuss many aspects of these peptides including binding and receptor sites, genetic regulation and functional roles.

(8) SUMMARY

There is now compelling evidence to suggest that the pineal gland contains a large number of bioactive proteins and peptides, some displaying circadian and/or seasonal fluctuations. However, so far no bioactive peptides have been identified which are pineal specific. The bioactive peptides described were found in extrapineal tissues such as the hypothalamus, pituitary, and other brain tissues. In view of the large number of peptides and proteins alleged to be present in the pineal, it is not surprising that there is a degree of confusion as to their precise role in the biological functioning of this once vestigal organ.

Chapter Three

Pineal Indoleamines as Bioactive Secretions

1. Introduction

The pineal indoleamines have been at the centre of pineal research for the last three decades and have created a vast data base which is subject to frequent review [Ariens-Kappers, 1976; Ariens-Kappers and Pevet, 1979; Birau and Schloot, 1981; Cardinali, 1983; Quay, 1974; Reiter 1982; Wurtman et al., 1963; Armstrong, 1989; Goldman, 1983; Lewy, 1983; Klein, 1985; Reiter, 1991]. The discovery of the indoleamine melatonin in 1958 by Lerner and coworkers is identified as seminal in the genesis of the "modern era" of pineal research. Complete coverage of the extensive literature published over the last 35 years on melatonin is no longer realistic and the present review focuses on the regulation of melatonin synthesis, sites and mechanisms of its actions.

2. MELATONIN SYNTHESIS

The essential amino acid L-tryptophan (TRP) is the precursor of all indoleamines. The pathways in the synthesis of melatonin and other indoleamines is shown in Fig (6). The structure of melatonin has been determined by x-ray diffraction and comprises a planar benzene ring and an indole.

Tissues shown to actively synthesize melatonin are the pineal gland, retina, Harderian gland, human red blood cells, rabbit platelets, peripheral nerves, rat hypothalamus and gastrointestinal tract [Launay et

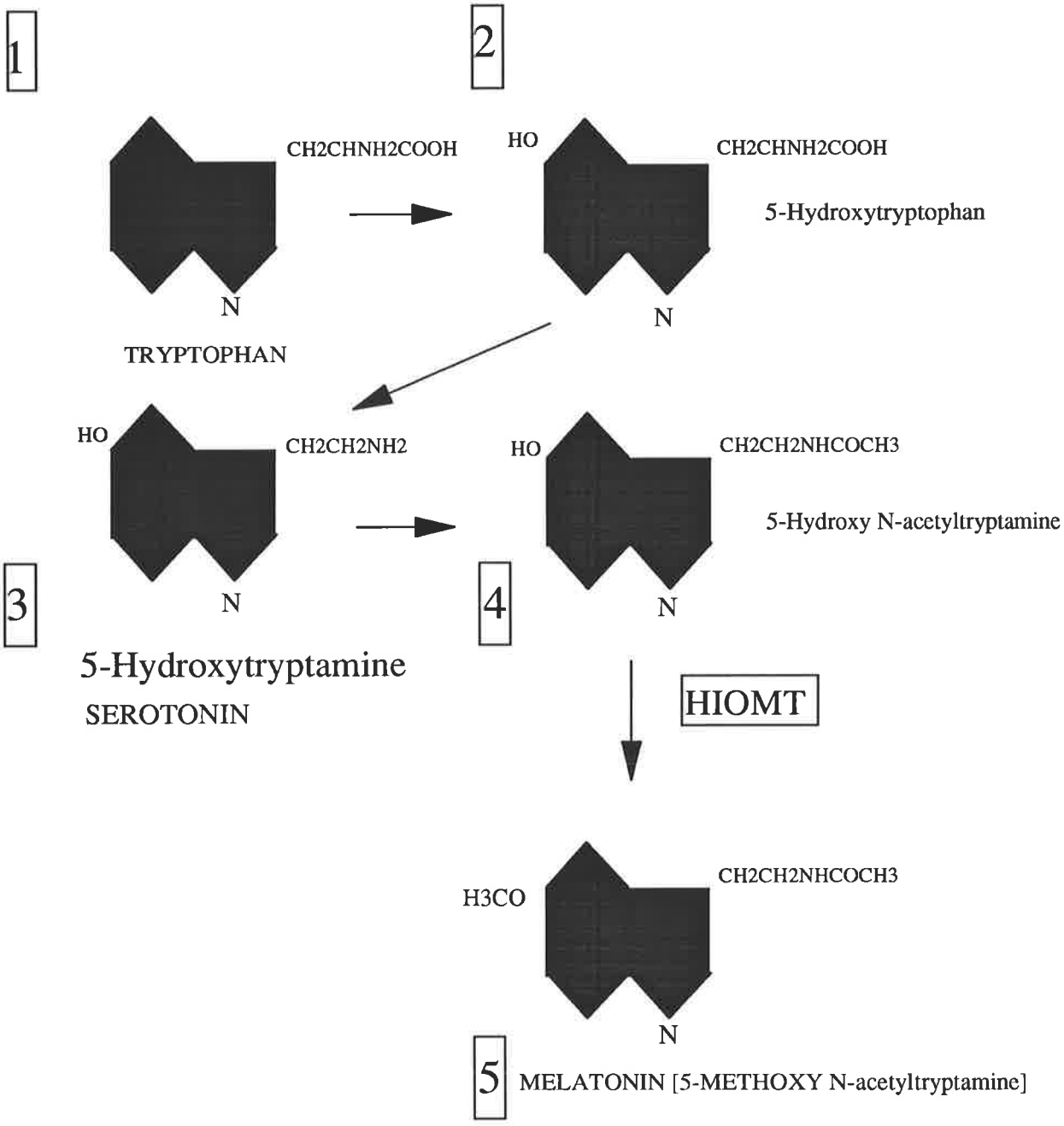

Figure 6. Biosynthetic pathway of melatonin

al., 1982; Ralph, 1981]. Fluids found to contain melatonin are the cerebrospinal fluid [Hewing, 1980; Welsh et al., 1989; Shaw et al., 1989], blood [Reiter et al., 1986, 1987], saliva [Vakkuri, 1985], ovarian fluid [Brzezinski et al., 1987], human amniotic fluid [Kivela, et al., 1989], seminal fluid [Bornman et al., 1989] and the anterior chamber of the eye [Yu et al., 1990].

3. PHOTOPERIODIC REGULATION OF MELATONIN SYNTHESIS

Early experiments demonstrated that melatonin synthesis by the pineal was regulated by the photoperiod [Wurtman et al., 1963]. The photoperiodic message was relayed to the pineal by sympathetic neurons [Kappers, 1960] arising from the superior cervical ganglion. Regulation was achieved through the control of essential enzymes involved in melatonin synthesis. Blinding and/or superior cervical ganglionectomy experiments abolished the effect of photoperiod on melatonin synthesis [Wurtman and Axelrod, 1965]. Reviews on circadian rhythms, melatonin biosynthesis, secretion and neuronal regulation are extensive [Moore et al., 1974; Goldman, 1983; Brainard et al., 1984; Klein, 1985; Reiter et al., 1990]

4. THE BIOACTIVE ROLES OF MELATONIN

Most of the literature on this topic of melatonin relate it to circadian rhythms and the photoperiod and to its correlation with the reproductive and neuroendocrine cycles.

Although there is considerable species variation in indoleamine secretions, rhythms are seen in all species and form the basis of markers

of photoperiodic time in seasonal reproductive mammals [Reiter, 1969;1972;1973;1980;1991]. Melatonin administered at critical time points during the 24 hour photoperiod can effect the entire gonadal cycle [Bittman, 1978; Tamarkin et al., 1976]. Blinding or pinealectomy prevents some species from responding to changing photoperiods [Hoffman and Reiter, 1966;Reiter, 1968]. Current hypotheses attempting to explain mechanisms of photoperiodic effects on melatonin are reported in recent reviews, Reiter 1990;1991. As discussed previously, much of the literature has focused on the pro- and anti-gonadotrophic effects of melatonin on a number of species [Reiter, 1991; Stetson and Whytmyre, 1984; Reiter et al., 1991], but these effects are species, age, sex, nutrition as well as time and dose related and although some trends are emerging the data are not simply interpreted.

In addition to a role in reproduction there is compelling data implicating melatonin in the regulation of other endocrine organs such as adrenal [Vriend, 1983; Lang and Sizonenko, 1988] and thyroid [Vriend and Steiner, 1988]. Melatonin has also been tentatively linked to several behavioural disorders such as depression [Wetterberg et al., 1990], schizophrenia [Miles and Grey, 1988], sleep disorders [Sack and Lewy, 1988] and even "jet-lag" [Arendt et al., 1987]. Possible roles for melatonin to cancer [Bartsch et al., 1990] and immunological phenomena [Pierpaoli and Maestroni, 1987] have been added to the list and potential clinical roles identified.

5. SITES OF MELATONIN ACTION

Presumptive target organs responding to melatonin are shown in Fig.(7). The pineal itself may also be a target organ as melatonin uptake is rapid [Quay, 1965, 1969],but this finding has not been explained..

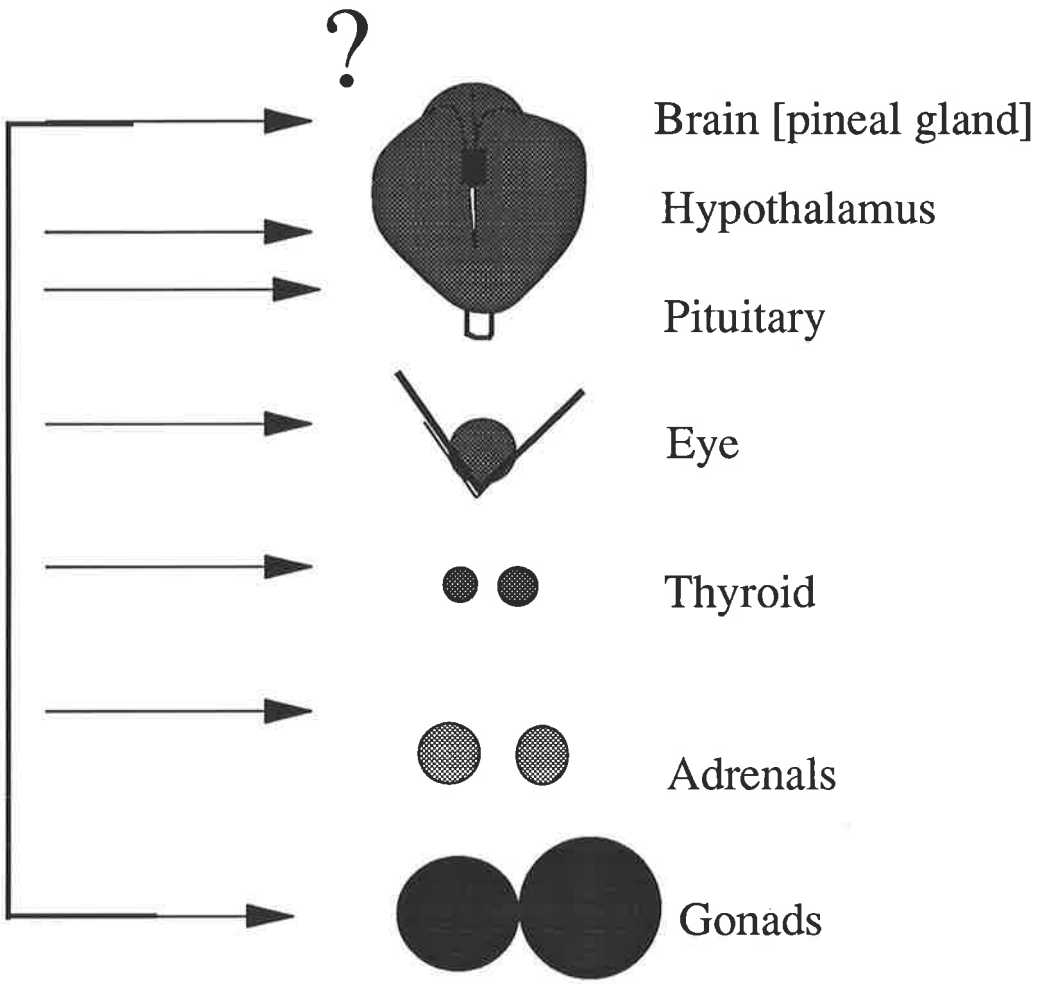

Figure 7. Presumptive pineal hormone target organs

In 1978, Cohen and coworkers provided evidence for melatonin specific binding site in rat and hamster ovaries, uteri, testes, liver and eyes. Cardinali et al., 1979 found melatonin binding activity in crude membrane fractions of bovine medial basal hypothalamic tissue. Although, these early reports disagreed on aspects of the cellular localization and tissue distribution of the presumptive melatonin receptors they provided important ground work for extensive studies [Cardinali et al., 1985; Dubocvich, 1988; Morgan and Williams 1989; Stankov and Reiter, 1990]. Melatonin binding sites have now been localized in a large number of neural and extraneural tissues. For example [^{3}H]-melatonin uptake sites have been identified in the central nervous system [including the pineal and hypothalamus] of the lamprey and lizard [Joss, 1981]. This has been confirmed by in-vitro autoradiographic studies using 2-[^{125}I]-melatonin [Vanecek et al., 1987; Weaver et al., 1988]. More recently, important melatonin binding sites have been localized in the pars tuberalis [Williams and Morgan, 1988] and SCN [Laitinen and Saavedra, 1990].

The localization of melatonin receptors in the SCN is considered of particular importance because it is the site of a major circadian "clock" involved in pineal melatonin rhythms [Davis, 1981]. However, there is some evidence suggesting that the SCN melatonin receptors are not involved in mediating its effects on reproduction since bilateral SCN ablation did not prevent melatonin-induced gonadal regression [Maywood et al., 1990]. The functional importance of melatonin binding site in the pars tuberalis remains unknown although there is some evidence to suggest gonadotrophic regulatory functions [Williams et al., 1989]

6. MECHANISM OF MELATONIN'S ACTION

Receptor sites for melatonin at both hypothalamic and pituitary levels are now acknowledged and several mechanisms have been advanced to explain how melatonin acts at these sites. These mechanisms involve interaction with other receptors such as serotonin receptors [Anton-tay and Wurtman, 1969], estradiol receptors [Roy and Wilson, 1981], benzodiazepine receptors [Marangos et al., 1981], and regulators of functions of endocrine cells as suggested by the findings of specific melatonin receptors in the medial basal hypothalamus [Cardinali et al., 1978, 1979], hypothalamic tubulin synthesis and axonal transport [Cardinali et al., 1975], monoamine oxidase inhibition [Urry and Ellis, 1975] dopamine regulation [Zisapel and Laudon, 1982], gamma-Aminobutyric acid regulation [Anton-Tay, 1974; Rosenstein et al., 1989] neuropeptide regulation [Kao and Weisz, 1977; Richardson et al., 1981; Hollander et al., 1978], prostaglandin synthesis [Cardinali et al., 1980; Leach et al., 1982], cyclic AMP and cyclic GMP synthesis, neuronal firing rates and catecholamine regulation [Demaine and Kann, 1979]. Those mechanisms affecting protein synthesis and secretion particularly at the pineal and other neural tissue such as the hypothalamus are pertinent to this thesis and will be reviewed here.

Part Two

General Methods

Chapter Four

Melatonin and Pineal Proteins

1. Introduction

Several early experiments supported the notion that exogenously administered melatonin was rapidly taken up by the pineal gland [Fiske and Huppert, 1968; Cady and Dilman, 1971], but its action within the pineal remains obscure. The effects of melatonin on pineal protein synthesis has not been previously documented. The pineal content of melatonin varies with the photoperiod [Fiske and Huppert, 1968], but whilst the protein content of rat pineal glands also vary diurnally [Nir et al.,1971; Merritt and Sulkowski, 1969] the maximum is found to be in the late afternoon of the light period, a time when melatonin content is suppressed. The peak in pineal protein content follows those of RNA content and nuclear size. Factors shown to affect pineal protein content and or its diurnal fluctuations are: inhibiting agents such as N-methyl-3-piperidyle benzilate [Merritt and Sulkowski, 1969], and stimulating agents such as p-chlorophenylalanine which causes an increase in morning pineal protein content in the rabbit [Smith, 1972], 17beta-estradiol also increases early morning protein content in female rat pineals [Nir et al., 1970]. Enviromental factors such as light and temperature have also been shown to influence pineal protein content, however their effects have been attributed to stress related mechanisms [Quay, 1974]. In studies on the incorporation of [^{14}C]-tryptophan into pineal indoles about 2% of label was incorporated into proteins [Wurtman et al., 1969]. Norepinephrine was shown to increase the intracellular content of radiolabeled tryptophan which was followed by increases in

protein and indoleamine synthesis. However, when pineal glands had increased levels of tryptophan, norepinephrine did not enhance [^{14}C]-protein synthesis. Other labeled amino acids such as [^{14}C]-leucine and [^{14}C]-methionine, did not show effects on protein synthesis. The involvement of cAMP in pineal protein synthesis is involved but precise mechanisms are not yet known. Indoleamine synthesis also involves norepinephrine and cAMP mechanisms in regard to increased levels of enzymes such as tryptophan hydroxylase and N-acetyltransferase. When the effects of indoles on pineal protein synthesis from [^{14}C]- tryptophan were examined by Wurtman et al., 1969, it was shown that melatonin in doses 10^{-4} and 10^{-5} M reduced protein synthesis by adult female rat pineals in organ culture by up to 30% but were considered not statistically significant.

Other evidence for pineal protein synthesis came from in-vitro autoradiographic studies using [^{3}H]-fucose incorporation and chromatography. A high percentage of pineal tissue proteins are glycoproteins with molecular weights in the range 20,000-200,000 daltons. The secreted proteins when chromatograpged showed that 50% were in the molecular weight range 30,000-50,000 daltons [Lott et al., 1971]. Incorporation of labeled fucose into glycoproteins was not affected by norepinephrine, however when another labeled product N-acetylglucosamine was used, norepinephrine stimulated an increase in the incorporation of this label into glycoproteins [Quarles, 1972]. The effects of melatonin on these proteins were not studied.

In 1972, Smith found four pineal specific protein bands on polyacrylamide electrophoresis gels confirming earlier claims by Pun and Lombrozo, 1964. One of the four pineal specific protein bands was shown to contain tryptophan and was localized in a specific pinealocyte cytoplasmic granule. Furthermore, diurnal variations in the content of

this tryptophan protein band was demonstrated with a peak at night. Inhibitors of tryptophan-hydroxylase and thus of melatonin synthesis such as p-clorophenylalanine resulted in an increase in tryptophan pineal protein specific bands which may reflect a metabolic diversion of tryptophan from indoleamine to protein synthesis [Quay et al., 1974], or build-up in a precursor pool. These results suggest the pineal gland synthesis of proteins may be dependent on melatonin synthesis, however detailed analysis of the interrelationship of the pineal synthesis of these two compounds was never fully evaluated.

2. MELATONIN AND PROTEIN SYNTHESIS IN EXTRAPINEAL SITES

Extrapineal effects of melatonin on protein synthesis include inhibition of hypothalamic tubulin [Cardinali and Freire, 1975; Prevedello et al., 1979], microtubule protein [Cardinali, 1980], nerve growth factor [Perez-Polo et al., 1978] and hypothalamic actin, [Ioanna et al., 1990], inhibition of calmodulin and phosphodiesterase activity [Benitez-King et al., 1991]. In a very recent study melatonin was shown to inhibit the incorporation of labeled amino acids into rat hypothalamic proteins in-vitro. The mechanism of melatonin's action was thought to involve either transcription or translation since mRNA levels were inhibited by melatonin [Ioanna et al., 1990]. Furthermore, these workers found that the effects of melatonin were time dependent and time related in rats. The inhibitory effect of melatonin [injected for 10 days at a dose of 100 micrograms /kilogram] on actin mRNA was found only in the morning, however the inhibition of [^{35}S]- methionine incorporation into actin decreased after morning or evening administration of melatonin. That melatonin administered in the evening affected incorporation of label into

protein but not mRNA levels was interpreted by these workers as a general effect on protein synthesis rather than a specific effect on actin. An alternative explanation offered was that melatonin had a post-translational effect on actin not accompanied by mRNA changes. In another totally unrelated system this type of effect is noted e.g. the synthesis of hepatic albumin during fasting is associated with disaggregation of membrane-bound polyribosomes but mRNA levels for the protein remaing unchanged [Yap et al., 1978]. Melatonin administered as single injections in the morning or in the evening did not affect hypothalamic actin mRNA levels or incorporation of labeled amino acid into proteins. This evidence suggested that the melatonin effect was not a direct one nor could it be explained as an effect on mitosis since total hypothalamic RNA and DNA levels were not changed by melatonin [Iovanna et al., 1990]. In other studies by the same workers, actin mRNA levels and incorporation of labeled amino acids into proteins varied with a 24 hour period in the adult male rat hypothalamus with a maximum mRNA level in the early part of the light phase and a maximum in the incorporation of labeled amino acids into actin in the midlight phase. These results suggest that melatonin interferes with structural protein biosynthesis and has been shown by others to occur at both neuronal and extraneuronal sites [Goldman, 1983; Kelly 1988].

3. Conclusion

The signal transduction mechanisms that mediate melatonin's effects on protein synthesis at their effector sites are only just beginning to be understood. That not much attention has been given to possible autocrine effects of melatonin on protein synthesis and secretion in the past may be a reflection of the misinterpretation of early work suggesting

that melatonin's actions may be mediated by pineal proteins. Furthermore, the possibility that the photoperiod effects on physiological events is mediated via melatonin's regulation of pineal protein synthesis and secretion becomes a viable hypothesis. The explanation of melatonin's mechanism(s) of action at extrapineal regulatory sites by protein inhibitory mechanisms whether at a transcriptional or translational level becomes more acceptable.

Chapter Five

Electrophoresis

I One dimensional electrophoresis

Tissue and medium samples were analyzed by one and two dimensional sodium dodecyl sulphate polyacrylamide slab gel electrophoresis [PAGE} essentially as described by Laemmli, 1970.

A. Reagents

Reagents for electrophoresis were electrophoresis grade acrylamide, bis-acrylamide, ammonium persulphate, TEMED [NNN'N-tetramethylenediamine], beta mercaptoethanol and bromophenol blue were purchased from Biorad, Richmond, CA, U.S.A; sodium dodecyl sulphate [SDS of highest purity] British Drug House, Tris(hydroxymethyl) aminomethane [Trizma-base] and glycine from Sigma Chemical Co; glycine and sec-butanol from Fisher.

B Equipment and Supplies

Glass plates, 20cm x 20cm, teflon spacers sample combs, electrophoresis chamber and power supply were from Biorad. Other equipment used were 50 ml beakers (2), Hamilton syringes (50 and 100 µl), syringes (20 ml) and 0.22 µm syringe filters.

C Stock Solutions

1. 30% (w/v) acrylamide and 0.8% bis-acrylamide
2. Running gel buffer stock: 1.5M Trizma base,0.4% SDS pH 8.8, at 23^0C
3. Stacking gel buffer stock: 0.5M Trizma base,0.4% SDS, pH 6.8 at 23^0C
4. Tank buffer: 0.025M Trizma base, 0.192M glycine, 0.1% SDS, to pH 8.3, but not with HCL, NaOH or salt
5. Sample buffer: 0.0625M Trizma Base, 2% SDS, 10% Glycerol, 5% beta-mercaptoethanol

D. Sample preparation for One-Dimensional SDS-PAGE

1. Unless otherwise stated, all samples were prepared from fresh tissue and medium. The amount of solubilization buffer added was optimized for each type of tissue and medium sample, in general 200 µl of sample buffer was added for every 100 µg of protein [determined by the method of Lowry et al., 1951].
2. The tissue samples were homogenized in a glass-glass homogenizer before heating for 10 mins at 95^0C in a waterbath and the medium samples were heated for 5 min at 95^0C. Screwcap tubes were used to ensure the samples did not evaporate during heating.

E Gel Casting Procedure

1. The plates were assembled as in Fig. (1) and were always washed and dried prior to assembling to ensure that any dust or contamination that may affect the running and detection of proteins was copletely removed.

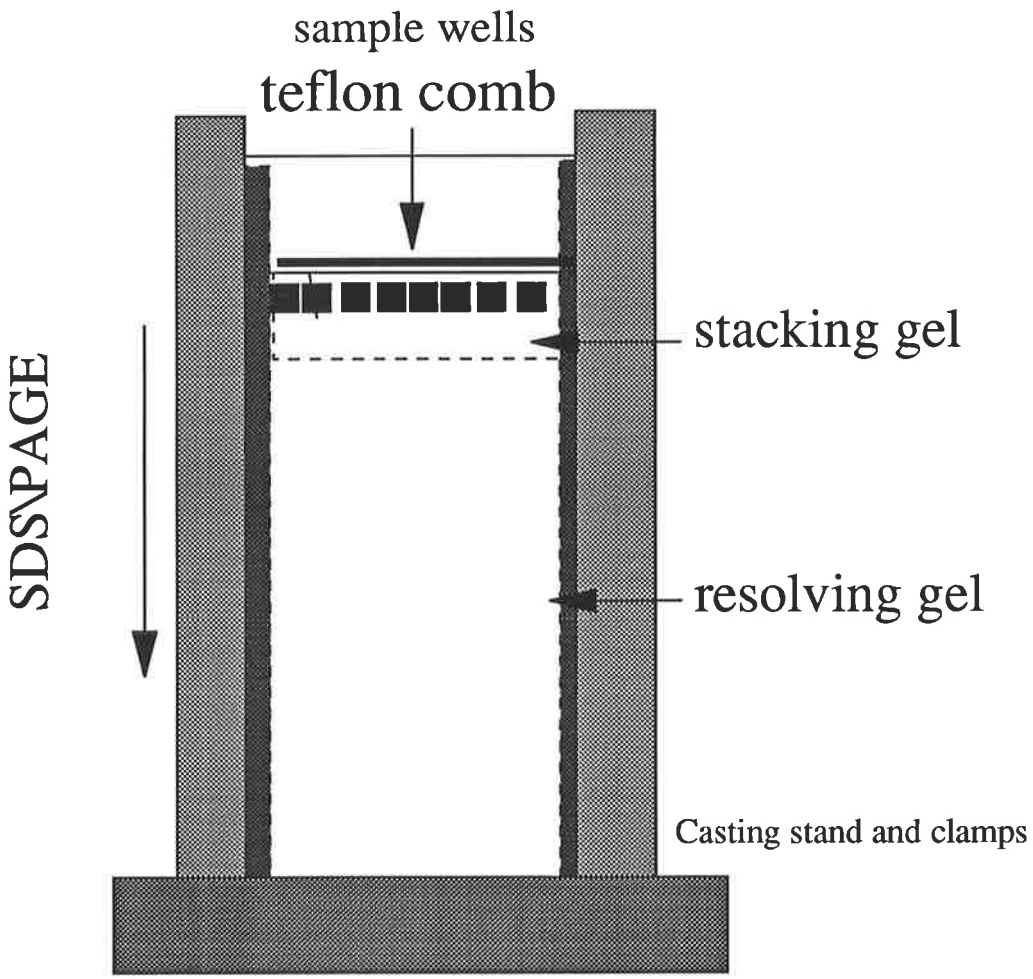

Figure 1. Assembly of electrophoresis apparatus

Protean II Electrophoresis Apparatus [from Biorad]

Generally, the procedure used to clean the plates was: soaked overnight in detergent, rinsed extensively with tap water, then with deionized water and finally soaked for about 15 minutes in 95% ethanol. Once dry, the plates were either assembled and used or stored in closed containers away from dust and handling. Contamination can cause improper polymerization of the gel and artifacts upon staining.

2. The majority of gels prepared were 10% for the running or separation gel and 2.5% for the stacking gel. A 10% running gel was prepared by mixing 9.5 ml acrylamide/bis-acrylamide stock (30:0.8), 7.1 ml running gel buffer pH (8.8), 11.8 ml H_2O, 105 µl 10% ammonium persulphate and finally 15 ml TEMED. Prior to the addition of ammonium persulphate and TEMED, the gel solution was filtered through a 0.22 µm filter and vacuum degased.

3. Once degassed, ammonium persulphate followed by TEMED was added and mixed gently by swirling. The mixture was then poured down one edge of the spacer of the assembled plates using a syringe attached to a 0.22 µm filter (the double filtration step helped eliminate any impurities and was found to enhance both the polymerization and the quality of the stained final gel). The gel solution was poured to within 4 cm from the top of the glass plates.

4. The gel solution was then carefully overlayed with water saturated sec-butanol and allowed to polymerize usually for 45-60 min. The sec-butanol step was important for the resolution of the proteins. Once the gel had polymerized the sec-butanol was carefully removed, the surface of the gel rinsed several times with water and allowed to drain for 2-5 min.

5. The preparation of the stacking gel was always the same regardless of the concentration of the running gel.The stacking gel was prepared by combining 1.9 ml acrylamide/bis-acrylamide (30:0.8), 3.1 ml stacking gel buffer pH 6.8, 7.5 ml H_2O, 60 µl ammonium persulphate and 10 µl TEMED. As for the running gel the solution was filtered and degassed prior to the last two additions.

6. The degassed mixture had ammonium persulphate and TEMED added and the carefully mixed solution was layered on top of the running gel. A teflon comb (see Fig.6) was inserted in the stacking gel solution taking care not to trap air bubbles and left to polymerize for 15-30 min. The comb was used to form the wells in which the samples are placed and come in various sizes (10-20 sample wells about 2 cm deep).

7. The gel was assembled in the chamber and tank buffer was poured in both the upper and lower chambers. Air bubbles that may have been trapped were removed from the base of the gel and from the sample wells.

8. Samples were loaded with the aid of a Hamilton syringe and electrophoresis was carried out at 100 mA/gel constant current (unless otherwise stated). The bromophenol blue within the sample served as a front marker and once this had reached the bottom of the gel the electrophoresis run was stopped, the gel removed and processed for staining.

F. Staining of gels

Proteins in the polyacrylamide gels were detected by a silver-based colour staining technique modified from Sammons et al.,(1981). This technique was chosen because it had been shown to be highly sensitive [a 100 fold increase over Coomassie Blue methods Merril et al., 1979; Switzer et al., 1979] and the colour provides an added dimension for identifying proteins in gels. For a more detailed account on this technique and its advantages see Dunbar (1987).

(1). Reagents

The following reagents were purchased from Sigma : silver nitrate, sodium hydroxide, sodium bicarbonate, double deonized water and formaldehyde.

(2) Equipment

Pyrex glass dishes (12 x 17 x 3 inches) for 8 x 8 inches gels were used for the staining procedure. Plastic dishes were avoided for silver staning procedures since they are known to absorb many impurities which will show up as artifacts on the stained gels. A variable speed shaker and glass storage bottles for the reagents and stock solutions were also used.

(3) Stock solutions

The fixative solution was made up prior to use from 5% acetic acid and 50% ethanol in water.

Silver stock was prepared by desolving silver nitrate 0.19% in deionized water prior to use and stored in extremly clean glass bottles.

A reducing solution made up from 3 % sodium hydroxide pellets in water and 0.75 % formaldehyde [added to the sodium hydroxide solution just prior to use]. It was important to keep to the same batch throughout the experiments in terms of source and age factors affecting reproducibility and colour effect of the staining.

Colour-enhancing solution was prepared by disolving sodium bicarbonate 0.7 % in water.

(4) Procedure

Gels were removed from the electrophoresis apparatus, placed in fixative solution in a volume 5.5 times that of the gel volume. The ratio of fixative to gel volume is important in ensuring that the fixative penetrated the gel. The gels were fixed overnight after which they were washed in three one hourly changes of water before incubating with the silver stock for one hour at room temparature whilst shaking at low speeds [silver stock solution : gel solution in ratio 1 : 3]. Following this the gel was washed quickly once for 20 seconds with water to remove excess silver grains which if left produce black grains on the gel surface. The reducing solution was added next [reducing solution : gel solution ratio 5.5 : 1] and left for 10 min while shaking gently at the end of this time colour formation was seen. Protein spots seen by five different colours against a yellow background stain of the entire gel were

enhanced further by placing the gels in sodium carbonate [5.5 : 1 ratio] for 3 one hourly periods. The yellow background which forms within the first 2-3 min of adding the reducing solution does not interfere with the interpretation, visualization or photography of proteins on the gels.
All gels were photographed using colour print or slide films and some gels were dried and stored or processed further for autoradiography [see later section materials and methods].

II Two dimensional electrophoresis

1. ISOELECTRIC FOCUSING THE FIRST DIMENSION

A. Reagents

Urea [ultrapure grade], acrylamide, bis-acrylamide, Nonidet P-40, ammonium persulphate, TEMED were purchased from Biorad. sodium hydroxide from Sigma, phosphoric acid from Fisher.

B Equipment

Cylidrical glass tubes were prepared from long lengths of tubing 1.5 mm internal diameter cut to 18 cm lenghths (12).
The tubes were thoroughly cleaned by soaking in detergent overnight, rinsing with distilled water and soaking in ethanol (95%) for 15 min, allowed to dry and kept free of dust and contamination until ready for use. The gel casting apparatus and tubes are shown in Fig (2a). The tube gel electrophoresis apparatus shown in Fig (2b) was purchased

A. Glass tubes B. Casting stand with tube gels. C. Sqeeze bottle with distilled water

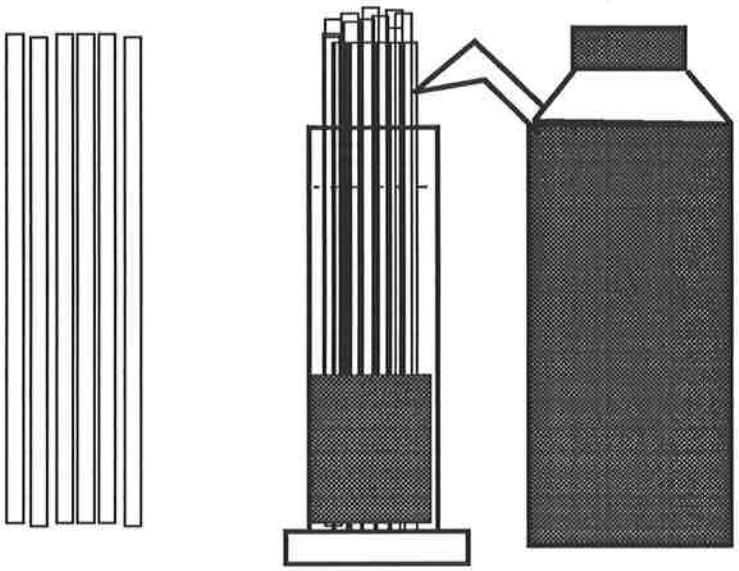

Figure 2[a]. Loading of tubes for isoelectric focusing

A. Tube stand and electrodes. B. Cover and lower buffer tank.

Upper Buffer Resevoir

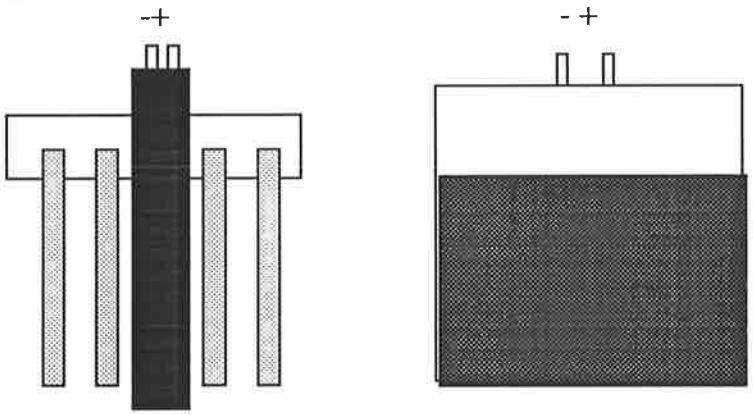

Figure 2[b]. Isoelectric focusing appparatus used to run tube gels
[Model 175 fro Biorad]

from Biorad and the power supply was the same as that used in the one dimensional PAGE.

C. Stock Solutions

1. 30% (w/v) acrylamide and 1.8% bis-acrylamide in water.
2. Upper cathode electrode buffer was prepared by degassing 200 ml H_2O and adding to it 0.4 ml of 10 N NaOH after degassing to avoid Na_2CO_3 formation.
3. Lower anode electrode buffer was 2.5 litres of 0.085% phosphoric acid in H_2O. which did not have to be degassed.
4. Second-dimension equilibration buffer: 0.125 M Tris base, 2% SDS, 10% glycerol, pH 6.8 at 23^0 C, bromophenol blue 0.1%, filtered through 0.2μm syringe filter and frozen in 15 ml aliqouts. 0.5% beta-mercaptoethanol was added prior to use.

D. Procedure for casting IEF tube gels

1. The tubes were held together with a rubber band and assembled as shown in Fig. (2a). The following solutions were mixed in a 50 ml glass beaker: urea 8.25 g, ampholytes 0.75 ml; [pH 3.5-10 Pharmacia], acrylamide/bis-acrylamide stock 2.0 ml, distilled H_2O 6.0 ml. this solution was mixed filtered through a 0.22 μm filter, and degassed. Finally 0.3 ml of Nonidet P-40, 70 μl ammonium persulphate and 10 μl of TEMED were added.

2. The gel mixture was then poured into the casting device Fig 2 (a) and the assembled tubes gently lowered into the solution. Water from a plastic squeeze bottle was run gently down one side of the casting device and the gel solution was pushed gradually into the tubes to within 2cm from the top. The volume of the gel solution was always adjusted so that there was sufficient to fill the 12 tubes with a small amount remaining in the bottom of the casting device. The gels were allowed to polymerize for at least one hour.

3. Once polymerized the tubes were removed separated carefully and any excess gel protruding from the bottom was cut off with a blade taking care not to cause the tube gel to be forced out of the tube. After rinsing with distilled water the tube gels were assembled into the electrophoresis chamber. Upper electrode and lower electrode buffers were added and the tubes were prefocused at 200 V for one hour. This step helped remove the sulphate ions from the ammonium persulphate which interfered with protein focusing.

4. Protein samples were loaded with the aid of a 50 µl Hamilton syringe and usually up to 30 µl of sample containing no more than 150 µg total protein [determined by the method of Lowry et al., 1951] was used.

5. The focusing was usually [unless otherwise stated] conducted at a fixed voltage for a total of 10,000 volt-hours [17 hrs at 500 V, followed by 2 hrs at 800 V].

6. After focusing, the gels were removed from the glass tubes with the aid of a syringe filled with water and a fine gauge long needle.

7. The tubes were equilibrtaed in 1 ml equilibration buffer per gel for 15 min which removed ampholytes and urea as well as recoating the proteins with SDS.

2. SDS-PAGE IN THE SECOND DIMENSION

The procedure outlined for the one-dimensional SDS-PAGE constitutes the second dimension of two-dimensional SDS-PAGE with slight modifications. Reagents, equipment, and stock solutions have been described and are identical as for 1D- SDS/PAGE. When casting the gel into the glass plates, the formation of sample wells was omitted and the running gel was poured to within 3 cm from the top. The tube gels were placed on top of the stacking gel taking care not to damage them [Fig 3] and fixed with 1 ml of overlay- agarose [0.025 M Trizma base, 0.192 M glycine, 0.1% SDS, 0.5% agarose]. The electrophoresis was carried out in the same chamber as for the first dimension using the same running buffers and running conditions. The bromophenol blue dye present in the equilibration buffer forms a front and gives an indication of the time needed for the separation to be completed. The subsequent procedures of fixing, staining and drying were those described for 1D- SDS/PAGE. Upon staining, all two dimensional gels were photographed and either stored or further processed for fluorography [see next section].

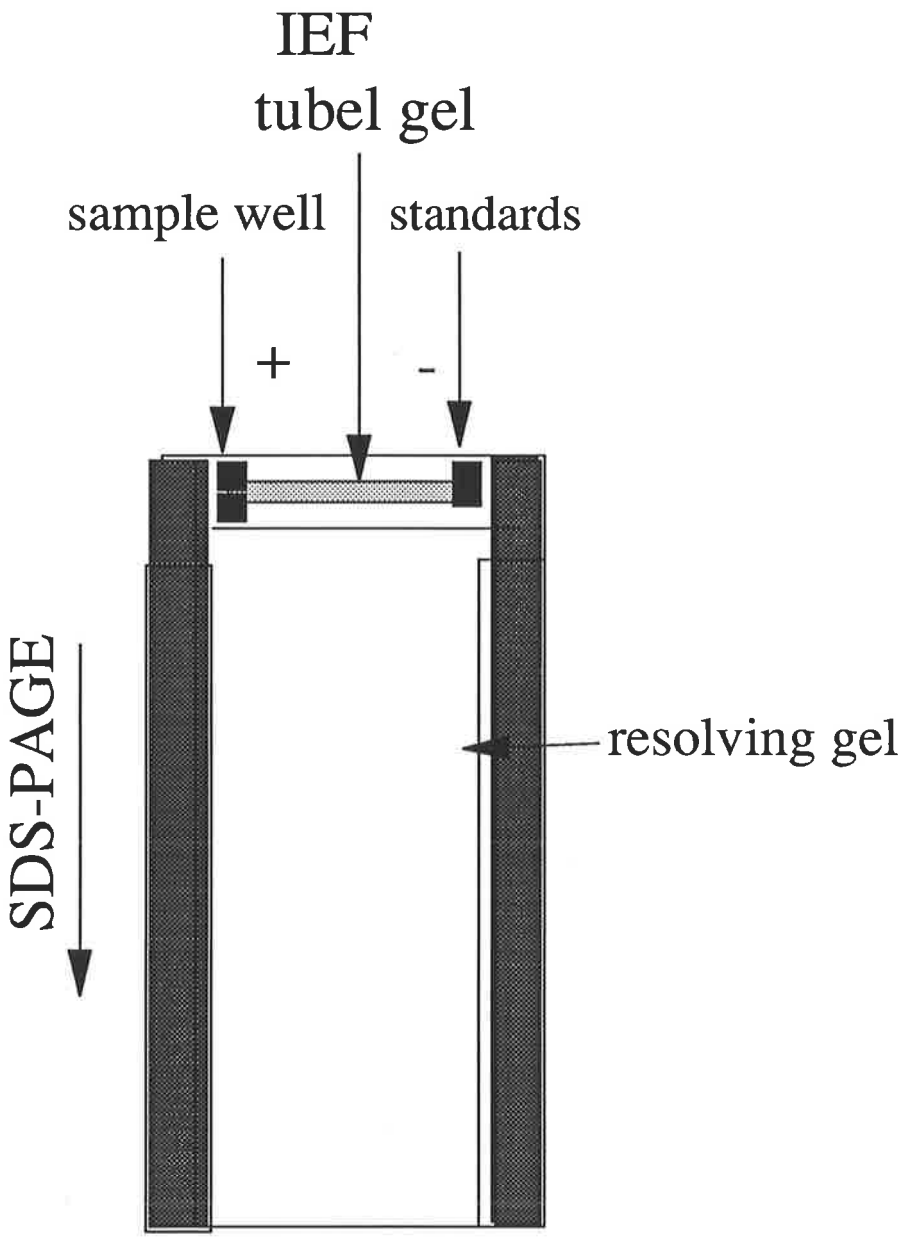

Figure 3. Assembly of plates for the 2nd dimension
plates assembled as in one dimensional SDS/PAGE
[without the multiple sample wells]

Chapter Six

Metabolic Labelling of Proteins In Organ and Tissue Culture

A. Reagents

1. L-[^{35}S]methionine, >800 ci/mmole, translation grade (Amersham)
2. ^{14}C-labeled amino acids (Amersham)
3. Minimum Essential Medium (modified) with Earle's salts with and without glutamine and methionine (Flow Laboratories)
4. L-Glutamine (Sigma)
5. 5% CO_2/95%O_2 gas mixture
6. Reagents for one and two dimensional SDS-PAGE (as above)

B. Equipment and Supplies

1. Sterile multi well organ and tissue culture dishes
2. Incubator
3. Laminar flow cabinet

C. Procedure for Labeling Proteins in Organ Culture

1. EMEM media warmed to 37^{0}C to which was added L-glutamine (4.35 mg/ml) and L-[^{35}S] methionine (1 mCi/100 ml). This was filtered through a 0.22 μm filter to maintain the media sterile.
2. O.1 ml/ well EMEM supplemented with methionine but without label was used in the initial incubation period. Fresh pineal glands were

placed individually/well and typically 2 mg of hypothalamic and cortical tissue per 0.1ml of medium was used in the incubations.

3. After an initial incubation period of 24 hr the pineal glands and tissues were placed in EMEM without methionine and labeled with L-[^{35}S]methionine (0.01 mCi/1ml) for unless otherwise specified 6 hr. Control tissue and medium that was incubated in the absence of label was always included in the preparations

4. At the end of the incubation periods the tissues were separated from the medium and processed for one and two dimensional electrophoresis and fluorography.

D. Procedure for preparation of labeled medium for SDS-PAGE

1. Immediately following labeling the medium was processed for SDS-PAGE by treating it with 55% ice cold acetone and resuspending the precipitated proteins in 50 µl of lysis buffer [2% SDS, 5% beta-mercaptoethanol, 20% glycerol, 2% ampholytes pH 3-10, 2% Nonidet P40].

2. Typicallly 30 µl aliquots of the medium sample preparation containing 6 x 10^5 cpm and 40 µg of protein were run on one and two dimensional SDS-PAGE.

3. All gels were silver stained, photographed, dried and processed for fluorography (see below).

E. Preparation of labeled tissues for SDS-PAGE

1. All tissue labeled were subsequently processed for one and two dimensional SDS-PAGE. The tissues were homogenized in the lysis buffer (100µl) and proteins precipitated with ice cold acetone (55%),

resuspended in 100 µl of lysis buffer and stored at -20^{0}C until analysed by one and two dimensional SDS-PAGE usually within a few days of preparation.

2. All tissue sample gels were silver stained, photographed and processed for fluorography.

D Fluorography

1. Prior to fluorography, stained gels were left overnight in 2% glycerol 10% acetic acid in water solution which helped prevent cracking of the gels upon drying.

2. Amplify was purchased from Amersham and used to soak the gels following silver staining. Usually 20 min in 300 ml of Amplify solution whilst gently shaking was sufficient. The gels were removed and dried for 2 hr at 80^{0}C on a gel dryer (Biorad).

3. Once dried the gels were placed in autoradiography cassettes with intensifying screens and Hyperfilm MP X-Ray film (Amersham) and placed at -70^{0}C for periods of between 5-10 days after which the films were developed in an automatic X-ray developing machine.

Part Three

Establishment and Evaluation of Techniques

Chapter Seven

Assessment of Two Dimensional Electrophoresis

1. Abstract

The development of a two-dimensional gel electrophoresis system [2D-SDS/PAGE] is described that provides superior performance in terms of resolving power and reproducibility. Isoelectric focusing [IEF] the first part of 2D-PAGE was performed as described in the previous section [chapter 6]. The majority of pineal proteins were found to focus in the pH range of 4 - 7.5. Factors found to be critical in the separation of proteins were conductivity, temperature, and quality of reagents. It was also shown that the maximal amount of information about proteins were obtained from large format gels [20cm X 20cm].

Analysis of the 2D-PAGE silver stained pineal cellular protein patterns by computerized image analysis showed that the molecular weights of polypeptides differed by approximately 300 daltons between gels, whilst the variation in isoelectric points waas in the order of .05 pH units. Computerized image analysis of the 2D-PAGE gels revealed as many as 1000 major and minor polypeptides could be repeatedly detected in the molecular weight range of 14,000 - 200,000 daltons and pI 3.5 - 10. This figure was increased dramatically [1.5x] when analysing fluorographs of ^{35}S-methionine labelled pineal proteins.

2. Introduction

Comprehensive evaluations of pineal protein secretions by 2D-SDS/PAGE is lacking. Extensive applications of this powerful technique to other biological fluids and tissues [reviews by Anderson etal,1982; Dunbar, 1985; Patton et al., 1990] has indicated that it would be highly applicable to assessing of pineal secretions and more important to secretions from single glands which would enable a more accurate assessment of the nature of pineal bioactive proteins.

Conventional one-dimensional SDS/PAGE and one-dimensional IEF are only capable of resolving approximately 100 of the most abundant proteins in a complex sample. Both techniques when employed in 2D-SDS/PAGE allows the detection of a greater number of proteins and provides information about both the relative molecular size and charge of the separated proteins. Every band on a one-dimensional SDS/PAGE gel may represent between 15 - 20 different polypeptides when separated by 2D-SDS/PAGE. In the past numerous problems were encountered in various aspects of the technique which prohibited its routine use [Dunbar 1985].

In this chapter high resolution 2D-SDS/PAGE in conjunction with sensitive color based silver staining and fluorography were optimized and assessed for their applicability to the assessment of pineal proteins secreted from individual glands in-vitro. Pineal protein data bases from which important pineal bioactive proteins could be identified, isolated, purified and sequenced would be a definite useful application of this powerful tool once established.

3. Materials and Methods

Pineal glands from male Hooded Wistar rats [150g] housed in a enviroment with lighting schedule 14:10 [L:D] food and water ad lib, were removed and individually cultured in EMEM as described in chapter 7. The secreted and cytosolic proteins were analysed by one and two dimensional SDS/PAGE [see chapter 5].

(1) Studies evaluating gel size.

In these studies, the influence of gel size on pineal protein resolution was assessed by using 3 tube sizes for running the IEF and 2 slab sizes for running the second dimension. Tube gels were run on 6cm x 1.5mm i.d., 12cm x 1.5mm and 18cm x 1.5mm. Slab gels of 8cm x 10cm x 1.5mm thicknes and 20cm x 22cm x 1.5mm were used.

(2) Studies evaluating buffers

To evaluate the role of buffer on the time required to perform the second dimension electrophoretic separations, sodium acetate was added to a final cocentration of 0.15M to the standard 0.025M Tris-base, 0.192M Glycine, 0.1% SDS, pH 8.3 running buffer and used as the anode buffer. The cathode running buffer consisted of 0.025M Tris-base, 0.192M Glycine. 0.1% SDS, pH 8.3 and was not altered by adding sodium acetate. The conductivity of the buffers was altered by these manipulations which could also be adjusted by NaCl.

(3) Studies evaluating the protein detection methods

Coomassie blue, silver staining [color-based] and fluorography were performed [see chapters 5 and 6] and the resulting one and two dimensional separation of pineal proteins compared by computerized image analysis . The pI's and molecular weights were determined aided by the addition of 5 standards ranging in molecular weight 14,000 - 200,000 daltons and pI range of 4.0 - 8.0.

(4) Evaluation of modification to second dimension slab gels

In order to enhance gel to gel reproducibility and to make between sample comparisons more practical a minor modification to the application of the sample tube gels to the slab gels was implemented [Fig 1]. Simply two sample tube gels were run on the same large format slabe gel side by side.

(5) Evaluation of pH

IEF was performed using a range of ampholytes [Pharmacia pH 5 -8 and 3 -10].

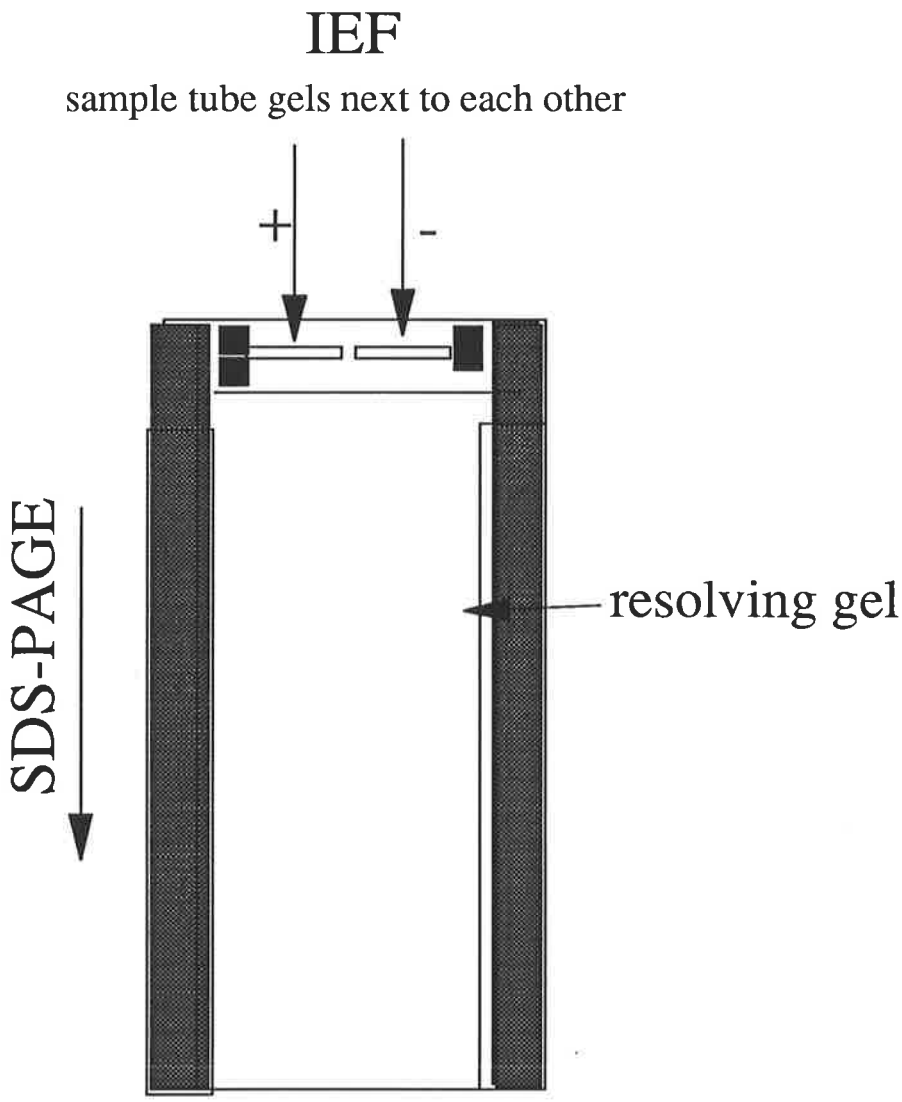

Figure 1. Modified 2nd dimension slab gel
allows the running of 2 sample tube wells concurrently on the same slab which enhances reproducibility and gel to gel comparisons.

5. RESULTS AND DISCUSSION

Resolution and Reproducibility in the Second Dimension

(1) Run Time

It was found that the optimal run time for separating pineal proteins in the second dimension was 5-6 hour [using 20W/gel]. The polypeptide area was less diffuse and more clearly defined at this run time than if increased to longer times and higher power [e.g, at 1W/gel and 17 hours the proteins spots were greatly diffused. It was further verified that the resolution was maximized by running the second dimesion gels at constant power rather than constant voltage. This confirmed findings by other investigators [Shaeffer and Johnson, 1973].

(2) Conductivity

It was found that conductivity influenced the running time and hence the resolution of the pineal protein separations on 2D-SDS/PAGE. Protein separations were considered completed when the dye bromophenol blue was about 1cm from the bottom of the gel. When the conductivity was altered by adding sodium acetate to the anode running buffer to a final concentration of 0.15M, the gels ran slowly almost taking twice the time and resulting in poorer resolution and changes to the rate of separation of respetive polypeptides. The R_f of some pineal proteins was reduced by nearly 50% in the lower conductivity buffers. The relationship of run time and conductivity was found to be linear.

(3) Reagent Quality

It was found that the highest quality electrophoretic grade reagents produced optimal run times and hence resolution of the pineal proteins in 2D-SDS/PAGE. Using lower grade reagents caused marked variations in both reproducibility and resolution of the polypeptides upon electrophoresis. For optimal results, the free base glycine instead of the glycine-HCL was used and any titrations to adjust the pH was not done by backtitrating with NaOH as this altered the conductivity of the buffers resulting in poor resolution and reproducibility.

(4) Temperature

Electrophoretic separations were performed at 3 temperatures: 4^0, 25^0 and 32^0 C. It was found that the optimal separations of pineal proteins were at temperatures 4 and 25^0 C and that the higher temperatures resulted in diffuse patterns making them difficult to analyse. Gels at both the 4 and 25^0 C were of comparable quality and it was decided to run all gels at 25^0 C.

(5) VALIDATION

Ten fluorographs of pineal protein patterns separated by one and two dimensional SDS/PAGE were evaluated for reproducibilty of polypeptide position. Molecular weight estimates of the pineal proteins differed on average by 0.3% or approximately 300 daltons. The pI estimates differed by a similar amount [0.35% or .05 pH units] These values represented very high level of reproducibilty.. The reproducibility of the 2D-SDS/PAGE pineal protein patterns were greatly enhanced to almost

zero differentiation by employing the modified system which ran two sample tube gels on the same slab gel [fig 1]. The system could be further modified to analyze more than 2 IEF samples concurrently.

6) Protein Detection

The number of pineal polypeptides detected depended on the method of visualization of proteins. A comparison of the coomassie blue, color-based silver staining and fluorographic methods of protein detection conclusively showed that the optimal method of detection of pineal secreted and cellular proteins is the latter two methods.

An average of 850 rat pineal protein spots from computer image analysis of silver stained 2D-SDS/PAGE gels [N=10] were detected and this figure was almost doubled by detection with fluorography of gels containing ^{35}S-methionine labelled pineal secreted proteins. By comparison, Coomassie blue staining detected very few proteins and would most decisively be excluded as the method for detecting pineal proteins especially those secreted from individual glands in-vitro. The colour based silver staining technique although very sensitive and useful because of the colors produced was suitable for cellular but not secreted pineal polypeptides. This method was able to detect about 85% of the proteins detected by fluorography.

(7) Size of the gels

The number of pineal proteins detected on 10% 2D-SDS/PAGE gels by silver staining and fluorography as a function of the area of the gels was examined and it was found that the larger format gels [20cm x 22 cm] resolved a significantly larger number of protein spots than did the

smaller. All 2D-SDS/PAGE separations of pineal proteins were performed on the large slab gels. A further observation revealed that 14% gels showed more protein spots than 10% gels particularly in the molecular weight range of 30,000 - 40,000 daltons.

6. CONCLUSION

These results indicated 2D-SDS/PAGE properly optimized and carefully performed in association with the sensitive protein detection methods described [colour-based silver staining and fluorography] would be a valuable tool for the assessment of the nature and pattern of pineal proteins.

Chapter Eight

Charecterization of Pineal Proteins Synthesized and Secreted In-Vitro

1. Abstract

There was a distinction between cellular and secreted pineal proteins with many of the cellular pineal proteins displaying similarities with other brain tissues such as hypothalamus and cortex. 2D-SDS/PAGE fluorographs of rat pineal secreted proteins revealed as many as 1000 protein spots between molecular weight range of 14,000 - 200,000 Daltons and pI range 4.0 - 7.5. Fluorography of ^{35}S-methionine incorporated pineal proteins gave the best measure of secretory activity. The rate of incorporation of label into cellular pineal proteins differed from that of the secreted rat pineal proteins. Uptake of label into the secreted proteins represented approximately 40% of the total incorporation of methionine into cytosolic proteins.

2. Introduction

The characterization of pineal bioactive secretions from individual pineal glands in-vitro is lacking. Previous results have been obtained from large numbers of glands pooled from more than one subject and detection of the proteins was by means of non-specific radioimmunoassays and bioassays.

The pineal gland is influenced by a large number of variables and it would be of immense benefit to assess its bioactive secretions with a method that was sensitive, specific and highly reproducible so as to detect activity from individual glands. In this chapter the techniques described in chapter 7 were applied to individual rat pineals with the aim of producing rat pineal protein 'maps' of both secreted and cellular fractions which could subsequently serve as aids for identifying factors affecting pineal protein secretions. It was also aimed at identifying key marker proteins that could be used to identify, characterize and eventually isolate and sequence important pineal bioactive proteins and peptides.

3. Aim

To obtain a 2D-SDS/PAGE profile of rat pineal secreted and cellular proteins, from which to charecterize key proteins. To determine the rates of incorporation of labelled methionine into secreted and cellular proteins from which to determine the optimal times of pineal culture.

4. Materials and Methods

10 Wistar-Hooded rats [21 day old; male] were housed in a light enviroment L:D 14:10, fed and watered ad-lib. After 3 weeks they were killed by cervical dislocation and their pineals, hypothalami and cortical tissue excised and either extracted for one and two dimensional SDS/PAGE or prepared for radiolabelling in culture with

35S-methionine [see chapter 6]. Hypothalami were obtained from the region 3 mm rostrally from the optic chiasma, caudally just behind the mammilary bodies, laterally through the optic tract and just beneath the anterior commissure. The cortex was obtained from a region adjacent to the striatum. Both hypothalamic and cortical tissues were always excised from the same regions and were about 1 mm^3 and had a wet weight of about 2 mg.

5. RESULTS AND DISCUSSION

Radioactive Incorporation into Medium and Cytosolic Proteins

Incorporation of radioactive methionine into protein was measured during incubations of rat pineal glands with EMEM. The time course of incorporation into acetone-precipitable proteins of cellular [Fig 1(a)] and secreted [Fig 1(b)] proteins revealed distinctive 1 and 2D-SDS/PAGE profiles. Incorporation of label into the intracellular pineal proteins was linear for 10 hrs although a further increase occured between 10 and 24 hr. Radioactive proteins were observed in the medium after a lag phase of 2-4 hours and then were secreted linearly for 24 hrs. Measurements of the rates of incorporation into cytosolic and secreted proteins observed during the linear phases of incorporation, demonstrated that uptake into secreted proteins represented approximately 40% of the total incorporation of label into cytosolic proteins.

Figure 1(a). Time course incorporation of ^{35}S-methionine into rat pineal cellular proteins.

Figure 1(b). Time course incorporation of ^{35}S-methionine into rat pineal secreted proteins.

1 [a] cytosolic

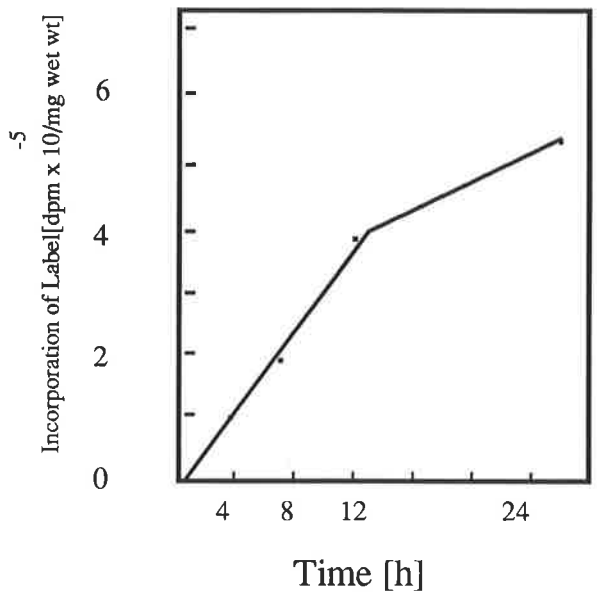

Figure 1[a] Time course of incorporation of label into cytosolic proteins

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1 [b] medium

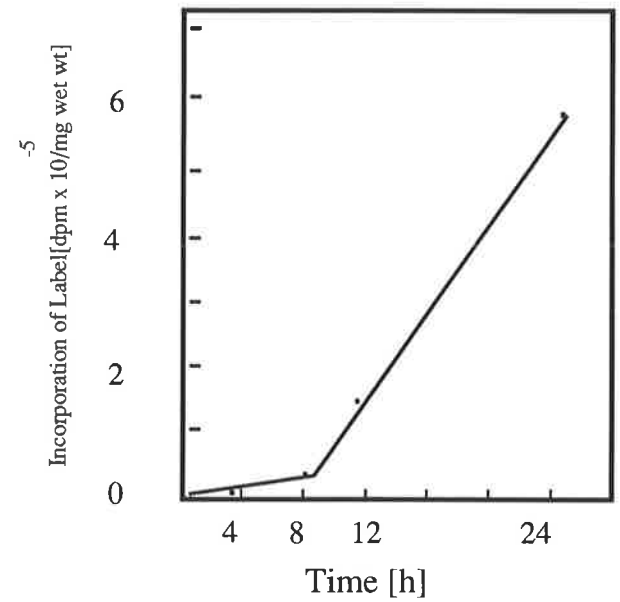


Figure 1[b] Time course of incorporation into pineal secreted proteins

Identification of Proteins

(a) Cellular

The 1D-SDS/PAGE silver stained cellular pineal protein patterns are shown in Fig 2(a). The silver stained 2D-SDS/PAGE profile [Fig 2(b) demonstrated a range of polypeptides many similar to those seen in other brain tissue [Fig 2(c)]. These represent cellular structural or "housekeeping proteins" common to all neural tissue. Their value is in acting as possible marker proteins and for determining possible cell lysis during culturing. It may be possible with further exploratory work to define key specific pineal celluar proteins with important bioactive functions.

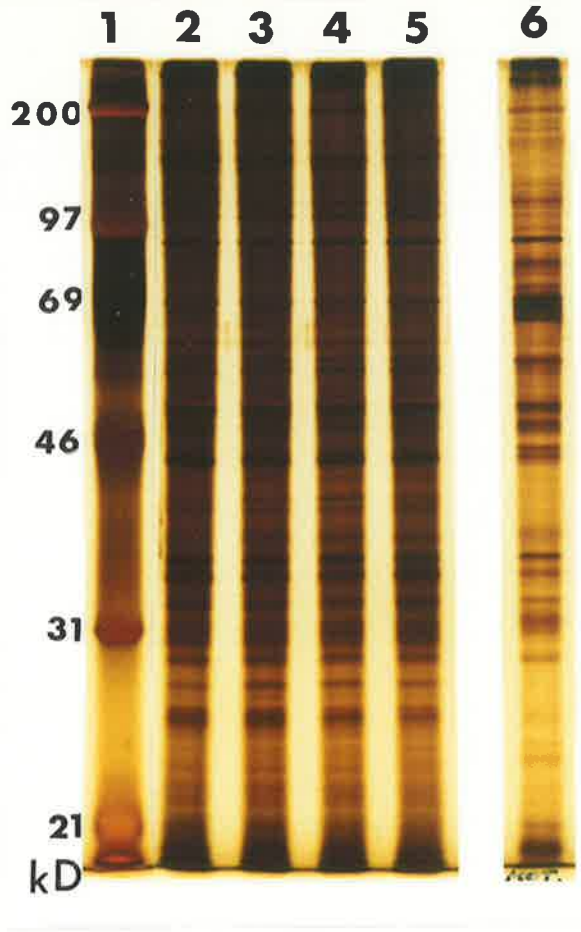
(b) Secreted

The secreted pineal proteins were best identified by fluorography of labelled pineal proteins. By this method newly synthesized pineal proteins can be evaluated whereas the silver stained secreted protein profiles would reveal both stored and newly synthesized fractions. Fig 5 shows representative fluorograph of labelled pineal secreted and cellular proteins and clearly highlights a large number of secreted proteins. The secreted profile is markedly distinct from the corresponding cellular profile as shown by silver staining and fluorography and also varies from both cortical and hypothalamic secreted 1 and 2D-SDS/PAGE protein profiles [Figs. 3 - 5]. The most significant feature of the pineal labelled secreted protein patterns is

Figure 2(a). Silver stained 1D-SDS/PAGE of rat cellular pineal proteins.

Lane 1	**Standards.**
Lane 2-5	**Profiles from 4 different subject animals.**
Lane 6	**Profile of rat secreted pineal proteins.**

Figure 2(b). Silver stained 2D-SDS/PAGE of rat cellular pineal proteins.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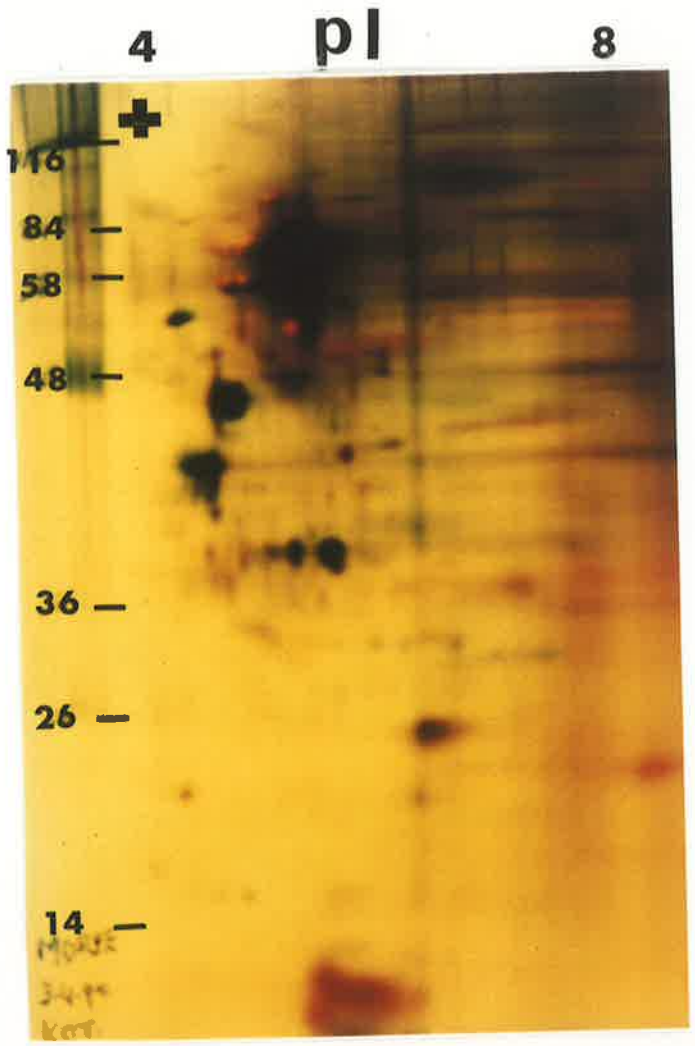
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84
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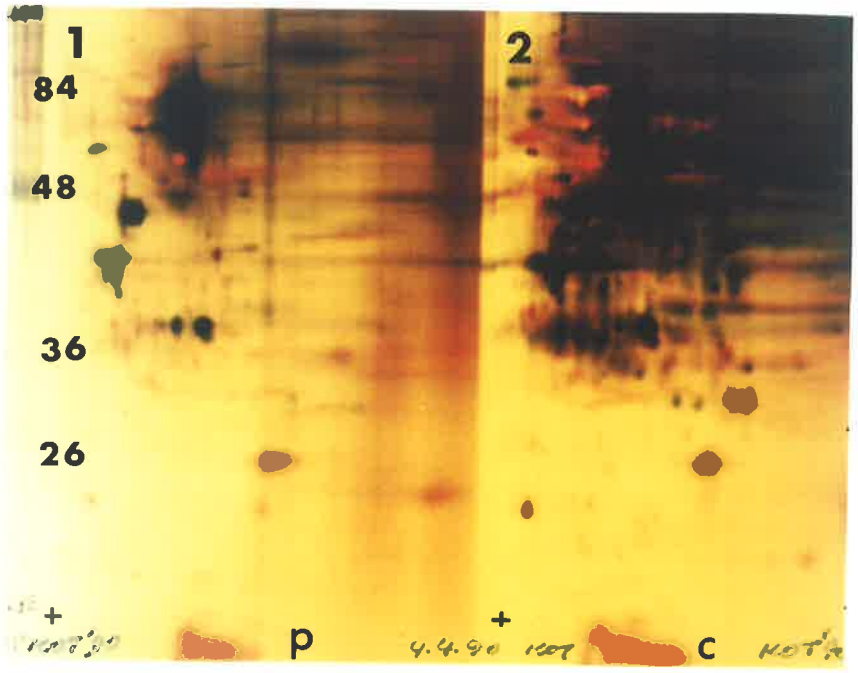
36
26
14

Figure 2(c). Silver stained 2D-SDS/PAGE of rat pineal cellular proteins

1. pineal [p]
2. cortex [c]
3. pineal [p]
4. hypothalamus [h]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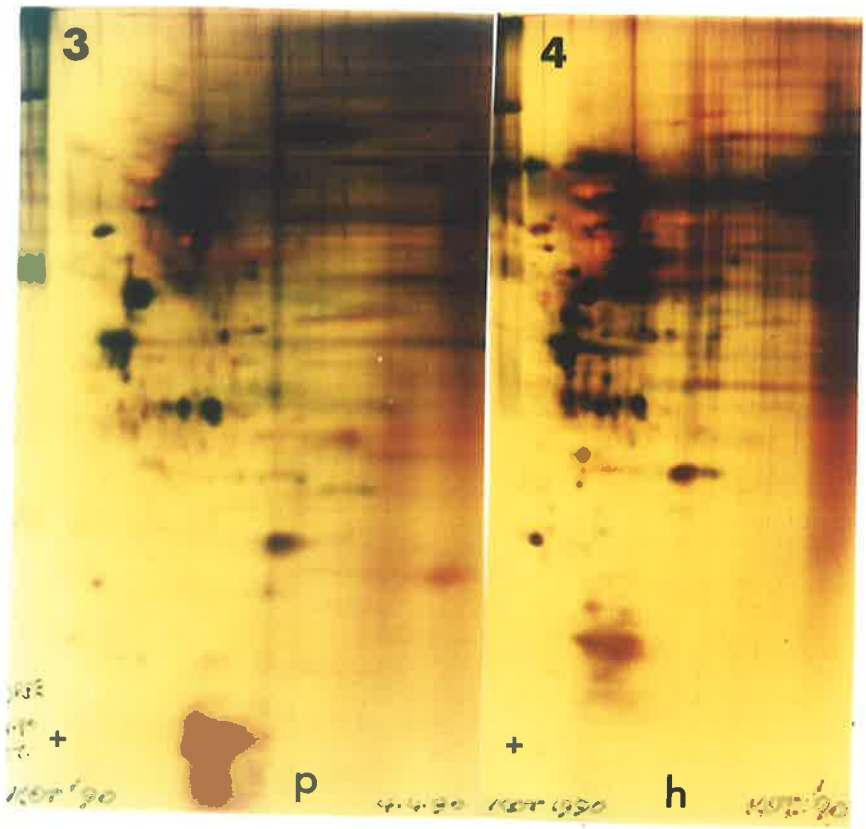
3
+
p
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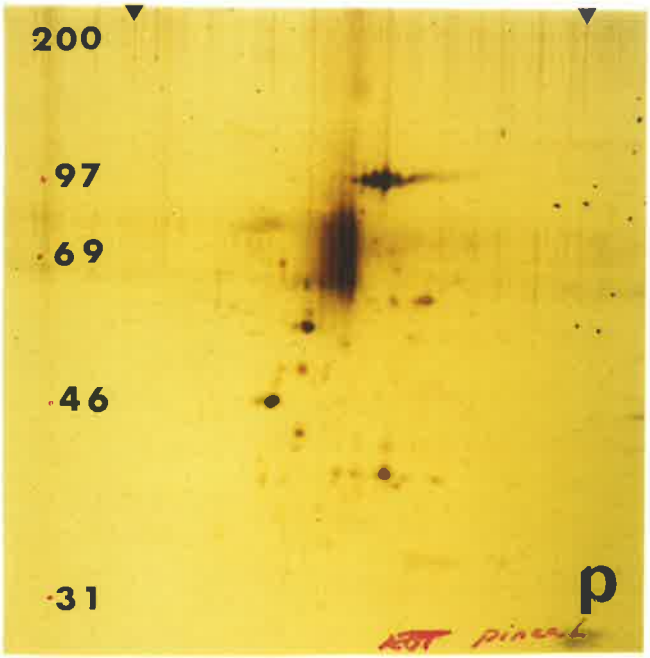
+
h

Figure 3(a). Silver stained 2D-SDS/PAGE rat secreted pineal proteins [p].

Figure 3(b). Silver stained 1D-SDS/PAGE rat secreted pineal proteins.
[Lanes 1-3; (p)] pineal.
[Lane 6; (c)] cortex.
[Lane 7; (h)] hypothalamus

Figure 3(c). Silver stained 2D-SDS/PAGE rat secreted hypothalamic proteins [h].

Figure 3(d). Silver stained 2D-SDS/PAGE rat secreted cortical proteins [c].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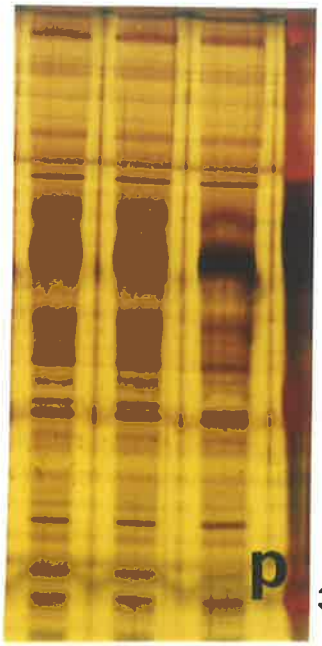
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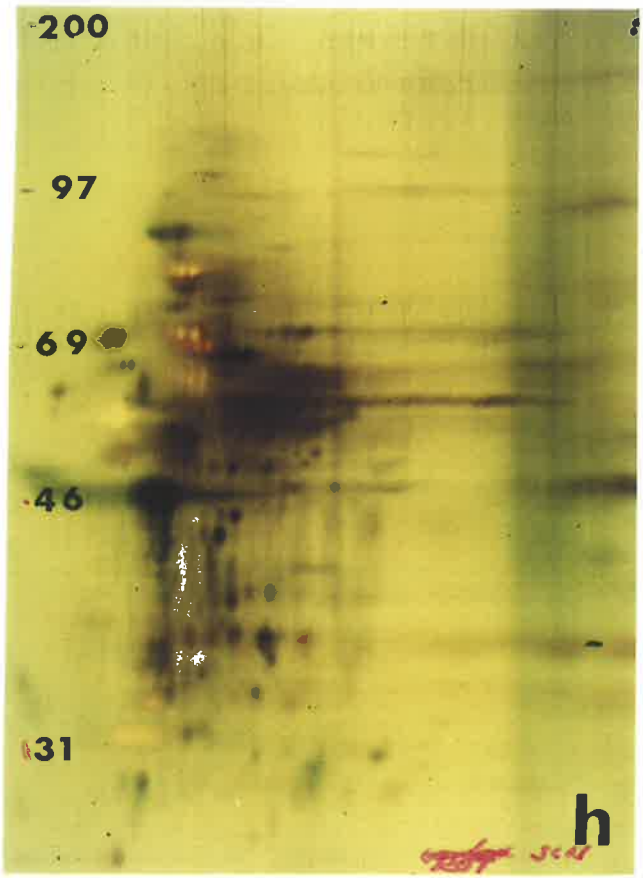
200
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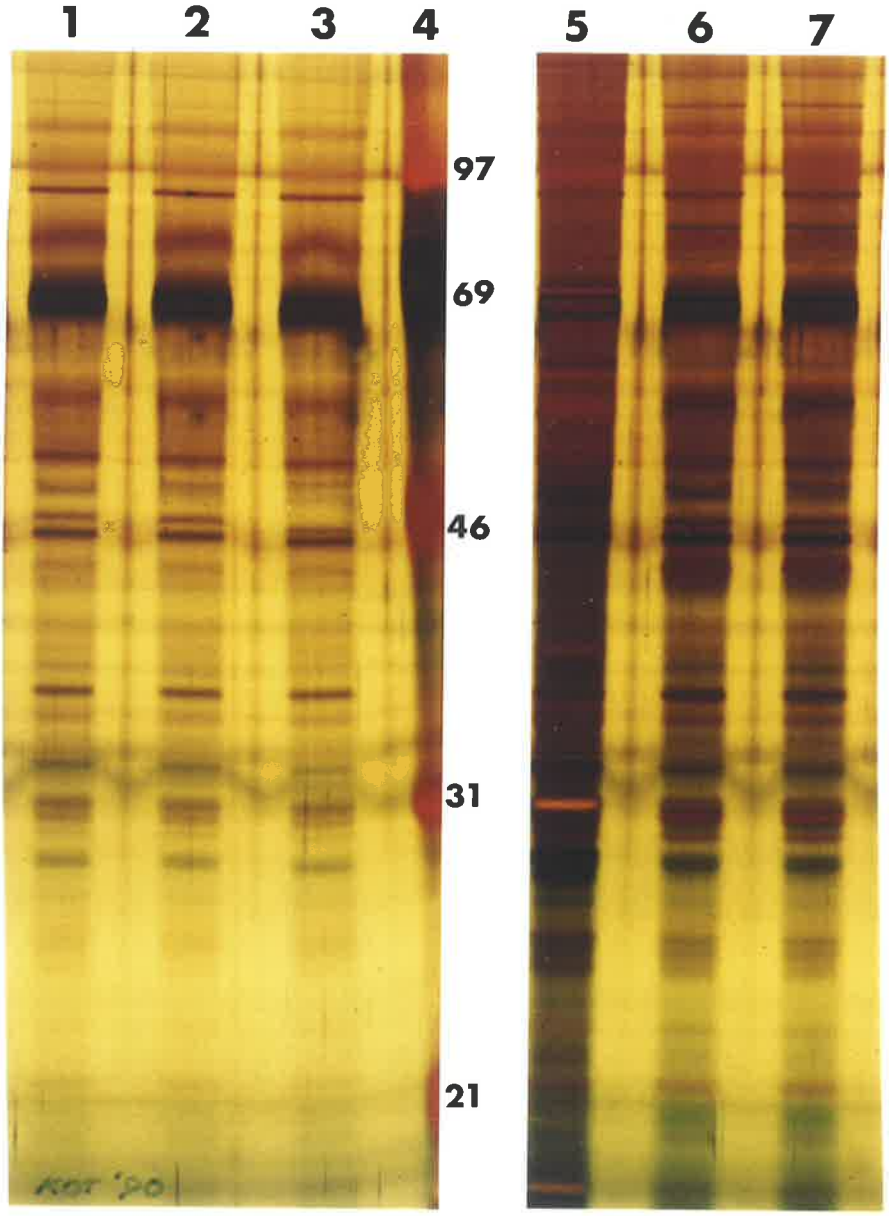
c

Figure 4. Silver stained 1D-SDS/PAGE of rat secreted:

Lanes [1-3] MD pineal proteins.
Lane [5] ML cortex proteins.
Lanes [6-7] ML pineal proteins.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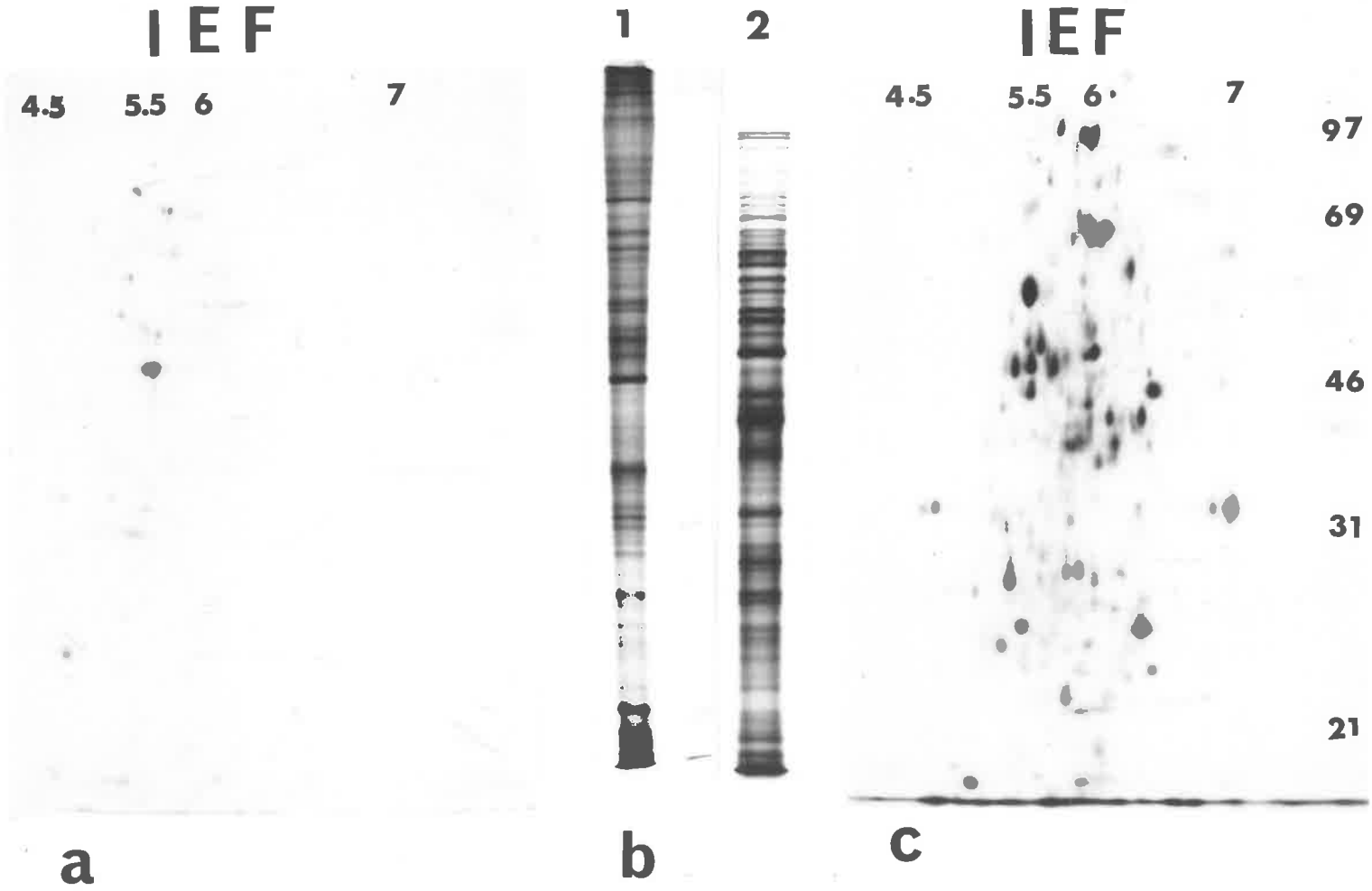
21

Figure 5(a). Fluorograph [2D-SDS/PAGE] of ^{35}S-methionine incorporation into rat pineal cellular proteins.

Figure 5(b). Fluorograph [1D-SDS/PAGE] of ^{35}S-methionine incorporation into cellular [lane-1] and secreted [lane-2] rat pineal proteins.

Figure 5(c). Fluorograph [2D-SDS/PAGE] of ^{35}S-methionine incorporation into rat pineal secreted proteins.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the abundance of proteins in the region between pI 4.5 - 7.0 and molecular weight range 14 - 200 kD This profile provides a much needed map of pineal secreted proteins and could prove valuable in the identification of key bioactive pineal polypeptides. Some of these proteins display features typical of glycoproteins [charge heterogeneity] as well as features typical of postranslational modification [glycosylation, phosphorylation]. Identification of pineal phosphoproteins could be achieved by using ^{32}P-orthophosphate and 2D-SDS/PAGE and for glycoproteins use can be made of Horseradish peroxidase [HRP] conjugated lectins, a project well worth considering for the future.

6. CONCLUSIONS

It was concluded from these experiments that the optimal culture time for studying secreted pineal secreted proteins from individual glands in-vitro was 6 hrs and the optimal method of detection was fluorography of ^{35}S-methionine labelled pineal proteins. The following chapters assess factors affecting rat pineal proteins synthesis and secretion by the methods of 2D-SDS/PAGE and fluorography.

Part Four

Factors Influencing Pineal Protein Synthesis and Secretion

Chapter Nine

Photoperiodic Influences on Pineal Secretions

1. Abstract

Diurnal patterns in rat pineal protein synthesis and secretion were assessed by incubating individual pineal glands with ^{35}S-methionine and analyzing the secreted [medium] and cellular [tissue] proteins by one and two dimensional electrophoresis [SDS/PAGE]. Pineal glands obtained from animals at 4 hourly intervals during the 24 hour photoperiod [L:D 14:10], revealed a photoperiod related incorporation of label, with secreted proteins showing a major peak at midlight [ML] and a second minor peak in the middark [MD] incubations. Both quantitative and qualitative differences were obtained in the patterns of labelled proteins secreted at the different times of the photoperiod, with major proteins of molecular weights 14 - 200 K Daltons displaying charge heterogeneity typical of glycoproteins. The rates of incorporation into the ML pineal proteins were greater than those into MD proteins. These data reveal that pineal protein synthesis and secretion is under the influence of the photoperiod and that the differences can be assessed by 2D-SDS/PAGE.

2. Introduction

Evidence from the previous chapter indicates that the rat pineal gland actively incorporates labelled amino acids into synthesized and secreted proteins. The exact nature their relationship to the endocrine function of the gland and factors that may influence these polypeptide secretions remain unknown. Indoles especially melatonin have been extensively linked to the photoperiod [Bittman, 1984; Reiter, 1991] and some aspects of photoperiodic influences on bioactive pineal proteins identified [see Literature Review Chapter 2]. This study re-examines the relationship of the photoperiod and pineal protein synthesis and secretion with the methods and techniques evaluated in this thesis.

3. Aim

The aim of this chapter is to answer questions relating to photoperiodic influences on rat pineal protein secretions. More specifically the aim is to assess whether or not 2D-SDS/PAGE in association with fluorography can detect changes to patterns of proteins synthesized and secreted from individual rat pineal glands in-vitro.

4. Materials and Methods

Hooded Wistar male rats [100-125g] were housed in a lighting schedule 14:10 L:D. They were sacrificed aftre 21 days by cervical dislocation in groups of 5 at 4 hourly intervals over a 24 hour period and the pineal, hypothalami and cortical tissue explants were removed [Chapter 8] cultured and analyzed by SDS/PAGE as described in Part Two [Chapters 5 & 6].

5. RESULTS

Incorporation of label into proteins

The incorporation of label into secreted and cellular pineal proteins showed a distinctive patten related to the time of the day the tissue was harvested [Fig. 1]. The times of maximum incorporation of label into secreted proteins was at the ML phase with a smaller but distinctive second peak at the MD phase. These points in the photoperiod correspond to the times when total pineal protein content were also at their maximum [Fig. 2]. These results indicate the pineal protein synthesis follows a bimodal pattern which is unexpected on the basis of reports [Nir et al., 1971] demonstrating a single peak in the protein content of rat pineals occuring in the late afternoon of the daily photoperiod.

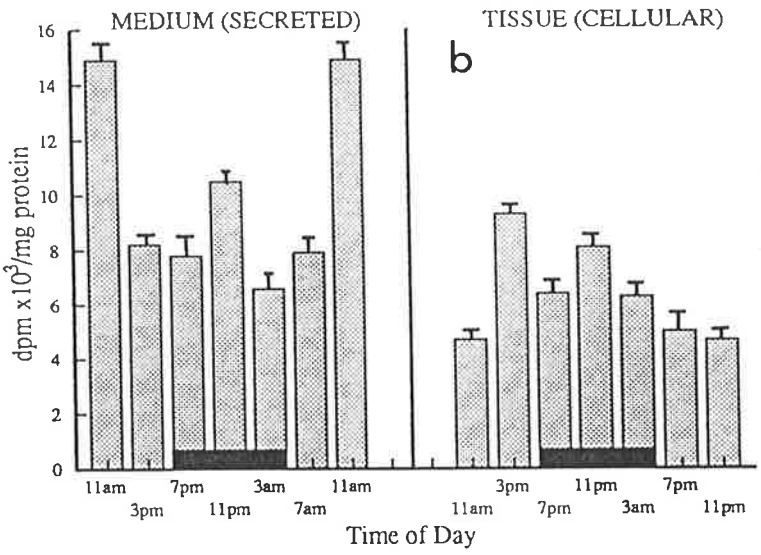
Protein 1D and 2D-SDS/PAGE Patterns

The 1D and 2D-SDS/PAGE protein patterns of ML and MD patterns showed marked diffrences in the incorporation of label into both secreted and cellular proteins. Figure 3 shows the silver stained 2D-SDS/PAGE cellular MD/ML protein patterns which upon analysis do not demonstrate substantial differences. The one apparent difference seen by visual and computer image analysis is the difference in amounts of some proteins between the two time points. One protein spot [molecular weight 36kD and pI 5.5] was 25% [$p<0.01$] more in the ML phase than in the MD phase. This pattern supports the diurnal fluctuations in total protein seen in Fig [2]. The 1D-SDS/PAGE

Figure 1. Incorporation of ^{35}S-methionine into rat pineal proteins during a 24 hour diurnal period [L:D 14:10]

(a) secreted
(b) cellular

Figure 2. The diurnal pattern of total rat pineal protein.[N=10 for each time point. Differences between ML and MD time points significant at $p<0.01$].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Time of Day

Fig 2.

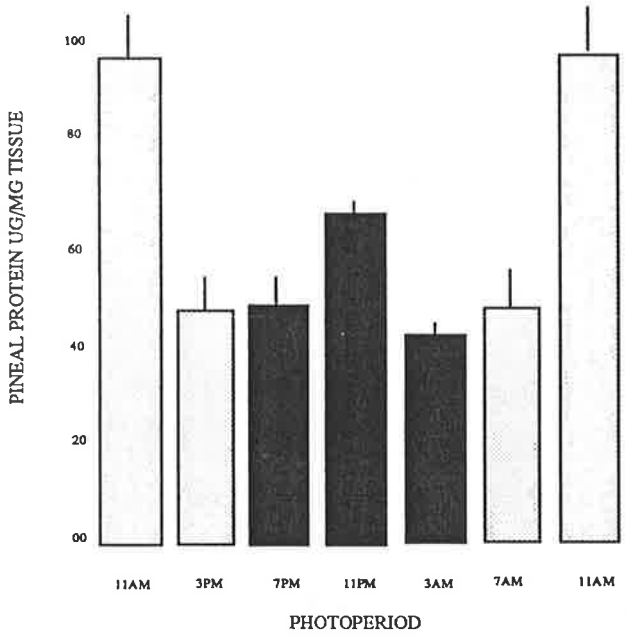
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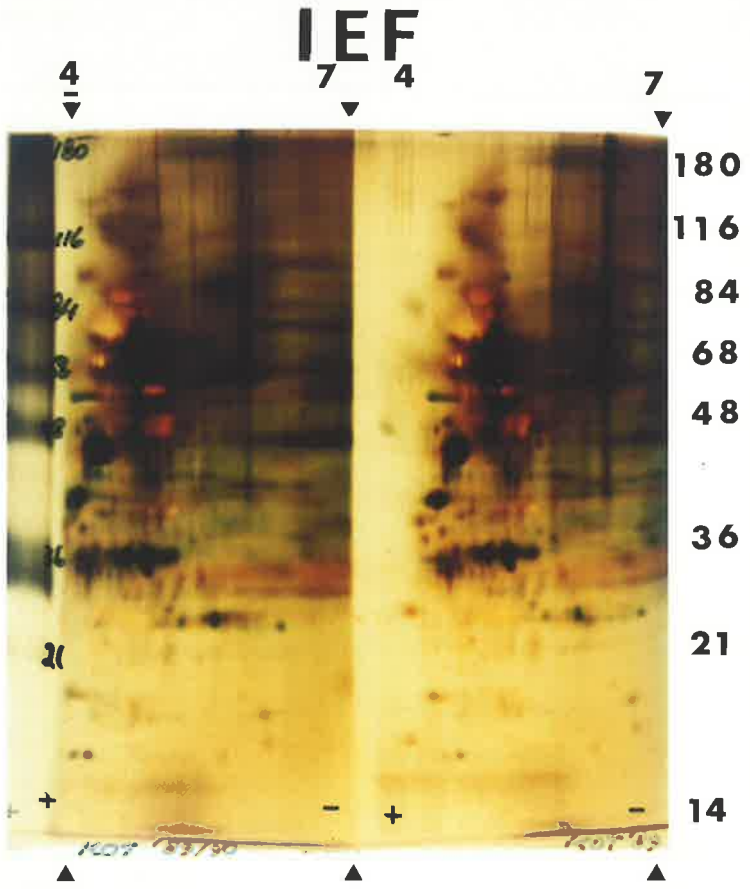
Figure 3. Silver stained 2D-SDS/PAGE of ML and MD rat pineal cellular proteins.

Figure 4. Fluorographs of 1D-SDS/PAGE ^{35}S-methionine incorporation into ML/MD cellular and secreted rat pineal proteins.

Lanes 1-2. MD cellular
Lanes 3-4 ML cellular
Lanes 6-7 ML secreted
Lanes 8-9 MD secreted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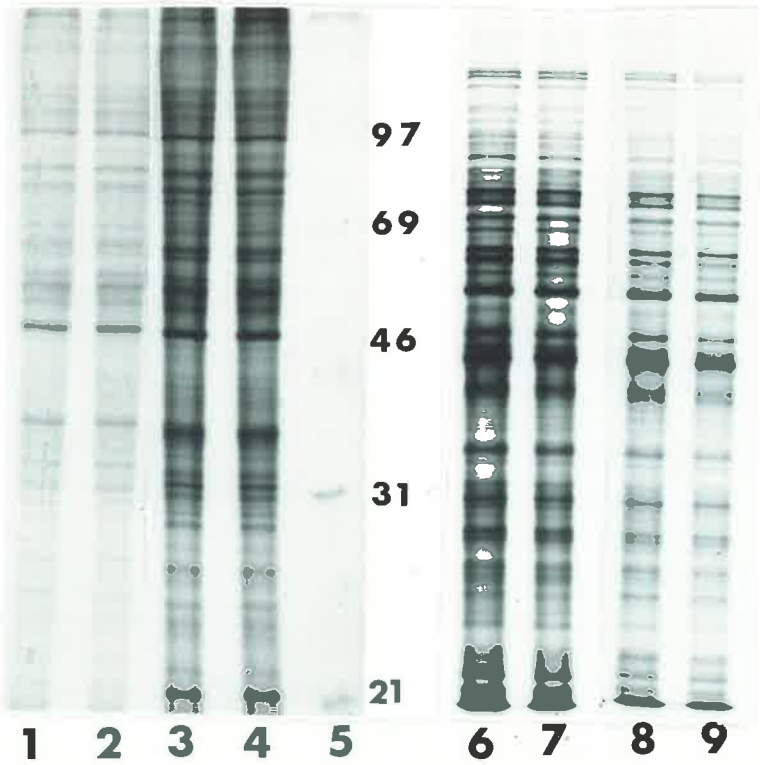
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fluorographs as seen in Fig [4] substantiate the diurnal fluctuation in the incorporation of ^{35}S-methionine into both cellular and secreted proteins. There is a significant difference [$p<0.001$] in the patterns between cellular and secreted labelled MD and ML proteins [Fig 4-Lanes 1-9].

The 2D-SDS/PAGE fluorographs of cellular and secreted MD/ML pineal proteins seen in Fig [5] further substantiate the differences in protein patterns [between time phases and between cellular and secreted] and display differences not seen in the silver stained protein patterns of the corresponding gels. The most significant effect of the photoperiod is the total ablation and variable inhibition of labelled proteins in the MD phase. Fig [6] graphically illustrates the percent change in the relative amounts of labelled pineal polypeptides between ML and MD phases. These percentage differences were shown in all cases examined. Analysis of hypothalamic and cortical tissue did not reveal similar diurnal influences on protein synthesis and secretion. Other brain tissues may have different patterns of protein synthesis and secretion to that of the pineal and would require separate detailed analysis in order to determine the optimal time points in the photoperiod in which to study them.

Rates of Incorporation

Fig. (7) shows the time course of incorporation of label into ML and MD cytosolic and medium pineal proteins.

The main difference was seen in the slope of the curves with ML rates of incorporation being greater than those of the MD pineal proteins.

Figure 5. Fluorographs of 2D-SDS/PAGE ^{35}S-methionine incorporation into rat pineal proteins.

[a]. ML cellular
[b]. MD cellular
[c]. ML secreted
[d]. MD secreted

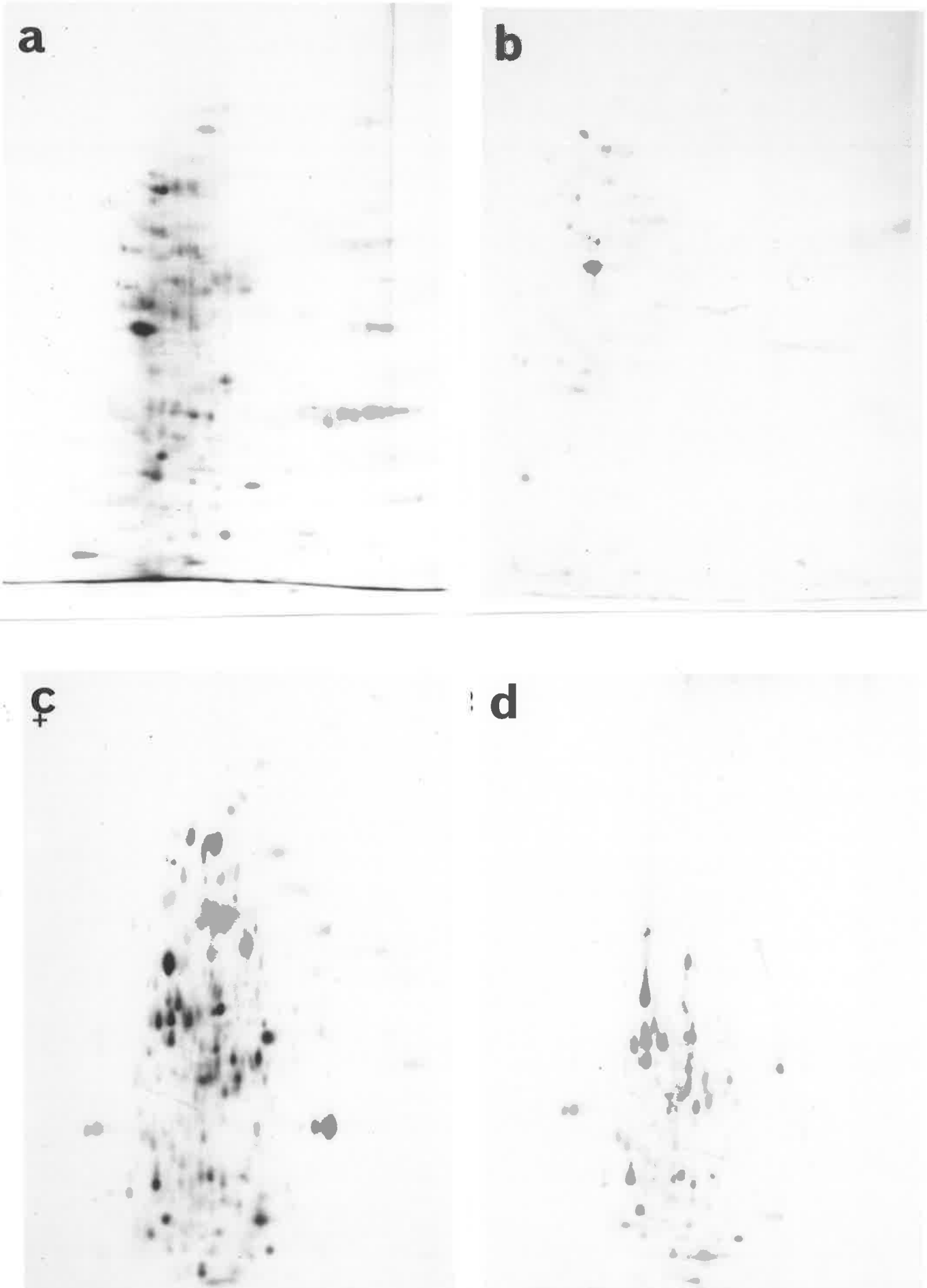

a
b
♀
d

Figure 6. ML/MD changes in the relative density of ^{35}S-methionine incorporation into rat pineal secreted proteins. Each point represents the average of 10 [2D-SDS/PAGE] fluorographs.

Fig 6.

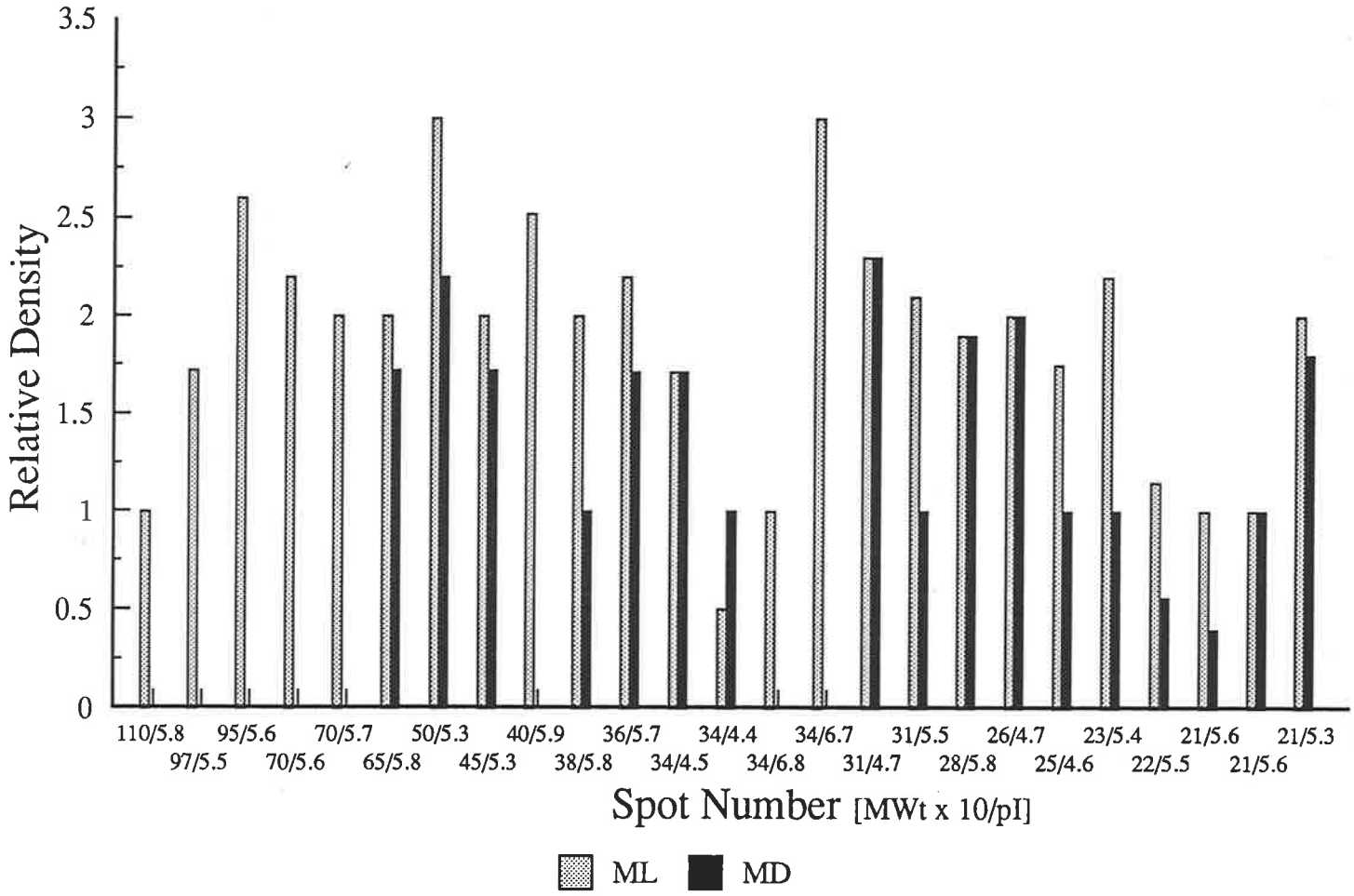
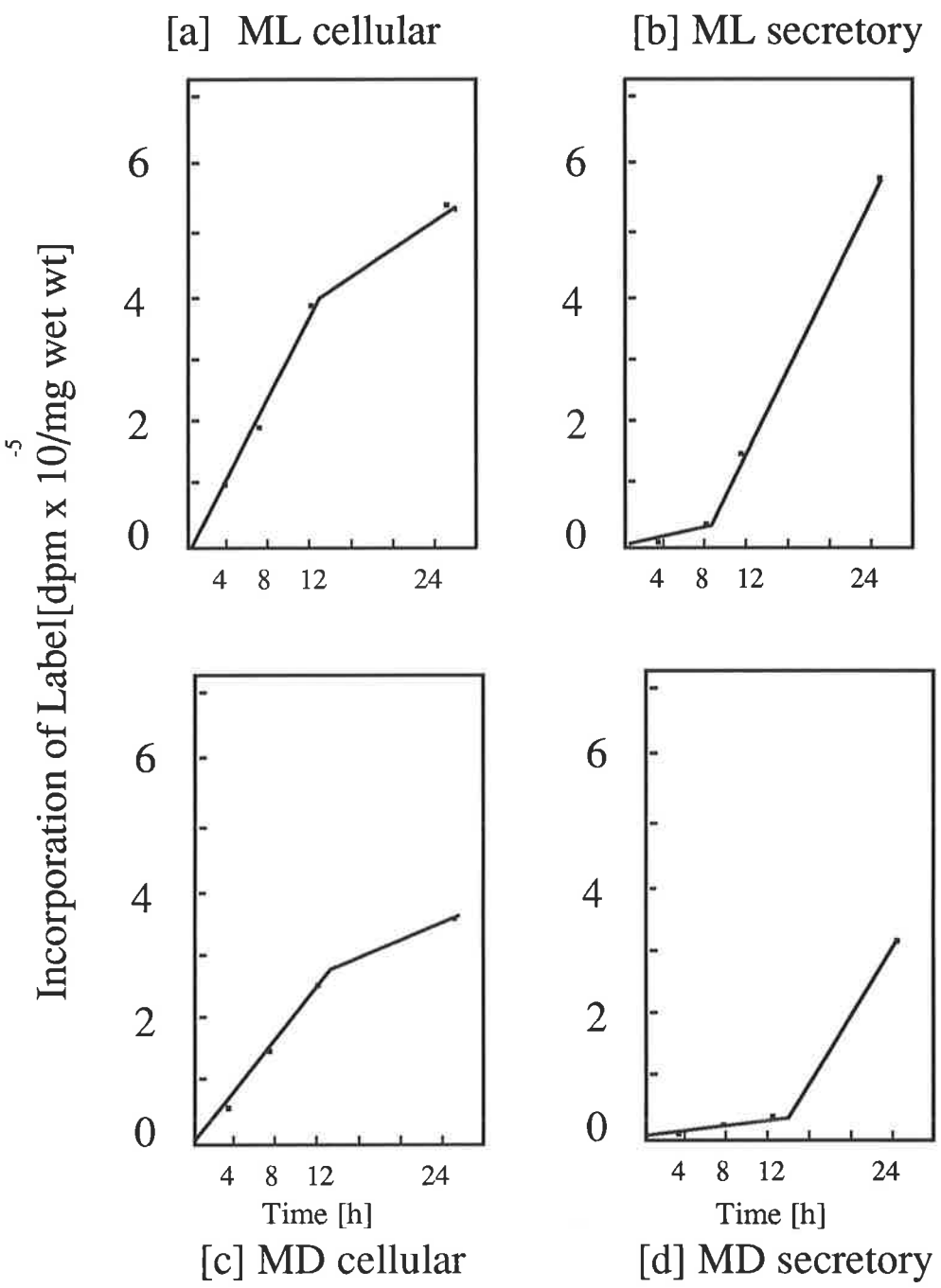

Relative Density
3.5
3
2.5
2
1.5
1
0.5
0
110/5.8
97/5.5
95/5.6
70/5.6
70/5.7
65/5.8
50/5.3
45/5.3
40/5.9
38/5.8
36/5.7
34/4.5
34/4.4
34/6.8
34/6.7
31/4.7
31/5.5
28/5.8
26/4.7
25/4.6
23/5.4
22/5.5
21/5.6
21/5.6
21/5.3
Spot Number [MWt x 10/pI]
ML
MD

Figure 7. Time course of incorporation of ^{35}S-methionine into ML/MD rat pineal proteins.

[a]. ML cellular
[b]. ML secreted

[c]. MD cellular
[d]. MD secreted


[a] ML cellular
[b] ML secretory
[c] MD cellular
[d] MD secretory
Incorporation of Label[dpm x 10/mg wet wt]
-5
0
2
4
6
4
8
12
24
Time [h]

Hypothalamic and cortical tissue did not show marked diurnal variations in the rates of incorporation of label into proteins.

6. CONCLUSIONS

These results demonstrate that the photoperiod has a profound effect on rat pineal protein synthesis and secretion. The ML pineal differs in its capacity to synthesize proteins from the MD pineal and both qualitative and quantitaive changes were identified by 2D-SDS/PAGE. The study indicates the optimal time to search for pineal bioactive secretions is in the ML phase, for to attempt identification of bioactive polypeptides at some other times in the photoperiod would result in negative results. Furthermore this study provides the first 2D-SDS/PAGE maps of ML and MD pineal proteins. Which of these proteins are bioactively important in terms of the functional significance of the pineal gland is another area of study and not the aim of this thesis. The next chapter deals with possible indole-protein relationships and is based on the result from this chapter which showed conclusively that the MD protein profile has many proteins either totally absent or partially inhibited, a time in the photoperiod when pineal melatonin is at its peak.

Chapter Ten

Melatonin Influences on Pineal Protein Secretions

1. Abstract

Melatonin [10^{-7} - 10^{-5}M] significantly reduced or totally ablated the incorporation of labelled methionine into both secreted and cellular proteins. The ML one and two dimensional SDS/PAGE protein patterns were transformed into MD protein patterns by melatonin [10^{-7}M] indicating pineal indoleamines may have a significant autocrine and/or paracrine role in modulating pineal protein synthesis and secretion. It is possible that the photoperiodic influences shown to affect pineal proteins may be mediated via melatonin. The mechanism of action of melatonin was identified as being at the transcription level of protein synthesis since melatonin [10^{-7} - 10^{-4}M] inhibited the incorporation of ^{3}H-uridine into RNA. This inhibitory effect of melatonin was dose related and could be reversed by removal of the indole from culture. Melatonin did not alter cortical tissue RNA however there was a moderate inhibition in hypothalamic incorporation of label into RNA but differed from that of the pineal.

Melatonin may act as a potent modulator of protein synthesis and this effect on the pineal proteins is a means of regulating pineal endocrine function.

2. Introduction

The multi-effector role of melatonin has been well documented [see chapter 5], however what remains unresolved is the mode of action of melatonin. Some of the stategies employed in the past to examine this enigma has been the assessment of nuerotransmitters effected by melatonin, particularly its effects on serotonergic, noradrenergic, dopaminergic and gabaergic pathways in the brain. Other stratergies have looked at signal transductive effects of melatonin and its interaction with a variety of receptors [review by Reiter, 1991]. What has also been well overlooked is the possible interrelationship of pineal bioactive secretions especially the relationship between pineal proteins and indoles. Early studies did not venture far enough and in view of the findings described in the previous chapter, it was important to address this area. This study assesses the effects of melatonin on pineal protein synthesis and secretion by incubating individual pineal glands with ^{35}S-methionine with and without melatonin of various doses and analyzing the secreted and cellular proteins by one and two dimensional SDS/PAGE. It further assesses the mode of action of melatonin by examining its effects on the incorporation of ^{3}H-uridine into RNA..

3. Aim

To determine the effects of melatonin on pineal protein synthesis and secretion and to determine whether transcription, translation or both are its mode of action.

4. Materials and Methods

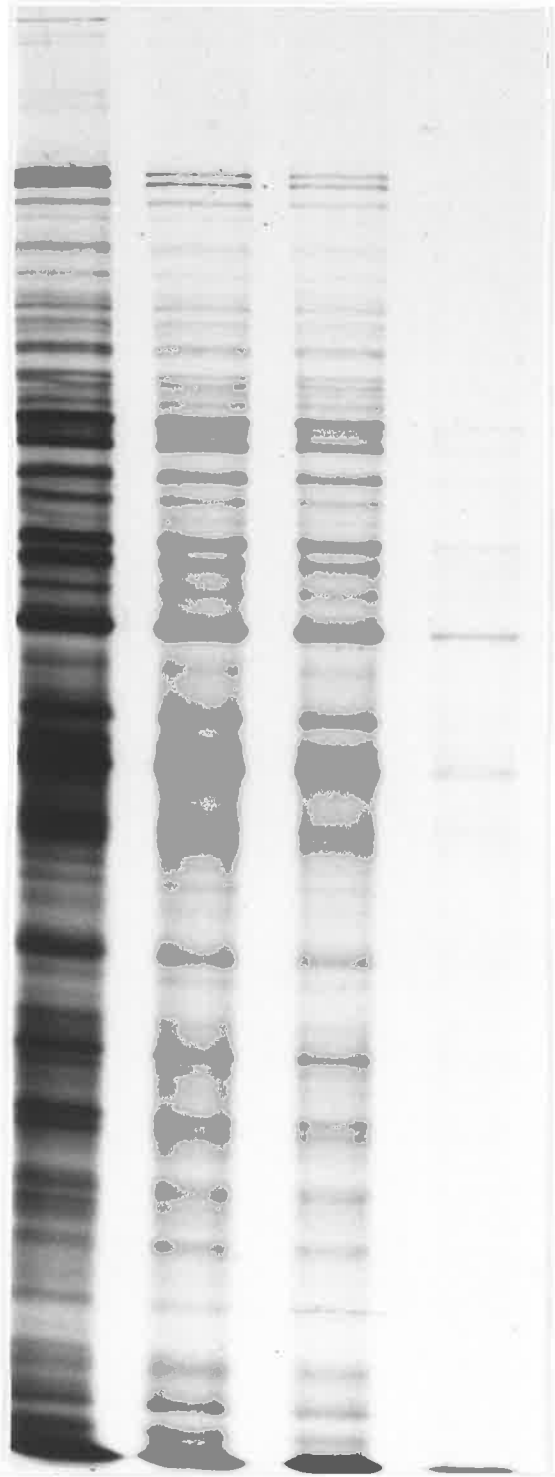
Animals, electrophoresis, tissues and pineal gland excision and culture was as for the previous chapter. Melatonin was added to the cultures at doses ranging from 10^{-4} - 10^{-8} M. All cultures were in quadruplicate and included control groups to which no melatonin was added. Another group of tissues were cultured with DRB [5,6-dichloro-1-b-D-ribobenzimidazole, from Sigma] a known reversible transcription inhibitor.

To measure the effect of melatonin on transcription, the incorporation of ^{3}H-uridine [Amersham] was measured as described by Raju et al., 1991 in TCA-precipitable RNA material. The experimental groups of tissues and pineals were treated with melatonin for 20 min before being exposed to labelled uridine [45 uCi/ml] and then exposed to melatonin for 2 hours. At the end of the experiments, the tissues were processed for TCA-precipitation and scintillation counting by the method described by Eskin et al., 1984. The method of Granick 1975. was used to correct for an effect of DRB and melatonin on the uptake of uridine.

5. RESULTS

Melatonin and ^{35}S-Methionine Incorporation

The effects of melatonin [10^{-7}M] on the incorporation of labelled methionine into cellular pineal proteins is shown in Fig. 1. Both one dimensional and two dimensional SDS/PAGE demonstrated distinctive inhibitory effects of melatonin on pineal proteins. The most distinctive effect of melatonin is the conversion of the ML pineal protein secretion pattern to a typical MD protein pattern [Figs 2-5]. The proteins mostly



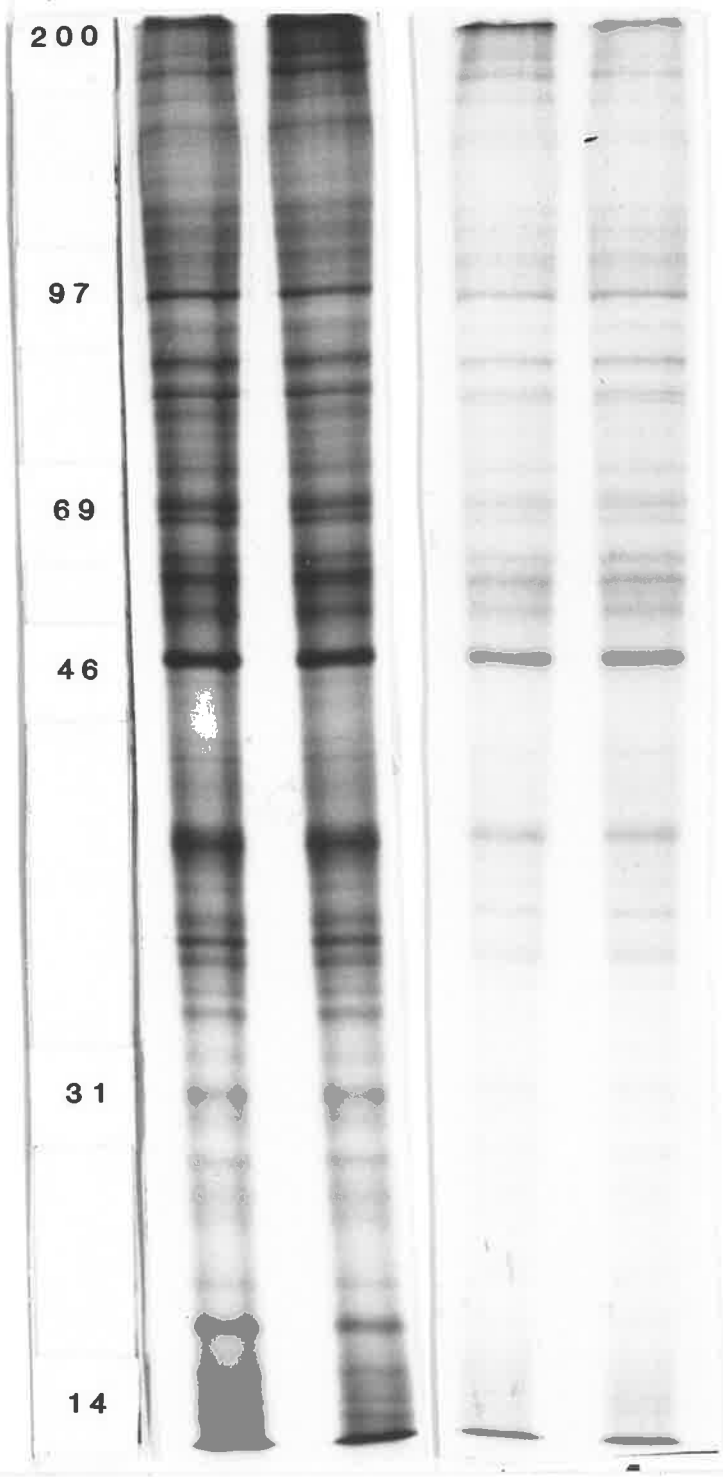

200
97
69
46
31
14

Figure 1. Representative 1D-SDS/PAGE fluorograph showing the effects of melatonin on the incorporation of ^{35}S-methionine into cellular rat pineal proteins.

Lanes [1-2]. ML control pineal tissue.
Lanes [3-4]. ML pineal treated with 1.6 X 10^{-7}M melatonin.

Figure 2. Representative 1D-SDS/PAGE fluorograph showing the effects of melatonin on the incorporation of ^{35}S-methionine into rat secreted pineal proteins.

Lane [1]. ML pineal control.
Lane [2-3]. ML pineal treated with 1.6 X 10^{-7}M melatonin.
Lane [4]. ML pineal treated with 1.6 X 10^{-6}M melatonin.

Figure 3. Representative 2D-SDS/PAGE fluorograph of ^{35}S-methionine incorporation into ML secreted proteins from an individually cultured rat pineal gland. Note protein spots marked with arrows and compare with the following figure [Fig 4].

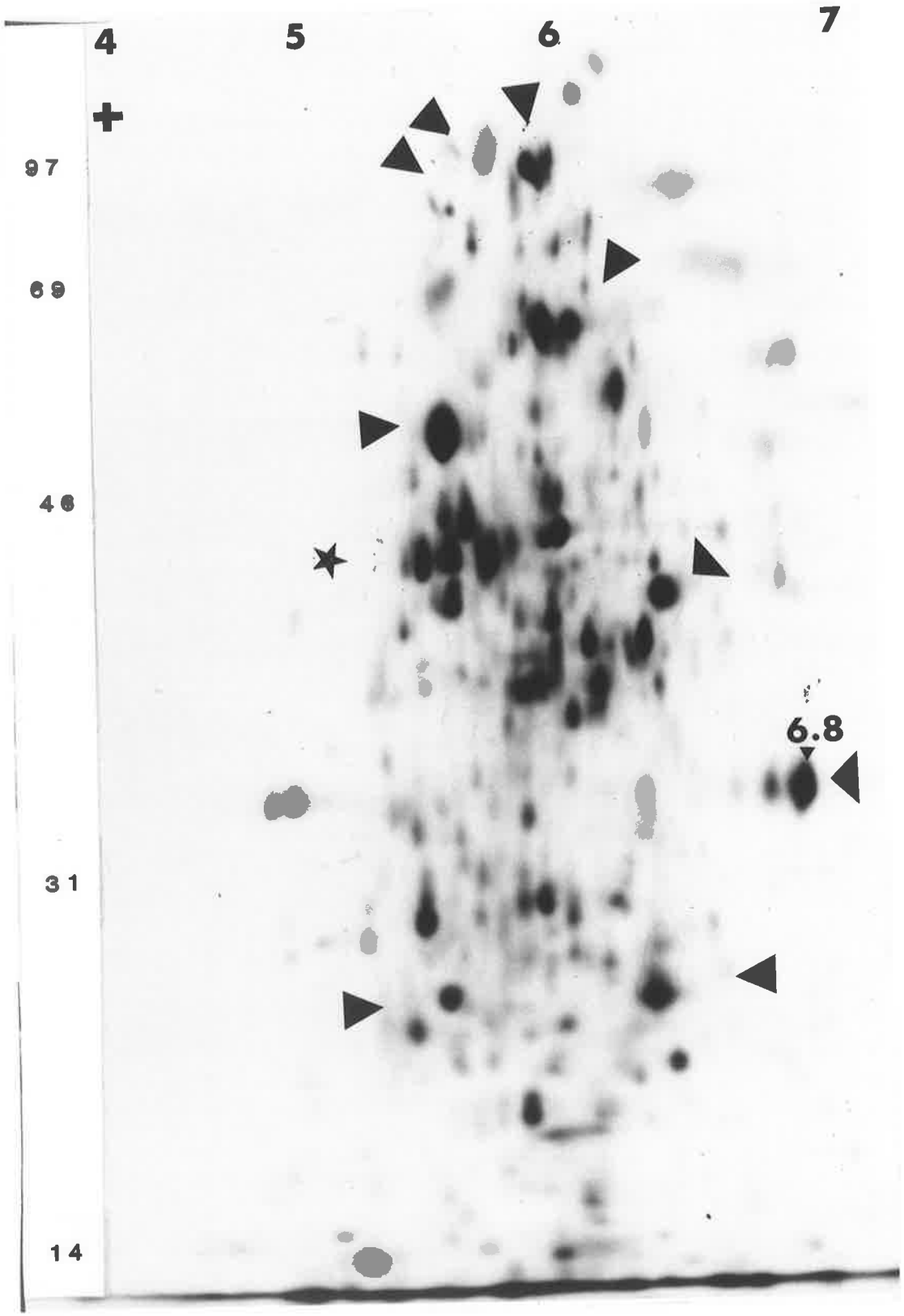
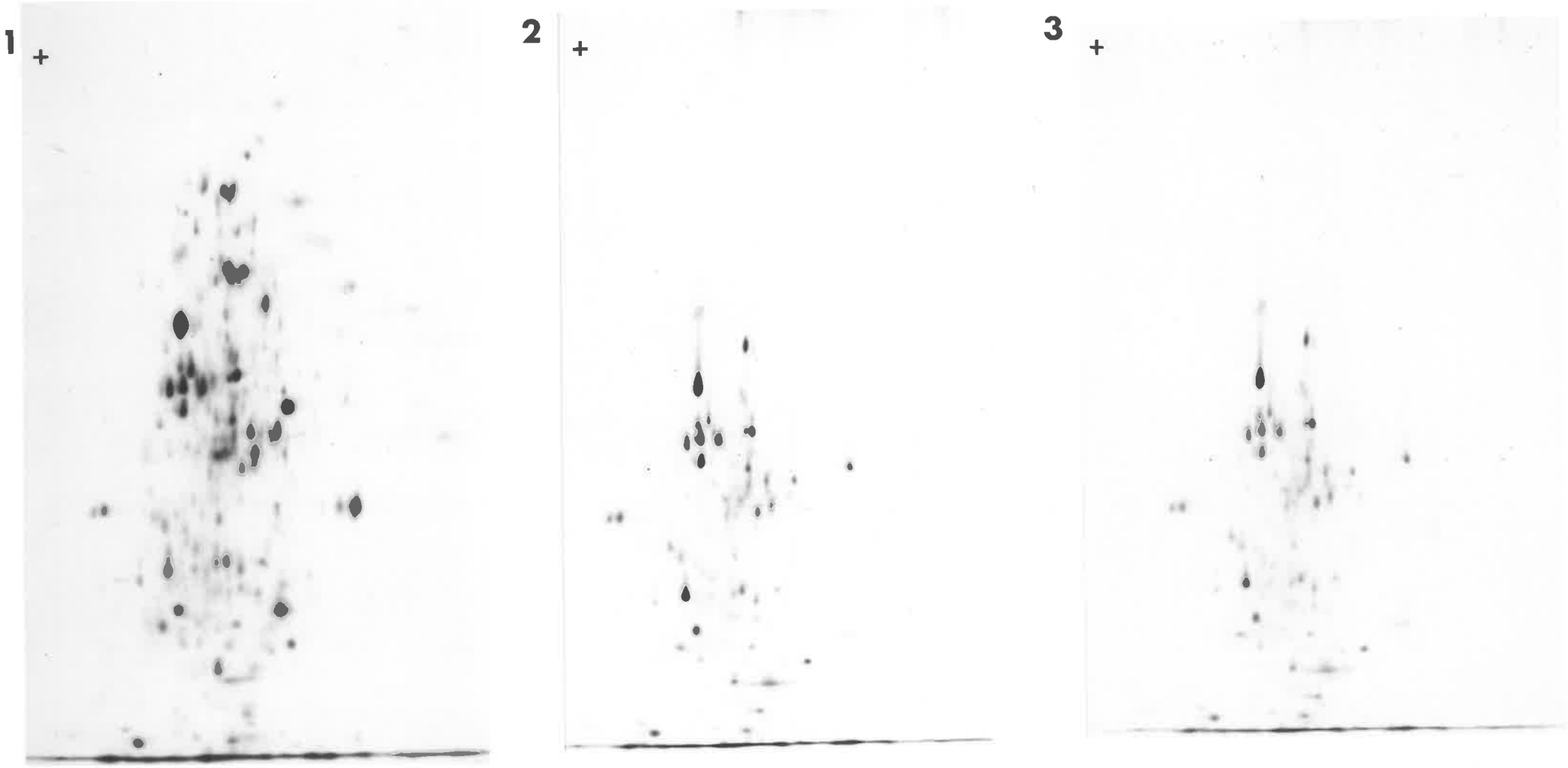

4
5
6
7
+
97
69
46
31
14
6.8

Figure 4. Representative 2D-SDS/PAGE fluorograph of the effects of melatonin [1.6 X 10^{-7} M] on the incorporation of ^{35}S-methionine into rat ML secreted pineal proteins in-vitro. [Note all the protein spots missing when comparing with Fig 3].


4
5
6
7
+
6.8

Figure 5. **A comparison of 2D-SDS/PAGE fluorographs showing the effects of melatonin and photoperiod on the incorporation of ^{35}S-methionine into ML rat pineal secreted proteins.**

[1]. ML pineal control.
[2]. ML pineal with melatonin [1.6 X 10^{-7} M].
[3]. MD pineal control.


1
+
2
+
3
+

affected by melatonin are in the molecular weight range 70-200 KDaltons. Below this range, proteins are variably affected some being marginally reduced in quantity others totally ablated, whilst some are qualitatively altered. One major group of secretory proteins of molecular weight 34 KDaltons and pI ranging from 4.5 - 6.8 are distinctly variable inhibited by melatonin [Figs 3-5]. Melatonin's inhibitory effect on the uptake of label by pineal secreted and cellular proteins is dose related over the range 10^{-7} - 10^{-5}M. [Fig. 6]. These results indicate an influential role for melatonin on pineal protein synthesis. That melatonin allows some protein synthesis may reflect on its ability to discriminate between day and night protein synthesis. It is posssible that melatonin can discriminate between pineal bioactive protein synthesis and secretion and protein synthesis for intra-cellular activities such as proliferation and cell repair.

Melatonin and ^{3}H-Uridine Incorporation

The effects of melatonin on the incorporation of labeled uridine into total RNA is shown in Fig. (7). Melatonin [10^{-7}M] inhibits the incorporation into ML pineal total RNA by about 50% [$p<0.001$]. When melatonin was added to MD pineal cultures it was revealed that labeled uridine incorporation into total RNA was even further reduced [10%;$p<0.01$]. Its effects on the other tissues examined were 25% [$p<0.01$] for hypothalamus and 5% [$p<0.01$] for the cortical tissue [Fig 8].

In experiments where the tissues were first cultured with melatonin followed by culture without melatonin, it was repeatedly shown that removal of melatonin reinstated the ability of pineal tissue to re-

Figure 6. Representative 1D-SDS/PAGE fluorograph showing the effects of 3 doses of melatonin on the incorporation of ^{35}S-methionine into rat pineal secreted proteins in-vitro.

Lanes [1-2]. pineal control.
Lanes [3-4]. pineal with 1.6 X 10^{-7}M melatonin.
Lanes [5-6]. pineal with 1.6 X 10^{-6}M melatonin.
Lanes [7-8]. pineal with 1.6 X 10^{-5}M melatonin.

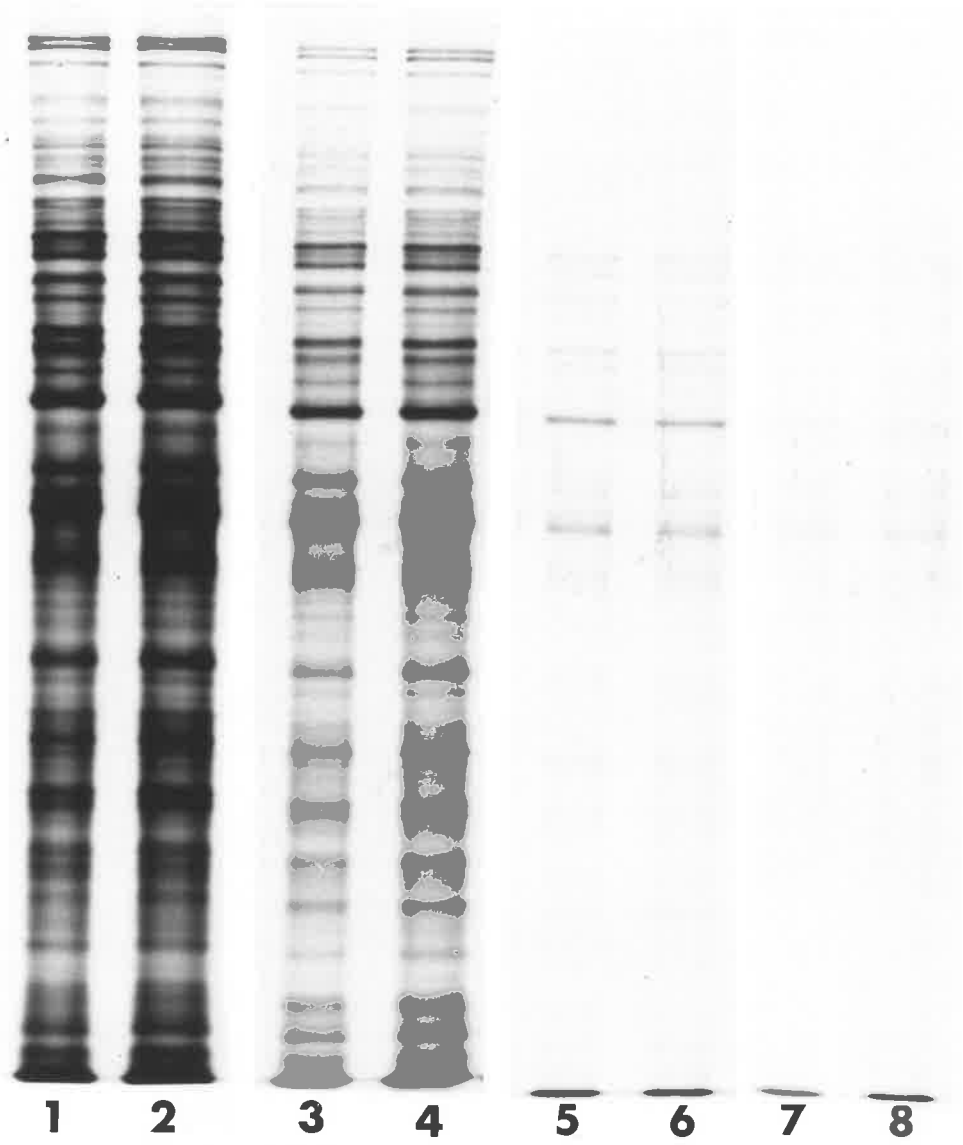

1
2
3
4
5
6
7
8

Figure 7.

The effects of melatonin [10^{-7}M] on the incorporation of ^{3}H-Uridine into total RNA in rat ML:

[a] pineal
[b] hypothalamus
[c] cortex.

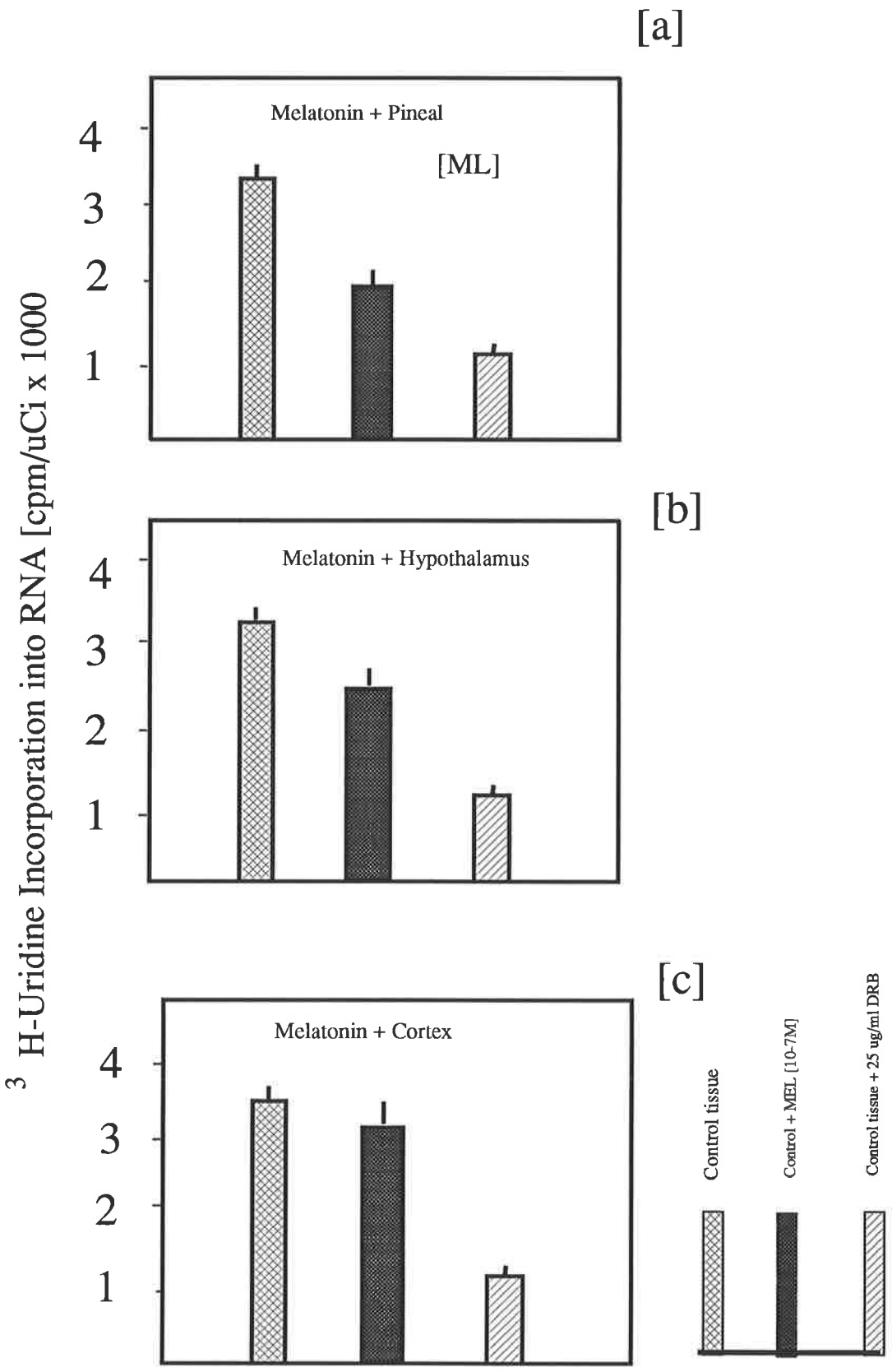

[a]
Melatonin + Pineal
[ML]
4
3
2
1
[b]
Melatonin + Hypothalamus
4
3
2
1
[c]
Melatonin + Cortex
4
3
2
1
Control tissue
Control + MEL [10-7M]
Control tissue + 25 ug/ml DRB
3 H-Uridine Incorporation into RNA [cpm/uCi x 1000

Figure 8

The effect of melatonin [10^{-7} M] on the incorporation of ^{3}H-Uridine incorporation into RNA of MD rat pineal glands. Compare this with melatonin effects on ML pineals and MD pineals treated with DRB.

Figure 9.

Melatonin effects can be reversed by removal of tissues from melatonin treatment. Further addition of melatonin later in the culture period again inhibits the incorporation of label into pineal and hypothalamic RNA. Cortical tissue is not affected.

Figure 8

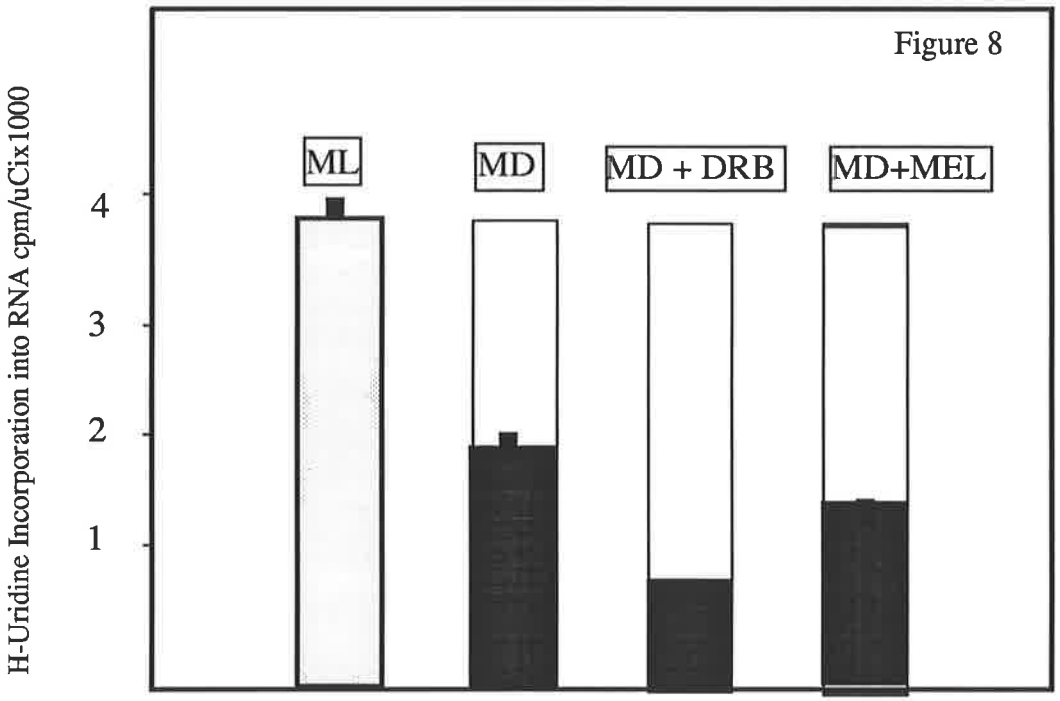
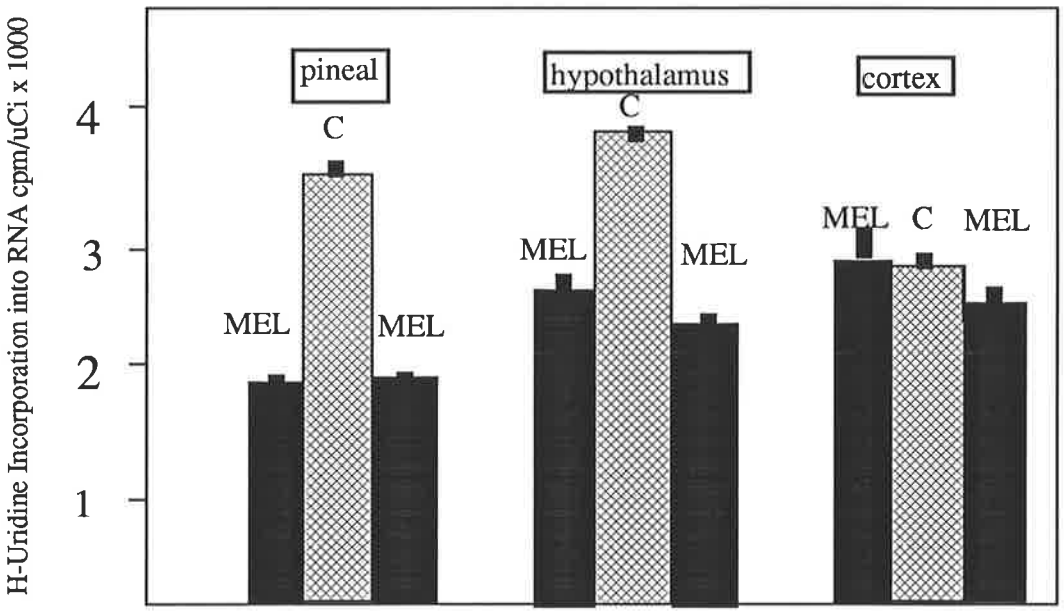


Figure 9



C= control tissue
MEL= melatonin

incorporated labelled uridine into total RNA. If tissues were rexposed to melatonin and culture period extended, further inhbition of the incorporation of label was evident [Fig. 9].

Recently in the literature there was a report by Joyce and Miller [1991], about the inhibitory effects of steroids [progesterone and 17b-estradiol] on mRNA transcription in the ovine pituitary an effect similar to melatonin on pineal RNA. Although similar in their effects, the steroid inhibitory mechanism may not be the same as that of melatonin. Other recent studies involving melatonin, inhibition of RNA transcription and protein synthesis at extrapineal sites were reported in the literature review [Chapter 3] and are worthy of re-emphasis, since they lend support to the results from this study.

6. CONCLUSIONS

These results strongly point to transcription as the protein synthetic process inhibited by the mode of action of melatonin although, translation should not be totally excluded. It would be possible by further experimentation to identify the precise ribosome(s) specifically affected by melatonin. The possibility that melatonin acts as a reversible transcription inhibitor adds new dimensions to the functional role of the pineal indole. Recent investigations have looked at reversible transcription inhibitors as protein regulators but have overlooked the possibility that melatonin in the pineal serves such a role. Further, if as the results suggest that melatonin can reversibly suppress or alter protein synthesis in putative target tissues within the brain including the hypothalamus, then this may provide a molecular basis for

understanding the role of the indoleamines in regulating circadian mechanisms.

Chapter Eleven

Miscellaneous Influence On Pineal Proteins

1. Abstract

Preliminary studies assessing the effects of age, sex, nutrition and species variations on rat pineal protein synthesis and secretion were performed. 2D-SDS/PAGE could discriminate between pineal protein secretions derived from an Australian native bush rat *[rattus fuscipes greii]* and the laboratory Hooded Wistar rat on the basis of 1 and 2D-SDS/PAGE protein pattern variations.

Sex, nutrition and age did not show any significant influences on the incorporation of ^{35}S-methionine into rat pineal proteins as determined by 1 and 2D-SDS/PAGE.

2. Introduction

It is accepted from the literature that the pineal is subjected to a number of influences [see Part One] and the present study was performed to determine whether these influences are translated to variations in pineal protein synthesis and secretion. However, it must be emphasized that these studies were only preliminary in that the subject animals were part of ongoing studies by other members of the department who were not interested in examining the pineal gland. The experiments in this study were divided into 4 groups: Species, Age, Sex and Nutrition.

I SPECIES AS AN INFLUENCE

The Bush rat is an Australian native species of rat from which very little pineal data is available. If 2D-SDS/PAGE could discriminate species variations in protein patterns, then this would be an excellent means of identification of species differences. It could play a role in resolving questions about the physiological function of pineal proteins in species specific events such as reproduction, hybernation and habitation.

Materials and Methods

The animals were captured in the wild and housed in laboratory conditions LD 14:10, food and water ad-lib. Upon sacrificing, pineals were removed and labelled in culture under conditions described in earlier chapters. Pineals obtained from Hooded Wistar rats were similarly cultured and the tissue and medium analysed by one and two dimensional SDS/PAGE

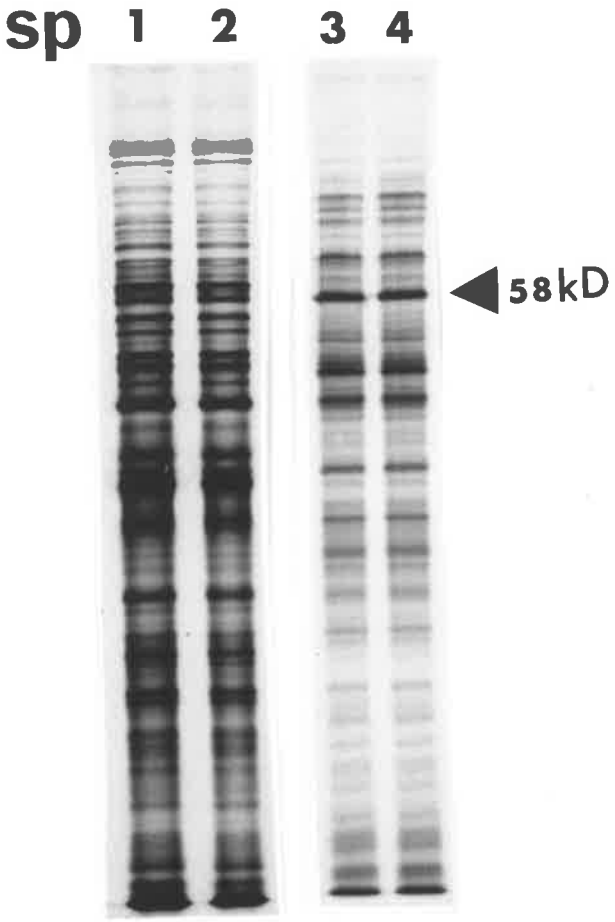
RESULTS AND DISCUSSION

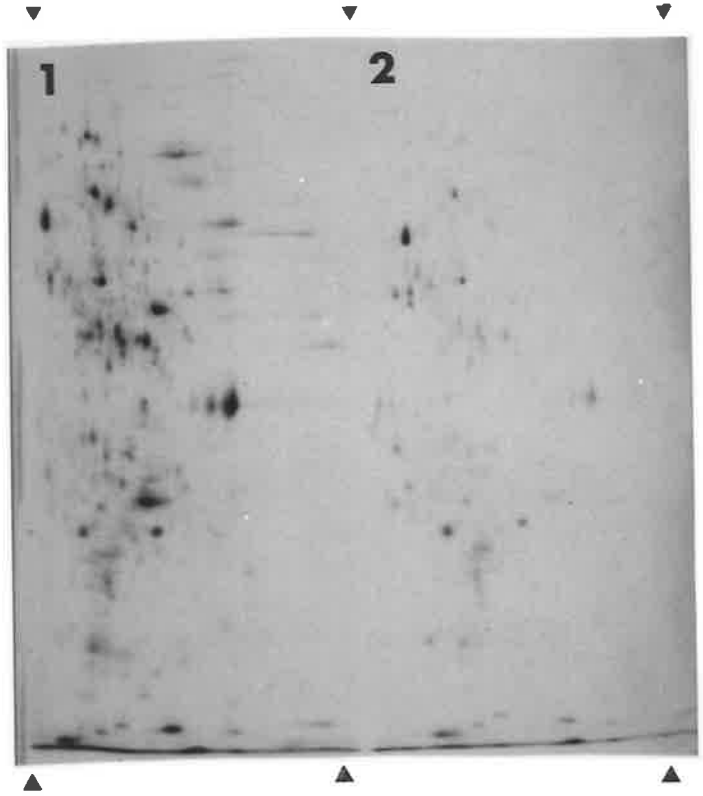
The one dimensional fluorographs of the two species of rat were compared [Fig 1]. Several protein band differences were seen but perhaps the most distinctive difference was the presence of a band at the 58 KDalton mark of the bush rat profile which was totally absent in the Wistar hooded animals. The experiment was repeated 5 times and in each case, the result was the same. The significance of this

Figure 1. Species Influences on Pineal Proteins. Comparison of the incorporation of ^{35}S-methionine into Hooded Wistar and Bush Rat *[rattus fuscipes greii]* pineal secreted proteins.

Lanes [1-2]. ML Hooded Wistar [male] pineal secreted proteins.
Lanes [3-4]. ML Bush Rat [male] pineal secreted proteins.

The 2D-SDS/PAGE fluorograph represents the ML Bush Rat pineal secreted proteins [part 1] and next to this the ML Bush Rat pineal secreted proteins treated with 10^{-7}M melatonin [part 2]. Bush Rat pineal protein synthesis and secretion is are also significantly reduced by melatonin.


sp
1
2
3
4
58kD


1
2

distinctive band in the native species is unknown but warrants further investigation. Speculation is that the native species pineal may have a different array of proteins to enable its existence and survival in the wild.

II AGE AS AN INFLUENCE

The effects of ageing on the pineal have been well documented. Calcification of pinealocytes as well as other morpological variations of pineal cell organelles suggests that protein secretion may also be influenced. This study made a preliminary assessment as to whether age was an important variable and whether the variations could be discriminated by one and two dimensional SDS/PAGE.

Animals

Three age groups [N=10] of male Hooded Wistar rats were housed in conditions described in earlier experiments. Each group of animals were sacrificed by cervical dislocation at the ML phase of the photoperiod and pineals cultured with labelled methionine and the medium and tissue processed for
1 and 2D-SDS/PAGE.

Results and DIscussion

A comparison of 4, 8 and 12 week old animals revealed no significant variations in the one or two dimensional SDS/PAGE protein profiles

[Fig.2]. Whether younger or older animals show any marked variatrions is not known. It may be possible that any variations in protein synthesis and secretion are in proteins and peptides outside the molecular weight range studied here.

III SEX AS AN INFLUENCE

Sex has been reported as an influence on pineal function. Gonadal steroid and other hormones upon feedback to the pineal may influence the pineal proteins. Any differences found by 2D-SDS/PAGE would be of value in explaining some of the possible sex variations reported in the past, such as estrogen receptors in the pineal. Female rat pineals may produce distinctive proteins to that of the males and may relate to differences in reproductive processes.

Animals

10 female and 10 male Hooded Wistar rats [100-125g] were housed in conditions as described earlier. Upon sacrifce their pineal glands were cultured with labelled methionine and the tissue and medium analyzed by one and two dimensional electrophoresis.

RESULTS AND DISCUSSION

No differences in 1 or 2D-SDS/PAGE protein patterns were revealed. [Fig. 2]. A more detailed analysis is required before making any conclusions about the influence of sex differences on the synthesis of

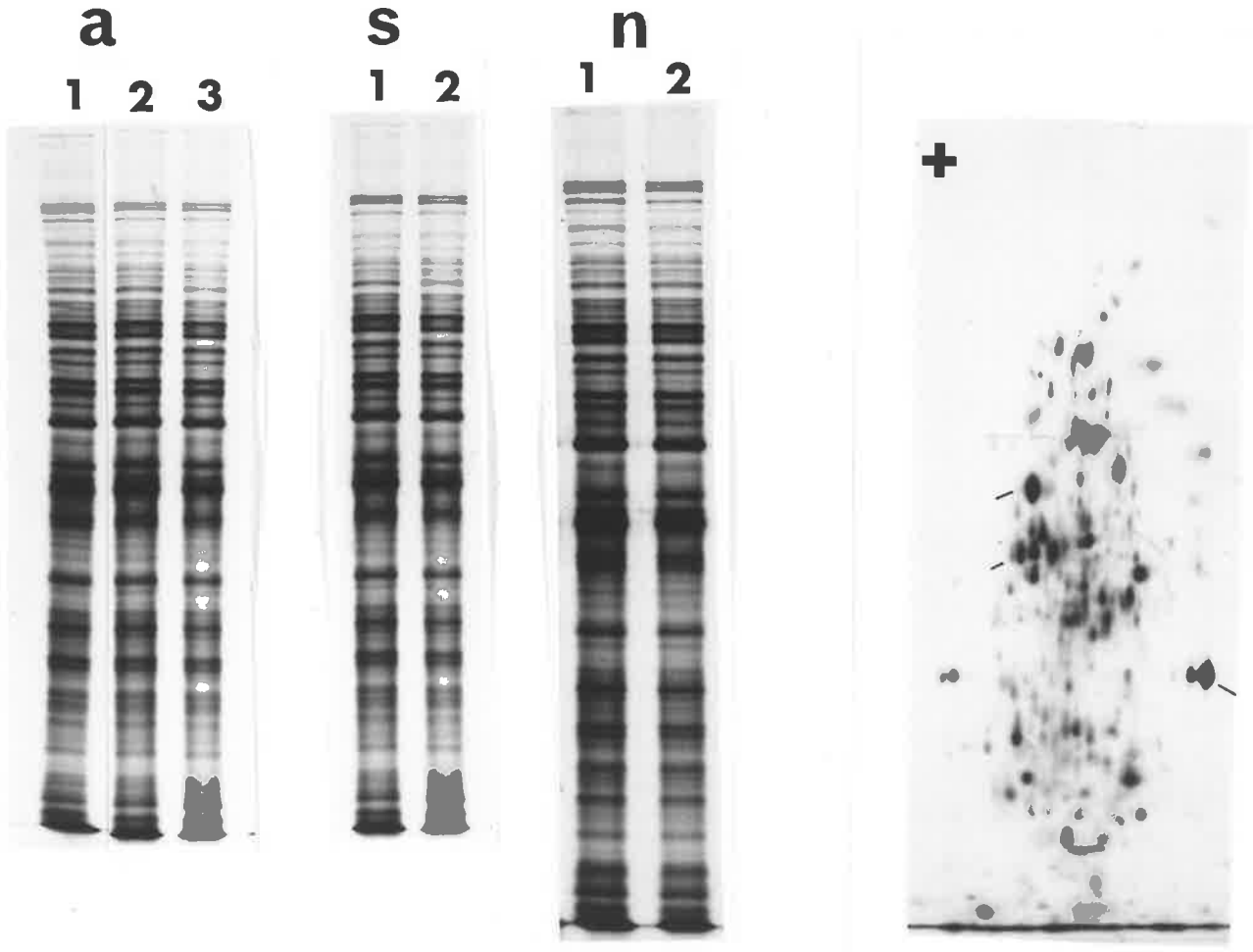
Figure 2. Age, Sex and Nutrition Influences on Pineal Proteins. There were no significant differences in the incorporation of label into rat pineal proteins of different ages [a], different sex [s] and between well fed and undernourished rats [n]. Both 1D and 2D-SDS/PAGE flourographs as seen opposite reveal protein profiles compatible with normal rat ML pineal secreted proteins.

[a] Lane 1. Four weeks.
Lane 2. Eight weeks.
Lane 3. Twelve weeks.

[s] Lane 1. Males.
Lane 2. Females.

[n] Lane 1. Normal protein diet.
Lane 2. Protein deficient diet.

The 2D-SDS/PAGE fluorograph is representative of the samples above.


a
1 2 3
s
1 2
n
1 2
+

rat pineal proteins. Factors such as estrous cycle and a combination of age-sex influences would need to be evaluated extensively by further experiments.

IV NUTRITION AS AN INFLUENCE

Once again several experiments in the past have linked nutrition to pineal function. In these experiments it was aimed at identifying nutrition as a factor causing marked changes to 1 and 2D SDS/PAGE proteins patterns.

Animals

Sprague Dawley male rats [150g] were housed in a lighting schedule of L:D 14:10 in four groups [N=5]. The diet to each group was as follows : one group was fed and watered ad-lib whilst a second group were fed a protein deficient diet [600g Kellogs Rice Bubbles; 12 ml cod liver oil; 12g glucose; 6g di-calcium phosphate] [water ad-lib].

Upon sacrifice [21 days later] by cervical dislocation, the pineals were excised and incubated with labeled methionine and the resulting medium and tissue prepared for one and two dimensional electrophoresis.

RESULTS AND DISCUSSION

The resulting protein profiles did not show any distinctive variations between the two different groups [Fig. 2]. The pineal may respond to protein deficient diets in other ways. It may be necessary to subject

animals to more extreme diets before observing any changes of significance in pineal protein synthesis and secretion.

CONCLUSIONS

Although these were only preliminary studies and apart from the species variation all the other findings were negative, the studies highlighted the extent of applicability of 2D-SDS/PAGE once established. These experiments warrant more detailed analysis before making any definite conclusions about the influences of age, sex, and nutrition on rat pineal protein synthesis and secretion.

Part Five

Thesis Summary and Bibliography

THESIS SUMMARY

The primary aim of this thesis was to initiate studies allowing the re-assessment of the relationship of the photoperiod to pineal protein synthesis and secretion. There is now compelling evidence derived from morphometric, cytological and cytochemical studies to indicate that the pineal is active in protein synthesis and secretion but, it is only since the advent of modern molecular biological techniques that a systematic study of the pineal products can be undertaken. In the present study, methods have been developed which allow characterization of pineal protein synthesis and secretion from individual rat pineal glands in-vitro in an attempt to identify and characterize pineal proteins with biological function in circadian events.

DIURNAL CHANGES IN RAT PINEAL PROTEINS

Diurnal changes in pineal protein synthesis and secretion were studied by removing glands from animals entrained to a 14:10 [L:D] lighting schedule at 4 hourly intervals over a 24 hour period and assessing the nature of the proteins secreted by one and two dimensional gel electrophoresis. The isolated glands were individually incubated with ^{35}S-methionine and both tissue [cellular] and medium [secreted] proteins analyzed by one and two dimensional electrophoresis. Proteins were detected by a highly sensitive colour-based silver stain and by fluorography. It was found that rat pineal proteins varied both quantitatively and qualitatively over the 24 hour photoperioid with a bimodal pattern

of incorporation of radiolabel. Maximum synthetic and secretory activity occured in the middle of the light phase [ML peak] with a second minor peak in the middle of the dark phase [MD peak]. Many of the secretory proteins displayed charge heterogeneity typical of glycoproteins with the ML pattern being significantly different from the MD protein pattern. By comparison with other brain tissues [hypothalami and cortex], this diurnal differentiation in rat pineal protein synthesis and secretion was not observed.

EFFECTS OF MELATONIN ON RAT PINEAL PROTEINS

The pattern of protein synthesis contrasts to the pattern of melatonin production which is restricted to the dark phase of the photoperiod. To examine whether melatonin influenced protein synthesis through autocrine or paracrine mechanisms, physiological doses of melatonin [10^{-7} - 10^{-4} M] were included in incubations of rat pineal glands and the effects on cellular and secreted proteins examined. It was found that melatonin [10^{-7}M] either totally inhibited or partially reduced the incorporation of labelled methionine into ML proteins and the effect was dose related. The inhibition of label into total precipitable protein was in the order of 25% [$p<0.01$]. Significantly, pineal protein patterns of labelled secreted proteins obtained from melatonin treated ML pineal glands resembled those obtained from untreated MD pineals. Melatonin also reduced the amount and altered the pattern of incorporation of labelled methionine into pineal cellular proteins. The inhibitory effects of melatonin on protein synthesis was not found with other brain tissues examined [hypothalami and cortex].

MELATONIN'S MECHANISM OF ACTION ON PROTEIN SYNTHESIS

To determine the mode of action of melatonin on protein synthesis experiments were carried out to determine whether melatonin acted at the transcription level of protein synthesis. Rat pineal glands obtained at ML were incubated with ^{3}H-uridine in the absence or presence of melatonin [10^{-7} - 10^{-4}M] for periods ranging from 30-180 min. and TCA-precipitable radioactivity measured. Significant dose related decreases were found in the incorporation of label into TCA precipitable material in pineals treated with melatonin, at 10^{-7}M melatonin the reduction being nearly 50% [p< 0.001]. This effect was rapid [< 30min] and could be reversed by removal of melatonin. With MD pineals the incorporation of label into the total RNA fraction was distinctively less than that of the ML but the suppressive effect of melatonin was still evident. A similar suppressive effect of melatonin on protein synthesis was observed for hypothalamic but not cortical tissues. Whilst these data do not totally rule out translational interference or protein-protein interaction mechanisms in the inhibition of pineal protein synthesis, they indicate that melatonin exerts its effects on protein synthesis via transcriptional mechanisms and that these effects are reversible.

MISCELLANEOUS FACTORS INFLUENCING PINEAL PROTEIN SYNTHESIS AND SECRETION

A series of preliminary studies were carried out to ascertain the effects of age, sex, nutrition and species variations on pineal protein synthesis and secretion. Age was identified as an important variable with nutrition and sex variations being less dramatic but these variations were minor compared to the effects of the photoperiod and melatonin. Major species differences were evident in the study which compared the Bush Rat [a native Australian species] with the laboratory Wistar Hooded Rat. A major pineal protein secretion of molecular size 58 kDaltons appearing on the SDS/PAGE profile of the Bush rat was absent in the laboratory species.

CONCLUSIONS

It was concluded that 2D-SDS/PAGE can provide useful insights into pineal protein synthesis and secretions. The study highlighted two major points, firstly, the major influence of the photoperiod and secondly, a novel role for melatonin as an autocrine and/or paracrine regulator of pineal protein synthesis. If as indicated melatonin acts as a reversible inhibitor of protein synthesis in the pineal and hypothalamus, then this may provide an important new pathway of enquiry into the action of melatonin on the regulation of circadian mechanisms.

Future Challenges

Many interesting questions arise from the data provided by the present studies. Foremost are those related to the role of the various proteins secreted by the pineal gland. Molecular biological techniques are now developed to the extent where it is possible and feasible to characterize selected proteins and through recombinant DNA procedures make them available in amounts suitable for pharmaceutical and immunocytochemical studies of their function.

Equally important are further studies aimed at determining the significance of melatonin's autocrine and/or paracrine regulatory affects on pineal protein synthesis. Questions immediately arise concerning the specificity of melatonin's actions - is it restricted to the pineal, or does it extend to other brain centres such as the SCN and pars tuberalis where melatonin " receptors " are found? Preliminary studies indicated that melatonin influences protein synthesis of hypothlamic but not the cortical brain tissue but this needs further elaboration.

Future studies may look at the effects and mechanisms of action of melatonin at tissues outside the brain where already reports are emphasizing melatonin's protein inhibitory actions. Can some of these actions be partially explained by data reported here and what are the implications of melatonin's protein inhibitory action in areas such as cancer and the immune system where it has been already shown to have a potent inhibitory effect on certain types

of tumor cells ? [Bartsch et al., 1990; Blask and Hill 1986]. It may act through transcription inhibitory mechanisms to in-activate tumor genes or it may influence the immune system by regulating the synthesis of potent bioactive proteins and peptides.

The list of questions for future appraisal is endless and undoubtedly the present study has generated more questions than it has provided answers for, but hopefully it has contributed to the reawakening of interest in pineal peptides and proteins and their interrelathionship(s) with melatonin - the much neglected arm of pineal gland research.

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