PINEAL GLAND FUNCTION DURING THE REPRODUCTIVE CYCLE - A MULTI SPECIES STUDY

by

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A thesis submitted to the University of Adelaide in fulfilment of the requirements for the degree of Doctor of Philosophy.

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This thesis is dedicated to my wife Roslyn

"Men who have excessive faith in their theories or ideas are not only ill prepared for making discoveries, they also make poor observations. They can see only a confirmation of their theory."

— Claude Bernard
(cited in Kitay and Altschule, 1954)

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DECLARATION

I hereby declare that to the best of my knowledge and belief, that this thesis contains no material previously published by another person, except when due reference is made in the text.

David J. Kennaway B.Sc. (Hons)

PREFACE

During the course of this study various aspects were published as follows.

a) Papers

1. "The occurrence of hydroxyindole-O-methyltransferase (HIOMT) activity in foetal sheep pineal tissue and its relationship to preparturient endocrine changes"

   D. J. Kennaway and R. F. Seamark
   J. Reprod. Fert. 45 529-531 (1975)

2. "A specific radioimmunoassay for melatonin in biological tissue and fluids and its validation by gas chromatography mass spectrometry"

   D. J. Kennaway, R. G. Frith, G. Phillipou, C. D. Matthews and R. F. Seamark
   Endocrinology 101, 119-127 (1977)

3. "On the presence of melatonin in pineal glands and plasma of foetal sheep"

   D. J. Kennaway, C. D. Matthews, R. F. Seamark, G. Phillipou and M. Schilthuis J Steroid Biochem 8 559-563 (1977)

4. "Changes in plasma tryptophan and melatonin content in penned sheep"

   D. J. Kennaway, K. J. Porter and R. F. Seamark
   Aust. J. Biol. Sci. (1978) In Press.

b) Abstracts

1. "Pineal function in foetal and neonatal lambs"

   D. J. Kennaway, C. D. Matthews, K. Umapathysivam and R. F. Seamark
   J. Reprod. Fert 36 468-469 (1974)

2. "Pineal changes during the period of blastocyst activation in the Tammar wallaby *macropus eugenii*.

   D. J. Kennaway and R. F. Seamark
   J. Reprod. Fert 46 503-504 (1976)

3. "Diurnal rhythm in plasma melatonin persists into late pregnancy in humans"

D. J. Kennaway, A. LeCornu, C. D. Matthews, A. Slavotinek and R. F. Seamark
Proc. Endocrine. Soc. Aust. 19 28 (1976)

4. "Increase in melatonin content of foetal sheep pineal tissue approaching term"

D. J. Kennaway and R. F. Seamark
Theriogenology 6 626 (1976)

5. "Plasma melatonin: an index of pineal function?"

D. J. Kennaway, C. D. Matthews and R. F. Seamark
Proc. Endocrine Soc Aust 20 (1977)

6. "The occurrence and synthesis of melatonin in the foetal sheep pineal gland"

D. J. Kennaway and R. F. Seamark
Proc. Int. Symp. Pineal Gland, Jerusalem p. 19 (1977)

7. "Pineal function in pregnant sheep"

C. D. Matthews, I. C. Kowanko, R. F. Seamark and D. J. Kennaway
Aust. NZ J. Obst. 16 250-251 (1976).

8. "Melatonin in pregnant humans and sheep"

C. D. Matthews, D. J. Kennaway, A. LeCornu and R. F. Seamark.
Aust. NZ J. Obst. 16 252 (1976).

9. "Melatonin in foetal and maternal ovine plasma"

C. D. Matthews, I. C. Kowanko, D. J. Kennaway and R. F. Seamark
J. Reprod. Fert. 46 498-499 (1976).

10. "Pineal gland function in pregnancy"

C. D. Matthews, D. J. Kennaway, M. Schilthuis and R. F. Seamark
Proc. VI International Cong. Endocrinology, Hamburg, p. 7 (1976).

11. "Plasma melatonin values in man and some domestic animals; initial observations on the effects of pregnancy in man and pinealectomy in sheep"

C. D. Matthews, D. J. Kennaway, R. G. Frith, G. Phillipou, A. Le Cornu and R. F. Seamark.
J. Endocrinology 73 41 P - 42 P (1977)

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Many aspects of my work would have been impossible without the help and enthusiasm of my collaborators. In particular I wish to thank Mr. Ken Porter for his excellent surgery and surgical teaching and his staff for their care of the animals. To Ann Le Cornu, Brenda Bell and Meg Kaethner, I extend my appreciation for their valuable assistance with some of the melatonin and steroid assays. I am indebted to Dr. G. Phillipou and Mr. G. Frith for their help with the validation of the radioimmunoassay. For providing samples and access to their patients and animals I wish to thank Drs. J. M. Obst, E. Dunstan, C. H. Tyndale-Biscoe, G. McCulloch, W. G. Breed, R. S. Tulsi, B. Firth, M. Wellby and Prof. W. V. McFarlane.

I wish to thank also Mr. J. Hadaway and his staff in the QEH clinical photography department for some of the photographs in this thesis.

SUMMARY

1) The thesis concerns a study of pineal gland function during the reproductive cycle of several species including the sheep, human and a scincid lizard *Tiliqua rugosa*.

2) The development and validation of a reliable radioimmunoassay for the presumptive pineal hormone melatonin is described and its application to the measurement of melatonin content of pineal tissue and blood plasma to provide an index of pineal function investigated.

3) Studies in the sheep and humans confirmed that plasma melatonin levels were higher during periods of darkness than during periods of light. The increase in plasma levels of melatonin was associated with the onset of darkness. In the sheep no relationship was apparent between this pattern of melatonin secretion and circulating levels of cortisol and tryptophan.

4) In the ewe there were no consistent alterations in circulating plasma melatonin which could be related to the stage of the oestrous cycle. Removal of the ovaries had no consistent effect on either the daytime concentrations of plasma melatonin or the diurnal rhythm. Depot injections of a synthetic progestin (medroxyprogesterone) had no significant effect on daytime concentrations of plasma melatonin in ovariectomised sheep, however, daily injection of oestradiol 17β raised the daytime plasma melatonin concentrations in some animals for the period of the injections.

5) Pregnancy was associated with alteration in the diurnal rhythm of maternal plasma melatonin in the sheep but not in the human. The nyctohemeral rise in plasma melatonin content of pregnant ewes of 90-150 days gestation was reduced. By contrast, plasma melatonin concentrations continued to exhibit a diurnal rhythm in the pregnant women.

6) Studies on foetal sheep showed that the pineal had an increased capacity to synthesise melatonin during the last 4-5 days of gestation. This increased synthetic capacity is associated with increased pineal gland content of melatonin but is not associated with increased plasma concentrations. Infusion of synthetic corticotrophin into the foetus induced parturition but did not cause any detectable changes in plasma melatonin concentrations. Infusion of melatonin (1-4 ug/hr) into the foetal circulation did not interfere with either synthetic corticotrophin induced parturition or normal parturition. By use of radioactive tracer substances it was shown exogenously administered melatonin can transfer from the maternal circulation across the placenta to the foetus. The reverse situation can also occur.

7) Plasma melatonin concentrations were monitored in pinealectomised sheep. An immunoreactive substance probably melatonin was detected in all sheep, often at concentrations greater than 200 pg/ml. Pinealectomised sheep did not show a diurnal rhythm in plasma melatonin. Plasma melatonin in sham operated and pinealectomised pregnant ewes was low and no changes in concentration appeared before or during parturition.

8) Plasma concentrations of melatonin were investigated in response to alterations in photoperiod and parietalectomy in the scincid lizard *Tiliqua rugosa*. A diurnal rhythm in plasma melatonin was discovered. Highest levels occurred during darkness. Peak melatonin concentrations tended to be lower during the breeding season. Shifting the photoperiod 6 hours and 12 hours resulted in a shift in the diurnal rhythm. Constant light and constant darkness abolished the diurnal rhythm. Parietalectomy had no significant depressive effect on the diurnal rhythm of plasma melatonin unless it was accompanied by shielding of the lateral eyes.

9) Analysis of plasma melatonin in rats, cattle, donkeys, chickens, camel and pig showed that nighttime concentrations were higher than daytime concentrations.

10) A study of pineal gland enzymes in the Tammar Wallaby indicated that a significant decrease in melatonin synthesis occurred at the time of blastocyst activation in free ranging animals. By contrast, very low concentrations of melatonin were apparent in possum pineal tissue and blood at different times of the year. No diurnal rhythm was evident.

11) During the course of investigations, two patients with radiographically identified pinealomas were investigated for anomalies in plasma melatonin content. Melatonin was undetectable at all times. Plasma gonadotrophins were in one case abnormally low (S.D.) while in the other case they were normal (E.S.). Prolactin levels were abnormally high (S.D.) and abnormally low (E.S.). Plasma corticoid levels were within normal limits.

GENERAL INTRODUCTION

The mammalian pineal gland is now thought to be an important endocrine organ. During the last twenty-five years, a considerable body of knowledge has accumulated which suggests that the pineal gland translates information about the environment into hormonal messages.

The nature of the pineal gland hormone(s) has remained controversial. One school of opinion proposes that indole derivatives of tryptophan, especially melatonin, are the pineal hormones, while another school proposes polypeptide hormones. At the time this study was initiated there was no strong evidence for either.

The aim of the study was to utilise the existing methods of monitoring pineal activity in an investigation of pineal function in a number of experimental animals. It was obvious, however, that new techniques were needed to investigate the endocrine potential of this gland. Thus considerable effort was expended in developing and validating a radioimmunoassay for one of the proposed hormonal products of the pineal, melatonin. Plasma and pineal levels of this compound were then used as an index of pineal function under various conditions in a number of species.

The following literature review discusses the "state of the art" of pineal physiology as it stood in the absence of the sophisticated monitoring techniques that are now becoming available, such as radioimmunoassay and gas chromatography-mass spectrometry. The knowledge gained during the past twenty-

five years has come from classical physiological studies utilising extirpation of the gland and replacement of its presumed hormones. It is hoped that assay methods such as that reported in this thesis will extend the knowledge of the pineal gland.

CHAPTER I

LITERATURE REVIEW

Historical Introduction

"Discussions about pineal systems have overutilized the term 'vestigial' more than any other single adjective. If such a description were to prove accurate then these organs would rate as among the most prevalent of vestiges" (Wurtman, 1967).

Such notions of the pineal being a vestige are relatively modern. The early Greek and Roman philosophers were the first to describe this small discrete cone-shaped organ of the brain and to attribute various functions to it. Galen (A.D. 139-201) spoke of it as "scolecoid" or worm-like and emphasised its intimate connection with the great cerebral vein bearing his name. Throughout this period the pineal (or epiphysis cerebri) was believed to have a role in the mental well-being of humans. Due to its central position, the pineal was considered to be a valve which controlled the flow of memories to the front of the brain. Magendie, in the nineteenth century, expanded this idea, suggesting that it regulated the flow of spinal fluid, which was responsible for the psychological requirements of the individual. Renee Descartes' impressions, however, have had the greatest impact. His notion that the pineal was the seat of the rational soul, which presided over the earthly machine (the human body) persisted for many years. Other philosophers apparently scorned the idea that the soul

should be placed in a "kernel".

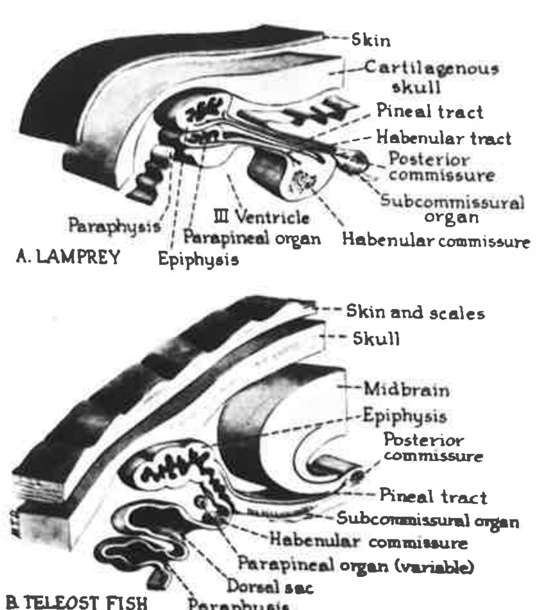
A physiological role of the pineal ("glandula pinealis") was proposed by Gibson (1682). Both he and Cowper (1666-1709) believed the pineal to be a lymph gland. Cowper further suggested that the "glandula pinealis which we take to be a lymphatic gland receives Lympha from the lymphe ducts which pass by way of the third ventricle of the brain to the infundibulum and the glandula pituitaria."

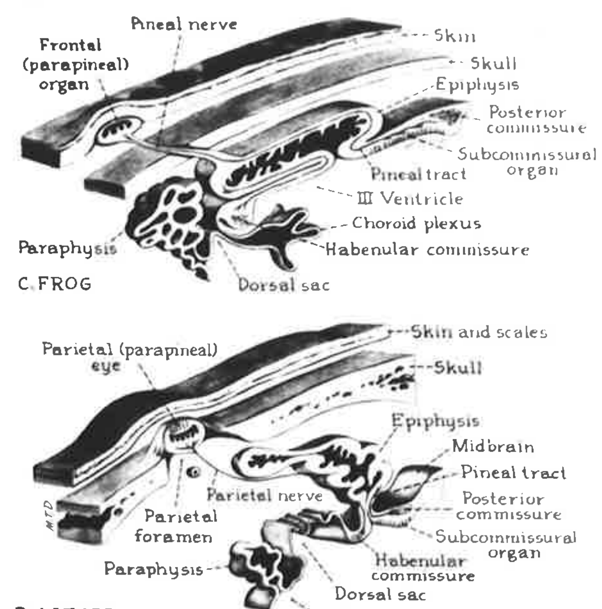
This endocrine role of the pineal was, however, discredited by the Cartesian philosophers expounding a relation to mental well-being. When their suggestion that the pineal calcification and madness were connected was permanently disproved, the pineal slipped into obscurity as a vestigial organ. This obscurity persisted until the 1940's when the pineal was again associated with an endocrine function, this time as a regulator of gonadal function. There is reason to suspect that nearly all present vertebrates have at least diffuse regions of pineal tissue, which suggests a long and continuing evolution. A discussion of the evolutionary significance and organisation of the pineal system is beyond the scope of this review. Figure 1, however, shows the complexity of the system in various vertebrates. For a definitive review of the subject, see Gladstone (1940).

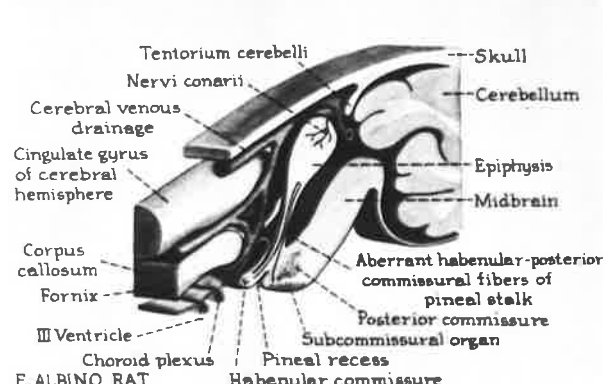
The pineal develops from the roof of the brain -- that is from the median region of the posterior end of the diencephalon. Like the third ventricle of the brain, this evagination is lined with ependymal cells. As differentiation proceeds, the pineal anlage becomes a sac-like structure with its

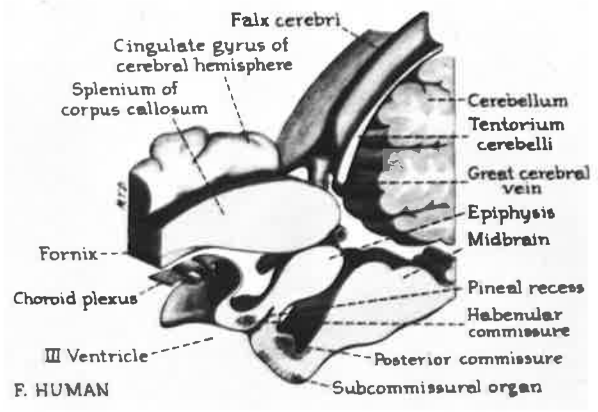
Figure 1

Schematic diagram showing the relationships between the pineal gland and other brain structures in representative animals. (From Wurtman, Axelrod and Kelly, 1968, with permission of the publishers).


Skin
Cartilagenous skull
Pineal tract
Habenular tract
Posterior commissure
Subcommissural organ
Habenular commissure
III Ventricle
Paraphysis
Parapineal organ
Epiphysis
A. LAMPREY
Skin and scales
Skull
Midbrain
Epiphysis
Posterior commissure
Pineal tract
Subcommissural organ
Habenular commissure
Parapineal organ (variable)
Dorsal sac
Paraphysis
B. TELEOST FISH


Pineal nerve
Frontal (parapineal) organ
Skin
Skull
Epiphysis
Posterior commissure
Subcommissural organ
Pineal tract
III Ventricle
Choroid plexus
Habenular commissure
Paraphysis
Dorsal sac
C. FROG
Parietal (parapineal) eye
Skin and scales
Skull
Epiphysis
Midbrain
Pineal tract
Posterior commissure
Subcommissural organ
Parietal nerve
Parietal foramen
Habenular commissure
Paraphysis
Dorsal sac
III Ventricle
D. LIZARD


Tentorium cerebelli
Skull
Nervi conarii
Cerebellum
Cerebral venous drainage
Cingulate gyrus of cerebral hemisphere
Epiphysis
Midbrain
Corpus callosum
Aberrant habenular-posterior commissural fibers of pineal stalk
Fornix
Posterior commissure
III Ventricle
Subcommissural organ
Choroid plexus
Pineal recess
Habenular commissure
E. ALBINO RAT


Falx cerebri
Cingulate gyrus of cerebral hemisphere
Splenium of corpus callosum
Cerebellum
Tentorium cerebelli
Great cerebral vein
Epiphysis
Midbrain
Fornix
Pineal recess
Choroid plexus
Habenular commissure
III Ventricle
Posterior commissure
Subcommissural organ
F. HUMAN

lumen continuous with the third ventricle from which it is derived. There is then extensive folding and production of minor lumina each continuous with the main lumen and in turn confluent with the third ventricle. Eventually in mammals the minor lumina are severed of their connection with the third ventricle. These so-called rosettes have been proposed as evidence of secretion during foetal life in sheep (Jordan, 1916).

Anatomy

The pineal in mature animals is situated between and connected by well defined superior and inferior peduncles with the habenular and posterior commissures. From studies on rodents a concept has developed that pineal innervation in mammals is almost entirely sympathetic, with the superior cervical ganglion providing one source of post-synaptic fibres (Kappers, 1969). Nerve fibres originating from the habenular and the commissures are considered by Kappers (1965) to be merely aberrant loops passing through the organ and lacking synaptic ribbons en route. There is, however, compelling evidence for para-sympathetic innervation of monkey (Kenny, 1961), ferret (David *et al*., 1973) and rabbit (Romijn, 1973) pineal glands. Specific pineal nerves have been described in sheep and rabbit foetuses (Moller *et al*., 1975) and human foetuses (Mollgard and Moller, 1973).

There is also evidence for a nervous connection with the brain in cats and monkeys (Nielson and Moller, 1975). Thus even within the mammalian species there appears to be some

diversity, with rats having strictly sympathetic innervation, with monkeys, ferrets and rabbits having both parasympathetic and sympathetic nerves and other mammals an even larger range of input mechanisms.

Blood Supply

The pineal is served by small arteriolar branches from off-shoots of the posterior choroid arteries. There is no specific pineal artery or any specific group of pineal arteries or arterioles. Scattered arterioles penetrate the pineal's connective tissue capsule but do not extend far into the organ. They are not accompanied closely by veins or venules and do not appear to be involved in the kind of portal supply system connecting the hypothalamus with the pituitary. Microscopic capillaries and venules drain into larger venules and eventually into larger veins or sinuses in the vicinity and finally drain into the jugular veins. Despite the lack of specific arteries the blood flow through the pineal gland of rats is exceeded only by the kidney and equalled only by the pituitary (Goldman and Wurtman, 1964).

Pineal Cells

The pineal gland contains two main cell types, astrocytes and pinealocytes. The pineal specific cells, the pinealocytes have been shown biochemically by phosphate incorporation studies to have high metabolic activity (Borrell and Orstrom, 1945). They possess all the structures normally associated with active endocrine cells, that is, abundant mitochondria,

rough and smooth endoplasmic reticulum and a well developed Golgi apparatus producing both dense core and lucent vesicles. These vesicles apparently migrate along the processes of the pinealocytes to the bud-shaped terminals. The lucent vesicles may also be pinched off from the smooth endoplasmic reticulum present in these terminals and thus could also be involved in the secretory process of the pinealocyte. Depletion of the content of the vesicles has been observed using Electron Microscopy (Lukasyk and Reiter, 1975).

Secretion of Pineal Hormones

The histological structure of the pineal gland strongly suggests that it is an endocrine organ. The physiological evidence which will be discussed later also supports this view. It is, therefore, pertinent to discuss the route of secretion of any pineal product in light of the above developmental characteristics and the anatomical position of the organ.

There is some light and electron microscopic evidence that the pineal products are released into the capillary blood via the capillary spaces and the endothelial wall and then reach the systemic circulation (Kappers *et al*., 1974). The pineal develops in close association with the cerebrospinal fluid and this medium must be considered. Reiter (*et al*., 1975b) have suggested five ways that cerebrospinal fluid could be involved in pineal secretion. 1). In some animals pineal secretory products may be released directly into the intraventricular cerebrospinal fluid without entering blood vessels within the pineal. This is apparently anatomi-

cally impossible in many animals but may be important in some rodents and lagomorphs. 2). Blood vessels draining the pineal gland may form a capillary plexus to the supra-habenular recess and secrete into the cerebrospinal fluid. 3). If reversal of blood flow within the great cerebral vein occurs the hormones may have ready access to, and be secreted into the cerebrospinal fluid via the choroid plexus. 4). The secretion of hormones via the choroid plexus into the third ventricle may involve the systemic circulation - that is the hormones leave the brain and return to the choroid plexus and 5). Tanycytes may transport the hormones from the systemic circulation to the third ventricle.

Once in the cerebrospinal fluid the pineal hormones could act on neurons or glial cells in the cerebrospinal fluid transmitting signals to the medial basal hypothalamus or they may act directly at the level of the median eminence to affect hypothalamic releasing factors. The secretory products could also act at the level of the pituitary itself.

Hormonal Products of the Pineal

Indolealkylamine Derivatives

The pineal gland contains high amounts of the tryptophan derivative 5- hydroxytryptamine and its metabolites. Particular emphasis has been placed on the 5- methoxylated derivatives, 5- methoxytryptamine, 5- methoxytryptophol and 5- methoxy-N-acetyl tryptamine. The last indole was found by Lerner and co-workers in 1958 to be responsible for the potent skin

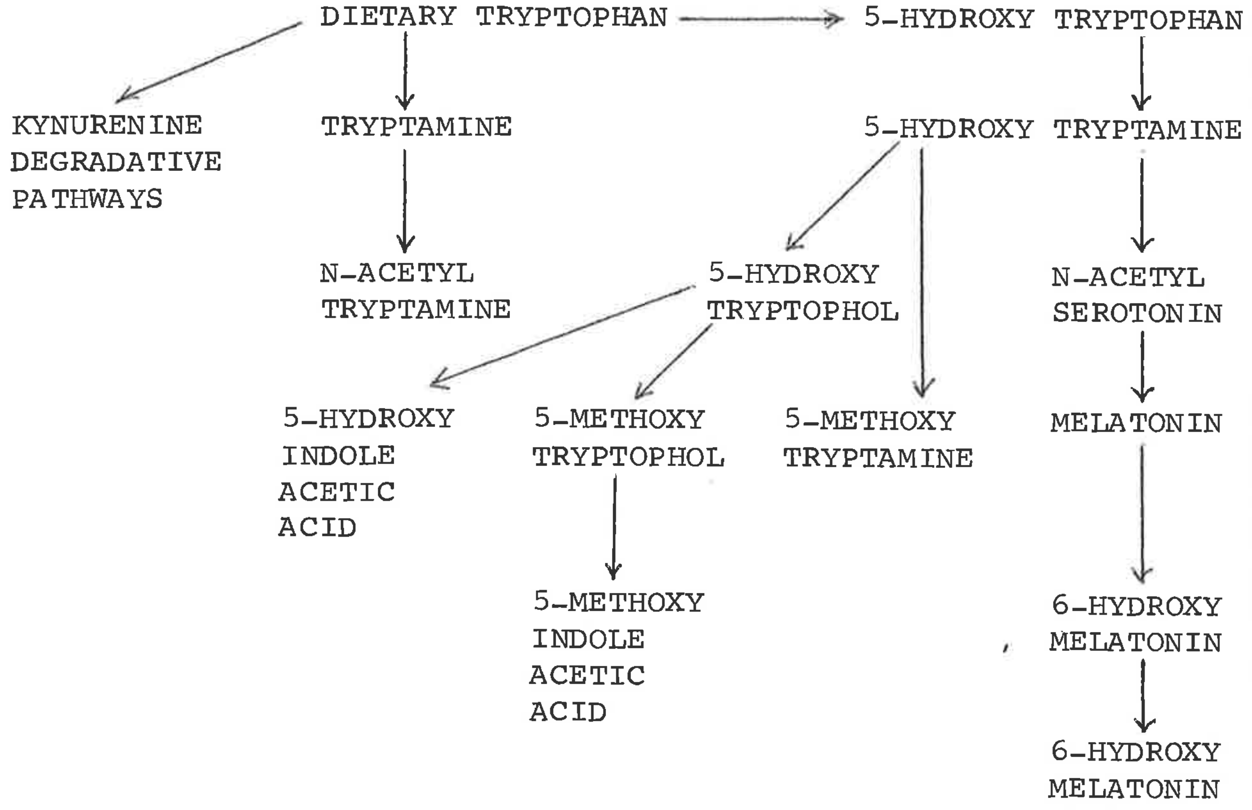
lightening activity which had been discovered 40 years earlier (McCord and Allen, 1917). This compound, called melatonin, has subsequently been associated with the pineal's reproductive effects.

In 1960, Axelrod and Weissbach discovered within the pineal an enzyme hydroxyindole-O-methyltransferase (HIOMT) which synthesises melatonin. Subsequently Weissbach et al., (1960) found an enzyme catalysing the formation of the immediate precursor of melatonin, N-acetyl 5-hydroxytryptamine (N-acetyl serotonin). The full indolic pathway of metabolism of tryptophan in the pineal is shown in figure 2. Tryptophan can also be metabolised by the kynurenine pathway.

There are several excellent reviews on the biochemistry of the pineal indoles (Quay, 1974, Axelrod, 1974, Brownstein, 1975). This review will cover those studies which indicate functional control of the synthesis of melatonin by external environmental factors and the internal hormonal milieu.

Axelrod et al., (1965) reported that pineal Hydroxyindole-O-methyltransferase activity was higher at night than during the day. Further investigation suggested that light influences the pineal via a nervous connection from the retina, inferior accessory tract, median forebrain bundle, superior cervical ganglion and sympathetic nerves (Moore et al., 1968. Moore and Klein, 1974). The nerves to the pinealocytes have an increased rate of firing during darkness resulting in the release of noradrenaline at the nerve terminal (Sakai and Marks, 1972). The noradrenaline acts on $_{1}$ adrenergic receptors on the

FIGURE 2 TRYPTOPHAN METABOLISM



pinealocyte membrane causing an elevation of adenylate cyclase activity (Weiss and Costa, 1967) and increased intracellular cyclic AMP production. This increase in cyclic AMP is followed by RNA production and synthesis of either a protein activator or de novo synthesis of the enzyme Serotonin N-acetyltransferase (Romero et al., 1975, Klein and Weller, 1970).

Sympathetic nerve release of noradrenaline increases tryptophan transport across the pinealocyte membrane (Wurtman et al., 1969) providing an increase in substrate for both protein synthesis and presumably increasing its metabolism to other indoles. The pineal content of the enzyme hydroxyindole-O-methyltransferase is 1-2 times higher at night than during the day (Axelrod et al., 1965). Superior cervical ganglionectomy decreases pineal hydroxyindole-O-methyltransferase suggesting that there is neural control of this enzyme (Nagle et al., 1973). Yang and Neff (1976) using immunological titration have shown that increases in hydroxyindole-O-methyltransferase activity in response to darkness and decreases due to superior cervical ganglionectomy are a result of alterations in the content of the enzyme and are not due to the presence or absence of an activator.

The induction of serotonin-N-acetyltransferase and hydroxyindole-O-methyltransferase and the subsequent synthesis of melatonin in response to darkness (Wilkinson et al., 1977) is very rapid and so is the deactivation and decrease in melatonin synthesis. If animals are exposed to light during the normal dark phase, Serotonin-N-acetyltransferase activity rapidly

decreases with a half life of 3 minutes (Klein and Weller, 1972). Enzyme activity and melatonin content begin to decrease before "lights on" the next morning - thus suggesting that there is endogenous control over rhythmic melatonin production (Wilkinson *et al.*, 1977).

The endogenous control is exemplified even more if animals are placed in constant light or constant dark conditions. Constant light results in a dimunition of rhythmic activity of enzymes and pineal melatonin content, whereas constant dark results in a free running rhythm (Nagle *et al.*, 1972). The endogenous rhythm arises from the suprachiasmatic nucleus (Moore and Klein, 1974) and apparently onset of darkness is used as a Zeitgeber.

There is some evidence that the internal hormonal milieu can influence melatonin synthesis at the level of the adrenergic receptor site of the pinealocyte. Lynch et al., (1973, 77) have shown that immobilisation stress or insulin induced hypoglycaemia induces serotonin-N-acetyltransferase and that adrenergic blocking agents prevent the induction. These workers suggest that in the absence of rhythmic changes in environmental lighting, other cyclical events such as sleeping, eating or locomotor activity may, by varying sympathetic tone generate continuing rhythms in pineal function. While the pineal gland is believed to influence gonadal function via the hypothalamo-pituitary axis it is now clear that the pineal is itself influenced by other endocrine organs. Various oestrogenic, progestogenic and androgenic steroids as well as pituitary gonadotrophins have been found to affect pineal biosynthetic

activity. Despite the fact that serotonin:N-acetyltransferase is the rate limiting enzyme in the melatonin pathway (Axelrod, 1974), gonadal factors do not regulate its levels (Illnerova, 1975). Hydroxyindole-O-methyltransferase levels are, however, apparently altered by gonadal hormones. Houssay and Barcello (1972) have shown that oestradiol 17β increased rat pineal hydroxyindole-O-methyltransferase, while Nagle et al. (1972) have provided conflicting evidence which shows that oestrogen treatment decreases hydroxyindole-O-methyltransferase activity. Nagle et al. (1972) have further shown that noradrenaline can attenuate the pineal response to 5 ug oestradiol 17β /day. Injections of oestradiol 17β of 0.05 ug-1 ug/day paradoxically increase hydroxyindole-O-methyltransferase (Cardinali et al., 1974a), but the effect of noradrenaline under these conditions has not been reported. Oestradiol 17β will stimulate melatonin production in tissue culture (Mizobe and Kurokawa, 1976). Progesterone administration inhibits hydroxyindole-O-methyltransferase activity in rats (Houssay and Barcello, 1972). Cardinali et al. (1974b) investigated the relationship between sheep pineal hydroxyindole-O-methyltransferase and plasma progesterone and found that the enzyme was lower during the luteal phase and suggested that it was due to progesterone inhibition. Castration of male rats decreases hydroxyindole-O-methyltransferase activity and this activity can be restored by testosterone injections of 0.1 mg-1 mg/day, while even higher doses inhibit the enzyme activity (Nagle et al., 1974). Noradrenaline apparently is required to maintain oestrogen and androgen

receptors in the pineal since superior cervical ganglionectomy decreases receptor populations and noradrenergic drugs restore them (Cardinali *et al.*, 1975a).

Recently Cardinali and co-workers demonstrated that the pituitary hormones LH, FSH and prolactin increase hydroxyindole-O-methyltransferase activity in rats (Cardinali et al, 1976). Since it is well established that castration in rats decreases hydroxyindole-O-methyltransferase, the physiological meaning of these results remains unclear. It has also been found that hypophysectomy in male rats also decreases pineal hydroxyindole-O-methyltransferase (Alexander *et al.*, 1970).

The discussion thus far has concentrated on the control of melatonin synthesis, however, another indole of some interest is 5-methoxytryptophol. This compound is the product of monoamine oxidase and hydroxyindole-O-methyltransferase. During periods of high pineal serotonin levels and low Serotonin-N-acetyltransferase, 5-hydroxytryptophol may be synthesised in preference to N-acetyl serotonin and then O-methylated to 5-methoxytryptophol. In some tissues monoamine oxidase is under hormonal control (Parvez and Parvez, 1973) but this is apparently not the case in the pineal gland.

Pineal Effects on the Pituitary

Pituitary function is known to be controlled by the secretion of monoaminergic nerves present in the hypothalamus. Stimulating various nerve tracts in the median eminence causes release of the releasing factors or release-inhibiting factors,

GnRH, TRH, PIF and/or PRF, MSHRH, MSHRIF and GHRH. Dopamine and noradrenaline have been shown to increase plasma LH secretion after injections into the third ventricle of rats (Kamberi et. al.,1971). Low doses (1.25 ug) dopamine caused 7.5 and 6.5 fold increase in LH and FSH plasma concentrations and reduced prolactin to one third of the controls. 100 ug noradrenaline was required for a similar effect. It was determined that these effects were caused by a direct action on the hypothalamic release of LHRH, FSHRH and PIF and not by a direct action on the pituitary. A generally held view is that the pineal interacts somehow with this nervous control.

Direct evidence that the pineal gland operates through the pituitary gland inhibiting gonadotrophin secretion was obtained by Fraschini et al. (1971). They found an increase in pituitary LH content in pinealectomised male rats and increased accessory organ weights. They subsequently demonstrated an increase in FSH under similar conditions. They thus suggested that the pineal gland inhibits LH synthesis as well as release.

Blinding plus anosmia in rats and blinding in hamsters has been shown to increase melatonin synthesising activity in the pineal gland (Reiter et al., 1971, Anton-Tay and Wurtman, 1968). It is found that these blind anosmic rats have elevated pituitary LH stores, depressed plasma LH and hypotrophic reproductive organs (Reiter et al., 1971). In hamsters blinding alone is sufficient to cause LH accumulation in the anterior pituitary (Reiter and Johnson, 1974a). LHRH injections in blind animals

lowered pituitary LH and increased plasma LH (Reiter and Johnson, 1974b) suggesting that the pineal does not prevent the action of LHRH or LH release, but possibly prevents LHRH from gaining access to the pituitary by inhibiting synthesis or release in the hypothalamus. Pinealectomy in rats made blind and anosmic at 25 days of age restores the ability of the pituitary to accumulate prolactin (Reiter, 1975). 100 days after the blinding and anosmia treatment the rats have restored pituitary prolactin despite an intact pineal gland. Thus whereas the pineal is capable of delaying the development of the hypothalamo-pituitary-gonadal mechanisms via inhibition of synthesis or release of prolactin inhibiting factor, it cannot prevent them from reaching the mature condition.

The actions of the pineal on the pituitary have been attributed to indolealklyamine derivatives especially melatonin and also to polypeptide factors.

Melatonin Effects

Kamberi et al. (1970, 1971) showed that intraventricular injection of serotonin or melatonin in doses of 1,5 or 50 µg/rat in anaesthetised male rats has an opposite effect to that seen with small doses of dopamine or larger doses of noradrenaline. Both indoles resulted in a suppression of the release of LH and FSH and simultaneously released prolactin. In unanaesthetised cycling female rats Kamberi (1972) demonstrated that intraventricular injection of melatonin or serotonin 1-5 µg/rat between 1300 - 1400 h on the day of pro-oestr us can suppress the pro-oestrous surge of LH and FSH and inhibit ovulation.

Ying and Greep (1973) using much higher doses of melatonin intracardially also suppressed ovulation at this time. Yamashita et al., (1973) found that chronic intraventricular injections of 1 and 10 mg melatonin into dogs resulted in a decrease in pituitary and plasma LH ten days later. They suggested that the cause of this phenomenon is an inhibition of synthesis of LH in the pituitary. Melatonin will also prevent compensatory ovarian hypertrophy following unilateral ovariectomy (Sorrentino 1968, Vaughan et al., 1971) an effect presumed to involve an increase in FSH (Benson et al., 1969). Smythe and Lazarus (1973) have shown that melatonin will suppress 5-Hydroxytryptophan stimulated increases in rat growth hormone and as well proposed an effect on human growth hormone (Smythe and Lazarus, 1974).

The early work of Kamberi and his co-workers (Kamberi et al., 1970, 1971) suggested that the site of action of melatonin is the hypothalamus. Moguilevsky et al., (1976) used clomiphene-induced release of LH to show that melatonin could suppress LH at the level of the hypothalamus but could not affect the response of the pituitary to injected LHRH. Cardinali et al. (1975b) have shown that melatonin decreases hypothalamic neurotransmitter uptake in a non-competitive (i.e. independent of transmitter concentration) manner while simultaneously causing a release of neurotransmitter. The effect was most potent on serotonin uptake and release. They concluded that melatonin may potentiate the effects of the neurotransmitter. This may be a partial explanation for the finding that both serotonin

and melatonin decrease LH and FSH release. Smythe et al., (1975) proposed that serotonin and melatonin compete at a common receptor site in the hypothalamus, but their results do not preclude inhibition of uptake and release of neurotransmitter.

The above studies implicate a hypothalamic site of action for melatonin, but there is some evidence that another site may well be the pituitary. Orsi et al., (1973) found that subcutaneous injections of 10 μg melatonin per day for 5 days significantly decreased protein synthesis in the hypothalamus and the pituitary 68 and 50 percent of control. Recently Martin and Klein (1976) and Martin et al. (1977) found that melatonin suppressed LHRH-induced release of LH from cultured neonatal rat pituitary glands at 1 nM concentrations. The effect was rapid and specific for disubstituted indolealkyamines like melatonin. Thus melatonin may act at the level of the hypothalamus and the pituitary.

Many early workers obtained equivocal results with melatonin injections in their experiments on pituitary function. For instance some workers (Adams et al., 1965, Narang et al., 1967, De Prospo and Hurley, 1971, Sorrentino et al., 1971) obtained decreased pituitary weight and increased pituitary LH, whereas others (Thieblot et al., 1966, Panda and Turner 1968, Ota and Hsieh, 1968, Singh and Turner, 1971) showed that melatonin does not affect the pituitary. It is now apparent that the route of administration and time of administration of melatonin are critical. The high doses required when using

subcutaneous or intraperitoneal injections (1 mg) when compared with intraventricular infusions (1 μg) or implants into the median eminence (30 μg) would suggest that high local concentrations of melatonin are required at its receptors. Acute subcutaneous intraperitoneal or intravenous injections of melatonin fail to take into account two critical observations. Firstly administered melatonin has a half life of 10 minutes in mice and rats (Kopin et al., 1961). Thus adequate concentrations of melatonin at the receptors following injection may either not be attained or attained only briefly. The second important consideration is that injection of melatonin has often been performed at 0900 h, ignoring the length of time before the next rise in endogenous melatonin production and secretion. At 0900 h the pineal target tissue(s) may either be refactory to its influence or subsensitive due to prior exposure to endogenous melatonin.

Reiter and his colleagues in a large series of papers (Reiter and Vaughan, 1975, Reiter et al., 1975 a,c,e, 1976 b,c, 1977 a,b) have attempted to overcome the problem of clearance of hormone by using chronic implants of melatonin: beeswax pellets to allow a steady secretion of melatonin during their manipulations. Using this approach they have found that weekly implants of 50 μg-1 mg melatonin prevents gonadal degeneration which is a result of exposing the animals to short daily photoperiods. The treatment has similar effects to pinealectomy. Turek et al., (1975) using silastic tubing implants of melatonin instead of beeswax achieved essentially similar results. It is proposed by Reiter that melatonin is not <u>the</u> pineal antigonado-

trophin, but that melatonin (1) interfered with mechanisms regulating the true pineal antigonadotrophin or (2) stimulated a pineal progonadotrophin or (3) rendered the neuroendocrine axis resistant to inhibition by pineal antigonadotrophin or (4) was directly stimulatory to the reproductive system.

Such an approach, however, fails to take into consideration the normal diurnal variation in melatonin levels. Bridges et al., (1976) and Tamarkin et al., (1976) found that the time of melatonin injection in hamsters was critical if there was to be an antigonadal effect. Injections of melatonin in the morning during a 14:10 L : D photoperiod were not effective in causing regression of testes in males or acyclicity in females, but subcutaneous injections of 10-25 µg melatonin later than 7.5 hours before the onset of darkness were effective. The delay in recovery of gonadal function after cessation of treatment was similar to their observations on gonadal function after withdrawal of hamsters from short photoperiods (Seegal and Goldman, 1975). Tamarkin et al., (1976) found also that late afternoon injections of melatonin into pinealectomised hamsters were ineffective. This is crude supportive evidence for Reiter's intrapineal action of melatonin. A report by Turek et al., (1976a) that silastic tubing implants which released 150 µg melatonin/day caused gonadal regression in 14:10 L:D and suppressed light induced testicular recrudenscence is difficult to explain. Turek et al., (1976b) have, however, found that melatonin implants are only successful in photoperiodic rodents and not in laboratory rats and house mice. Reiter

et al., (1976a) has also recently reported that melatonin beeswax implants prevent the gonadal regression induced by late afternoon melatonin injections.

Tamarkin et al., (1977) concluded "It will be desirable to determine whether the presently reported diurnal production of the compound such that photoperiodically-induced gonadal responses in the hamster may be influenced by phases shifts in the rhythm of melatonin synthesis". This hypothesis is extremely important for the understanding of why melatonin production is rhythmic. It suggests that the time of onset of melatonin production and secretion is critical. The administration of melatonin via continuous release implants may dampen or abolish the endogenous rhythm and thus pharmacologically pinealectomise the animal.

The complex control of melatonin synthesis and the demonstration of antagonistic activity on the pituitary at relatively low doses when administered at specific times of the day, suggests that this indole is one of the pineal hormones.

Physiological actions of methoxytryptophol have not been intensively studied despite the demonstration by McIsaac et al. (1965) and Ebels and Horwitz-Bresser (1976) of measureable quantities of the indole in pineal tissue. Methoxytryptophol does possess some antigonadotropic activity (McIsaac et al., 1964, Fraschini et al., 1971). Reiter et al., (1975c) have shown that methoxytryptophol is as effective as melatonin in reversing the gonadal regression due to exposure to short photoperiod. The reproductive effects of methoxytryptphol

require further scrutiny.

Pineal Peptide Factors

While it is well established that appropriately timed melatonin administration to animals can suppress hypothalamic-pituitary function, there is a large body of evidence in favour of non-indolic pineal hormones.

Arginine vasotocin (AVT) is one such proposed peptide hormone. It is a nonapeptide bearing structural similarities to both vasopressin (AVP) and oxytocin (OT).

AVT - Cys-Tyr-Ileu-Glu(NH_2)-Asp(NH_2)-Cys-Pro-Arg-GlyNH_2

AVP - Cys-----Phe--------------------Cys-----Arg-GlyNH_2

OT - Cys-----Ileu-------------------Cys-----Leu-GlyNH_2

While arginine vasotocin is predominantly found in non-mammalian vertebrates, the peptide is present in many mammalian species as well. Milcu, Pavel and Neacsu (1963) characterised biologically and chromatographically extracted a peptide from bovine pineal glands which they proposed to be arginine vasotocin. In subsequent work Pavel utilised a specific bioassay to identify arginine vasotocin in human cerebrospinal fluid (Pavel, 1970) and noted that the ependymal cells were involved in it's secretion in bovine (Pavel, 1971), human (Pavel, 1975a) and rat foetuses (Pavel et al., 1977). Conclusive evidence of the occurrence of arginine vasotocin in pineal tissue was advanced by Cheesman (1970) using massspectrometry. Arginine vasotocin has also been detected in pineal glands by radio-

immunoassay (Rosenbloom and Fisher, 1975). Foetal rat pineal arginine vasotocin content is higher than adult levels (Pavel et al., 1975a), perhaps reflecting a decrease in secretory ependymal cells within the pineal with maturity. Adult male rat pineal arginine vasotocin exhibits a diurnal rhythm (Calb et al., 1977). There is increasing evidence also that the pineal arginine vasotocin is associated with neurophysin-like proteins similar to those found in the pituitary and involved in vasopressin and oxytocin secretion (Reinharz et al., 1974, Legros et al., 1975).

Since it's discovery in high amounts in the pineal, arginine vasotocin has been scrutinised as a possible pineal antigonadotropin. Initially Pavel and Petrescu (1966) found that 0.1 IU of pure synthetic or pineal arginine vasotocin injected subcutaneously would prevent Pregnant Mare Serum Gonadotrophin (PMSG) induced stimulation of the ovaries and uteri of mice. Cheesman and Forsham (1974), however, found that arginine vasotocin was incapable of preventing ovulation following Human Chorionic Gonadotrophin (HCG) injections. Extracts of human foetal pineals and arginine vasotocin (50uIU, intraperitoneal) inhibit compensatory ovarian hypertrophy in mice (Pavel et al., 1973/74).

Acutely administered arginine vasotocin (100ng, intravenous) is capable of stimulating prolactin release in male rats while having no significant effect on LH (Vaughan et al., 1976a). Arginine vasotocin can also stimulate prolactin _and_ LH, however, when incubated _in vitro_ with pituitaries (Vaughan et al, 1976a).

Pavel et al. (1975b) demonstrated that intraventricular injection of 0.0002pg arginine vasotocin will prevent the pinealectomy-induced rise in pituitary prolactin.

Arginine vasotocin administered intraventricularly (1uIU) will inhibit compensatory adrenal hypertrophy following unilateral adrenalectomy, thus apparently blocking ACTH secretion (Pavel, 1975b). Arginine vasotocin will also stimulate ACTH if injected intrapituitarily (Pavel, 1975b), but this may be a non-specific effect since a number of other basic nonapeptides have similar properties.

The high levels of arginine vasotocin detected in pineal tissue and the demonstration that as little as 0.2 phemtogram is physiologically active when injected into the third ventricle of the brain suggests that it cannot be ignored as a possible pineal hormone. Indeed it may be the true pineal hormone since Pavel (1973) showed that melatonin injected intraventricularly in cats results in release of arginine vasotocin into cerebrospinal fluid.

Ebels and her co-workers have been very active over the last ten years, fractionating sheep pineal glands and testing for biological activity. They use mild extraction procedures, followed by column and paper chromatography, paper electrophoresis, thin layer chromatography, fluorescence and mass spectroscopy. In a large series of papers (Zurburg and Ebels, 1974, 1975, Ebels et al., 1972a,b, 1973, 1975, Ebels and Horwitz-Bresser, 1976, Moszkowska et al., 1976, van der Havekirchberg et al., 1977), melatonin, methoxytryptophol, hydro-

xytryptophol, hydroxyindoleacetic acid and methoxyindoleacetic acid together with 6-erythrobiopterin have been identified. They have also detected both pro and anti-gonadotrophic peptide factors, some of which apparently act on the hypothalamus and others on the pituitary (Ebels et al., 1975). It is difficult to calculate the content of these peptide factors. They start initially with 100 g of sheep tissue and achieve physiological responses in their test systems at doses of 1.25 gram equivalents of pineal tissue, i.e. approximately 25 sheep pineal glands. None of the peptide fractions have been purified to the extent that amino acid analysis could be performed. They also note that certain cerebral cortex extracts are active in their test systems (Moszkowska and Ebels, 1971) and so the physiological importance of many of their results is open to question.

Organic Solvent and Acid Extracted Factors

Acetone extracts of pineal glands have been shown to possess antigonadotrophic activity by many workers but usually at very high doses. For example, inhibition of compensatory ovarian hypertrophy in mice has been demonstrated with 25 mg eq bovine tissue or 7 rat pineal glands, (Benson et al., 1972), and 2 g eq of bovine tissue (Vaughan, 1972). Benson et al. (1972) prepared isobutanol extracts of bovine pineal tissue which inhibited compensatory ovarian hypertrophy at a dose of 430 mg eq. Orts et al. (1975a) prepared isobutanol and acetic acid extracts of bovine tissue which inhibited compensatory ovarian hypertrophy and fecundity in doses ranging from 500 mg eq to

10,000 mg eq! Melatonin could block the inhibitory effect of one of their fractions (Orts et al., 1975b). They also found that one fraction devoid of antigonadotrophic activity in a previous report subsequently inhibited compensatory ovarian hypertrophy (Orts et al., 1975b). Cheesman and Forsham (1974) prevented PMSG and HCG induced ovulation with 600 mg eq of bovine tissue. Ota et al., (1975) also inhibited PMSG and HCG induced ovulation with a partially purified acetone extract called F4 at 180 mg eq. 360 mg eq was, however, lethal! These studies mentioned above highlight the high doses of extract required to achieve a response (usually in excess of 10 bovine pineals injected into mice). It is also worth noting that in the majority of bioassays used to test for antigonadotropic activity saline injections are used as controls rather than similarly fractionated tissues from non-endocrine organs. It is obvious also that some experiments are difficult to repeat. In contrast to much of the work is the report by Blask et al. (1976) identifying prolactin releasing factors and release-inhibiting factors in human, rat and bovine pineals, which were effective both _in vivo_ and _in vitro_ at very low levels.

Progonadal Factors

In 1974, White _et al._ identified immunoreactive gonadotrophin releasing hormone and TRH in porcine, bovine and ovine pineal glands. On the basis of this evidence, White suggested that the pineal is a supplemental source of releasing factors. While the existence of pineal gonadotrophin releasing hormone has been disputed (Carson _et al._, 1976) it does seem that the

pineal contains immunoreactive gonadotrophin releasing hormone, but not in the high concentrations first reported, (Araki *et al.*, 1975, Clemens *et al*, 1975, Gradwell *et al.*, 1976, Duraiswami *et al.*, 1976, and Morris *et al.*, 1975). It has been proposed that the pineal gonadotrophin releasing hormone could influence pituitary function via the cerebro spinal fluid. Evidence supporting this notion is that gonadotrophin releasing hormone injected into the third ventricle will cause luteinising hormone release from the pituitary (Ben Jonathan *et al.*, 1974). Attempts to identify luteinising hormone releasing activity in bovine cerebrospinal fluid (Gradwell and Symington, 1975) and immunoreactive GnRH in rat, ovine cerebrospinal fluid have been unsuccessful (Cramer and Barraclough, 1975 and Coppings *et al.*, 1977).

The pineal then does appear to possess non-melatonin anti-gonadotrophic activity and prolactin releasing and release-inhibiting activity in a number of species. Only one compound, arginine vasotocin, has been completely characterised. The physiological actions of this peptide are in concurrence with it's measured content unlike many of the other "antigonadotrophic factors". Arginine vasotocin has been shown to be secreted into the cerebrospinal fluid in response to certain stimuli. Using the most commonly used test for antigonadotrophic activity, compensatory ovarian hypertrophy, arginine vasotocin has been estimated to be 1 million times more potent than melatonin and Benson's peptide factor 60-70 times more potent. Until studies of metabolic clearance of these factors are performed, such

statements have limited application. Until these factors are chemically characterised, until they are shown to have effects at low doses, and until they are shown to be secreted in response to either environmental or hormonal stimuli, the relevance of the antigonadotrophic activity of pineal peptides apart from arginine vasotocin must be questioned. Pineal progonadotrophic factors must undergo the same scrutiny.

Extra-Pituitary Sites of Action of the Pineal

A number of investigators have attempted to demonstrate that the pineal indoles especially melatonin act at the level of the gonads, altering gonadal steroidogenesis. Melatonin depresses androgen production and œstrogen production (Peat and Kinson, 1971 and Balestreri et al., 1969). It has no effect on progesterone production by bovine luteal tissue (Ewig and Wickersham, 1968), but stimulated progesterone synthesis in human ovaries (MacPhee et al., 1975). The doses required to achieve changes in steroidogenesis (1-100 ug/ml) raise doubts about the physiological significance of the results, even though melatonin is known to accumulate in ovarian tissue (Wurtman et al., 1964b).

Melatonin also is capable of preventing serotonin and oxytocin induced contractions of rodent uterus (Davis et al., 1971). Pineal peptides have not been investigated for direct gonadal actions.

The pineal gland has been implicated in the control of growth, thyroid function, adrenocorticoid function and parathyroid function (Reiter et al., 1975a). Results in these

areas are inconsistent and few meaningful conclusions can be drawn.

CHAPTER 2

GENERAL MATERIALS AND METHODS

A. 1) Indole Derivatives

Indolic tryptophan derivatives and kynurenine were obtained from Sigma, St. Louis, Mo., U.S.A. O and N-acetylindoles, unavailable commercially were prepared using acetic anhydride/pyridine (Fieser and Fieser, 1967) and their purity confirmed by thin layer chromatography. N-Formyl-L-Kynurenine was purchased from Calbiochem, San Diego, Ca., U.S.A. Stock solutions (1 mg/ml) of indoles were prepared in either ethanol, dilute acid or dilute alkali. Melatonin standard for the radioimmunoassay was prepared in ethanol (1 mg/ml) and diluted to 10 ug/ml in ethanol. This dilution was prepared every 2 months and kept at 4 C.

2) Steroids

All steroids were purchased from Steraloids Inc., Wilton, N.H., U.S.A.

3) Radioactive Compounds

S-Adenosyl-L-methionine-methyl-^{14}C, specific activity 50.8-60.4 m Ci/mmole was obtained from New England Nuclear Corporation (NEN) Boston, Mass., U.S.A. or 56-60 mCi/mmole from the Radiochemical Centre (RCA) Amersham, England. S-Adenosyl-L-methionine-methyl-^{3}H, specific activity 8.5 Ci/mmole was obtained from NEN and 8.1 and 11.4 Ci/mmole from RCA.

Tryptamine bisuccinate (side chain-2-^{14}C), specific activity 47-60 mCi/mmole was obtained from NEN.

5-Hydroxytryptamine binoxalate (2-^{14}C), specific activity 51.7-53 mCi/mmole was obtained from NEN.

N-Acetyl-5-methoxy-tryptamine (2-Aminoethyl-2-^{3}H), specific activity 24.3 Ci/mmole was obtained from NEN.

(1,2,6,7(n)-^{3}H) Cortisol, specific activity 82 Ci/mmol, (1,2,6,7(n)-^{3}H) progesterone, specific activity 101 Ci/mmol and (2,4,6,7(n)-^{3}H) oestradiol 17β specific activity 93 Ci/mmol were obtained from RCA.

4) Solvents

Analytical reagent grade solvents were used after 1 x distillation. Solvents were obtained from Merck, Darmstadt, Germany or Ajax Chemicals, Sydney, Australia.

Ethanol

Chloroform

Toluene

Petroleum Spirit b.p. 60 - 80

Methanol

5) Glassware

Pyrex culture tubes with screw caps (16 mm x 125 mm) were used for the hydroxyindole-O-methyltransferase assay and for extracting plasma in the melatonin radioimmunoassay. For the monoamine oxidase assay, 16 mm x 100 mm Pyrex screw capped culture tubes were used. The tubes used for evaporation of the chloroform extract in the melatonin assay were 15 mm x 125 mm Pyrex rimless test tubes, while 13 mm x 100 mm borosilicate rimless test tubes were used to collect the column eluate prior to radioimmunoassay.

All glassware was rinsed 2 times in tap water, and sonicated in Extran Flussig detergent (Merck, Darmstadt, Germany) for 30 minutes, rinsed 3 times in tap water and sonicated in ethanol for 30 minutes. The glassware was dried in an oven at 60 C.

6) Scintillation Counter and Scintillation Fluids

A Nuclear Chicago Isocap 300 liquid scintillation spectrometer was used to record radioactivity.

The scintillation fluids used were -

a) Enzyme assays and melatonin radioimmunoassay - 5 ml Toluene scintillator consisting of 5 g PPO (2,5 Diphenyl oxazole and 0.3 g POPOP (1.4-bis-2-(5-Phenyloxazolyl)-Benzene in 1 litre of Toluene.

b) Corticoid assay - 10 ml toluene/triton scintillator (2 parts toluene to 1 part Triton X-100 detergent).

B. Steroid Assays

1) Corticoid Assay

The method of Bassett and Hinks (1969) was used without significant modification. Plasma (50 ul-200 ul) was diluted to 2 ml with ethanol in disposable plastic centrifuge tubes. After centrifugation to compact the denatured protein in aliquot of the supernatant was transferred to similar incubation tubes. The solvent was evaporated in a stream of warm air and 400 ul of a 0.8% dilution of dog plasma added. (The dog plasma was filtered through glass wool to remove clots, and stored in 200 ul aliquots until required. On the day of assay the plasma was diluted to 25 ml (0.8%) with 0.05 M borate buffer

pH 7.6 and ^{3}H-cortisol (4x10^{5}cpm) added). After incubation at 4 C for 3-18 hours, 300 ul was loaded on to short columns of Sephadex G-25 fine, to separate the fraction containing cortisol bound to the corticosteroid binding globulin. Standard cortisol was run through the assay procedure. Cortisol content of unknown plasma was estimated by plotting the reciprocal of the bound counts versus the standard doses.

The corticosteroid binding globulin binds a number of steroids with differing efficacy. The cross reacting steroids compared to cortisol (100%) are 17α hydroxyprogesterone 81%, corticosterone 64%, progesterone 54%, cortisone, testosterone 5%, oestradiol 5%, oestriol 5%, oestrone 5%. In the sheep the major circulating corticosteroid is cortisol (Bassett and Hinks, 1969). In humans, there are a wider range of corticosteroids and so total plasma corticoid is estimated.

2) Oestradiol Radioimmunoassay

An oestradiol antibody was raised against oestradiol 17β-6-(O-carboxymethyl) oxime conjugated to bovine serum albumin. A dilution of 1:16,000 was used to assay an ether extract of 0.5 ul plasma. Steroid bound to antibody was separated using polythylene glycol 6000. Assay sensitivity was 25 pg. The cross reactivity of the oestradiol antibody was, oestrone 17%, oestriol 0.6%, testosterone 0.2% and progesterone 0.1%.

3) Progesterone Assays

Plasma progesterone was assayed by Competitive Protein Binding (CPB) assay using corticosteroid binding globulin or by radioimmunoassay. The CPB assay involved extracting 0.5 ml

plasma with petroleum spirit. The solvent was evaporated in incubation tubes and 0.8% dog plasma containing ^3H cortisol added. Separation of bound steroid was performed with Sephadex columns as in the cortisol assay.

The specificity of this assay is progesterone 100%, 5α pregnane-3,20-dione 100%, desoxycorticosterone 100%, 5α-pregnane-3,20-dione 50% and testosterone 50%. The assay is specific for progesterone in non-pregnant sheep but the high levels of cross-reacting steroids makes the assay unsuitable in pregnant sheep.

The progesterone antibody was raised against progesterone conjugated to bovine serum albumin at the 11 position. A dilution of 1:2000 was used to directly assay 20 μl plasma. Steroid bound to antibody was separated using polyethylene glycol 6000. Assay sensitivity was 50 pg. The cross reactivity of the progesterone antiserum was 5α pregnanedione 5%, 20α hydroxyprogesterone 1%, 17α hydroxyprogesterone 0.2%, testosterone 0.6%, oestradiol 17β, pregnanediol and pregnenolone all 0.1%.

C. Plasma Tryptophan Assay

The method of Wapnir and Stevenson (1969) was used without modification. 20 μl plasma or standard was applied to the centre of 2 x 1 cm filter paper cards (Whatman 3MM) and air dried. The card was rolled and placed in a disposable plastic centrifuge tube. 0.8 ml of 78% (v/v) ethanol was added, the tubes stoppered and allowed to stand for at least 30 minutes. An aliquot of the ethanolic extract was diluted 1:10 with

0.02 M Tris (hydroxymethyl) aminomethane base solution (pH 10), mixed and read immediately in an Aminco Bowman spectrophoto fluorometer using a quartz flow cell. Excitation wave length was 290 nm and emission wave length 360 nm.

Tryptophan standards (10,20,30 and 50 ng/ml) were prepared in Tris base fresh for each assay. To test whether circulating indole contributed to the fluorescence, some filter paper cards were washed with cyclohexane prior to ethanol extraction. No reduction in the fluorescence was evident.

CHAPTER 3

ASSESSMENT OF PINEAL FUNCTION

A. Pineal Enzyme Assays

1) Introduction

In 1958 Lerner and co-workers purified a skin lightening compound from bovine pineal glands. Analysis of the purified compound identified it as 5-methoxy N-acetyltryptamine (Melatonin). In 1961 Axelrod and Weissbach isolated an enzyme (Hydroxyindole-O-Methyltransferase) which catalysed the formation of melatonin from 5 hydroxy N-acetyltryptamine (N-acetyl Serotonin) and S-adenosyl methionine. This enzyme has been used as a marker of pineal function in the absence of specific sensitive assays for melatonin itself. Hydroxyindole-O-methyltransferase was originally thought to be specifically located within the pineal but subsequent studies have identified the enzyme in retina (Cardinali and Rosner, 1971), harderian gland (Vlahakes and Wurtman, 1972) and blood cells (Rosengarten _et al._, 1972).

The two enzymes investigated in this study, Hydroxyindole-O-methyltransferase and monoamine oxidase were assayed in crude homogenates. Following incubation with the appropriate substrates, one of which was radioactively labelled, the products were extracted and quantitated by liquid scintillation spectrometry. In the case of pineal hydroxy indole-O-methyltransferase, activity has been demonstrated to change in response to physiological stimuli. In using this enzyme as a marker for pineal function the assumption is made that

production of melatonin reflects secretion of melatonin.

2) Assay of Hydroxy Indole-O-Methyltransferase

The method of Axelrod, Wurtman and Snyder (1965) was used without substantial modification. The assay involves incubation of S-adenosyl methionine-^{14}C and N-acetyl serotonin with homogenate. The ^{14}C melatonin formed is extracted quantitatively with chloroform.

Pineal glands were homogenised in ice-cold sodium phosphate buffer (0.05M pH 7.9), using a Kontes glass homogeniser. As a standard procedure tissue was homogenised to a concentration of 2.5 mg/wet weight per ml of buffer. Use of higher tissue concentrations inhibited melatonin production probably because of exhaustion of substrate. A 200 µl aliquot (i.e., 0.5 mg pineal) of the homogenate was transferred to a cold culture tube using a Finn pipette. 50 µg (230 n moles) N-Acetyl serotonin and 890 moles S-adenosyl methionine-^{14}C (90,000 d.p.m.) both in phosphate buffer were added to give a final volume of 300 µl. Estimations were performed in duplicate and controls were incubated in absence of homogenate. After incubating in air at 37 C for 30 minutes the reaction was inhibited by addition of 1 ml of potassium tetraborate buffer (o.2M pH10) followed by 8 ml chloroform. The tubes were then stoppered and shaken mechanically for 20 minutes. The aqueous phase was removed by aspiration and a 5 ml aliquot of the chloroform transferred to glass scintillation vials. The chloroform was then evaporated in a stream of air or nitrogen, and the residue dissolved in 1 ml distilled 96% ethanol. 5 ml toluene-based

scintillant was added, and the radioactivity measured. Recovery of melatonin is 96%. Using the supplied specific activity of the S-adenosyl methionine-^{14}C, 1 enzyme unit is defined as 1 pmole of melatonin produced per hour at 37 C. Under the conditions of assay incorporation of label was linear with time and sigmoidal with respect to substrate concentrations. Chromatography of the chloroform extracts showed that melatonin was the only labelled compound extracted.

3) Purification of HIOMT

28 g sheep pineal glands were homogenised in 100 ml of 0.15 M KCl for 30 seconds in a Sorval Omnimixer at top speed. The final volume was 110 ml. The homogenate was centrifuged at 48000 x g x 60 min in a Sorvall RC-2 centrifuge. 8 x 0.4 ml aliquots were removed and retained leaving a final volume of 100 ml. Preliminary experiments showed that most activity appeared in the 30-60% ammonium sulphate fractions. 19.4 solid ammonium sulphate was added with stirring at 4°. The cloudy solution was centrifuged at 48000 x g x 30 min and the pellet discarded. Another 11.8 g ammonium sulphate was added to the supernatant followed by stirring and centrifugation at 48000 x g for 30 min. The pellet was dissolved up in 0.005M phosphate buffer pH 7.9.

The clear solution was then dialysed against 20 volumes of the same buffer with changes at 4,6 and 22 hours. Aliquots (200 ul) were frozen in glass ampoules.

Using a N-acetyl serotonin concentration of 5.7 x 10^{-4}M and S-adenosyl-methionine-^{14}C 2.2 x 10^{-12}M and incubation for

30 minutes incorporation of label was linear up to 8 ul of dialysate.

4) Michaelis-Menten Kinetics

In order to investigate the probability of 5-methoxy-tryptophol and 5-methoxy tryptamine being valid pineal constituents determination of kinetic parameters were undertaken, using the partially purified enzyme. Preliminary experiments with the enzyme showed linear incorporation of label into methylated products with time up to 10 minutes. Subsequent experiments used the activity at 10 minutes as an estimate of velocity.

The results of the first study showed a Km app for N-acetyl serotonin of 1.25 x 10^{-5}M and a Km app for S-adenosyl ethionine of 2 x 10^{-5}M. In the experiment to investigate substrate specificity it was found that the Km app for N-Acetyl erotonin was 1.8 x 10^{-5}M for hydroxytryptophol 1 x 10^{-3}M. Serotonin did not appear to act as a substrate. It would appear then that under most circumstances hydroxyindole-O-methyltransferase would produce melatonin from N-acetyl serotonin, but in the absence of this substrate methoxytryptophol may be produced from hydroxytryptophol.

Comparison of kinetic parameters between partially purified enzyme and the supernatant starting material showed a Km app for N-acetyl serotonin approximately 3 times higher for the supernatant than the dialysate. This may be due to the contamination of the supernatant with substrate.

5) Pineal Monoamine Oxidase

Monoamine oxidases deaminate a wide range of phenylethanolamines and indolealkylamines. The enzyme is extremely widespread but generally reflect the presence of nerves. The pineal monoamine oxidase is located in nerve terminals and pinealocytes and serves to deaminate serotonin to 5 hydroxy tryptophol as well as deaminating catecholamines. Monoamine oxidases elsewhere in the body are influenced by physiological stimuli (Parvez and Parvez, 1973) and it was of interest to investigate this enzyme in pineal tissue particularly the foetal sheep pineal.

Method

The method of Wurtman and Axelrod (1963) was used without modification. Tissue was homogenised in cold isotonic potassium chloride to a concentration of 2.5 mg (wet weight/ml). 50 ul homogenate was incubated with ^{14}C-tryptamine (20000 cpm) and 200 ul Sodium phosphate buffer, (0.5 M pH 7.4) for 30 minutes at 37 C. The reaction was inhibited by addition of 300 ul 2 M hydrochloric acid and the ^{14}C-indole acetic acid extracted into 6 ml toluene by vigorous mechanical shaking. 4 ml of the toluene extract was added to scintillation vials followed by 5 ml toluene based scintillant. The formation of ^{14}C-indole acetic acid by tissue homogenate was linear with respect to time up to 30 minutes and linear with respect to tissue weight up to 1 mg. Thin layer chromatography of the toluene extract indicated that ^{14}C-indole acetic acid was the main product extracted. Clorgyline, a type A monoamine oxidase inhibitor, (Hall et al., 1969) was found to inhibit the formation of

^{14}C indole acetic acid by pineal homogenates. One unit of activity is defined as 1 pmole of ^{14}C indole acetic acid produced per hour at 37 C.

B. Radioimmunoassay of Melatonin

1) Introduction

The foregoing chapter described *in vitro* methods of determining the extent of biosynthetic activity in pineal tissue. Inherent in the approach are major difficulties. Firstly investigations are restricted to acute effects. Secondly, as noted initially in the introduction to this chapter, it is assumed that synthesis of the indoles is reflected by release, an assumption which in some cases is not strictly true. Thirdly, the enzymes can synthesise more than one product. It is known that serotonin N-acetyltransferase is rate-limiting in the melatonin pathway and levels are low during the day. Serotonin and its metabolising enzyme monoamine oxidase, are high during the day. Thus during the day hydroxy tryptophol may well be present in much higher concentrations than N acetyl serotonin and so despite the high Km app of hydroxyindole-O-methyltransferase for hydroxy tryptophol, methoxytryptophol may be produced and secreted during the day. Thus levels of hydroxyindole-O-methyltransferase during the day may reflect methoxytryptophol production.

What is, therefore, needed is an assay specific for the indole of interest. Early attempts at fluorometric assays were suitable for measurement of pineal levels, (Miller and Maickel, 1970). Melatonin can be assayed using a bioassay

with good specificity and sensitivity in pineal and blood (Ralph and Lynch, 1970), however, the method is extremely tedious and unsuitable for routine use. Gas liquid chromatography-mass spectrometry has very high sensitivity and specificity, but is also unsuited to large sample numbers. Several groups have now produced radioimmunoassays for melatonin which are sensitive and specific enough to measure melatonin in pineal, blood and cerebrospinal fluid. The following section describes the development of a specific melatonin radioimmunoassay.

2) Synthesis of Antigen

The method of Grota and Brown (1974) was used without modification to produce an N-acetylserotonin-bovine serum albumin conjugate. 183 mg Bovine serum albumin (Cohn Fraction V, Sigma) dissolved in 9 ml water, 37.7 mg N-acetyl serotonin dissolved in 6 ml water were mixed with 6 ml 2M acetate and 6 ml 8% formalin for 70 minutes. At this stage the reaction mixture was a light purple colour but not turbid. ^{14}C-Serotonin (970,000 cpm) was added to allow quantitation of the reaction - serotonin and N-acetyl serotonin undergo the Mannich reaction with similar efficacy. The reaction mixture was dialysed against distilled water for 4 days at 4°C. After dialysis the mixture was slightly turbid. The dialysate was centrifuged at 4000 rpm and an aliquot taken for counting. The remainder was lyophillised and stored in a desicator at 4°C. Assuming a similar incorporation of ^{14}C-serotonin and N-acetyl serotonin the molar ratio of hapten to protein was 49:1.

3) Immunization

The N-acetyl serotonin-bovine serum albumin antigen was dissolved (21 mg/6 ml) in 0.05 M phosphate buffer pH 7.9 and mixed with 3 ml Freund's complete adjuvant. 3 ml of this was injected subcutaneously into 3 or 4 sites on the back of 3 rabbits. One month later each animal received 2 mg antigen. 10 days later the rabbits were bled from an ear vein into lithium heparin coated tubes. Booster injections followed by bleeding 10 days after were continued for 6 months. 12 months after the initial injections rabbit 8 was injected with 2 mg antigen and sacrificed 10 days later to obtain the maximal volume of blood. Blood was centrifuged at 4000 rpm for 15 min. at 4 C plasma divided into 1 ml aliquots and stored at -20 C. All 3 original animals produced antibodies, but one, R8 gave useful antibodies on two occasions 17/6/75 and 25/2/76, i.e. 3 and 12 months after initial injection. The antibody designated R8 17/6 was used at a titre of 1:4000, which represented 40% binding of ^{3}H melatonin. It was found that R8 25/2 could be used at a titre of 1:1000 at which dilution it gave 40% binding. Antiserum R8 25/2 was not characterised as extensively as R8 17/2 and was used only towards the end of the study. No direct comparison between the two antibodies was made. For routine radioimmunoassay utilising the antibodies, it was necessary to precipitate plasma albumin with Rivanol (2-ethoxy-6, 9 diamino acridine lactate, K & K Laboratories, Plainview, New York). 1 ml plasma was treated with 4 ml 0.4% Rivanol in water and allowed to stand for 10 minutes (Abraham, 1969). Then 300 mg activated charcoal was added and the mixture

allowed to stand at room temperature for 10 minutes. 5 ml distilled water was added and the tubes centrifuged at 4000 rpm. The 1:10 dilution of plasma was aliquoted into vials and stored at -20 C. Once thawed the antibody was kept at 4 C.

4) Assay Method

The antibody used in this study recognises two biologically active indoles melatonin and N-acetyl serotonin, both of which could be present in tissues of interest. Direct radioimmunoassay was not feasible because little is known about the circulating levels of indoles apart from melatonin. It was found that solvent extraction followed by Lipidex 5000 chromatography on short columns removed most of the potential immunoreactive indoles from the samples. An added benefit was a reduction of the blank. Table 1 shows comparison of chromatographed vs chloroform extraction of 10 different plasma samples using R8 17/6.

5) Plasma Extraction

1-2 ml plasma was aliquoted into extraction tubes. An equal volume of 0.5 M potassium tetra borate buffer pH 10 was added, followed by 8 ml redistilled chloroform. Tubes were shaken with a gentle rocking motion for 30 minutes on a Paton extractor (Paton Industries Pty Ltd., Beaumont South Aust.) adapted for this purpose. Tubes were centrifuged at 1000 rpm for 10 min. inverted to break up the phases and centrifuged again at 1000 rpm for 10 min. The aqueous phase was aspirated and 7 ml chloroform pipetted into rimless test tubes. The chloroform was evaporated at 37 C under a stream of nitrogen. 0.5 ml chloroform/petroleum spirit (Boiling range 60° - 80° C)

TABLE 1

EFFECT OF CHROMATOGRAPHY ON THE PLASMA MELATONIN RADIOIMMUNOASSAY

Sample	Without Chromatography (pg/ml)	With Chromatography (pg/ml)
Ewe 18 Day	77	42
Ewe 18 Night	174	115
Ovariectomised ewe	215	127
Ewe 596 Day	82	46
Ewe 20 Day	93	64
Ewe 106 Day	61	39
Ewe 110 Day	28	22
Ewe 110 Pinealectomy	14	5
Ewe 14 Night	87	72
Blank (Water)	31	12

1:1 was added and the tubes vortexed briefly.

6) Pineal Tissue Extraction

5-50 mg pineal tissue was homogenised in 1-2 ml 1M NaOH in a Kontes glass homogeniser and the contents transferred to extraction tubes. 1-2 ml borate buffer pH10 was added followed by 8 ml distilled chloroform. The procedure was then as above.

7) Preparation of Columns

2 ml (175 mm) glass pipettes (i.d. 3.2 mm) with an expanded glass reservoir (7-9 ml) were set up in a length of Dexion angle iron. A glass bead and 1 cm of sand was placed in each column and the tip was fitted with 2 cm silastic tubing (Dow Corning Midland, Mich) and 10 cm of thin glass tubing (end of a long Pasteur pipette). A paper clip on the silastic tubing regulated solvent flow. Lipidex 5000 (Packard Instrument Co., Downets Grove, Ill.) which had been thoroughly washed with $CHCl_3$: petroleum spirit (1:1) was added to each column and allowed to settle by gravity up to the top of the column. $CHCl_3$: methanol 2:1 (20 ml) was passed through the columns followed by $CHCl_3$: petroleum spirit 10 ml before they were first used in assays. Columns prepared in this way were viable for up to six months even when stored in $CHCl_3$: petroleum spirit (1:1).

It is necessary to stir the columns prior to use for every assay to maintain acceptable flow rates.

8) Sample Application

0.5 ml of the chloroform extract was transferred to the top of Lipidex columns which had been stirred and equilibrated

in fresh $CHCl_3$: petroleum spirit (1:1). Clips were removed and the eluate discarded. The clips were replaced when the sample had been passed into the column. 2-5 ml of solvent were added and the eluate discarded. 5 ml of solvent were added and the eluate collected into 13 x 100 mm borosilicate rimless culture tubes. This fraction was then evaporated in a stream of nitrogen at 37°C.

9) Immunoassay Procedure

500 µl Phosphate buffer (0.1 M, pH 7.4, 0.9% sodium chloride, 0.1% sodium azide and 0.15% gelatin) was added to each sample tube. A standard curve 30 pg - 1000 pg was set up in buffer and volumes adjusted to 500 ul. Using a Hamilton repeating syringe 100 µl buffer, 100 µl antibody dilution and 100 µl ^3H melatonin (10,000 cpm) melatonin were added in sequence to the tubes. Antibody was diluted from the 1:10 stock to 1:500 for R8 17/6 and 1:125 for R8 25/2 with buffer using an "auto zero" pipette. ^3H-Melatonin (26 Ci/mmole) was diluted 1:100 with ethanol for storage and further diluted 1:100 prior to use with buffer. Tubes were incubated at 4°C overnight. 800 µl of ice-cold saturated ammonium sulphate was added in the cold, the tubes briefly vortexed and centrifuged at 3000 rpm for 10 minutes. The supernatant was poured into scintillation vials in the cold room, 5 ml toluene scintillant added and the vials vortexed. Vials were allowed to equilibrate overnight and counted.

10) Sensitivity

The sensitivity of the assay was defined as that amount of standard giving a response significantly different from

zero concentration. The sensitivity of both anibodies, R8 17/6 and R8 25/2 was 30 pg. Tables 2 and 3 show the mean percentage binding and the derived statistics for the two antibodies in 10 consecutive assays over a period of less than a month. The higher amount of variation for R8 17/6 was not common and the reason for it in these 10 assays is unknown.

11) Accuracy

Melatonin (50-500pg) was added to plasma and equilibrated overnight at 4°C and then assayed. A linear response curve was obtained indicating quantitative recovery. The slope of the regression line was 0.83, with an intercept of 142 pg, which represents the endogenous concentration of melatonin in the sample. The correlation coefficient was 0.966. The within-assay coefficient of variation over the range of 100-600 pg varied between 2.5 and 14%. Mean recovery was 86% (Table 4).

12) Quality Control

Plasma obtained from sheep at night was used as a quality control in each assay. Inter-assay coefficients of variation were 17% at 83 pg, 22% at 133pg and 23% at 162pg.

13) Parallelism

One of the criteria which must be satisfied in any radioimmunoassay or bioassay is that the substance being measured must react in an identical manner to the standard. Thus in a radioimmunoassay, serial dilutions of test samples must give response curves parallel to the standard curve. Two plasma pools from sheep taken at midlight and middark, together with pineal tissue taken during the day were used. Following

TABLE 2

STANDARD CURVE OF MELATONIN RADIOIMMUNOASSAY

Compiled from 10 separate standard curves assayed over a period of 1 month.

Antibody R8 17/6 (dilution 1/4000)

Amount of Melatonin (pg)	Percentage ^{3}H Melatonin Bound						
	Mean	SD	CV	SEM	n	t	P
0	42.6	2.9	6.8	0.5	40	4.2	<0.05
30	39.3	2.5	6.3	0.6	20	2.2	<0.05
50	37.5	2.7	7.2	0.6	20	2.7	<0.05
70	35.1	2.7	7.7	0.6	20	1.8	NS
100	33.5	2.4	7.1	0.5	20	7.4	<0.05
200	27.7	2.4	8.6	0.5	20	4.3	<0.05
300	24.2	2.5	10.3	0.55	20	2.0	NS
400	22.4	3.0	13	0.7	20	2.5	<0.05
500	19.8	3.2	16	0.7	20	4.3	<0.05
1000	15.7	2.5	16	0.55	20		

TABLE 3

STANDARD CURVE OF MELATONIN RADIOIMMUNOASSAY

Compiled from 10 separate standard curves assayed over a period of 1 month.

Antibody R8 25/2 (dilution 1/1000)

Amount of Melatonin (pg)	Percentage ^{3}H Melatonin Bound						
	Mean	SD	CV	SEM	n	t	P
0	40	1.9	4.7	0.3	40		<0.05
						5.3	
30	37.5	1.3	3.5	0.3	20		<0.05
						5.5	
50	35.2	1.3	3.7	0.3	20		NS
						1.6	
70	34.4	1.7	4.9	0.4	20		<0.05
						3.6	
100	32.6	1.3	4.0	0.3	20		<0.05
						10.5	
200	27.6	1.6	5.8	0.4	20		<0.05
						6.9	
300	24.0	1.6	6.7	0.3	20		<0.05
						3.2	
400	22.2	1.9	8.6	0.4	20		<0.05
						2.4	
500	20.8	1.7	8.2	0.4	20		<0.05
						7.2	
1000	16.9	1.6	9.5	0.4	20		

TABLE 4

RECOVERY OF MELATONIN FROM EWE PLASMA

Melatonin was added to 2 ml of normal ewe plasma, extracted and assayed by radio-immunoassay.

Amount of melatonin added (pg)	Amount of melatonin measured (pg) Mean ± SEM(n)	CV	Amount Recovered (pg)	Amount Recovered (%)
0	142 ± 6 (3)	6%	-	-
50	184 ± 15 (3)	14%	42	84
100	250 ± 8 (3)	6%	108	108
200	302 ± 19 (3)	11%	160	80
300	359 ± 6.5 (3)	2.5%	217	72
400	488 ± 18 (3)	6.3%	346	87
500	564 ± 40 (3)	12%	422	84
Mean ± SEM				86 ± 4.9

chromatography, the extracts were serially diluted and assayed using the R8 17/6 antibody. Figure 3 shows the results plotted as Logit % bound versus log dose. These data were computed using a Wang 700 programmable calculator. The slope of the dose response curves of the plasmas and the pineal were not significantly different from the standard curve of melatonin ($p < 0.05$). Midlight plasma (-0.95 ± 0.1 SD), middark plasma (-0.91 ± 0.04) and sheep pineal (-0.95 ± 0.5), standard melatonin (-0.94 ± 0.02).

14) Specificity

The antibodies produced in this study were specific for N-acetyl indoles as originally demonstrated by Grota and Brown (1974). Table 5 shows the percentage cross-reaction of various commercially available indoles and some derivatives synthesised in this laboratory, compared with authentic melatonin. The degree of specificity of the antibodies could be predicted from information about the site of coupling of the N-acetyl-serotonin to the bovine serum albumin. Using a model system it was found that position "4" of the indole was the site of the Mannich addition (see appendix). Thus it is likely that substitution at position "5" would not be detected due to the proximity to the point of conjugation. While the reaction was expected to occur mainly at the position "4" it was also likely that some substitution could occur at the other ortho position i.e., position "6". The cross reaction profiles confirm this with 6-hydroxy melatonin cross reacting to a greater extent than most other indoles (Table 5).

Figure 3

Standard curve of melatonin radioimmunoassay, plotted as Logit % bound *vs* Ln dose of melatonin (★). ▲ denotes a serial dilution of pineal extract (the amount of pineal tissue giving the response was multiplied by 10 to prevent an overlap with the melatonin standard curve. ● denotes a serial dilution of an extract of plasma sampled at mid-dark. ☆ denotes a serial dilution of an extract of plasma sampled at midlight.

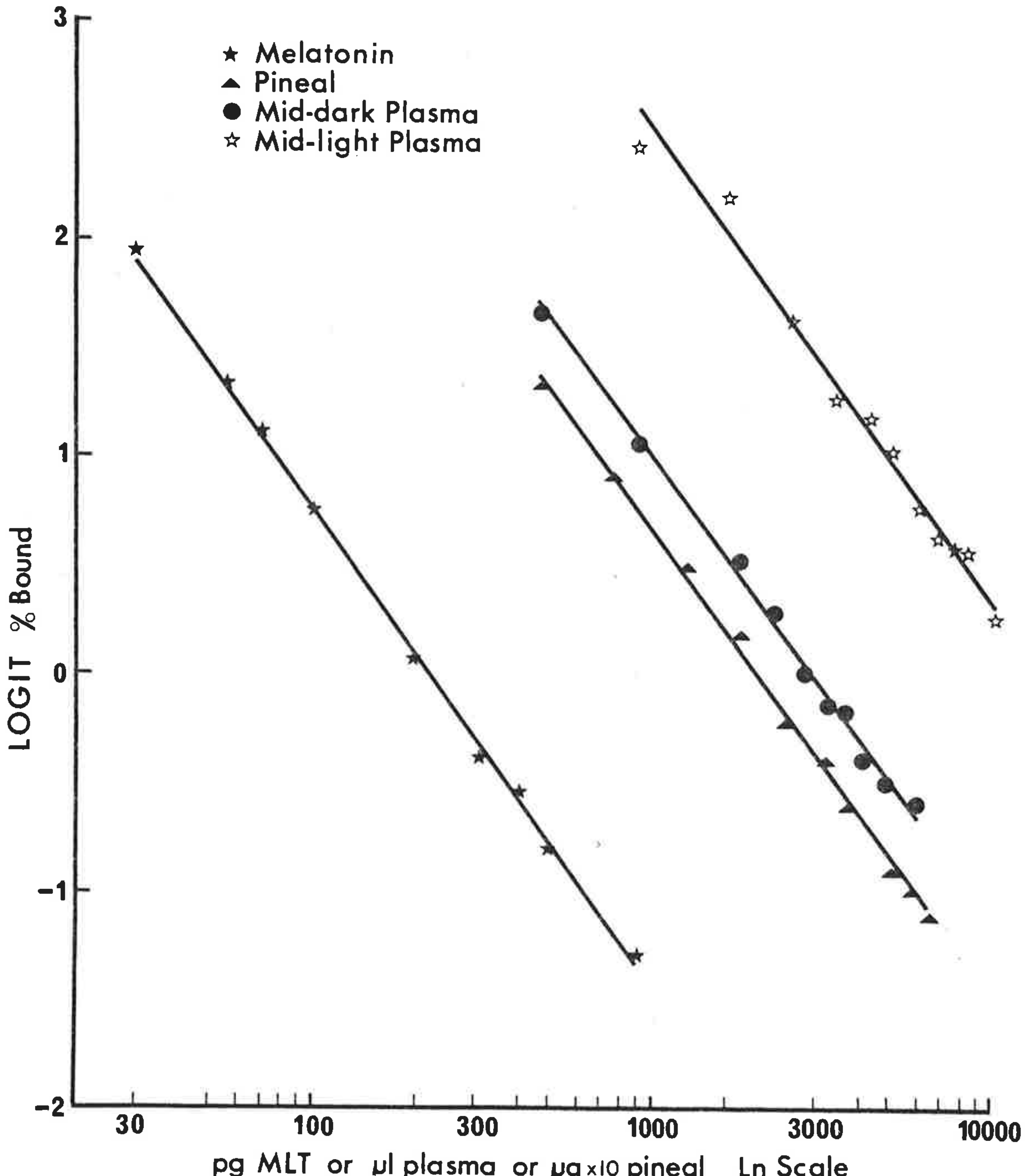

Melatonin
Pineal
Mid-dark Plasma
Mid-light Plasma
3
2
1
0
−1
−2
LOGIT % Bound
30
100
300
1000
3000
10000
pg MLT or µl plasma or µg x10 pineal Ln Scale

TABLE 5

CROSS REACTIVITY OF VARIOUS INDOLES

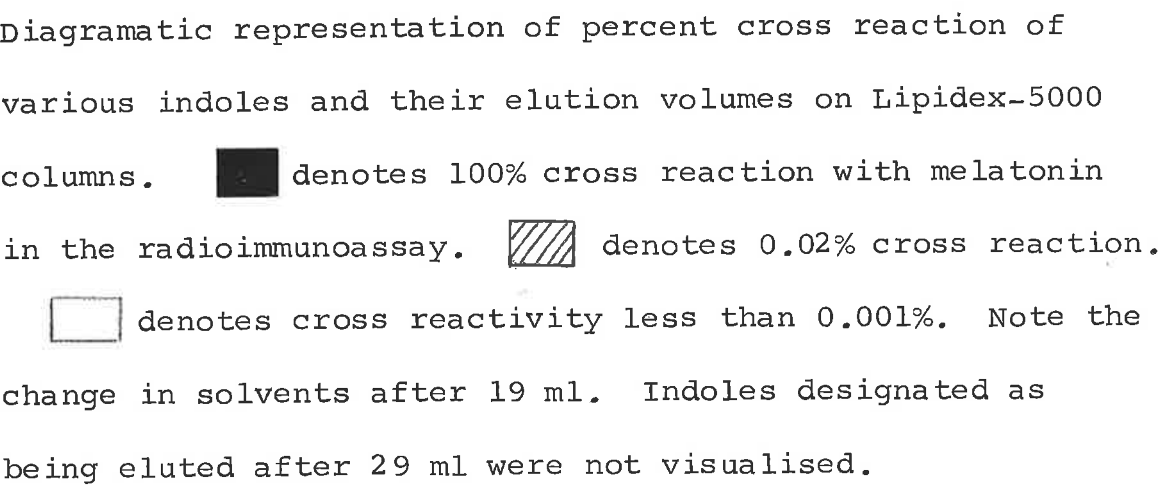
Indole	R8 17/6 % Cross Reaction	R8 25/2 % Cross Reaction
Melatonin	100	100
6-Hydroxy Melatonin	0.02	0.1
5-Methoxy Tryptamine	0.0002	0.01
5-Methoxy Tryptophol	0.0015	0.01
5-Methoxy Indole Acetic Acid	0.0001	0.01
5-Methoxy Tryptophan	0.0001	-
5-Hydroxy N-Acetyl Tryptamine	100	100
5-Hydroxy Tryptamine	0.004	0.01
5-Hydroxy Tryptophol	0.0001	-
5-Hydroxy Indole Acetic Acid	0.0008	-
5-Hydroxy Tryptophan	0.0001	-
N-Acetyl Tryptamine	100	100
Tryptamine	0.006	-
Tryptophol	0.0005	-
Indole Acetic Acid	0.0002	-
Tryptophan	0.0001	-
Kynurenine	0.0001	-
N-Formyl Kynurenine	0.0002	-
5-Methyl Tryptamine	0.002	-
5-Methyl N-Acetyl Tryptamine	100	-
O-Acetyl-5 Methoxy Tryptophol	0.0010	-
O-Acetyl-Tryptophol	0.005	

For the assay of pineal and plasma melatonin it was thus important to separate 5 and 6 substituted N-acetylindoles from melatonin prior to radioimmunoassay. This was achieved by solvent extraction from a high pH, followed by Lipidex 5000 chromatography. Using the procedure outlined in the method, N-acetylserotonin and 6-hydroxymelatonin were separated from melatonin. Other N-acetylated indoles could not be separated from melatonin in this system and so must remain as possible contributors to melatonin immunoreactivity (Figure 4).

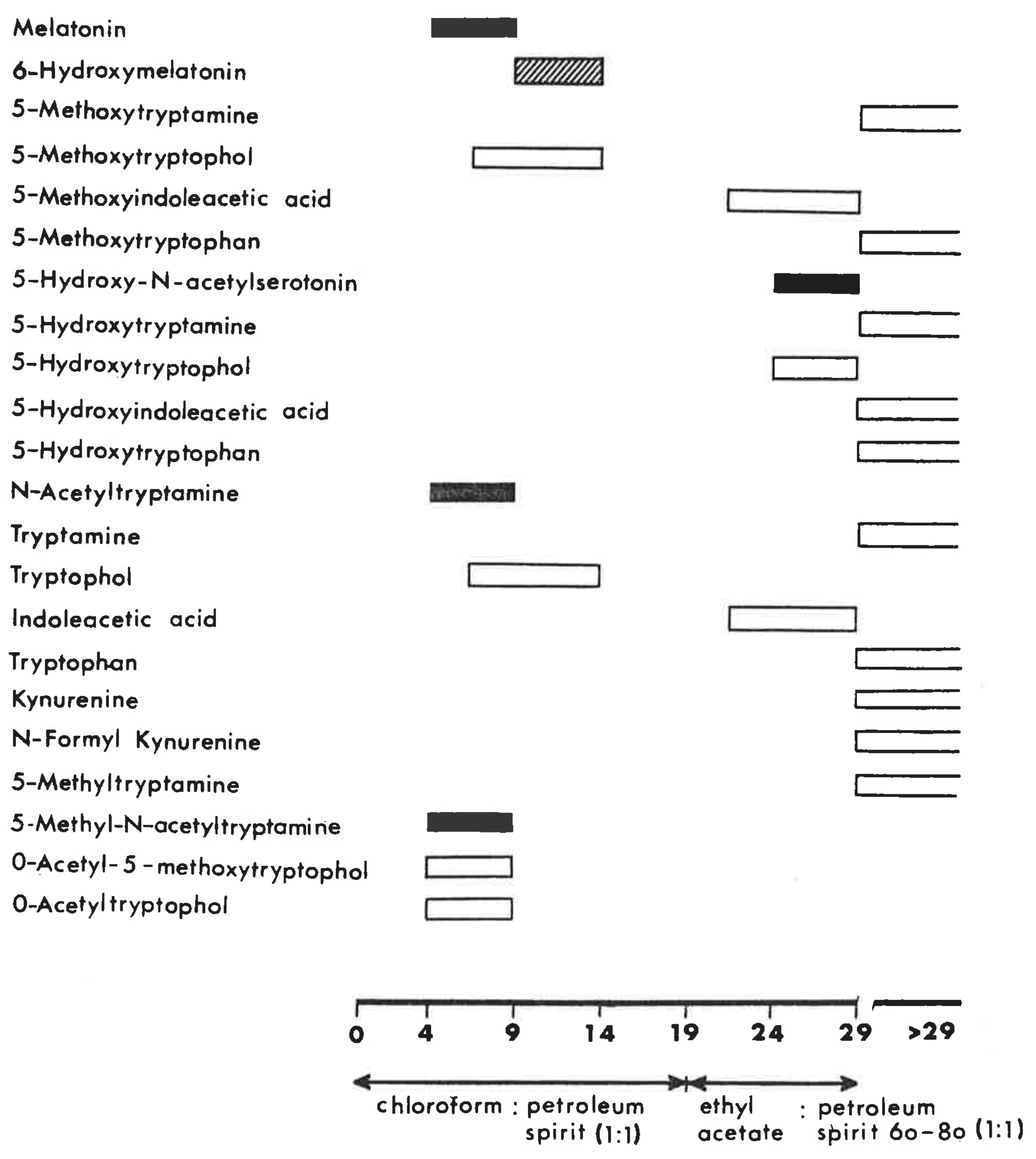
The specificity of the assay system has been further checked using a number of standard procedures. A sheep plasma pool was extracted with chloroform and chromatographed on a short column of Lipidex 5000 and assayed for melatonin by both radioimmunoassay and gas chromatography-mass spectrometry (see appendix for the GC-MS method). The values obtained by each method were comparable. The specificity of the assay in sheep pineal glands was similarly tested. Nine sheep pineal glands (collected at 1100h) were homogenised, extracted and chromatographed as per methods. A portion (1 ml) of the column eluate was assayed by radioimmunoassay and after the addition of deuterated melatonin as an internal standard, 3 ml of the eluate was assayed for melatonin by gaschromatography-mass-spectrometry. The correlation coefficient for the relationship was 0.986, indicating that the radioimmunoassay method is specific for melatonin in pineal glands. Indeed in this experiment the radioimmunoassay tended to slightly underestimate by approximately 5% the content of melatonin (Figure 5).

Further chromatographic evidence that the substance being measured in plasma was melatonin was obtained by co-chromato-

Figure 4



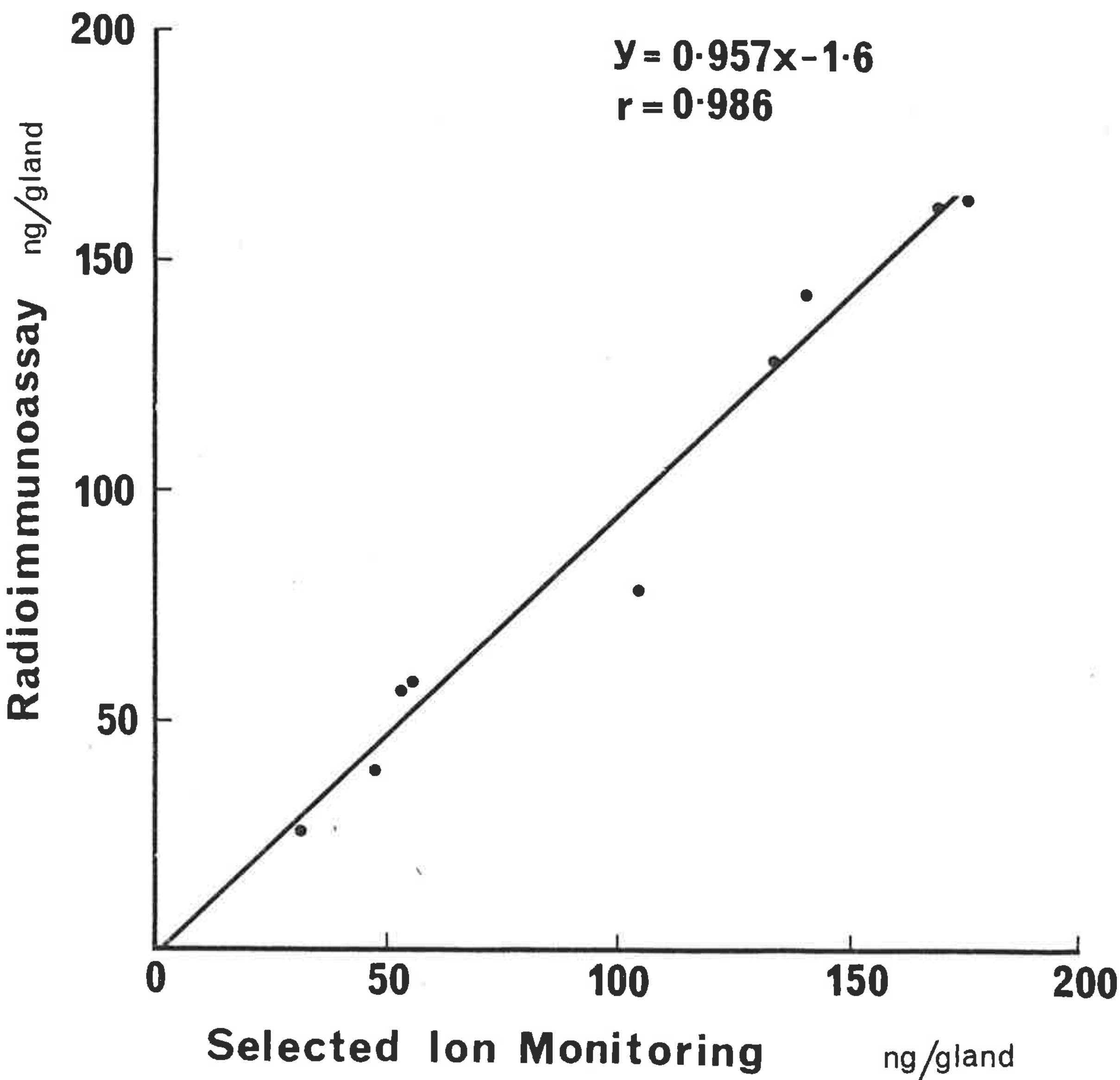
Diagramatic representation of percent cross reaction of various indoles and their elution volumes on Lipidex-5000 columns. ■ denotes 100% cross reaction with melatonin in the radioimmunoassay. ▨ denotes 0.02% cross reaction. □ denotes cross reactivity less than 0.001%. Note the change in solvents after 19 ml. Indoles designated as being eluted after 29 ml were not visualised.



Melatonin
6-Hydroxymelatonin
5-Methoxytryptamine
5-Methoxytryptophol
5-Methoxyindoleacetic acid
5-Methoxytryptophan
5-Hydroxy-N-acetylserotonin
5-Hydroxytryptamine
5-Hydroxytryptophol
5-Hydroxyindoleacetic acid
5-Hydroxytryptophan
N-Acetyltryptamine
Tryptamine
Tryptophol
Indoleacetic acid
Tryptophan
Kynurenine
N-Formyl Kynurenine
5-Methyltryptamine
5-Methyl-N-acetyltryptamine
0-Acetyl-5-methoxytryptophol
0-Acetyltryptophol
0
4
9
14
19
24
29
>29
chloroform : petroleum spirit (1:1)
ethyl acetate : petroleum spirit 60-80 (1:1)
Elution Volume (ml)

Figure 5

Comparison of the radioimmunoassay for melatonin and a gas chromatography-mass spectrometry method (selected ion monitoring). See text for details.


y = 0·957x - 1·6
r = 0·986
Radioimmunoassay ng/gland
200
150
100
50
0
50
100
150
200
Selected Ion Monitoring ng/gland

graphy of nighttime plasma pools with ^{3}H-melatonin on 0.97 x 34 cm Lipidex 5000 columns. Figures 6 and 7 show the results of chromatography of two separate plasma pools and subsequent radioimmunoassay using R8 17/6 and R8 25/2 antibodies. In both cases the immunoreactivity corresponded with authentic radioactive melatonin. Using this column it was shown conclusively that 6-Hydroxymelatonin and 5-methoxytryptophol do not contribute to the immunoreactivity of plasma extracts.

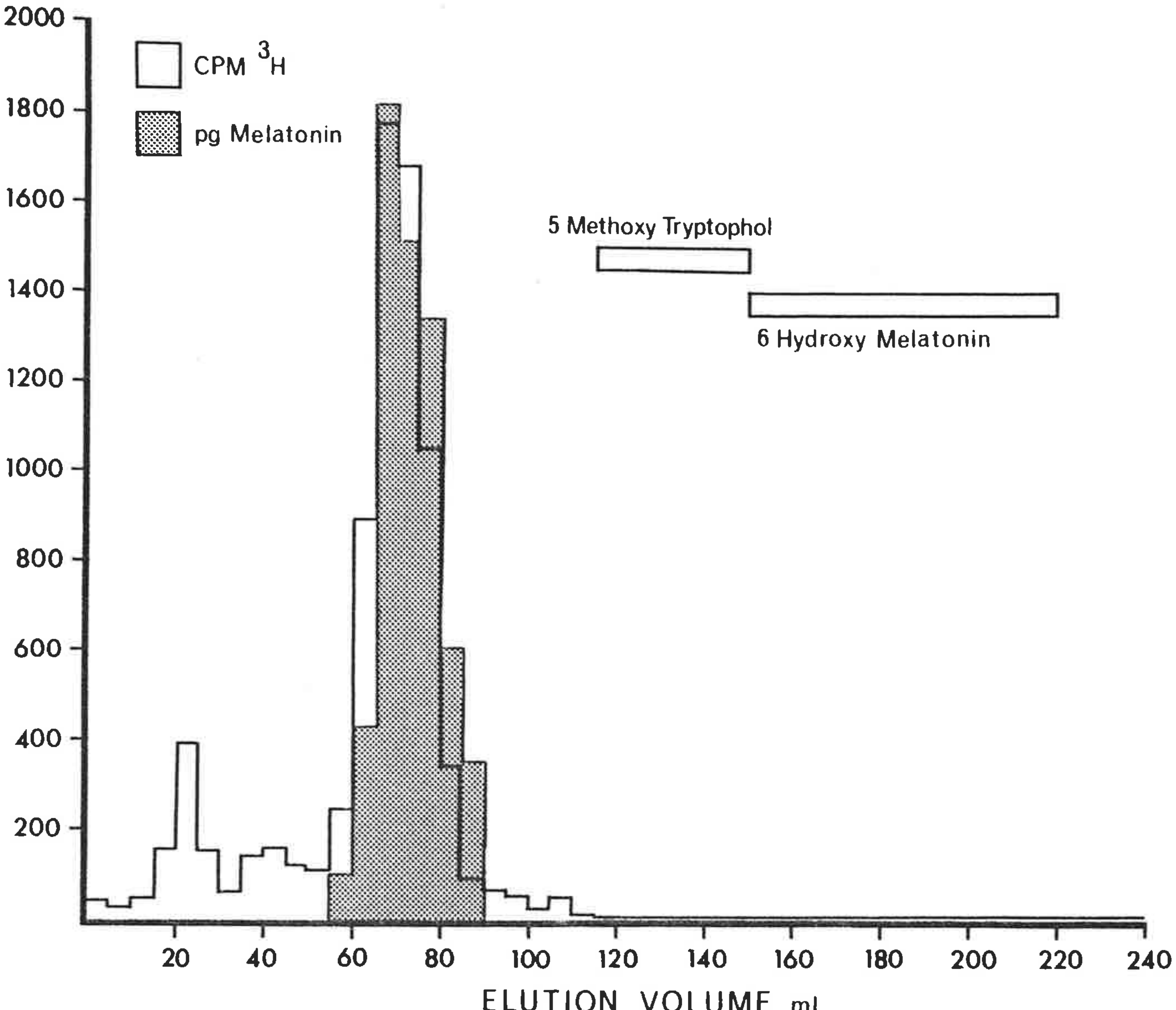
15) Collaboration in World Wide Cross-Validation Study

In December 1976, Professor L. Wetterberg initiated a world wide validation study for melatonin assays. Seven laboratories were sent a sample (60 ml) of lyophilised calf serum to be assayed for melatonin. Participants were told only that the melatonin content was in the mid-range of human nighttime levels. Each participating laboratory was asked to assay the sample using their standard method.

Six vials, containing 3 ml lyophilised serum were reconstituted with 3 ml distilled water. The 3 ml serum was assayed at two levels (2 ml and 1 ml) after the addition of 2100 cpm ^{3}H melatonin as an internal standard. Part (3 ml) of the Lipidex column eluate was assayed for melatonin and 1 ml melatonin reported to be in the sample was 239 pg/ml $\pm$ 35 $\pm$ 14.4 (mean $\pm$ SD $\pm$ SEM). The results have been corrected for recovery (93%). A comparison of the results obtained in this study is given in Table 6. The serum supplied for this study was also assayed by gas chromatography-mass spectrometry and a value lying between 210-240 pg/ml was obtained which is in good agreement with the radioimmunoassay results.

Figure 6

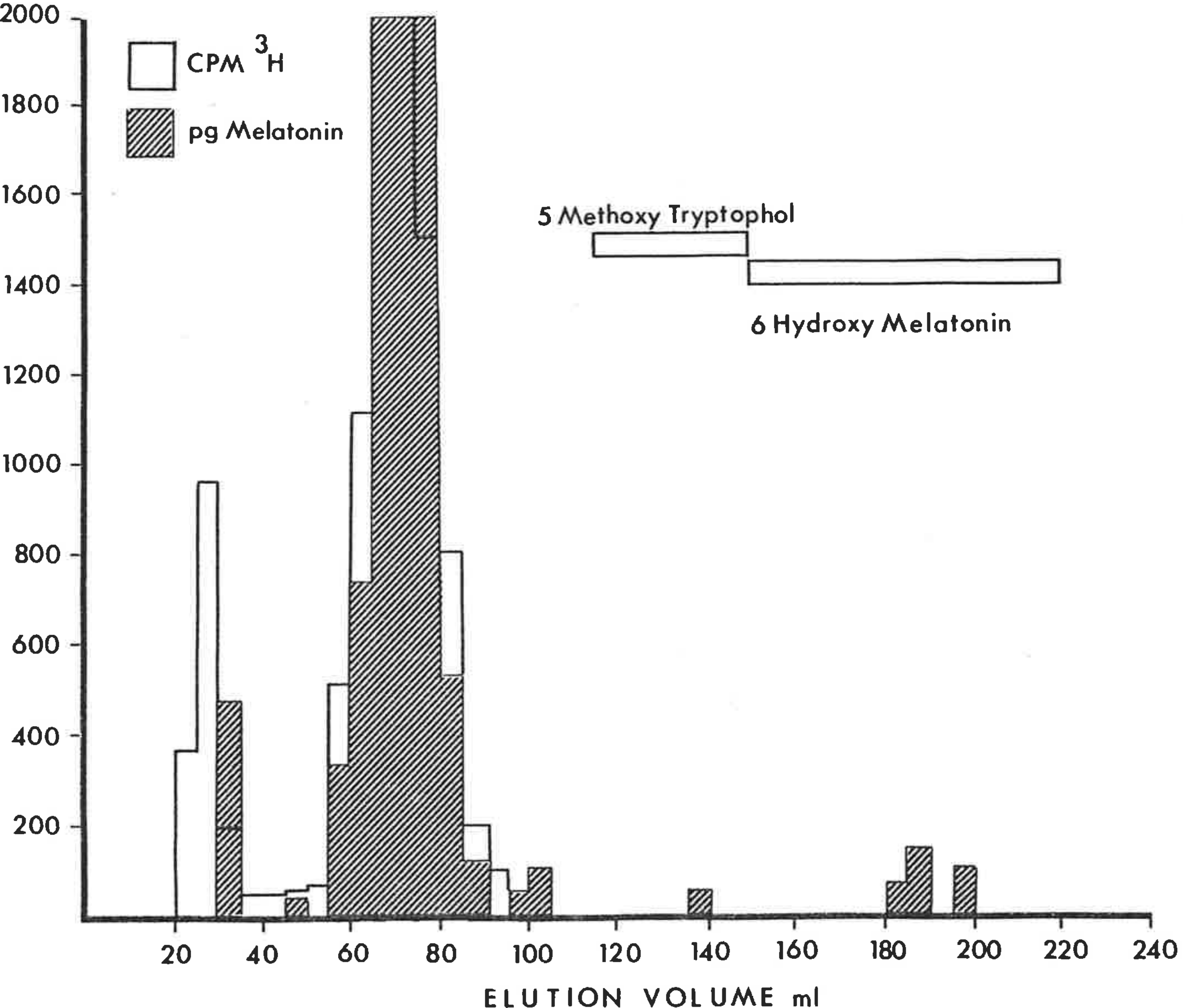
Chromatography of a plasma pool on a 0.97 x 34 cm column of Lipidex-5000 followed by radioimmunoassay using antibody R8 17/6. Aliquots of the eluate were also assayed for radioactivity. The open rectangles designated 5-methoxy tryptophol and 6-hydroxy melatonin indicate the elution volumes of these two indoles. The solvent was chloroform petroleum spirit (1:1).



2000
1800
1600
1400
1200
1000
800
600
400
200
CPM ³H
pg Melatonin
5 Methoxy Tryptophol
6 Hydroxy Melatonin
20
40
60
80
100
120
140
160
180
200
220
240
ELUTION VOLUME ml

Figure 7

Chromatography of a plasma pool on a 0.97 x 34 cm column of Lipidex-5000, followed by radioimmunoassay using antibody R8 25/2. Aliquots of the eluate were also assayed for radioactivity. The open rectangles designated 5-methoxy tryptophol and 6-hydroxy melatonin indicate the elution volumes of these two indoles. The solvent was chloroform: petroleum spirit (1:1)



CPM ^{3}H
pg Melatonin
5 Methoxy Tryptophol
6 Hydroxy Melatonin
2000
1800
1600
1400
1200
1000
800
600
400
200
20
40
60
80
100
120
140
160
180
200
220
240
ELUTION VOLUME ml

TABLE 6

COMPARISON OF DIFFERENT RADIOIMMUNOASSAYS FOR MELATONIN

Laboratory No.	Melatonin concentration of reference calf serum	
	pg/ml	nmol x L^{-1}
1 Kennaway et al	239 ± 35	1.03 ± 0.15
2	214 ± 5	0.92 ± 0.02
3	210 ± 41	0.90 ± 0.18
4	190 ± 28	0.82 ± 0.12
5	188 ± 10	0.81 ± 0.04
6	135	0.58
7	128 ± 15	0.55 ± 0.06

All values are mean ± S.D. except no. 2 (S.E.M.) Different laboratories have assayed different number of samples.

16) Discussion

The preceding section has documented the development and validation of a melatonin radioimmunoassay. It was obvious from theoretical considerations as well as from the results of Grota and Brown (1974) that the antibody raised for this study would not be specific for melatonin. Conjugation at the 4 position (see appendix) has resulted in an antibody which is specific for N-acetylated indoles provided there is no substitution at the 6 position. Separation of possible cross reacting indoles from the melatonin prior to immunoassay was considered to be of prime importance.

Lipidex 5000, a lipophilic hydrophobic derivative of Sephadex LH-20 has proved extremely useful for the separation of steroids prior to radioimmunoassay (Apter et al., 1975, 1976). Lipidex 5000 has also proved useful for separating various classes of indoles as well. Retention by the gel is determined by the polarity of the steroid or indole. Thus highly polar indoles such as serotonin are considerably retarded while the relatively neutral indoles such as melatonin are not retarded (at least not in solvents such as chloroform: petroleum spirit (1:1). By changing the polarity of the eluting solvents, various indole-rich fractions can be obtained. Combination of differential organic extraction and Lipidex 5000 chromatography on short columns results in an essentially pure melatonin extract for immunoassay.

An obvious advantage of a non-specific antibody is that it can be used to measure a number of related compounds pro-

vided a good separation procedure exists. While it has not yet been tested, the combination of Lipidex 5000 chromatography and the antibody reported here should enable analysis of melatonin, N-acetylserotonin, 5-methoxytryptamine and serotonin in the same sample.

While the chromatography step eliminated the two most likely cross reacting indoles, N-acetylserotonin (the melatonin precursor) and 6-hydroxymelatonin (melatonin metabolite), it was important to establish that unknown indoles were not responsible for at least some of the immunoreactivity. N-acetyltryptamine for instance is a possibility, even though tryptophan is known to be hydroxylated prior to being decarboxylated. Simultaneous analysis of sheep plasma pools and sheep pineal glands has, however, failed to identify any discrepancies between the absolutely specific mass spectrometer assay and the radioimmunoassay. Since most of this thesis is concerned with sheep pineal function, all the validation studies have been performed with tissues and plasma from those species. Unless there are unusual N-acetylated indoles present in other species, however, it is likely that this assay is universally applicable between species.

Four melatonin radioimmunoassay methods have been published since this work began (Arendt et al., 1975, Levine and Riceberg, 1975, Rollag and Niswender, 1976 and Wurzburger et al., 1976). The antibodies used in these assays were directed to recognise different areas of the indole structure. All the authors have claimed that their assays are highly specific for

melatonin in both plasma and pineal tissue following solvent extraction or direct assay of pineal tissue homogenate (Wurzburger et al., 1976). The claims for specificity have been based solely on cross reactivity of synthetic indoles and in some cases, co-chromatography with authentic melatonin. Even with so few workers involved in melatonin radioimmunoassays there has been considerable discrepancy in reported values of normal human plasma melatonin levels between groups. For example Arendt et al. (1975) and Wetterberg et al. (1976) reported plasma melatonin levels up to 10 times the levels reported by Vaughan et al. (1976b) who used a highly specific bioassay. Subsequently Arendt has reported much lower plasma melatonin levels using antibodies produced in another rabbit (Arendt et al., 1977). It was this type of discrepancy which no doubt prompted Professor Wetterberg to initiate the cross-validation study.

After all the groups had submitted their results, Wetterberg disclosed the method that was employed to prepare the serum which was distributed (Wetterberg, 1977). Calf serum was sampled in the morning and found to contain less than 10 pg/ml melatonin. To this serum was added melatonin to a final concentration of 200 pg/ml. Table 6 indicates that 5 of the 7 cooperating laboratories reported values close to the expected 200+pg/ml melatonin, while the other two were rather lower. It is indeed encouraging that there was so little discrepancy between groups in this study, however, it is also hardly surprising. Firstly, there is no doubt that all of the

antibodies can detect melatonin; thus given adequate recoveries in the methods, all assays should have detected the 200 pg that was added unless there is a serum factor that interferes with melatonin binding. Secondly, there has been no dispute over calf melatonin levels; thus far human studies have produced the discrepancies. If there is a biological reason for some antibodies detecting high melatonin concentrations rather than a methodological reason, then validation studies such as that initiated by Professor Wetterberg should be performed in the species of immediate interest using endogenous melatonin rather than synthetic. Hopefully the validation study planned for 1978 will consider these points.

CHAPTER 4

PINEAL FUNCTION THROUGH THE SHEEP REPRODUCTIVE CYCLE

A. Diurnal Changes in Plasma Melatonin

1) Introduction

To date investigations of pineal function have been restricted to laboratory rats and hamsters. There are a number of problems involved in the study of rodents, the most significant being that experiments utilising each animal as its own control are difficult. The sheep was chosen for this study because they are readily available in Australia, surgical manipulations and serial blood sampling are very easy to perform and finally the functioning of the reproductive axis of this animal has been well investigated. There have been few previous investigations of pineal function in this animal. The study has been restricted to investigations of pineal function in female sheep. Pineal function has been assessed in normal cycling, ovariectomised and pregnant ewes using plasma melatonin and pineal enzymes as markers.

2) Animals

Merino cross-bred ewes 3-4 years old were brought from the Mortlock Experimental station to the Animal House facilities of the Q.E.H. Lighting conditions in the rooms were 14:10 (L:D) and temperature 22°C.

3) Cannulation Procedure for Automated Blood Collection

Because melatonin levels are highest at night in humans (Vaughan et al., 1976) and rats (Pang and Ralph, 1975), a

practical means of collecting blood from sheep at various times during a 24-hour period was required. An automated programmable sampling system utilising a double lumen cannula was developed for this purpose.

A double lumen cannula was constructed from 2 m of Portex size 5E or 5 vinyl tubing (o.d. 4 mm, i.d. 2.9 mm; Boots Co. Australia) by inserting Portex size 2 vinyl tubing (o.d. 2 mm, i.d. 1 mm) into the lumen through a small hole in the wall 8 cm from the end. The hole was then sealed with silastic adhesive (Dow Corning Corp., Midland, Michigan, U.S.A.) and the adhesive allowed to cure for at least 24 hours. Prior to use, 14G and 18G blunted needles which served as connections for the outer and inner cannulae, were fitted and the cannula sterilised by immersion in a chlorhexidine-cetrimide alcoholic solution for 10 min. The cannula was then flushed with sterile saline.

To insert the cannula, the sheep was anaesthetised with sodium pentobarbitone (17 mg/kg, May and Baker Pty. Ltd., West Footscray, Victoria, Australia), and the cannula introduced into the jugular vein (4 cm towards the heart) via a maxillary vein. The cannula was tied in place with black braided silk (2/0), the wound sprayed with antibotic and closed with size 0 Dexon thread (American Cyanimid Company, Pearl River, New York, U.S.A.) The sheep was then returned to its usual pen to recover. The cannula, suspended above the sheep by rubber bands, was attached to a pumping system during the recovery period (24 hours) by connecting the outer tubing to one channel of a double channel peristaltic pump which was arranged to continuously deliver sterile saline containing 250 μ/ml heparin

(Weddel Pharmaceuticals Ltd., Sydney, Australia) at a rate of 3 ml/hour. The inner tubing was attached to the second channel which delivered sterile saline at 3 ml/hour. Figures 8, 9, 10 show some of the equipment used in the blood collection technique. The sheep pictured had two cannulae implanted.

4) Blood Collection Configurations

Figure 11 A & B show the two configurations utilised in this study. Configuration A was used for collection periods of 24-48 hours. After the recovery period the flow of the inner tubing was reversed and increased to 12 ml/h and the pump tubing connected to a fraction collector (Paton Industries Pty. Ltd., Stepney, South Australia). Blood was collected into clean glass tubes which were periodically removed, centrifuged at 4000 rpm for 10 min. Plasma was stored at -10°C.

Configuration B was used for collections longer than 48 hours and where excessive blood loss was to be avoided. The inner tubing in this configuration was connected by a T piece to the double channel pump as well as another single channel pump. Flow through the inner tubing was reversed and increased to 12 ml/hour. The flow through the outer tubing was maintained at 3 ml/hour, but the heparin concentration was altered to 500U/ml. When blood was not required, a programmable timer switched on the single channel peristaltic pump, resulting in a flow of 25 ml/hour sterile saline through the T junction. As the double channel pump remained operating during this time, 12 ml saline was delivered to the fraction collector and 13 ml into the sheep each hour, thus maintaining the patency of the cannula.

Figure 8

Photograph showing the programmable timer and the second peristaltic pump. This is the arrangement of tubing for configuration B.

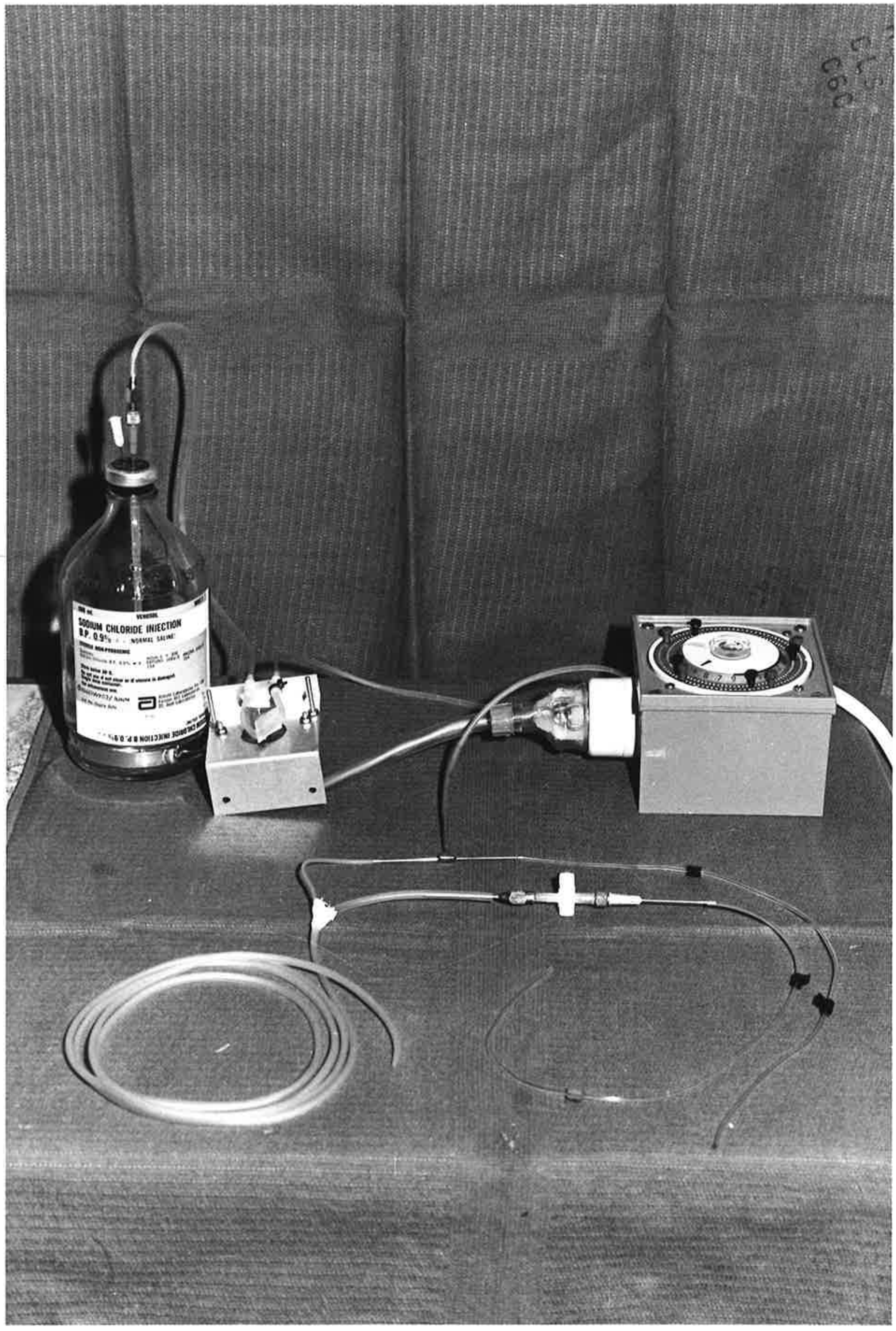

SODIUM CHLORIDE INJECTION
B.P. 0.9%
'NORMAL SALINE'

Figure 9

Photograph of a sheep being sampled simultaneously from the left and right jugular veins. The device on the white box is the multichannel peristaltic pump. Note; the sheep was restrained in a half size pen for photographic purposes. This preparation was set up in configuration A.

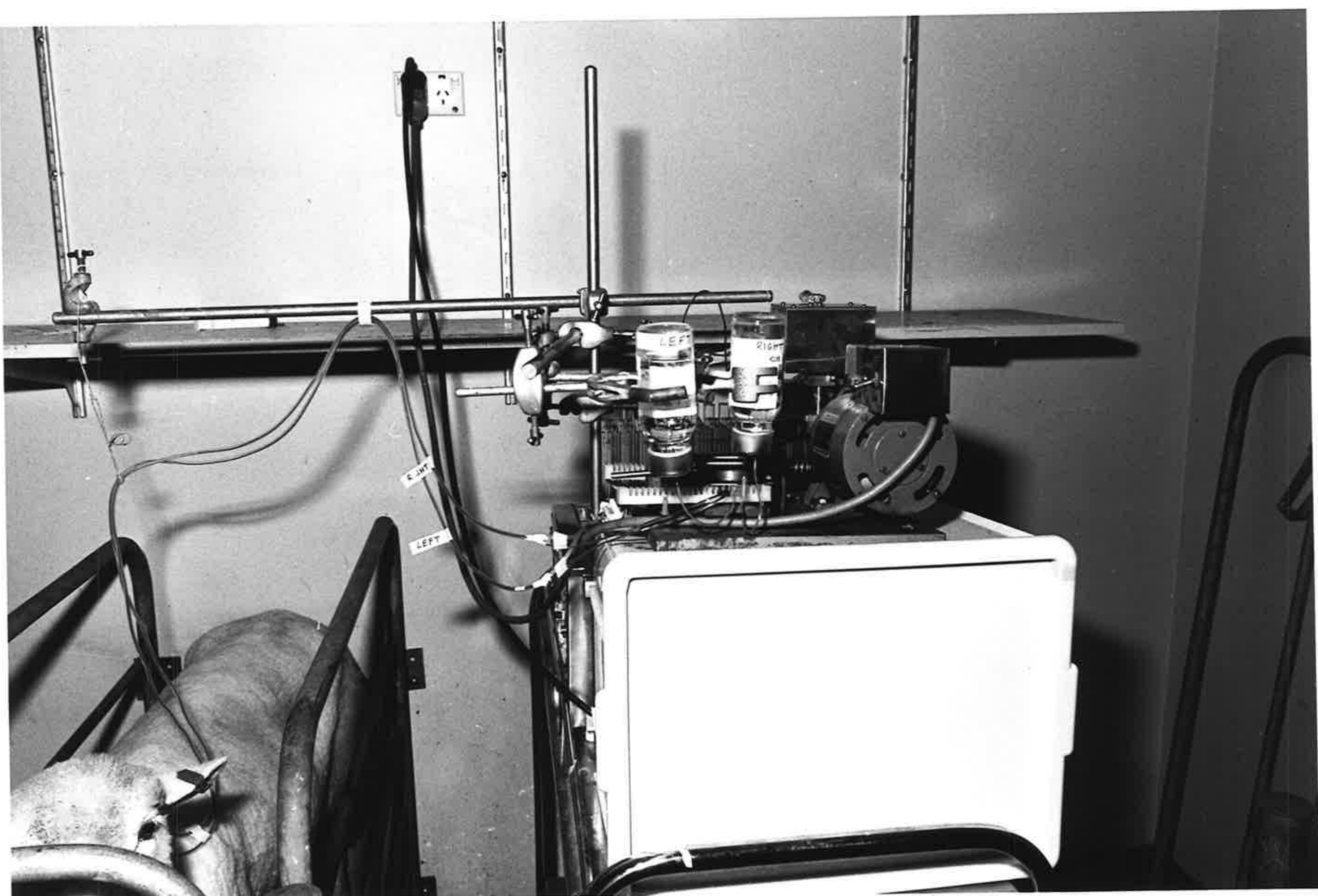

LEFT
RIGHT
LEFT

Figure 10

Photograph of multichannel peristaltic pump and fraction collector being used for simultaneous collection of blood from right and left jugular veins. Note: only one fraction collector was required. This preparation was set up in configuration A.

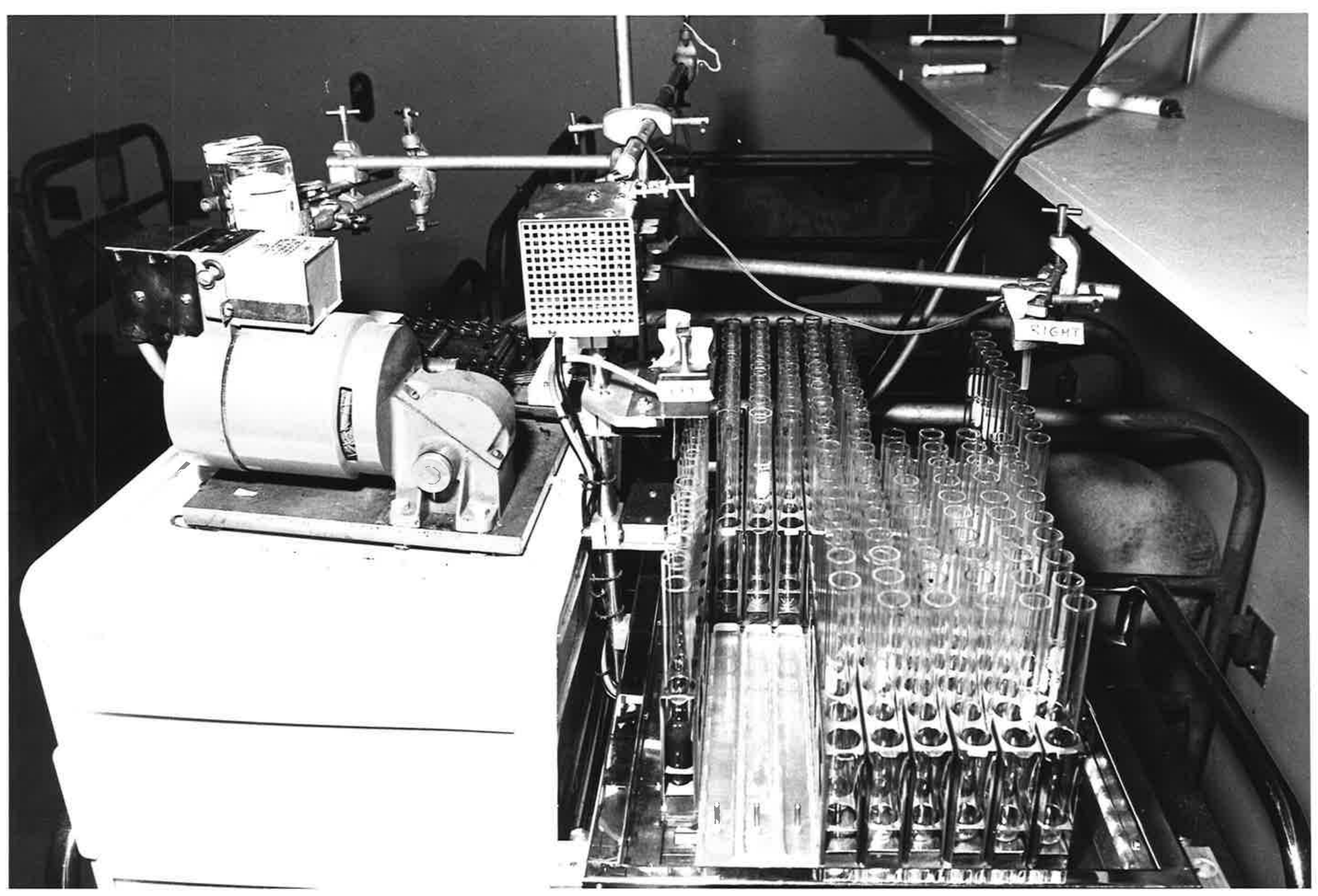

RIGHT

Figure 11(A)

Schematic diagram of the blood collection system in configuration A. See text for details.

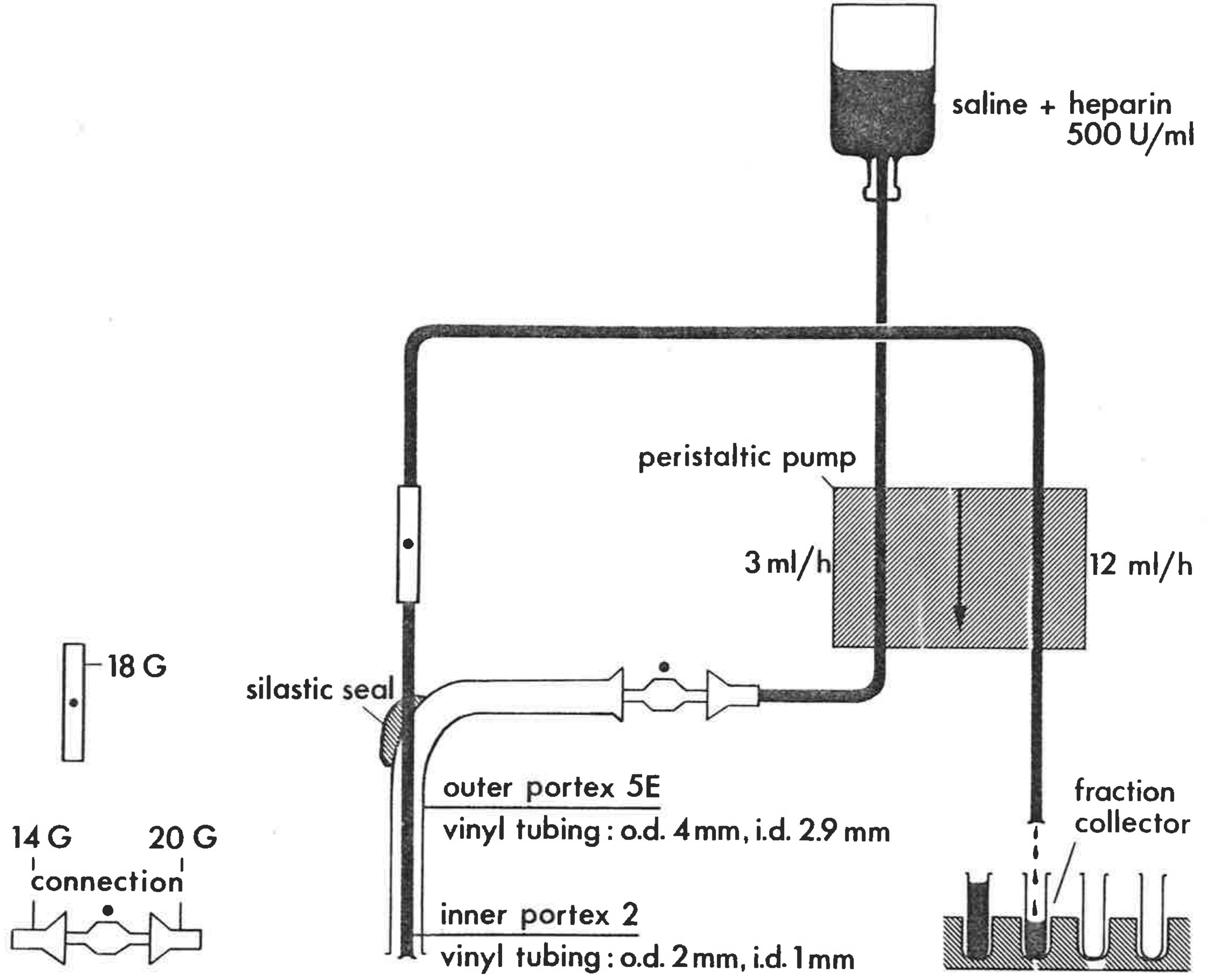
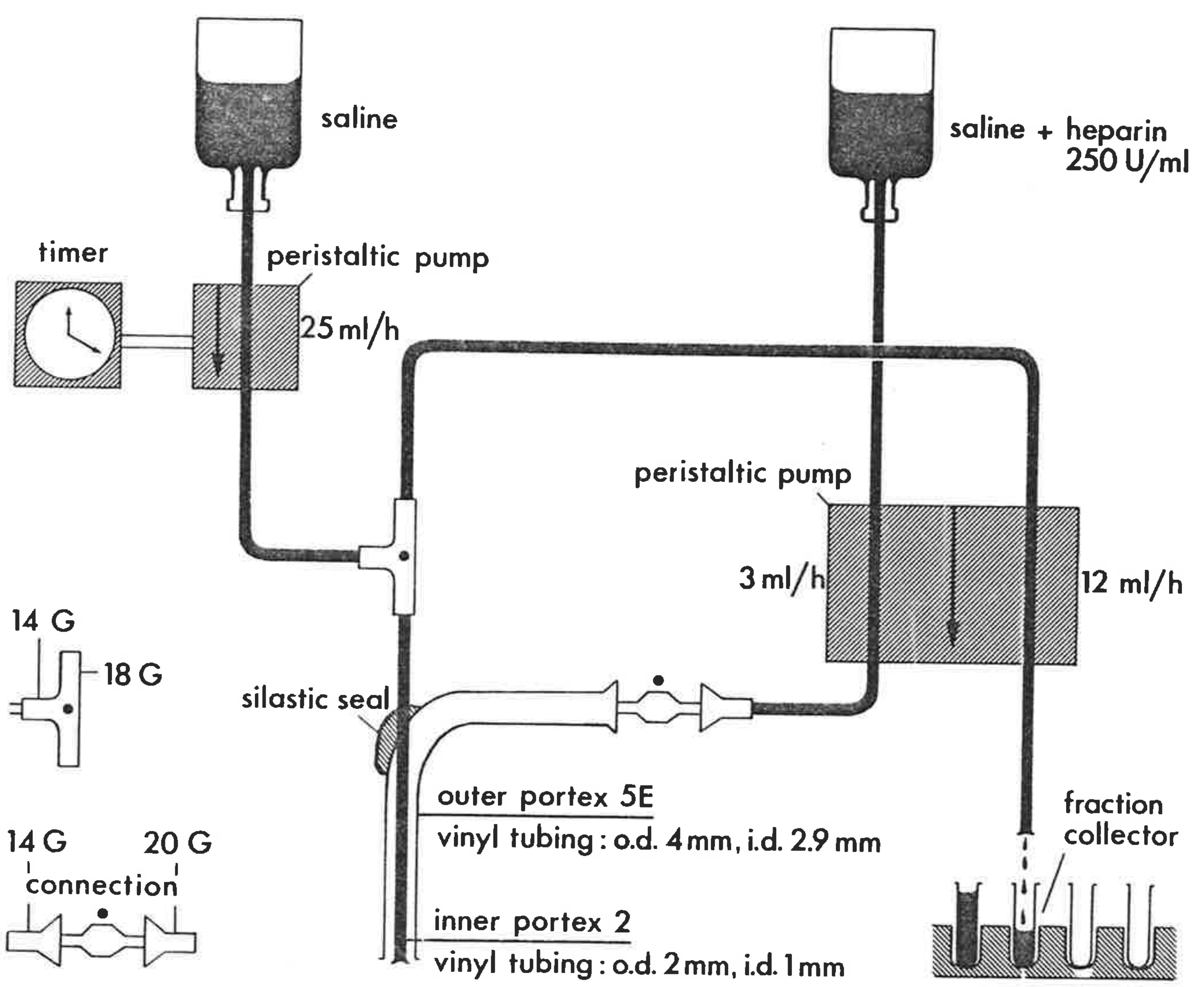

saline + heparin
500 U/ml
peristaltic pump
3 ml/h
12 ml/h
18 G
silastic seal
outer portex 5E
vinyl tubing : o.d. 4 mm, i.d. 2.9 mm
inner portex 2
vinyl tubing : o.d. 2 mm, i.d. 1 mm
fraction
collector
14 G
20 G
connection

Figure 11(B)

Schematic diagram of the blood collection system in configuration B. See text for details.


saline
saline + heparin
250 U/ml
timer
peristaltic pump
25 ml/h
peristaltic pump
3 ml/h
12 ml/h
14 G
18 G
silastic seal
outer portex 5E
vinyl tubing: o.d. 4 mm, i.d. 2.9 mm
inner portex 2
vinyl tubing: o.d. 2 mm, i.d. 1 mm
fraction
collector
14 G
20 G
connection

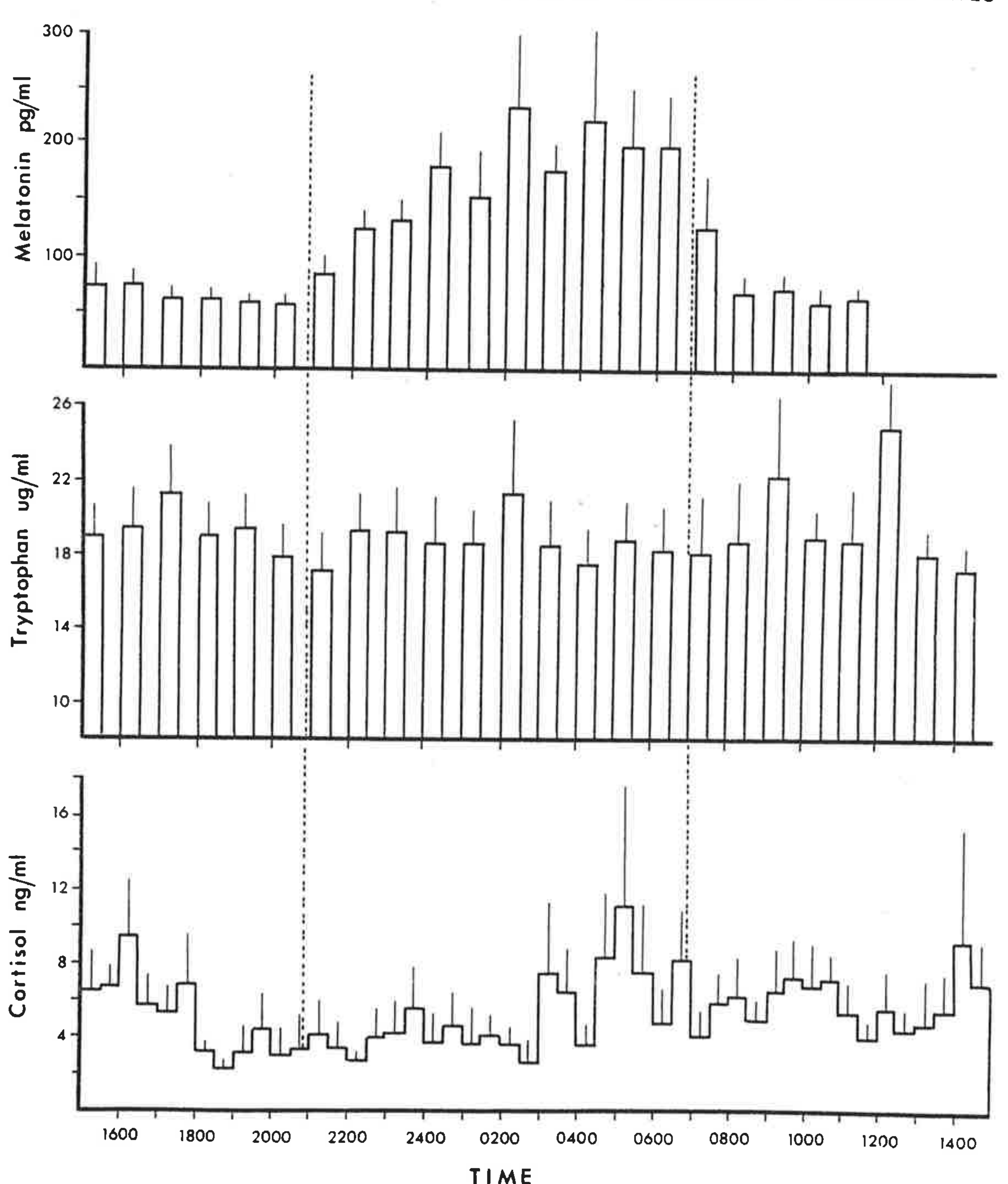
The amount of heparin solution contaminating samples could be monitored by measurement of changes in haematocrit, or by admixing a dyestuff (Indocyanine Green, Aynson, Wescott and Dunning Inc., Baltimore, Maryland) with the heparin. Contamination of blood with heparinised saline was always less than 5%. Clotting of blood within the system was not a problem provided adequate concentrations of heparin were used and attention paid to cleanliness of the system. Of the 33 cannulations performed 3 failed to collect for at least 24 hours due to clotting. Two preparations were ruined by the test ewe or it's neighbour biting through the suspended cannula.

5) Diurnal Rhythm of Plasma Melatonin, Cortisol and Tryptophan

Utilising the automated collection technique (configuration A) blood was collected over a period of 24 hours and assayed for melatonin, cortisol and tryptophan. Melatonin and tryptophan were assayed in alternate 30 minute collections, while cortisol was assayed in successive 30 minute collections. Figure 12 shows the means ± SEM for the 3 circulating compounds. Of the 8 sheep studied in this manner 7 showed significantly higher dark period than light period concentrations of melatonin. It is unknown whether the length of time that the sheep were acclimatised to the new photoperiod influenced these results. The mean plasma cortisol concentrations of the 6 sampled continuously, varied between 2.5 and 11 ug/ml. There was a suggestion of a diurnal rhythm in these sheep with maxima occuring around 1600h and 0500h. This phenomenon was also apparent when the data was expressed as a percentage of indi-

Figure 12

24 hour profiles of circulating hormones in normal ewes. The values represent mean levels $\pm$ SEM for 30 minute collection periods. The vertical broken line indicates lights out (2050h) and lights on (0650). Sample sizes were 8 for melatonin and 6 for both tryptophan and cortisol.


24 HOUR PROFILES OF CIRCULATING HORMONES IN NORMAL EWES
Melatonin pg/ml
300
200
100
Tryptophan ug/ml
26
22
18
14
10
Cortisol ng/ml
16
12
8
4
1600
1800
2000
2200
2400
0200
0400
0600
0800
1000
1200
1400
TIME

vidual daily mean levels (table 7). Mean plasma tryptophan concentrations varied between 17 and 25 ug/ml. A significant elevation in the circulating tryptophan occurred at 1200h. There was no difference between day and night levels of tryptophan in 5 out of 6 ewes studied. One ewe did have significantly ($P<0.05$) higher levels of tryptophan during the day (18.1 ± 1 ug/ml cf. 12.5 ± 2.2 ug/ml). Expressing the tryptophan levels as percentages of daily means (table 8) failed to uncover a significant change during the dark period.

6) Effect of Dim Red Light on Diurnal Rhythm of Melatonin

Exposure of animals to light during the dark phase of the photoperiod results in a rapid decrease in serotonin N-acetyltransferase activity (Klein and Weller, 1972). Red light exposure, however, is not inhibitory to the pineal gland (Cardinali et al., 1972). During the course of the present study, it was important to enter the animal house holding rooms to take blood samples, check the automatic pump, etc., during the dark phase. To test whether red light exposure during darkness affected the dark-induced plasma melatonin increase, a sheep was cannulated with the double lumen cannula and sampled using configuration A, before and during exposure. Control samples were obtained for 24 hours of normal photoperiod (14th light: 10h dark). The following day an Ilford LR 915 Safelight fitted with a 25 watt incandescent globe was installed and programmed to be switched on when the room lights were extinguished. On the second night of red light exposure, blood was collected for a further 24 hour period.

TABLE 7

NORMAL EWES: PLASMA CORTISOL VALUES CALCULATED AS PERCENTAGES OF DAILY MEANS

Time	Cortisol (% daily mean ± SEM)	Time	Cortisol (% daily mean ± SEM)
1500h	135 ± 27	0300h	117 ± 29
1530h	148 ± 21	0330h	97 ± 20
1600h	181 ± 41	0400h	66 ± 12
1630h	111 ± 10	0430h	119 ± 27
1700h	129 ± 35	0500h	224 ± 67
1730h	154 ± 41	0530h	120 ± 30
1800h	79 ± 16	0600h	78 ± 14
1830h	56 ± 15	0630h	148 ± 36
1900h	81 ± 33	0700h	79 ± 8
1930h	78 ± 22	0730h	104 ± 30
2000h	41 ± 16	0800h	112 ± 24
2030h	61 ± 28	0830h	93 ± 7
2100h	73 ± 20	0900h	117 ± 42
2130h	57 ± 18	0930h	132 ± 29
2200h	59 ± 9	1000h	139 ± 56
2230h	69 ± 19	1030h	146 ± 12
2300h	63 ± 19	1100h	95 ± 22
2330h	83 ± 19	1130h	78 ± 13
2400h	67 ± 17	1200h	120 ± 40
0030h	82 ± 15	1230h	88 ± 26
0100h	47 ± 20	1300h	78 ± 30
0130h	73 ± 12	1330h	116 ± 33
0200h	85 ± 32	1400h	136 ± 58
0230h	51 ± 14	1430h	128 ± 21

TABLE 8

NORMAL EWES: PLASMA TRYPTOPHAN VALUES CALCULATED AS PERCENTAGES OF DAILY MEANS

Time	Tryptophan (% daily mean ± SEM)
1500h	103 ± 7
1600h	104 ± 3.5
1700h	112 ±+4.5
1800h	102 ± 2
1900h	104 ± 8
2000h	93 ± 3
2100h	92 ± 6
2200h	102 ± 7
2300h	99 ± 4
2400h	82 ± 12
0100h	95 ± 2
0200h	105 ± 11
0300h	90 ± 8
0400h	96 ± 8
0500h	94 ± 5
0600h	92 ± 8
0700h	91 ± 8
0800h	93 ± 10
0900h	111 ± 15
1000h	101 ± 12
1100h	100 ± 15
1200h	135 ± 22
1300h	100 ± 4
1400h	97 ± 6

Due to technical difficulties, plasma melatonin from each 24 hour profile could not be assayed within a single assay. Therefore, direct comparison of the absolute levels in this experiment may not be valid. Nevertheless, it was evident (Figure 13) that there was a significant increase ($P<0.05$) in plasma melatonin concentration in response to the room lights being extinguished on both occasions. The intensity of light during exposure to the red light at the level of the sheep's head was 22 lux compared to 2800 lux during the light phase. Even in light of this intensity it was feasible to work in the room.

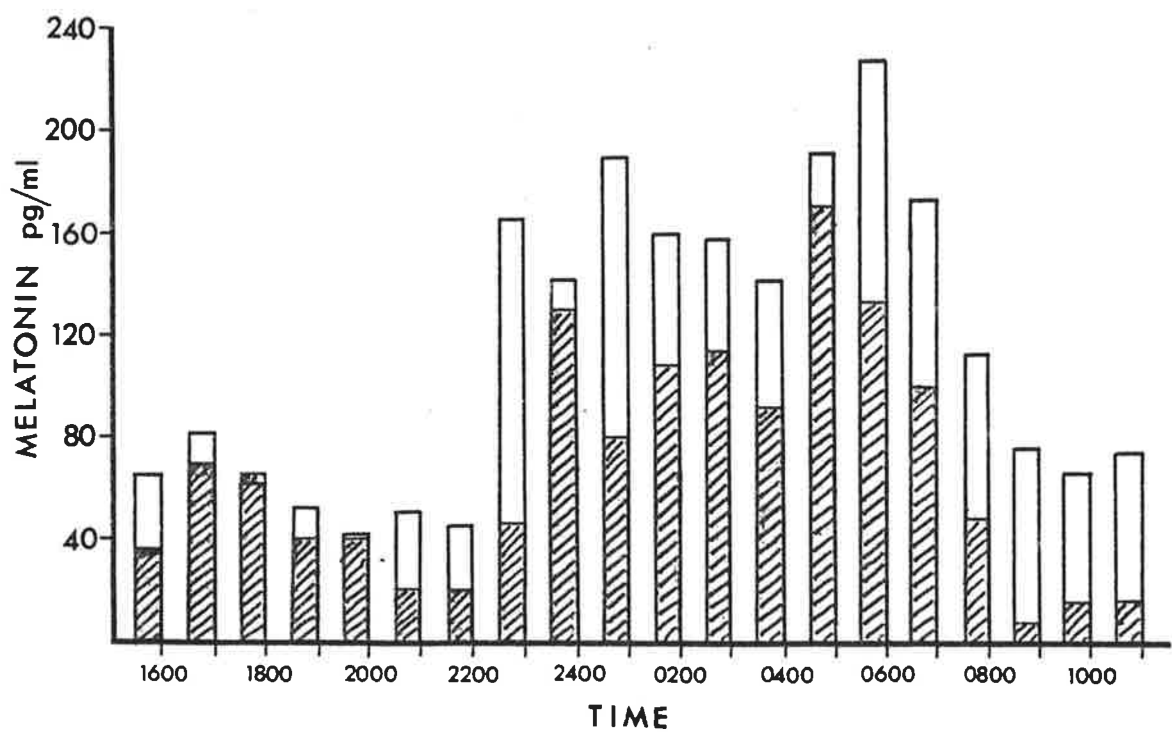
7) Effect of Chlorpromazine on Daytime Melatonin

While this work was in progress a report appeared (Ozaki et al., 1976) demonstrating that chlorpromazine elevated circulating melatonin in rats during the light phase. The response was due in part to a decrease in metabolism by the liver, but also involved stimulation of the pineal. Such a drug effect promised to be useful as a pineal function test. Chlorpromazine was thus injected into sheep and plasma melatonin assayed before and after administration of the drug.

Two ewes were injected intramuscularly with either 2 ml saline or 2 ml (50 mg) chlorpromazine. Blood samples (jugular venepuncture) were taken prior to injection and at 30 minute intervals afterwards for a total of 150 minutes. Three weeks later the same ewes were given higher doses of the drug (100 mg and 150 mg) and bled to the same regime. An obvious tranquilising effect was observed with the higher doses - both

Figure 13

The effect of dim red light on plasma melatonin. The open boxes represent melatonin concentrations from 30 minute collection periods under normal conditions of light and dark. The shaded boxes represent melatonin concentrations from 30 minute collection periods during a light and red light regime. Room lights were out from 2050h to 0650h. See text for details.


ROOM LIGHTS OUT
DARKNESS
DIM RED LIGHT
240
200
160
120
80
40
MELATONIN pg/ml
1600
1800
2000
2200
2400
0200
0400
0600
0800
1000
TIME

sheep were listless and failed to respond to loud noises. Maximum sedation had occurred after about 90 minutes.

Table 9 gives the melatonin levels in this experiment. At no time did the melatonin levels become elevated above a baseline of about 30 pg/ml. Thus intramuscular injections of chlorpromazine up to 150 mg are incapable of raising plasma melatonin levels in the short term in sheep.

8) Discussion

There is now considerable evidence to suggest that pineal gland synthesis of melatonin is higher during darkness than during daylight in a large variety of species.

From extensive studies of the rat pineal a general hypothesis of control of melatonin synthesis by environmental lighting has arisen. Light acting upon the retinae activate nerve bundles passing along the optic tract to the superior cervical ganglion. The sympathetic nerves from this ganglion which innervate the pineal gland have a reduced rate of firing during periods of light and increased rate of firing at night. The increased nerve activity results in an elevation of Norepinephrine levels in the post synaptic junction with the pinealocyte membrane. The transmitter activates the adenylate cyclase system resulting in cyclic AMP production, N-acetyltransferase and hydroxyindole-O-methyltransferase induction. The net effect of these events is an increase in pineal melatonin synthesis and content. Little is known about the subsequent secretion of melatonin because of the lack of sensitive assays. While it is apparent that exposure to light during the darkness results in a precipitous drop in enzyme activity, the effects

TABLE 9

EFFECT OF CHLORPROMAZINE ON PLASMA MELATONIN

(Melatonin pg/ml)

	Ewe 188	Ewe 033	Ewe 033	Ewe 188
Time After Injection	Saline	50 mg	100 mg	150 mg
0 min.	41	12	25	21
30 min.	15	33	14	21
60 min.	28	40	11	32
90 min.	29	25	11	24
120 min.	35	29	15	27
150 min.	21	36	31	26
180 min.	-	-	-	41

on circulating melatonin are less clear. Rollag and Niswender (1976) have reported a decrease in plasma melatonin in sheep exposed to light for a short period but definitive evidence in other species is lacking.

The role of environmental light in the melatonin diurnal rhythm appears to be that of a Zeitgeber. Moore and Klein (1974) found that selective lesions in the rat brain were ineffective unless they interferred with the suprachiasmatic nucleus. Animals exposed to constant darkness have a maintained rhythm in enzyme activity (Yochim and Wallen, 1974) and plasma melatonin (Rollag and Niswender 1976). Constant light tends to remove this rhythmicity.

The results reported here confirm the report of Rollag and Niswender (1976) who used a different sampling procedure and a different radioimmunoassay. Sheep kept under diurnal lighting conditions show elevated plasma levels of melatonin at night. The elevation of plasma melatonin levels occurs in response to darkness. Cardinali et al., (1972) reported that exposure of rats to continuous red light had the same effect as continuous exposure to darkness. Exposure of a sheep to two nights of red light of low intensity (<22 Lux) did not remove the diurnal rhythm in melatonin as would be expected after two nights exposure to high intensity white light. Thus the sheep pineal is activated by the absence of light in a manner which may well be similar to the rodents.

The levels of tryptophan found in plasma are comparable to those previously reported by Schwiegert et al., (1946).

The 24 hour profile of circulating plasma tryptophan indicates that fluctuations in availability of the amino acid are not responsible for the melatonin rise at night. The effects of periodic fluctuations in the plasma content of neutral amino acids cannot be excluded as these are known to compete with tryptophan for entry into the brain (Fernstrom and Wurtman, 1973).

Plasma cortisol fluctuated throughout the 24 hour period of sampling and examination of individual data indicated periods of secretory activity. Calculation of mean levels during the 24 hour period indicated a possible rhythm with two maxima (1600h and 0500h). McNatty et al., (1972) and Holley et al., (1975) have reported similar increases in plasma cortisol prior to the time of "lights on". Holley et al., (1975) suggested that the 1600 h increase was related to feeding, a proposition supported by the present study. A temporal relationship between the falling melatonin levels during early morning and the increasing cortisol levels is suggested by the data but further experiments are required to prove a causal relationship.

B. Pineal Function in Cycling and Ovariectomised Ewes

1) Introduction

The pineal gland has been implicated in the control of ovulation via an inhibitory action on the hypothalamo - pituitary - gonadal axis (Reiter, 1974a). It was of interest to investigate the changes in plasma melatonin during the oestrous cycle as well as the effects on pineal function of the removal of circulating ovarian steroids. The effects of

a synthetic depot progestin, medroxy progesterone and oestradiol 17β were also investigated.

2) Melatonin During the Oestrous Cycle

Two ewes had blood samples taken at 0930h every second day for a total of 11 consecutive cycles (7 cycles and 4 cycles). Day of oestrous was determined by running a vasectomised ram with the ewes. Plasma melatonin and progesterone were assayed as per the methods section. Both ewes had regular 18 day cycles and so the data was pooled for analysis. Mean progesterone values were highest midcycle and lowest at oestrus (Figure 14). Mean melatonin levels were, however, remarkably constant throughout the cycle. There was considerable day to day variation within animals but no rhythm with a period greater or less than 18 days could be distinguished.

3) Protocol for the Ovariectomy Experiments

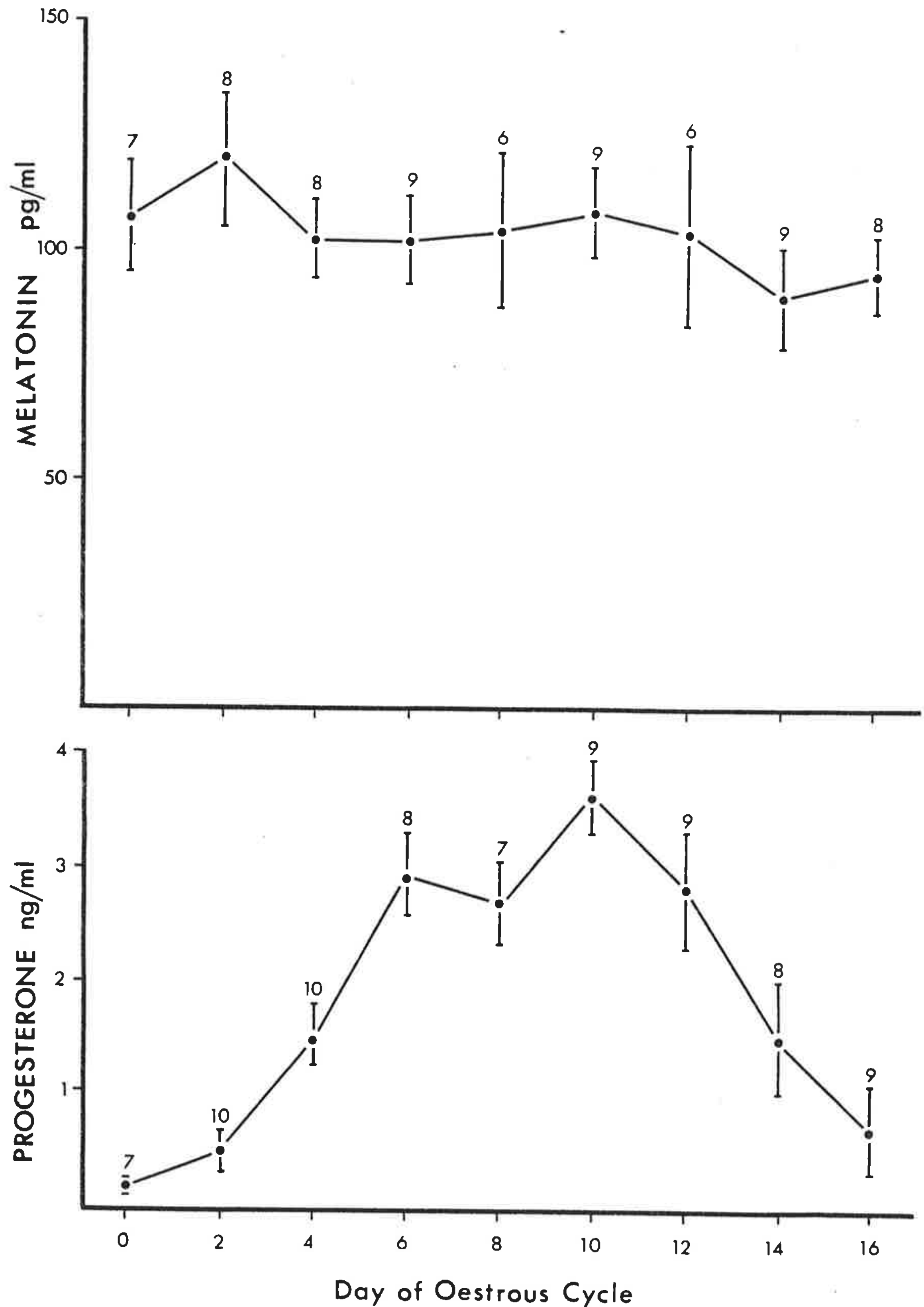
Five ewes were subjected to preliminary daily blood sampling, 24-hour blood sampling, bilateral ovariectomy, daily blood sampling, another 24 hour blood sampling, medroxy progesterone depot injection and oestradiol 17β injection. The timing of these various treatments is shown in Table 10.

4) Effects of Sampling from the Right and Left Jugular

Because the experimental protocol required two periods of intensive blood sampling a month apart, for consistency the first samples were taken from the left jugular and the second lot of samples from the right. (It was found that recannulation via the maxillary vein was impossible once the original cannula had been removed). Thus it was important to determine

Figure 14

Plasma melatonin during the oestrous cycle. The values represent mean $\pm$ SEM melatonin normalised to an "ideal" 18 day cycle. Numbers at each point represent the number of samples.



PLASMA MELATONIN during the OESTROUS CYCLE
MELATONIN pg/ml
150
100
50
7
8
8
9
6
9
6
9
8
PROGESTERONE ng/ml
4
3
2
1
7
10
10
8
7
9
9
8
9
0
2
4
6
8
10
12
14
16
Day of Oestrous Cycle

TABLE 10

PROTOCOL FOR THE OVARIECTOMY EXPERIMENTS

Ewe	802	058	156	024	034
No. of days monitored prior to ovariectomy.	25 d	45 d	-	25 d	25 d
No. of days prior to ovariectomy of first 24hour profile.	5 d	5 d	-	18 d	11 d
No. of days after ovariectomy of second 24hour profile.	37 d	36 d	26 d	37 d	43 d
Depot medroxy progesterone	70 d & 83 d	55 d	38 d	50 d	50 d
Time of oestradiol 17β injection.	175 d	125 d	111 d	70 d	70 d

whether there were any gross differences in the secretion of melatonin into the two jugular veins. A sheep was cannulated on both sides simultaneously and sampled for 20 hours. Figure 15 shows that the plasma levels of melatonin obtained from left and right jugular veins were identical, both during the day and during the night.

5) Effect of Ovariectomy on Daytime Levels of Plasma Melatonin

Figure 16 shows the individual results of the effect of ovariectomy on plasma melatonin. Blood samples were always taken between 1530h - 1600h. One ewe (156) was not sampled prior to ovariectomy. Removal of the ovaries had no apparent effect on circulating melatonin. Only one sheep (802) had higher levels after ovariectomy but these fluctuated considerably. Ewe 024 which had high irregular levels of melatonin prior to ovariectomy maintained a similar pattern after the ovaries were removed.

6) Effect of Ovariectomy on the Diurnal Rhythm of Plasma Melatonin

Figure 17 shows individual 24-hour profiles of melatonin in blood sampled from the left jugular prior to organ removal and the right jugular after organ removal. In two cases (Ewes 058 and 802), the rhythm was absent following ovariectomy. Both had had significantly higher nighttime levels than daytime levels prior to ovary removal. The other two ewes sampled before and after ovariectomy showed an opposite response i.e., lack of a diurnal rhythm prior to ovariectomy. This lack of a dark induced rise of melatonin in normal sheep is unusual

Figure 15

Comparison of melatonin levels in a ewe sampled simultaneously from left and right jugular veins. Open boxes represent melatonin levels from 30 minute collection periods taken from the right jugular. Shaded boxes represent melatonin from the left jugular. Lights were off from 2050h until 0650h.

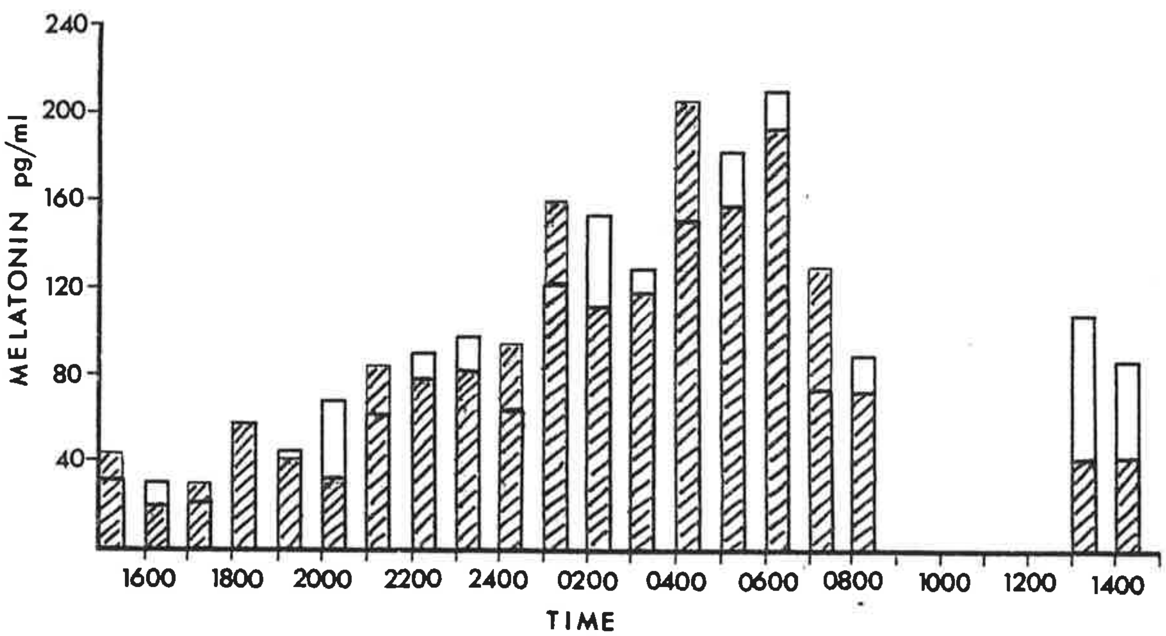

DARKNESS
RIGHT JUGULAR
LEFT JUGULAR
240
200
160
120
80
40
MELATONIN pg/ml
1600
1800
2000
2200
2400
0200
0400
0600
0800
1000
1200
1400
TIME

Figure 16

Effect of ovariectomy on plasma melatonin. Individual melatonin levels are plotted in relation to the day of ovariectomy (vertical broken line). Note: no samples were obtained from Ewe 156 prior to ovariectomy.

EFFECT OF OVARIECTOMY ON PLASMA MELATONIN

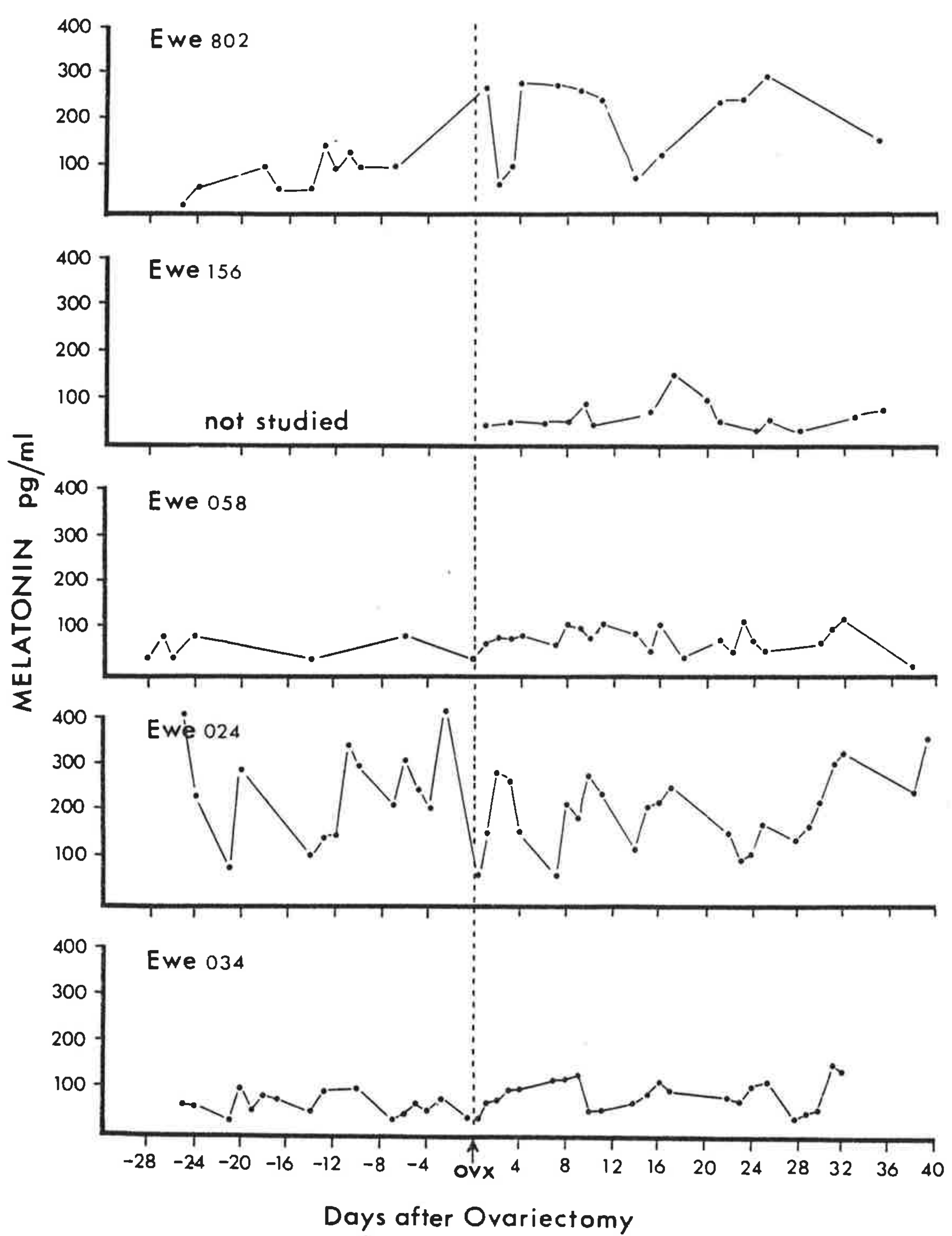
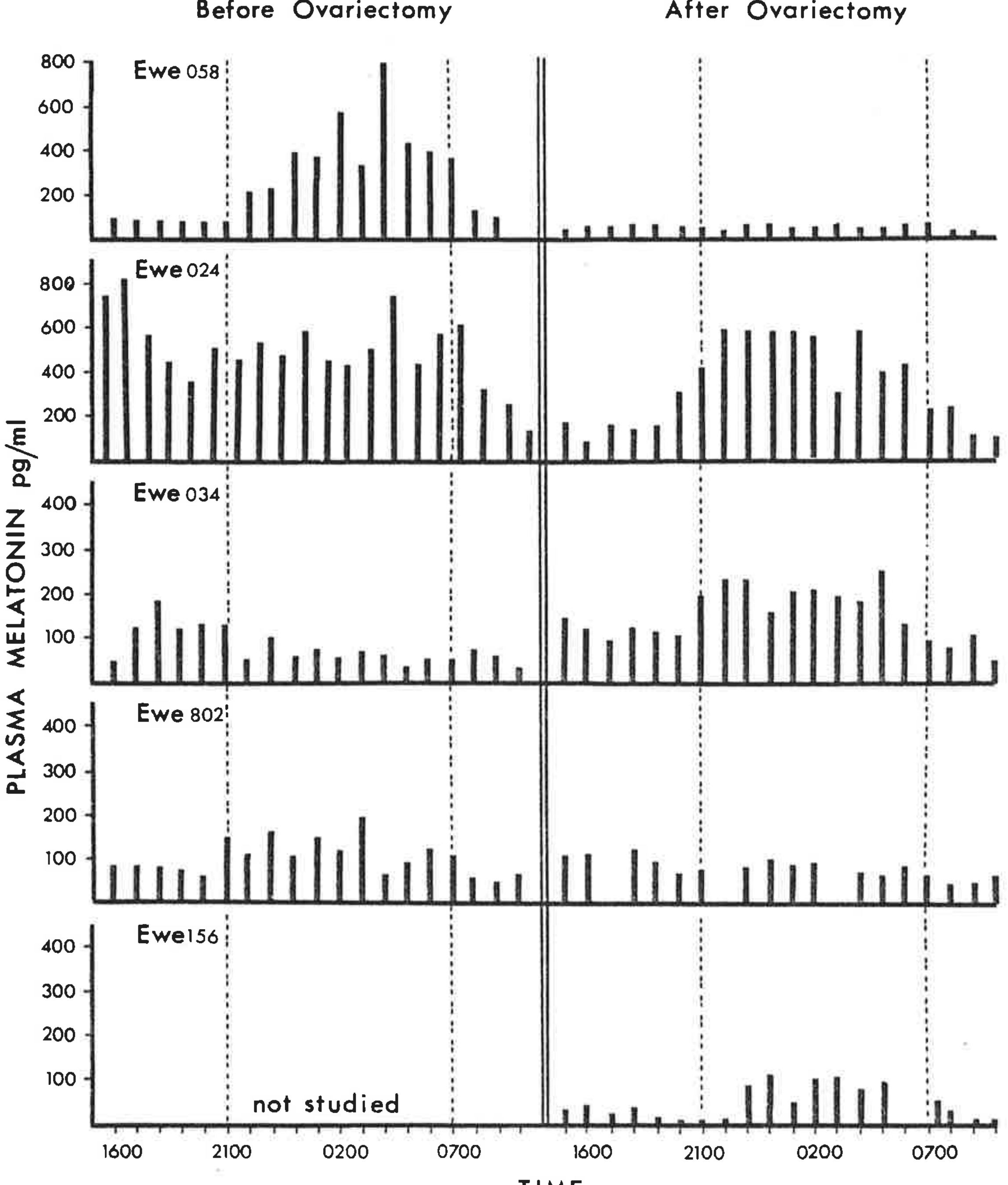


Figure 17

24-hour profiles of melatonin before and after (1 month) ovariectomy. The bars represent melatonin levels from 30 minute collection periods. The broken vertical lines designate lights out (2050h) and lights on (0650h). Ewes were sampled from the left jugular prior to ovariectomy and from the right jugular after. Ewe 156 was not sampled prior to ovariectomy.



Before Ovariectomy
After Ovariectomy
Ewe 058
Ewe 024
Ewe 034
Ewe 802
Ewe 156
not studied
PLASMA MELATONIN pg/ml
800
600
400
200
400
300
200
100
1600
2100
0200
0700
TIME

and no explanation is available. The two ewes had normal low cortisol concentrations during the sampling period. Ewe 156 which was not sampled prior to ovariectomy exhibited a significant diurnal rhythm after organ removal.

7) Effect of Depot Medroxy Progesterone Injection on Plasma Melatonin

There is some evidence from rat studies that gonadotrophins can stimulate pineal enzymes (Cardinali et al., 1976). Three ewes were, therefore, subjected to an intramuscular injection of 150 mg medroxy progesterone acetate (Depo Provera) to test whether lowering circulating gonadotrophins would affect plasma levels of melatonin. Unfortunately, it is not known to what extent the gonadotrophins were affected in this series due to the unavailability of Luteinising Hormone and Follicle Stimulating Hormone assays in this laboratory. Depot medroxy progesterone acetate had neither an inhibitory nor a stimulatory effect on plasma melatonin levels in ovariectomised sheep. See Table 11.

8) Effect of Oestradiol 17β Injection on Plasma Melatonin

Low doses of oestrogen injected into ovariectomised rats increase pineal synthetic activity (Cardinali et al., 1974a). To test this point in sheep, five ewes were injected with 100 ug oestradiol 17β (intramuscular) in almond oil at 0900h each day for 5 days. Blood samples were taken daily for one week prior to treatment (1530h), just prior to each injection and 6 hours later during the treatment period and then daily for one week after injections were terminated. Oestradiol 17β injections

TABLE 11

EFFECT OF DEPOT MEDROXY PROGESTERONE ON PLASMA MELATONIN

Days prior to injection	Ewe 802	Ewe 158	Ewe 156
	- Melatonin pg/ml -		
- 10d	77	61	27
- 9d	-	41	-
- 8d	-	-	-
- 7d	93	-	-
- 6d	148	-	-
- 5d	148	30	57
- 4d	-	52	62
- 3d	468	73	69
- 2d	-	-	-
- 1d	-	-	-
0D.P.	245	21	19
+ 1	279	24	62
2	279	25	37
3	479	-	-
4	59	43	47
5	-	-	-
6	-	-	-
7	-	9	16
8	140	51	-
9	93	-	-
10	211	-	-
11	417	89	-
12	-	-	-
13	-	-	-
14 D.P.Ewe 802	320	0	36
15	408	45	90
16	320	-	92
17	148	-	127

of 100 ug/day have been shown to increase fallopian tube secretion in this breed of sheep (Warnes, 1976) despite the fact that peripheral levels are less than 25 pg/ml. Figure 18 shows the individual responses to oestradiol injections. The mean results (Table 12) suggest an increase in plasma melatonin in response to the treatment, but because of the different baseline levels, the increase does not reach significance.

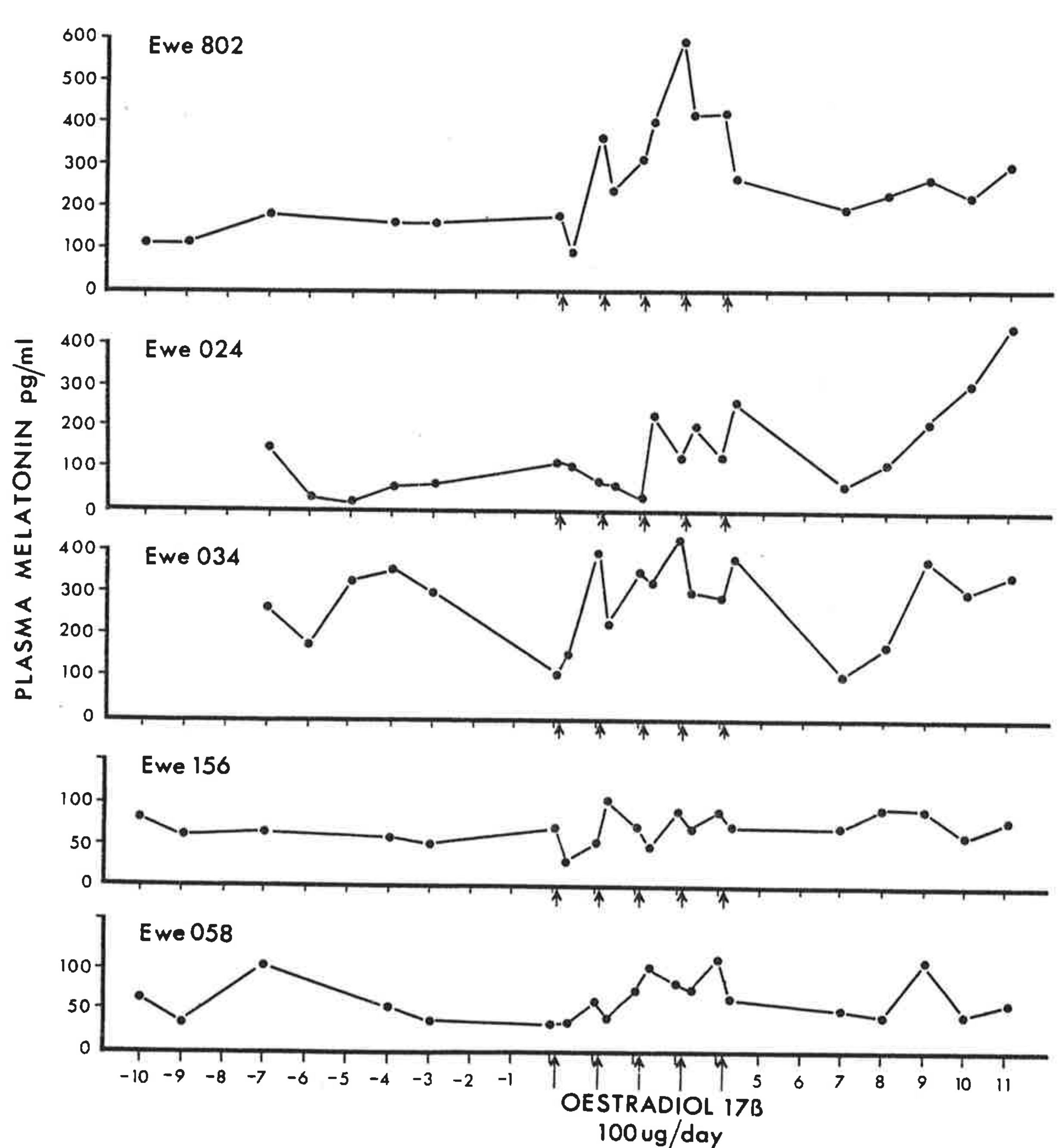
9) Discussion

There is a considerable amount of data suggesting that the pineal gland influences ovulation during the rat oestrous cycle. The ability of melatonin injections given during the critical period of pro-oestrous to suppress ovulation (Ying and Greep, 1973) and the reports that pineal hydroxyindole-O-methyltransferase activity was low during pro-oestrous (Wurtman et al., 1965, Cardinali et al., 1974a) and high during oestrous, specifically implicated melatonin in this process. Subsequently Wallen and Yochim (1974) demonstrated that there is a dampening of the nighttime levels of pineal hydroxyindole-O-methyltransferase during the oestrous-metoestrous interval in rats. Pineal adenylate cyclase activity is lowest during pro-oestrous, and fails to respond to noradrenaline (Weiss and Crayton, 1970).

Removal of the ovaries has significant effects on the synthesis of melatonin in the pineal gland. Pineal hydroxyindole-O-methyltransferase activity decreases following ovariectomy, (Cardinali et al., 1970, Wallen and Yochim, 1974) but is restored by low doses of oestrogen (o.05 - 1 ug per day oestradiol or 10 ug per day oestrone). It is to be noted that

Figure 18

Effect of oestradiol (100 ug/day) on plasma melatonin in ovariectomised ewes. Individual melatonin levels are plotted in relation to the first injection. The daily injections are denoted by arrows.



OVARIECTOMY : EFFECT OF OESTRADIOL 100ug/day
PLASMA MELATONIN pg/ml
Ewe 802
Ewe 024
Ewe 034
Ewe 156
Ewe 058
600
500
400
300
200
100
0
50
-10 -9 -8 -7 -6 -5 -4 -3 -2 -1 5 6 7 8 9 10 11
OESTRADIOL 17β
100 ug/day

TABLE 12

EFFECT OF OESTRADIOL INJECTION
ON PLASMA MELATONIN

Days Prior to Injection	802	- Ewe Number - 058	156	024	034	Mean ± SEM
	- Melatonin pg/ml -					
- 4d	165	54	60	358	54	138 ± 58
- 3d	163	37	49	300	61	122 ± 50
0	178	37	60	110	115	101 ± 23
0 + 6h	92	36	30	153	102	82 ± 23
1d	367	62	56	406	67	191 ± 79
1d + 6h	244	39	103	232	60	135 ± 43
2d	322	75	69	355	32	170 ± 69
2d + 6h	398	102	48	334	226	221 ± 66
3d	616	83	88	443	131	272 ± 108
3d + 6h	417	73	68	300	197	211 ± 67
4d	425	115	92	389	129	230 ± 72
4d + 6h	270	65	67	385	258	209 ± 62
7d	194	51	70	105	64	97 ± 26
8d	234	42	94	177	114	132 ± 33
9d	273	110	92	381	212	213 ± 53
10d	228	37	61	306	300	186 ± 58
11d	304	57	81	344	444	246 ± 75

Wurtman et al., (1965) observed no change in pineal hydroxy-indole-O-methyltransferase activity and a decrease in activity following 10 ug per day oestradiol. Ovariectomy of rats does not alter pineal adenylate cyclase activity but does increase the stimulatory effects of noradrenaline. Oestradiol in low doses inhibits the response of adenylate cyclase to noradrenaline (Weiss and Crayton, 1970). With respect to other pineal enzymes, Illnerova (1975) has shown that doses of oestradiol of 10 µg/100g body weight/day are incapable of altering the response of serotonin: N-acetyltransferase to either darkness or noradrenaline. Progesterone administered to ovariectomised rats can inhibit hydroxyindole-O-methyltransferase activity (Houssay and Barcello, 1972).

While there are certain contradictions in the literature discussed above (i.e., reduction of adenylate cyclase sensitivity to noradrenaline following oestrogen treatment and the increase in hydroxy indole-O-methyltransferase after oestrogen treatment), the concept which has evolved is that LH secretion and ovulation occur because melatonin production (and secretion) is decreased during the pro-oestrous period of the rat.

This then is the general background against which the present sheep experiments were designed. There has been a paucity of investigations into sheep pineal function thus far. Cardinali et al. (1974) did report significant changes in pineal hydroxyindole-O-methyltransferase during the sheep oestrous cycle. Highest levels of the enzyme occurred around the periovulatory period and day 4 corresponding to periods

of high oestrogen secretion. Lowest levels occurred during the luteal phase of the cycle. Tissue was apparently obtained during daylight.

The results of the present study do not indicate a major role of circulating melatonin in the ovine oestrous cycle. Certainly there is no change in daytime levels of melatonin throughout the cycle. Analysis of nighttime levels during the cycle may be required to demonstrate active changes in melatonin secretion. Ovariectomy of the sheep results in no consistent alterations in the daily pattern of circulating melatonin or the diurnal rhythm. Likewise the synthetic progestin, medroxy progesterone does not alter the daytime levels of melatonin. The increase in plasma melatonin levels in response to oestradiol injections in some sheep are at variance with the lack of change in daytime levels during the cycle. Since comparable vehicle injected controls were not included it could be argued that the rise during the injection period was a stress induced one. While this possibility cannot be ignored it should be noted that the ewes used in this study were accustomed to being handled, having been bled 5 days a week for up to seven months. A reason for the discrepancy may be that in the oestrous cycle study the sampling times (0900h) may have missed the preovulatory surge of oestradiol. The effect of oestradiol administration on the diurnal rhythm of melatonin after ovariectomy remains to be determined.

These results reflect a possible action of the ovary on pineal secretion of melatonin but the role of the pineal in

the control of the sheep ovary remains obscure. It is conceivable that the pineal has no role in the day to day control of the cycle since pinealectomised sheep appear to cycle normally and become pregnant (C.D. Matthews, personal communication). The pineal gland of sheep is, however, likely to be involved in seasonality of reproduction possibly altering the sensitivity of the hypothalamus to feedback from ovarian steroids.

C. Pineal Function During Pregnancy

1) Introduction

The pineal gland has been implicated in the control of the timing of the endocrine changes around ovulation (Chu et al., 1964, Ying and Greep 1973) and puberty (Kincl and Benagiano, 1967). The pineal is also apparently involved in the sexual differentiation of the rat brain (Hyyppa et al., 1973). It was of interest to investigate the role of the pineal gland in the timing of another important endocrine event, parturition. The sheep was chosen for this study because the size of the developing foetus allows various surgical manipulations to be performed. It undergoes sexual differentiation prenatally (Short, 1975) as opposed to postnatally in the rat and because a great deal is known of the peri-parturient endocrine changes in this species (Liggins et al., 1972). Assessment of pineal function during pregnancy included diurnal rhythm studies of plasma melatonin in ewes and determination of foetal pineal enzyme and melatonin levels. Chronic cannulation of foetal blood vessels were used to follow plasma melatonin levels during normal and adreno corticotrophin induced parturition.

2) Animals

In the acute studies of foetal pineal function, pregnant uteri from sheep of mixed breed (mainly Merino Cross) were obtained within 20 minutes of maternal death from the South Australian Meat Corporation abattoirs or from the Mortlock Experimental Station. Animals were slaughtered between 0900h and 1300h. The weight, crown-rump length and sex of the foetus were noted. The pineal, adrenal and thyroid glands as well as the testes and kidneys were removed and blood was collected by cardiac puncture, transferred into lithium Heparin tubes (125 u/ml) and kept on ice until centrifuged. Pineal glands were placed into coded containers and immediately frozen on solid carbon dioxide. The pineals were weighed and assayed for enzymes within 48 hours. Gestational age of foetuses obtained from the abattoirs was estimated using a nomogram which was derived from sheep of known mating date.

For the chronic experiments, merino crossbred ewes were obtained from the Mortlock Experimental Station and transported to the Queen Elizabeth Hospital Animal House. Ewes were mated at Mortlock with entire rams, fitted with raddling harnesses and crayons. The day of first marking of the ewes was designated day 1 of pregnancy. In the animal house facility the ewes were kept in individual 0.8 m x 1.1 m pens, 7-8 sheep per room. Room lighting temperature and feeding were the same as for the non-pregnant sheep.

3) Diurnal Rhythm of Plasma Melatonin in Pregnant Ewes

A total of 6 pregnant ewes had a double lumen cannula placed into the left jugular vein and blood was sampled for

24 hours. The range of gestational ages was from 90 days up to parturition. Ewe 21 had blood collected for only 10 hours due to a technical failure. Ewe 186 was cannulated on the left side at 120 days and the right jugular at 146 days. Ewe 150 was sampled using configuration B for 3 days.

Figure 19 shows the 24 hour plasma melatonin profiles at various stages during the last trimester of pregnancy. The nocturnal rise of melatonin appears suppressed - only one ewe (Ewe 191) had a high amplitude diurnal rhythm. Figure 20 shows data from a non-pregnant ewe (198) a pregnant ewe 177 and its foetus over a 20 hour period. Samples in this experiment were taken by indwelling cannulae in the jugular vein, every hour during brief exposure to dim red light. The darkness was stimulatory to the non-pregnant ewe, but caused only a modest increase in melatonin levels in the pregnant ewe. The foetus had very low circulating melatonin levels and no discernable rhythm.

4) Foetal Sheep Pineal Gland Function - Enzymes

The hydroxy indole-O-methyltransferase assay of Axelrod, Wurtman and Snyder (1965) was established in adult sheep pineal tissue. Incorporation of label into melatonin was linear with respect to time and was proportional to by N-acetyl serotonin and S-Adenosyl Methionine. This assay was then applied to foetal tissue but because of the scarcity of foetal material, no validation was performed. When sufficient pineal tissue became available, it was established that homogenates incubated for 1 hour with N-acetyl serotonin concentration of 7.6×10^{-4}M

Figure 19

24 hour profiles of melatonin at various stages of sheep pregnancy. The bars represent melatonin levels from 30 minute collection periods using configuration A (except ewe 150, configuration B). Lights were off from 2050h until 0650h.

MATERNAL PLASMA MELATONIN

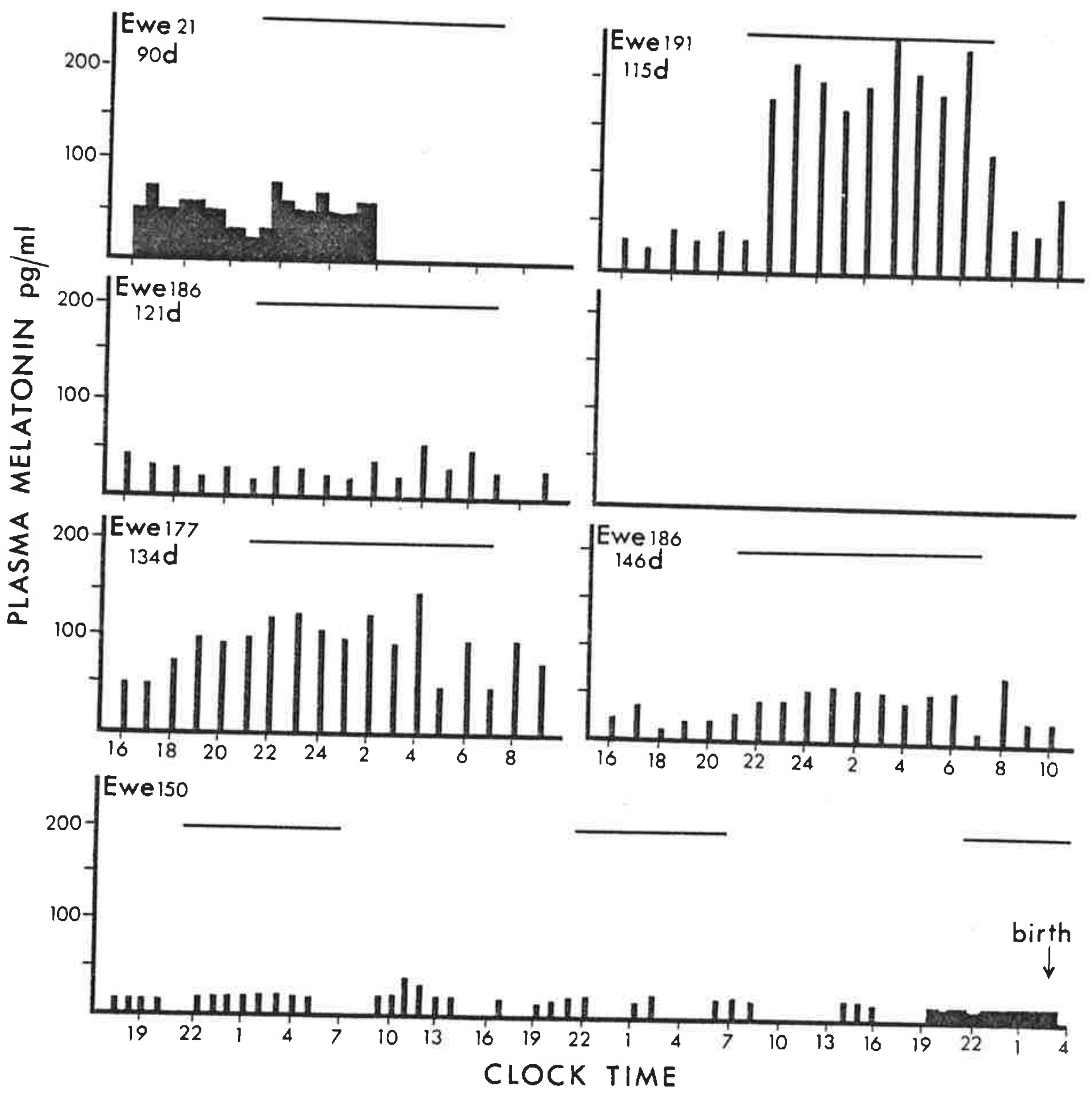
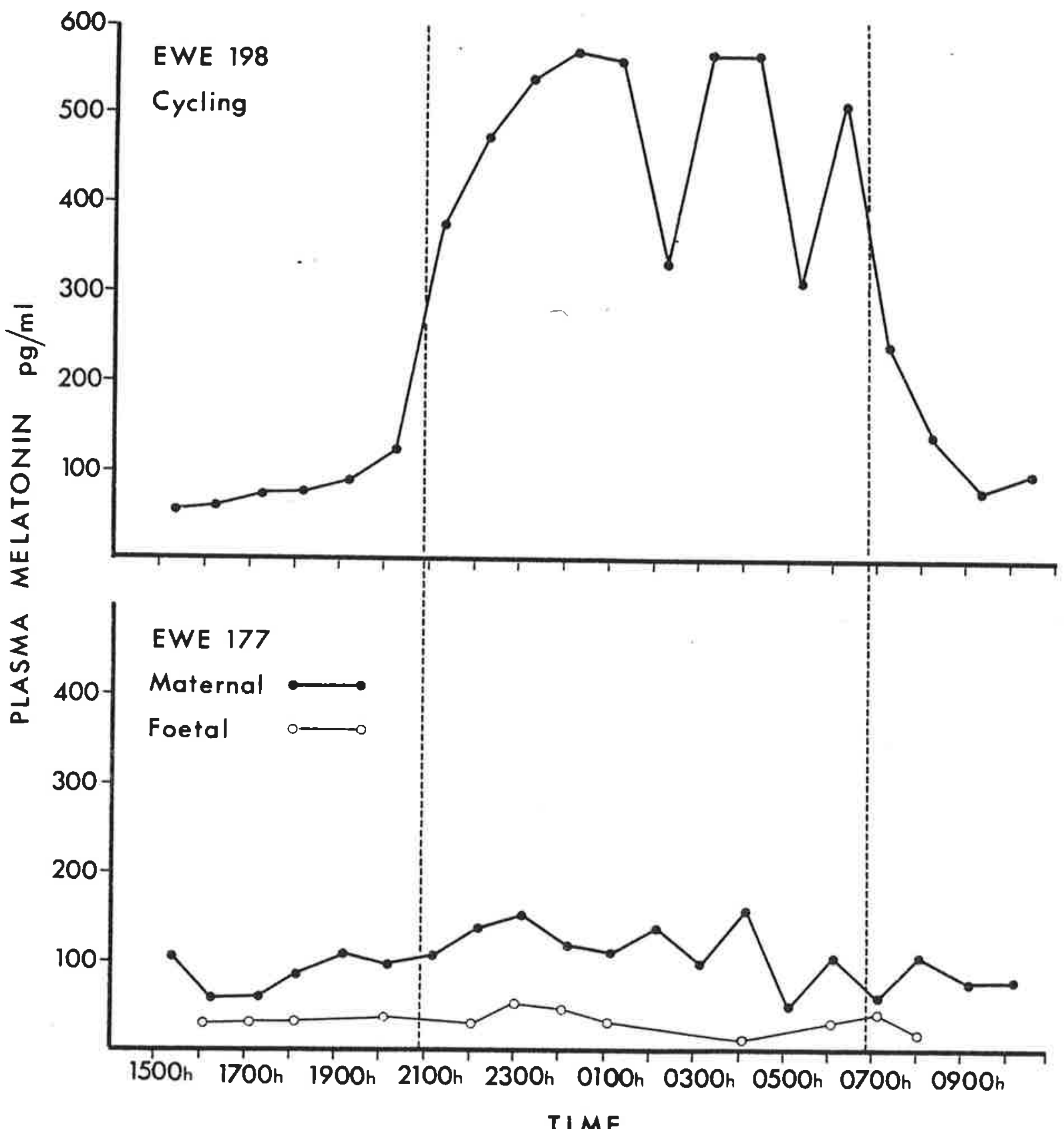


Figure 20

24 hour profile of melatonin in a normal ewe, a pregnant ewe and it's foetus (130d). Samples were taken every hour by indwelling cannulae. The vertical broken line denotes lights out (2050h) and lights on (0650h).


EWE 198
Cycling
600
500
400
300
200
100
PLASMA MELATONIN pg/ml
EWE 177
Maternal
Foetal
400
300
200
100
1500h
1700h
1900h
2100h
2300h
0100h
0300h
0500h
0700h
0900h
TIME

and S-adenosyl methionine 2.3 x 10^{-6}M showed apparent product inhibition. Incubations with ^{3}H-S adenosyl methionine at a concentration of 3 x 10^{-8}M did not show evidence of inhibition. Foetal pineal hydroxy-indole-O-methyltransferase activities thus may be underestimated by up to 40%.

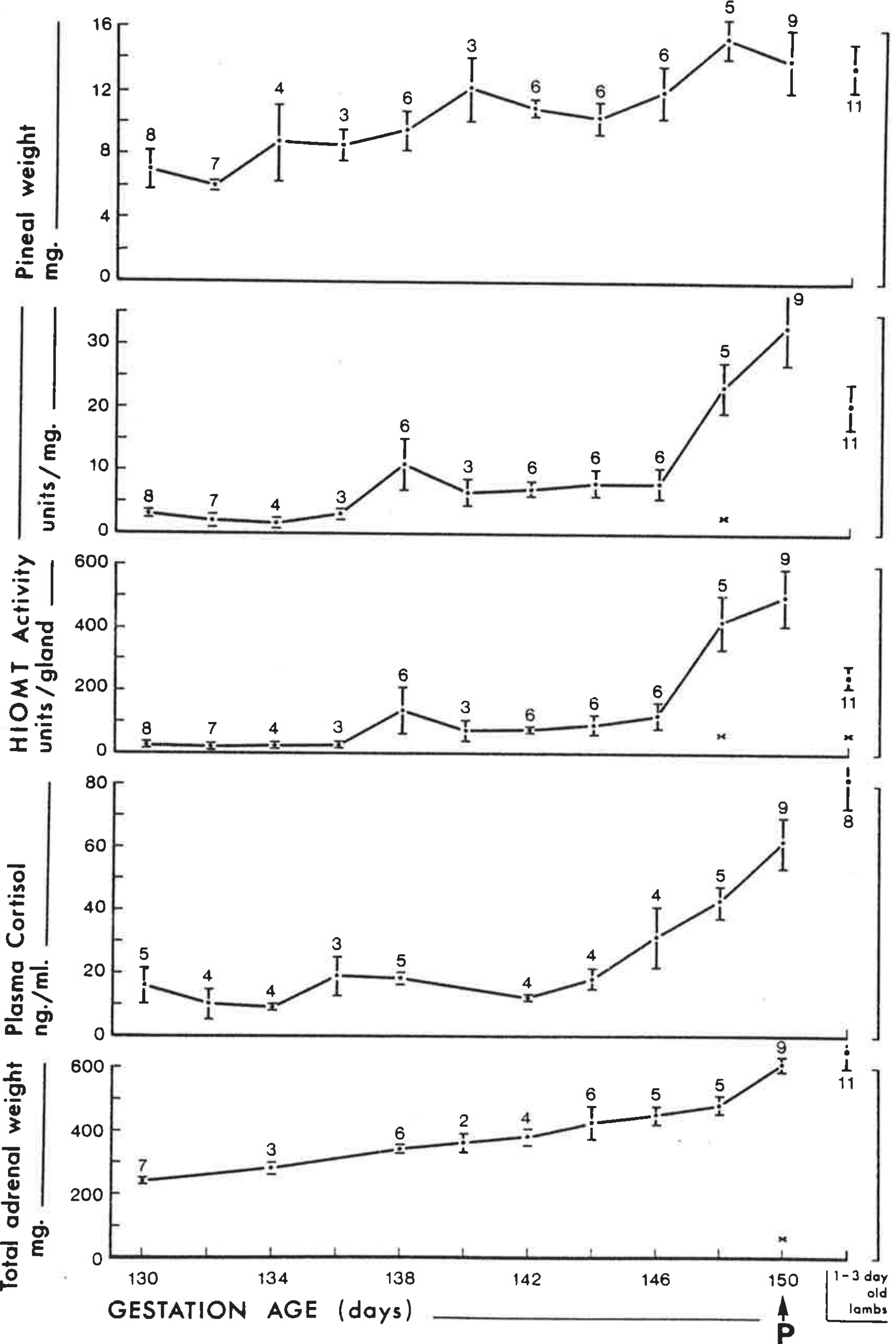
Figure 21 shows the ontogeny of pineal hydroxyindole-O-methyltransferase activity in relation to foetal pineal and adrenal weight and plasma cortisol. Hydroxyindole-O-methyltransferase was detectable (albeit low), in some pineal glands at 100 days gestation when the foetal pineal weighed approximately 1 mg. Low levels of hydroxyindole-O-methyltransferase were maintained until about 4-5 days prior to birth when both hydroxyindole-O-methyltransferase specific activity and total activity increased 4-5 fold. Prior to birth pineal hydroxyindole-O-methyltransferase specific activity is approximately 1/3 non-pregnant adult daytime levels (74.9 $\pm$ 7.8 units/mg n=18). There was a significant decrease ($p < 0.05$) in hydroxyindole-O-methyltransferase total activity in early neonatal pineals. In this series of animals there were significant increases in adrenal weight and in plasma cortisol concentration at the time of the high hydroxyindole-O-methyltransferase activity.

5) Foetal Sheep Pineal and Plasma Melatonin

Figure 22 shows the mean ($\pm$ SEM) values for weight, hydroxyindole-O-methyltransferase activity, monoamine oxidase activity and melatonin content of the pineal gland, the plasma melatonin and cortisol and adrenal weight of the fetal lamb at various

Figure 21

Ontogeny of pineal enzymes in foetal sheep. Values are means ± SEM. Numbers above each bar represent sample size. X denotes significantly different from preceding group ($p < 0.05$). Data was normalised to 2 day intervals. P denotes expected date of birth.



Pineal weight
mg.
units/mg.
HIOMT Activity
units/gland
Plasma Cortisol
ng./ml.
Total adrenal weight
mg.
GESTATION AGE (days)
P
1-3 day old lambs

Figure 22

Ontogeny of pineal enzymes, pineal melatonin and plasma melatonin in foetal sheep. Values are means ± SEM. Numbers above each bar represent sample size. Data were normalised to either 4 day or 2 day groups. The shaded area denotes the lower limits of the melatonin assay. P denotes the expected date of birth.

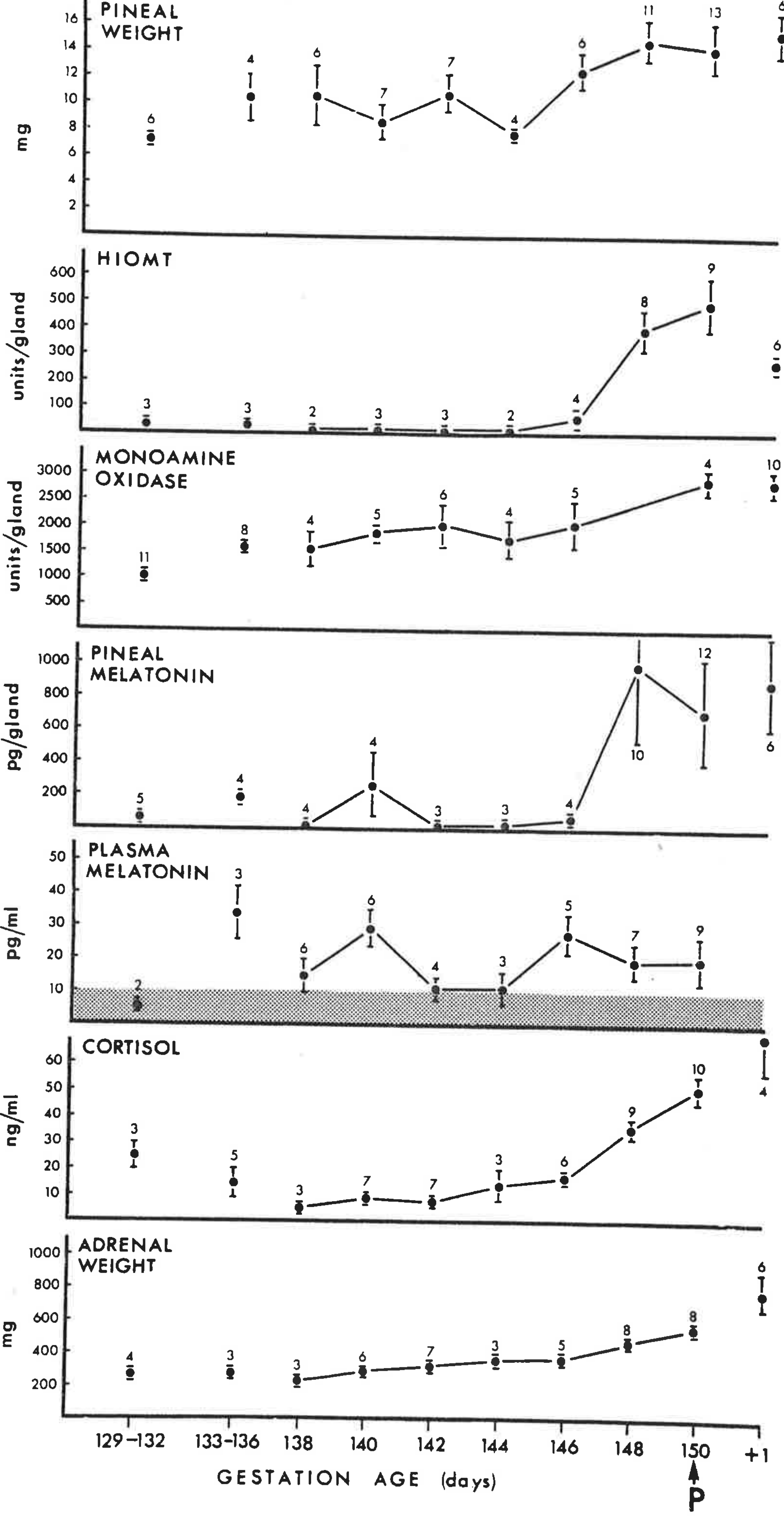
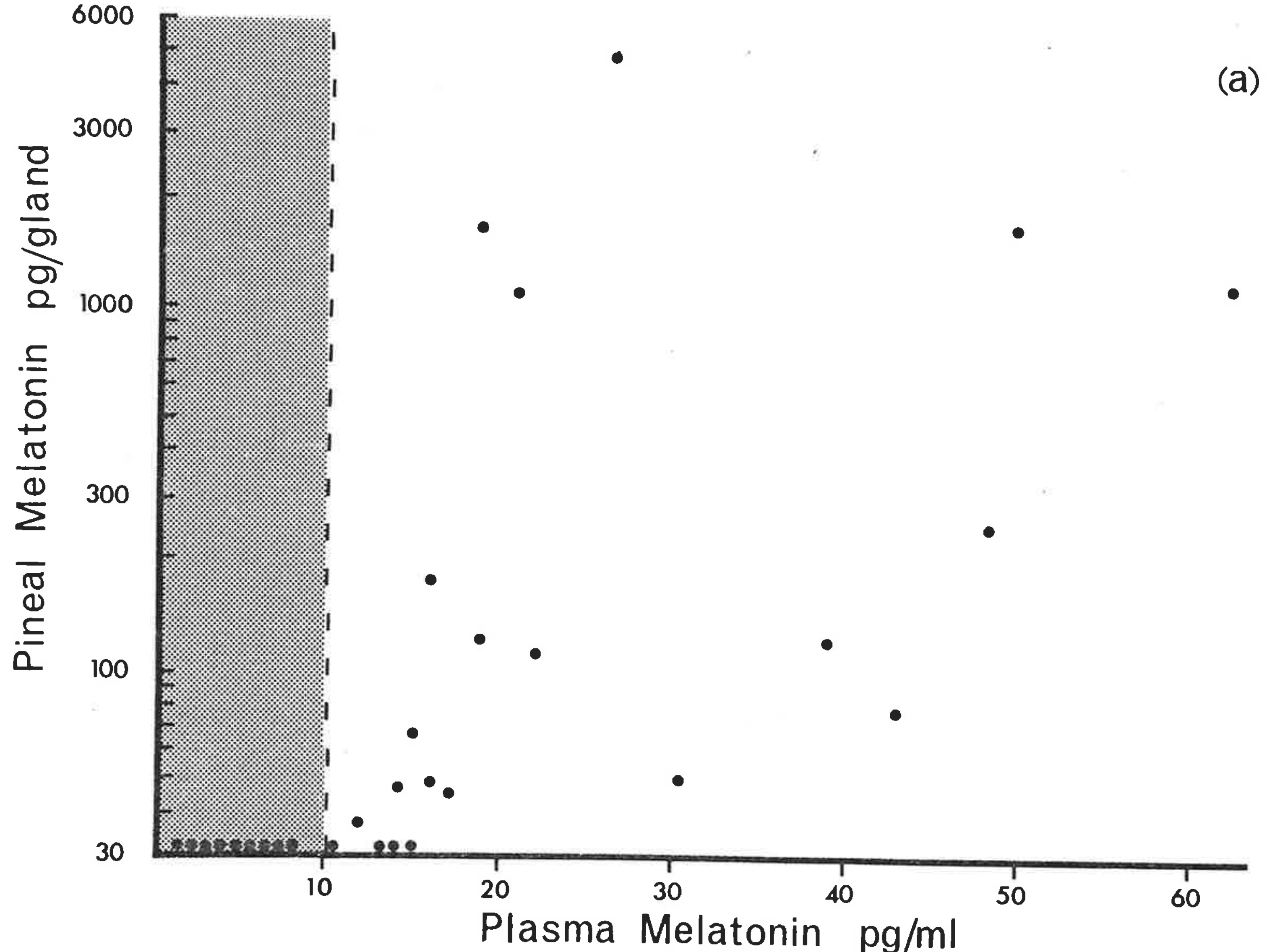

PINEAL WEIGHT
mg
HIOMT
units/gland
MONOAMINE OXIDASE
units/gland
PINEAL MELATONIN
pg/gland
PLASMA MELATONIN
pg/ml
CORTISOL
ng/ml
ADRENAL WEIGHT
mg
129–132
133–136
138
140
142
144
146
148
150
+1
GESTATION AGE (days)
P

Figure 23

Relationship between foetal pineal melatonin and foetal plasma melatonin. The hatched area encloses values not differing significantly from zero.



6000
3000
1000
300
100
30
(a)
Pineal Melatonin pg/gland
10
20
30
40
50
60
Plasma Melatonin pg/ml

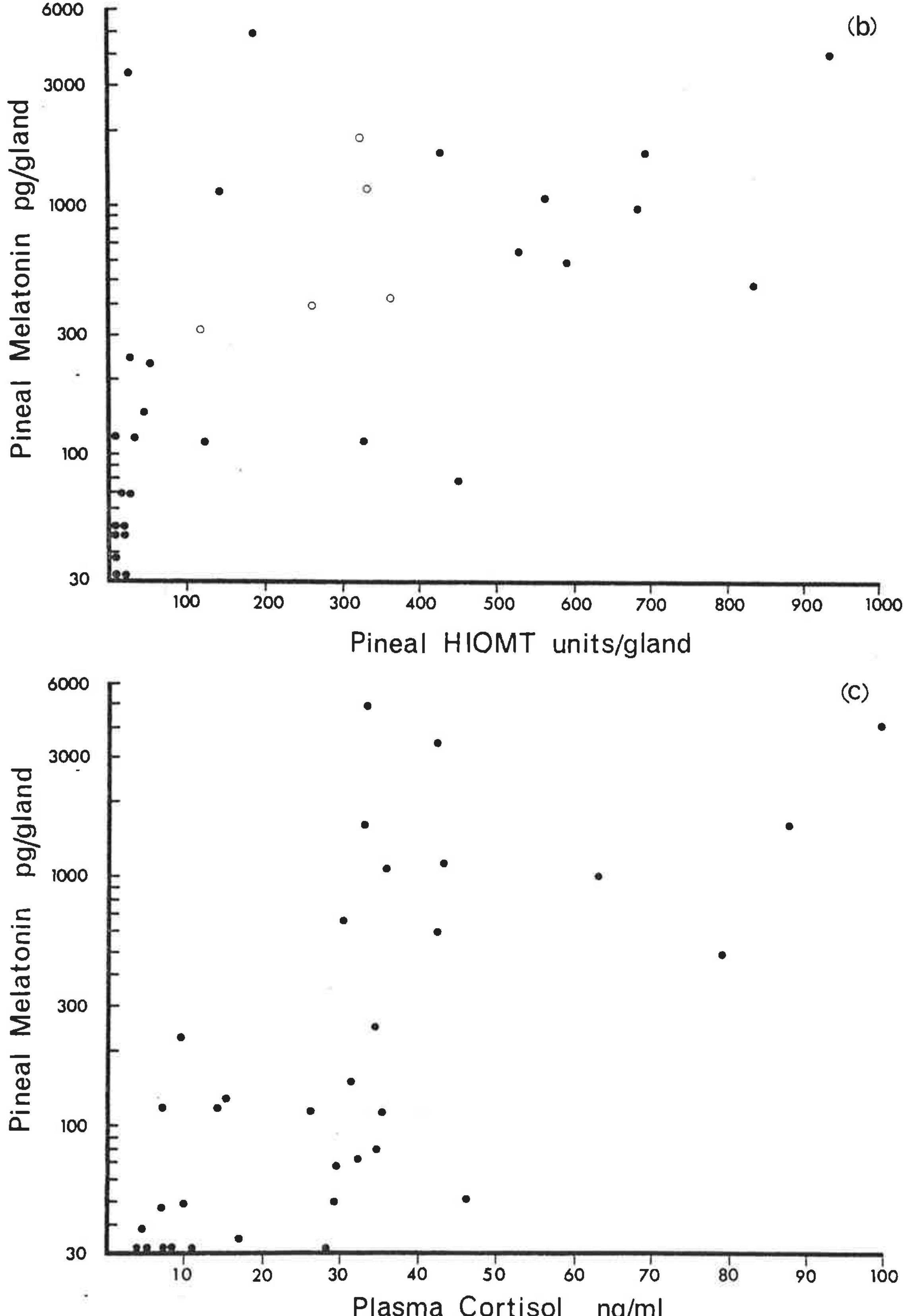
stages of gestation. In this series of animals the rise in hydroxyindole-O-methyltransferase activity which occurred over the last few days of gestation corresponded to a rise in pineal melatonin content. The mean $\pm$ SEM fetal melatonin content was 79 $\pm$ 25 pg per gland for 30 foetuses obtained from days 124-145 and 836 $\pm$ 286 pg per gland for 21 foetus judged to be within 5 days of birth. Neonatal pineal melatonin content was 899 $\pm$ 280 pg per gland (n=5). The positive correlation between pineal hydroxyindole-O-methyltransferase activity and melatonin content is illustrated in figure 23. No direct relationship was apparent between the pineal hydroxyindole-O-methyltransferase activity or melatonin content and the monoamine oxidase activity. The simultaneous occurrence of hydroxyindole-O-methyltransferase activity and monoamine oxidase could provide conditions for methoxy tryptophol production. Incubation of 146-150 day foetal pineals with 7×10^{-5}M hydroxy tryptophol and ^{3}H-S adenosyl methionine (3×10^{-8}M) resulted in no metabolic conversion to methoxy tryptophol.

Figure 22 indicated no apparent relationship between mean plasma melatonin content and gestational age. Figure 24 indicates a correlation between pineal melatonin content and plasma melatonin levels. High pineal melatonin content is associated with high plasma melatonin levels.

The relationship between pineal melatonin and plasma cortisol was studied in 35 foetuses with the results shown in Figure 23. Plasma cortisol values exceeding 25 ng/ml were used to provide an indication that birth was imminent within

Figure 24

Relationships between foetal pineal melatonin and foetal pineal HIOMT and pineal melatonin and plasma cortisol. Open circles are 1-3 day neonatal lambs.


(b)
Pineal Melatonin pg/gland
6000
3000
1000
300
100
30
100
200
300
400
500
600
700
800
900
1000
Pineal HIOMT units/gland
(c)
Pineal Melatonin pg/gland
6000
3000
1000
300
100
30
10
20
30
40
50
60
70
80
90
100
Plasma Cortisol ng/ml

5 days (Liggins et al., 1972). Of the 21 foetuses in which the plasma cortisol levels exceeded this figure, 12 had significantly elevated pineal melatonin content when compared with the foetuses with plasma levels below 25 ng per ml.

6) Foetal Cannulation Procedure

After acclimatisation in the animal house for at least 7 days, pregnant ewes were sedated with intramuscular xylaxine (0.35 mg/kg, "Rompun", Bayer Aust.) intramuscularly. After about 15 minutes they were taken into the operating theatre where lumbosacral epidural anaesthesia was induced with 10 ml 2% xylocaine (Astra Chem., Aust). The abdomen was clipped free of wool and the entire abdomen washed thoroughly with soap and rinsed. Chlorhexidine-cetrimide-alcohol was swabbed over the abdomen and the ewe placed in a wooden cradle and strapped to the operating table.

Strict aseptic procedures were maintained during the following procedures. The lower abdomen of the ewe was opened by a 8 cm mid-line incision and the pregnant uterine horn exposed. The foetus was located by palpation and an incision made in the uterus. For femoral vessel cannulation a hind limb was delivered and the appropriate vessels located by palpation and exposed. Portex polyvinyl tubing No. 2 (o.d. 2mm, i.d. 1mm) was used for femoral arteries and Portex No. 1 (o.d. 1.4 mm, i.d. 0.63 mm) generally used for femoral veins. These cannulae previously sterilised with ethylene oxide were fitted with disposable 18G or 20G needles and a 3 way stopcock and filled with sterile saline solution. The cannulae were placed a few cm

into the vessels and tied in place with 2/0 black braided silk thread and the foetal incision closed with 0 Dexon (American Cyanamid Co., New York). For jugular cannulation the foetal head was delivered and immediately covered with a tight fitting sterile glove to prevent the foetus from swallowing air. The jugular veins were located by palpation and an incision made 2 cm from the point of the jaw where the maxillary vein joins the jugular vein. The left and right maxillary veins were cannulated with Portex No. 2 tubing and passed a few centimetres into the jugular veins. The cannulae were tied in place with 2/0 black braided silk thread. The foetal incision was closed also with 0 Dexon. Both femoral and jugular cannulae were sewn on to the skin at several sites with 2/0 black braided silk thread.

The foetus was then returned to the uterus together with about 15 cm cannula (to allow for foetal movement) and the uterine incision closed with 0 Dexon and oversewn. The peritoneal cavity was closed and oversewn with 0 Dexon. The area was sprayed with Neotracin spray (Bacitrocin, Neomycin, poly-mixin, Ethnor, Aust.) and then the skin incision closed with 0 Dexon. Before returning to its normal pen the ewe was given 800,000 u Benzylpenicillin (intramuscular) and 200,000 u (intravenous) to the foetus. Ewes were fitted with girths and pockets to store the plugged ends of the cannulae.

Immediately after surgery the ewes were returned to their pens, given access to food and water. To minimise foetal infection during manipulations of the cannulae the open ends

of the three way stopcocks were filled with 96% ethanol and capped.

Cannulae were flushed daily with sterile saline containing 250 u Heparin/ml and 1×10^4 u/ml Benzyl penicillin. There were some foetal deaths during the study and these were generally due to intra uterine infection.

7) Foetal Plasma Melatonin During Normal Pregnancy - Chronic Studies

The acute studies of plasma melatonin in the foetal sheep suggested that a rise in circulating melatonin could occur around parturition. Two foetuses were implanted with jugular cannulae and samples were obtained daily at 1000h. One ewe (122) experienced normal labour and delivered a live lamb 155 days after the estimated day of conception. Samples were taken from this animal during labour. The other ewe went into labour but apparently experienced difficulties in expulsion and a fresh dead foetus was delivered. It is probable that the jugular cannulae had become tangled and physically prevented proper expulsion in this case. The delivery of the foetus of ewe 177 was 8 days premature and may have been precipitated by the stress of blood sampling over the 24 hour period 6 days before (Figure 20). The results of plasma melatonin and cortisol analyses are given in Table 13. In the two foetuses studied there were no consistent changes in jugular venous plasma melatonin before, during or after parturition. Generally the plasma melatonin was below 30 pg/ml. Both animals had elevated plasma cortisol at the time of delivery and total adrenal weights of 1070 mg (Ewe 122) and 700 mg (Ewe 177) indicating that

TABLE 13

PLASMA MELATONIN AND CORTISOL
AT PARTURITION

	Ewe 122		Ewe 177	
Days prior to birth	Melatonin pg/ml	Cortisol ng/ml	Melatonin pg/ml	Cortisol ng/ml
- 10d	-	16	38	9
- 19d	-	20	9	11
- 8d	24	30	-	-
- 7d	48	32	13	11
- 6d	25	20	65	-
- 5d	7	38	36	12
- 4d	-	-	22	20
- 3d	12	106	32	39
- 2d	33	72	47	40
- 1d	19	98	21	64
- 3 hours	15	56	-	-
- 1 hour	7	82	-	-
- 10 min	8	76	-	-
+ 10 min	5	40	-	-
Adrenal weight	1070 mg		700 mg	

appropriate hypothalamic signals had been sent to the pituitary-adrenalaxis.

8) Foetal Plasma Melatonin During Adrenocorticotrophin Induced Parturition

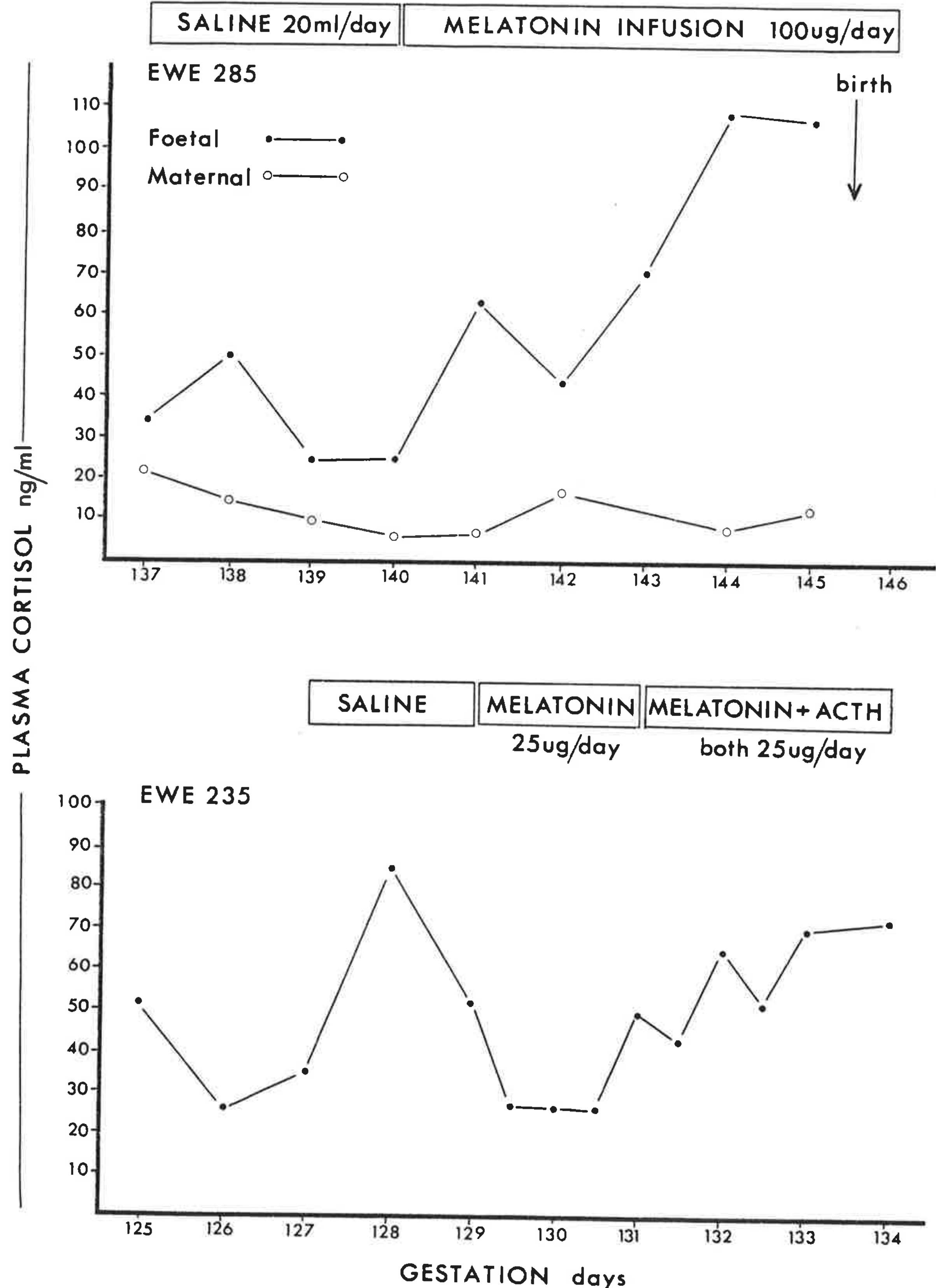
The increase in the melatonin synthesising enzyme activity of the pineal gland and the accumulation of melatonin which occurred at the time of foetal adrenal maturation suggested an inter-play between pineal and hypothalamic systems. Either the initiation of melatonin production (and secretion?) could result in the adrenal changes or the increase in endogenous corticotrophin and/or rise in plasma cortisol could result in the pineal changes.

Preliminary experiments by Dr. C. D. Matthews in this laboratory have indicated that infusions of melatonin into the foetus do not consistently result in early onset of parturition (C.D. Matthews, personal communication). Nor does melatonin seem to inhibit parturition. Infusion of saline (0.8 ml per hour for 3 days) into a 137 day foetus followed by a 5 day infusion of melatonin (4 ug/hour) failed to alter the course of the pregnancy. Throughout the melatonin infusion period, plasma cortisol increased until 145½ days gestation when a lamb was delivered. Maternal cortisol remained below 20 ng/ml (Figure 25). Another foetus received saline infusion for 2 days (0.8 ml/hour) followed by 2 days melatonin infusion (1 ug/hour) and 3 days melatonin (1 ug/hour) plus corticotrophin (Synacthen, CIBA-GEIGY, 1 ug/hour). At least at this dose level, melatonin was not able to prevent the cortisol increase during the corticotrophin infusion or alter the adrenal weight

Figure 25

a) Cortisol response to a melatonin infusion into a 140 day sheep foetus. Saline was infused from day 137 - day 140 while melatonin was infused from day 140 until birth.

b) Cortisol response to a melatonin and synacthen (ACTH) infusion into a 131 day sheep foetus. Saline was infused from day 127-129, melatonin 129-131 and melatonin +ACTH 131 - 134 when the experiment was terminated.


SALINE 20ml/day
MELATONIN INFUSION 100ug/day
EWE 285
birth
Foetal
Maternal
110
100
90
80
70
60
50
40
30
20
10
137
138
139
140
141
142
143
144
145
146
PLASMA CORTISOL ng/ml
SALINE
MELATONIN
25ug/day
MELATONIN+ACTH
both 25ug/day
EWE 235
100
90
80
70
60
50
40
30
20
10
125
126
127
128
129
130
131
132
133
134
GESTATION days

response (1146 mg at autopsy 134 days).

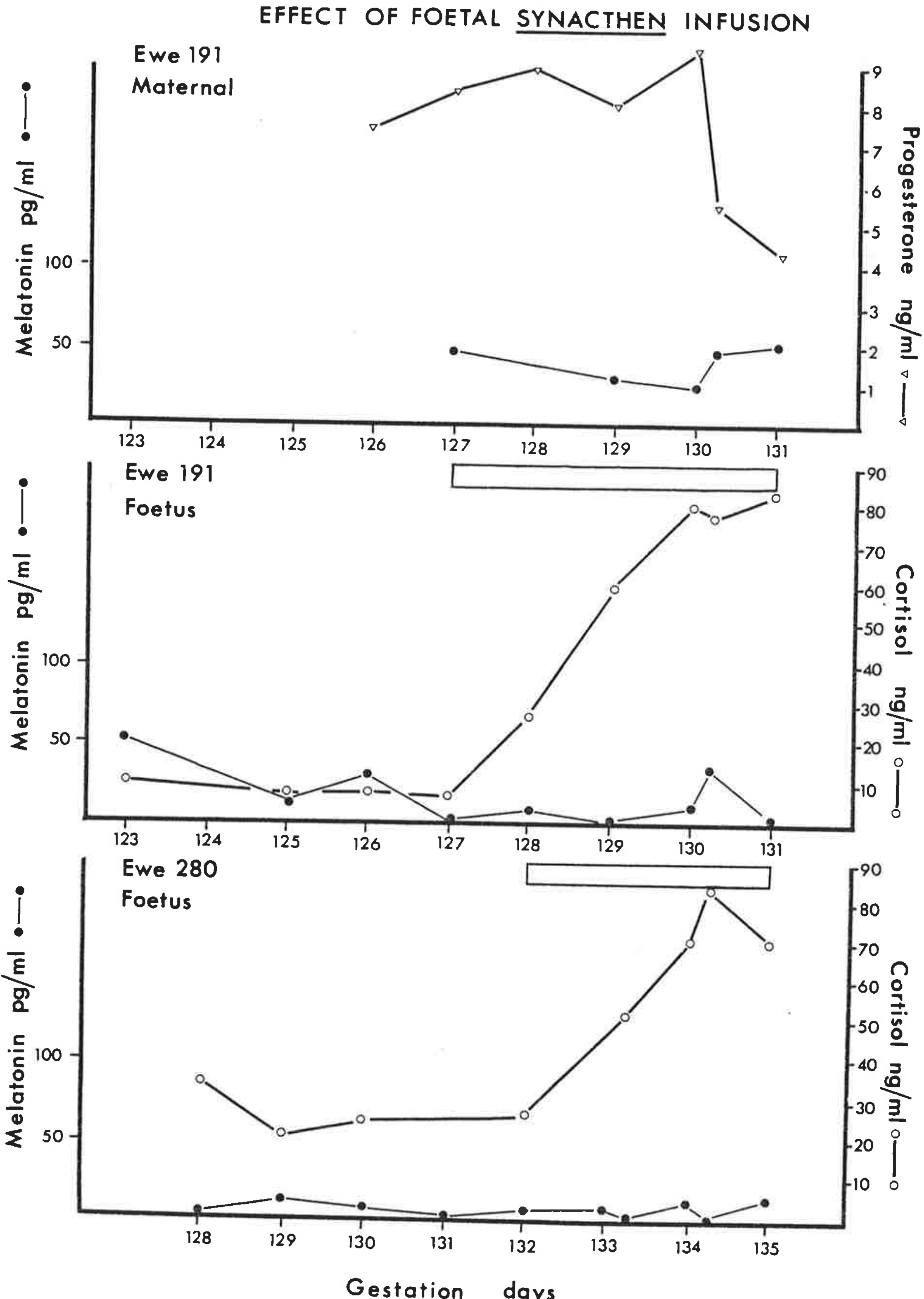
To test whether the pineal gland changes were caused by increased pituitary-adrenal secretions, synthetic corticotrophin was infused into 4 foetuses and plasma samples assayed for both melatonin and cortisol. The four foetuses (122-125 days gestation) had femoral artery and vein cannulae implanted, and after a recovery period of 4-5 days, were infused with synacthen (1 ug/hour) for 3-4 days. Figure 26 shows the foetal cortisol and melatonin concentrations before and during the infusion period in 2 of the foetuses. There was a stimulation of cortisol production in these foetuses comparable to that seen in normal parturition. Adrenal weights at autopsy in these two animals were 1300 mg and 863 mg, indicating that considerable hypertrophy had occurred. Plasma melatonin levels were low throughout the experiments. In one case (Ewe 191) maternal blood was sampled and as indicated in Figure 26, plasma progesterone decreased during the infusion while melatonin levels remained low. Plasma oestradiol (maternal) in this animal increased during the last day of infusion from 50 pg/ml to 260 pg/ml.

When the foetal pineal glands were assayed for hydroxyindole-O-methyltransferase activity, the foetus of ewe 191 had 4.8 units/mg (25 units/gland) while the foetus of ewe 280 contained 10.6 units/mg (123 units/gland). These activities are within the range obtained from unoperated foetuses of the same gestational age. Pineals were not assayed for melatonin content.

Of the two other foetuses cannulated, one lamb was born

Figure 26

Melatonin and cortisol responses to synacthen infusion into foetal sheep. The period of infusion is denoted by the open boxes.


EFFECT OF FOETAL SYNACTHEN INFUSION
Ewe 191
Maternal
Melatonin pg/ml
Progesterone ng/ml
Ewe 191
Foetus
Melatonin pg/ml
Cortisol ng/ml
Ewe 280
Foetus
Melatonin pg/ml
Cortisol ng/ml
Gestation days

alive after 3 days infusion, while the other died *in utero* after 4 days infusion. The gestational age for both foetuses at the end of the experiment was 133 days. Both foetuses showed plasma cortisol increases during the infusion period but melatonin levels were always less than 20 pg/ml. The hydroxyindole-O-methyltransferase of the sacrificed lamb (12h) was 12.5 units/mg (121 units/gland) which was not grossly different from normal lambs of this age. The adrenal weight was 2023 mg.

9) Maternal-Foetal and Foetal-Maternal Transfer of Melatonin

To test whether melatonin of maternal origin could transfer to the foetal circulation and possibly affect parturition, the following experiment was performed. A pregnant ewe was operated 131 days after mating and foetal femoral artery and bladder cannulae (B. Pudney, 1977) implanted. After a 4 day recovery period, two jugular vein cannulae were implanted in the ewe and a urethral catheter inserted. ^{3}H-melatonin was infused at a rate of 6.7 uCi/minute for 30 minutes into the right jugular. Blood (left jugular) and urine were sampled at various times during and after infusion of melatonin from both the ewe and the foetus. Plasma and urine were stored at -10°C.

Plasma and urine were treated with equal volumes of borate buffer pH 10 and extracted into chloroform. The chloroform extracts were evaporated under a stream of nitrogen. The dry residue and the aqueous fractions were assayed for radioactivity after addition of scintillants. Toluene based scintillant was used for the evaporated chloroform extract while commercial PCS solubiliser was used for the aqueous samples. The results,

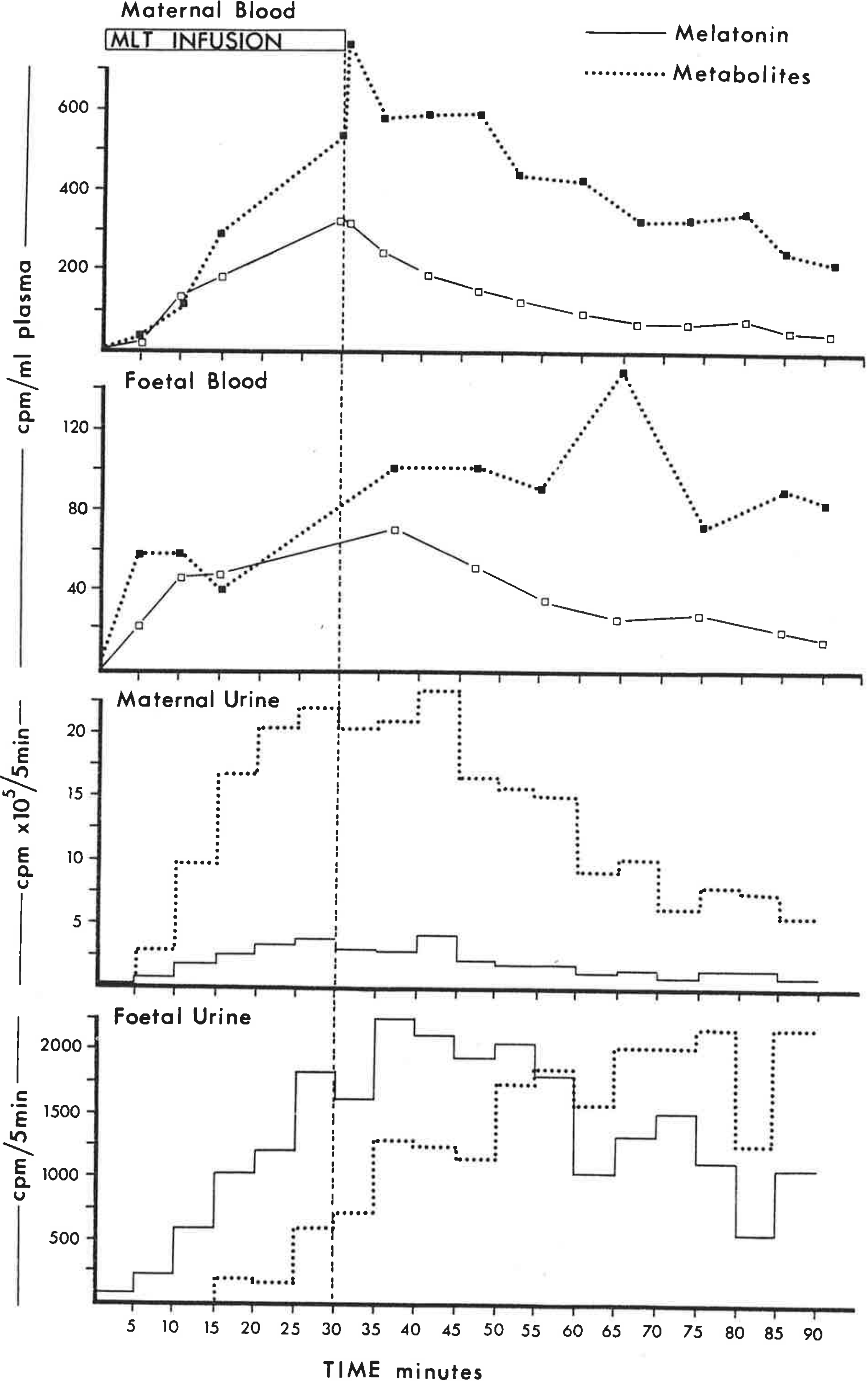
corrected for quenching and volume are graphed in Figure 27. The chloroform extractable radioactivity is designated melatonin since the major metabolite of melatonin, 6-hydroxy melatonin is not extracted into chloroform at pH 10. The unextracted radioactivity is presumed to be sulphate or glucuronide conjugates of 6-hydroxy melatonin as well as free 6-hydroxy melatonin.

^{3}H-melatonin content of maternal blood increased throughout the infusion period and did not reach a steady state. When the infusion was terminated ^{3}H-melatonin in plasma decreased steadily with an apparent half-life of 17 minutes. At all times following the cessation of the infusion, the content of metabolites of melatonin were at least twice as high as melatonin itself. ^{3}H-melatonin also appeared in the foetal circulation albeit in lower concentrations than the ewe and metabolised melatonin content was higher than unmetabolised hormone. The appearance of peak levels of metabolised melatonin in the foetal circulation appeared to be delayed.

^{3}H-melatonin in maternal urine increased throughout the infusion period, peaking at the time the infusion ceased. Urinary metabolites also peaked at this time, and then declined with an apparent half-life of about 30 minutes. Foetal urinary ^{3}H-melatonin excretion increased steadily for 40 minutes, plateaued and then 25 minutes after cessation of the infusion, the levels began to decrease. Foetal urinary metabolites were delayed in their appearance and had reached a plateau by 60 minutes after the infusion ceased.

Figure 27

Maternal - foetal transfer of ^{3}H-melatonin. ^{3}H-melatonin was infused for 30 minutes and samples taken during and after this procedure. The vertical broken line indicates the end of the infusion period. Urinary results are plotted as integrated concentrations.



Maternal Blood
MLT INFUSION
Melatonin
Metabolites
600
400
200
cpm/ml plasma
Foetal Blood
120
80
40
Maternal Urine
20
15
10
5
cpm x10⁵/5min
Foetal Urine
2000
1500
1000
500
cpm/5min
5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90
TIME minutes

These results indicate that exogenously administered melatonin can cross from the mother to the foetus. The results also indicate that either the foetus is slow to metabolise melatonin or there is a slow transfer of metabolites of melatonin across the placenta.

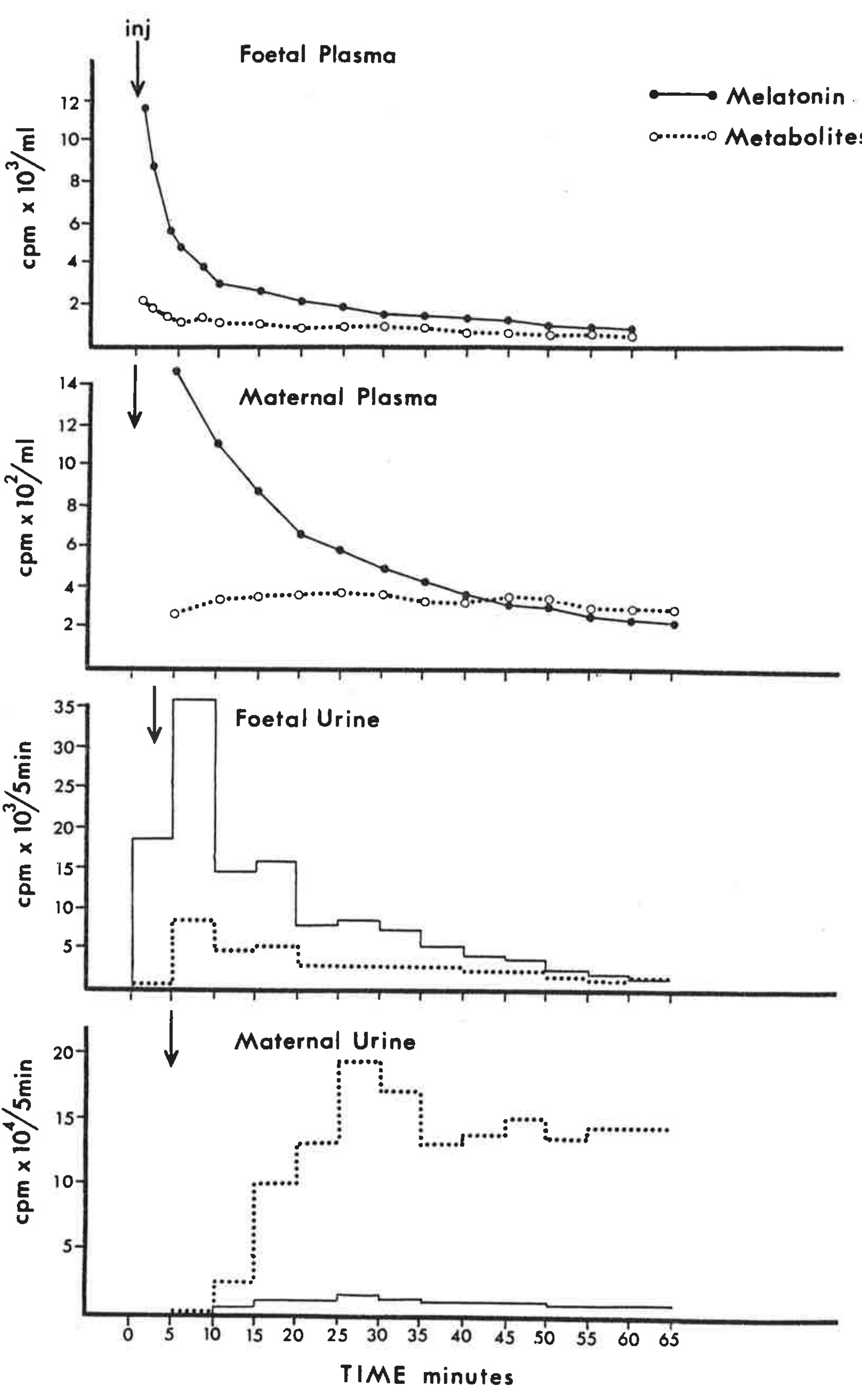
In a second experiment a 140 day foetus was cannulated acutely via the left and right jugular veins and bladder. The maternal ewe had a cannula placed in a uterine vein and a urethral catheter inserted. The exposed foetus was kept warm and moist for the duration of the experiment using wet towels (40 minutes after injection of the melatonin urinary output steadily decreased, indicating either compensation for fluid loss or the effects of stress). 50 uCi ^{3}H-melatonin was rapidly injected into the right jugular vein of the foetus and chased with saline. At various time intervals, foetal (left jugular) and maternal blood were collected and foetal and maternal urine obtained over 5 minute intervals. Figure 28 shows the extracted ^{3}H-melatonin and metabolite contents in the various fluids.

Injected melatonin has a very short half-life in the foetus (of the order of a few minutes). Metabolites of the injected melatonin were highest 1 minute after injection but declined very slowly over the next 60 minutes. ^{3}H-melatonin content was highest in uterine vein blood in the first sample (5 minutes post injection) and declined over the next 60 minutes with an apparent half-life of 15 minutes. Metabolites remained relatively constant for the duration of the experiment.

Foetal ^{3}H-melatonin was highest in urine 5 minutes after injection and then declined. Metabolites were considerably

Figure 28

Foetal-maternal transfer of ^{3}H-melatonin. ^{3}H-melatonin was injected into the foetus at time zero and samples taken from various sites. The arrows represent the time of injection after adjustments were made for dead space in the cannulae. Urinary results are plotted as integrated concentrations.



FOETAL-MATERNAL TRANSFER OF MELATONIN
inj
Foetal Plasma
Melatonin
Metabolites
cpm x 10³/ml
12
10
8
6
4
2
Maternal Plasma
cpm x 10²/ml
14
12
10
8
6
4
2
Foetal Urine
cpm x 10³/5min
35
30
25
20
15
10
5
Maternal Urine
cpm x 10⁴/5min
20
15
10
5
0 5 10 15 20 25 30 35 40 45 50 55 60 65
TIME minutes

lower than melatonin but exhibited a similar pattern. Maternal urine contained high levels of metabolised melatonin with a plateau occurring at about 30 minutes after injection. Unmetabolised melatonin represented about 5% of the total counts in urine during this period.

These results indicate that melatonin can cross from the foetus to the ewe extremely rapidly and also indicate that the foetus has a low capacity for metabolising and excreting exogenously administered melatonin.

10) Discussion

> "We may be said to be in the dark as to why the uterus after remaining for months subject only to futile contractions, is suddenly thrown into powerful and efficient action, and within it may be a few hours or even less gets rid of the burden which it has borne with such tolerance for so long a time." Foster (1891)

A number of hypotheses have been advanced in recent years purporting to explain the induction of parturition in the sheep. One of the more popular hypotheses is directed towards the removal of a progesterone block of myometrial activity which precipitates the birth. Others include changes in progesterone: oestrogen ratios, increases in uterine volume, rises in oestrogen secretion, release of oxytocin from the pituitary and local uterine factors such as prostaglandins and catecholamines. There is little doubt that these particular phenomena are of special significance in the trigger of delivery in the sheep. The earliest endocrine changes which are possibly involved in the initiation of parturition occur in the foetal

lamb itself. Hopkins and Thorburn (1971) recorded decreases in foetal plasma thyroxine 5-10 days prior to parturition. Indeed the actual trigger of parturition may originate from the foetus, since Liggins, Kennedy and Holm (1967) demonstrated that an intact foetal pituitary gland was required for parturition to occur. Since that report, an hypothesis has been developed (See Liggins et al., 1972) that an activation of the foetal hypothalamus is the trigger for the beginning of the parturition process in sheep.

The sheep foetus is involved in a remarkable series of endocrine events during the periparturient period. The effect of foetal hypothalamic activation is to cause an increase in foetal pituitary adrenocorticotrophin (ACTH) secretion. The ACTH then acts on the foetal adrenal to stimulate cortisol production and secretion which in turn acts upon the placenta to promote oestrogen biosynthesis. Certain aspects of this hypothesis have not survived rigorous testing. Rees et al., (1975) and Jones et al., (1977a) for instance have been unable to detect a rise in foetal plasma ACTH before the rise in plasma cortisol. There is, however, a change in sensitivity in the adrenal to the influence of ACTH near term, an effect which occurs independently of ACTH (Jones et al., 1977b). Other hormones may be involved in this change in sensitivity.

The result of the increased adrenal activity is a progressive rise in plasma cortisol which promotes placental oestrogen production (Flint et al., 1976). During this period maternal plasma progesterone levels fall (Basset and Thorburn, 1969).

Within 72 hours of labour a number of dramatic changes occur in hormone levels of both the foetal and maternal circulations. Foetal plasma androgens decrease (Strott et al., 1974) while maternal and foetal oestradiol 17β increase many fold (Thorburn et al., 1972). Just prior to or at birth, foetal and maternal plasma noradrenaline are very elevated (Phillipo et al., 1975) and may be causally related to the final late burst of secretion of prostaglandins F2α (Phillipo et al., 1976).

How does the foetal sheep pineal gland fit into this scheme? Is it possible that the foetal hypothalamus is stimulated by pineal melatonin, or has it's sensitivity to feedback by cortisol altered and this allows parturition to proceed? Or is melatonin acting at the level of the adrenal and changing it's sensitivity to ACTH?

The observation that the foetal sheep pineal gland has the enzyme necessary for synthesis of melatonin from at least day 120 gestation and the 4-5 fold increase in this activity 4-5 days prior to parturition do suggest a role of the pineal in birth. Analysis of the pineal content of melatonin in a similar series of animals confirmed that melatonin was indeed synthesised and accumulated during the last five days of gestation. Further experiments designed to determine whether an increase in plasma melatonin occurred during this period were inconclusive.

A number of reasons for this lack of correlation could be advanced. Firstly the experiment in which ^{3}H-melatonin was injected into the foetus demonstrated a very fast foeto-maternal transfer. Thus the melatonin may not have reached detect-

able levels in the periphery. The use of jugular cannulae (in which jugular blood flow should have been unaltered) should have enabled increased melatonin secretion to be detected since the site of sampling was close to the site of production. No increase during normal induction of labour was observed.

Secondly, the sampling times may have been inappropriate for detecting the presumed release of melatonin. Samples were taken daily except during labour itself and so it is possible that a surge in plasma melatonin may have occurred outside the sampling times. While there is no evidence to suggest a dirunal rhythm in foetal melatonin secretion (at least at 130 days) the initiation of the birth process may have occurred one night. One could speculate that the ewe participated in this event. Maternal plasma melatonin levels appeared to be lower during late pregnancy and this lower secretion may have contributed to an increase in foetal capacity to synthesise (and secrete) melatonin.

What is more likely on the basis of the above experimental results is that the pineal gland in the foetus does not receive an adequate stimulatory signal to secrete melatonin during intrauterine life. The changes observed in pineal synthesis and storage of melatonin occur during a period of positive interactions of endocrine glands and hormones which were in part initiated by the hypothalamus and pituitary gland. Melatonin is usually associated with an inhibition of pituitary function (Reiter, 1974a). Nor is there a rise in melatonin once labour is established even when hormones which are stimulatory in other species such as oestradiol (Cardinali et al.,

1974a) and noradrenaline (Klein and Weller, 1970) are circulating in high concentrations. The failure of melatonin infusions to initiate or delay natural parturition or to attenuate synacthen induced labour also suggests a minimal involvement in the birth process. Thus on the basis of these experiments it would appear that the pineal gland enzymes of the foetus are probably induced by the hormones of pregnancy in preparation for extra-uterine synthesis and secretion of melatonin.

There have been relatively few studies performed in other species which could help to clarify the role of the pineal in pregnancy. In rats, significant amounts of hydroxyindole-O-methyltransferase activity appear in pineal glands 10 days after birth (Zweig and Snyder, 1968, Klein and Lines, 1969). Serotonin: N-acetyltransferase activity is apparent at about 4 days prior to birth and a diurnal rhythm is established about 4 days after birth (Ellison et al., 1972). A logical conclusion then, is that melatonin production and secretion does not occur until around day 10 of post natal life. Thus while the foetal rat pineal may not be involved in parturition it is sensitive to the influence of steroid hormones until day 10, probably playing a role in sexual differentiation of the brain (Hyyppa et al., 1973).

Melatonin injections into pregnant rats have no effect on the outcome of the pregnancy (Tigchelaar and Nalbandov, 1975). Furthermore, pinealectomy of the foetal rat has resulted in changes in intestinal epithelium but no other discernable effects (Owman, 1963). Dale et al., (1976) reported premature neonatal testis development following pineal coagulation in

utero but their experimental design precluded any investigations into the birth process. Clearly it would be desirable to test the effect of pinealectomy in foetal sheep on the outcome of the pregnancy. To date numerous technical difficulties have prevented successful foetal pinealectomies (C.D. Matthews, personal communication).

In conclusion, the foetal sheep pineal gland has an increased capacity to synthesise and store melatonin during the last few days of intrauterine life. There is, however, no evidence to indicate a profound rise in secretion of melatonin during this period. Thus it is unlikely that the pineal gland of the foetus plays a role in the initiation of parturition. The apparent decrease in circulating maternal melatonin concentrations requires further investigation in order to determine whether this phenomenon precipitates the birth processes.

CHAPTER 5

EFFECT OF PINEALECTOMY ON CIRCULATING MELATONIN IN SHEEP

1) Introduction

The pineal gland has been assumed to be the sole or major source of melatonin since the discovery of the hormone. Surgical removal of the gland should remove all melatonin from the circulation and bring about physiological changes in the animal which should be reversed by melatonin administration. This study was instituted to determine whether pinealectomy does remove melatonin from blood.

2) Operative Procedure

The pinealectomy technique of Roche and Dzuik (1969) as modified by Drs. J. Obst and E. Dunstan (unpublished results) was used. I am grateful to Dr. C. D. Matthews of the Department of Obstetrics and Gynaecology, The Queen Elizabeth Hospital, for making the animals available for this study.

1 - 4 year old sheep (Merino Cross) were obtained from the Mortlock Experimental Station and acclimatised in the animal house for 1 week. Anaesthesia was induced with intravenous sodium pentobarbitone (17 mg/kg, Sagatal, May and Baker, Vic, Aust.). The head wool was clipped and the scalp washed with a chlorhexidine-cetrimide-alcohol solution.

The sheep was restrained by leather straps in the kneeling position with the head over the end of the operating table. The head was held in position by adjustable lateral clamps and a mouth bar between the jaws. A 7 cm scalp incision was made

0.5 cm lateral to the midline and the scalp undercut to free it from the periosteum. The periosteum was incised in the midline and dissected from the bone in an intact layer. A circular trephine (2.5 cm diameter) was centralised on the left parietal bone so that the medial edge was 0.7 to 1 cm from the midline. Care was taken not to damage the midline longitudinal sinus during trephining. When possible the bone flap was hinged to facilitate replacement.

The dura was incised longitudinally avoiding the superficial brain blood vessels. A flat smooth metal spatula was used to depress the occipital region of the cerebral hemisphere to gain access to the midline. The connecting inter-cerebral vein was often ruptured during this procedure and suction was required. A rounded concave retractor was then used to separate the hemispheres so that the cerebellum could be visualised. Exposure continued anteriorly until the left superior colliculus was observed and finally until the pineal was visualised between the left and right superior colliculi. The pineal was grasped with forceps and the gland removed. The pineal was always checked for any obvious tearing. Sham pinealectomy involved visualisation of the gland but the pineal was left _in situ_.

Following removal of the pineal the area was sucked clear of blood until active bleeding ceased. The dural flap was replaced but not sutured and the bone flap positioned. The periosteum and scalp were sutured with chromic 1 catgut.

Temporary loss of vision was often apparent due to minor compression of the occipital cortical area. Some animals

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demonstrated a degree of extensor neck muscle spasm due possibly to subdural haematoma tracking down the spinal canal. Both effects were usually evident for 48-72 hours post surgery.

Most of the animals used in this current study have had the completeness of pinealectomy checked by macroscopic examination of the pineal recess and in some cases histologically by serial sections. No pineal remnants were found.

3) Plasma Melatonin Following Pinealectomy in Rams and Ewes.

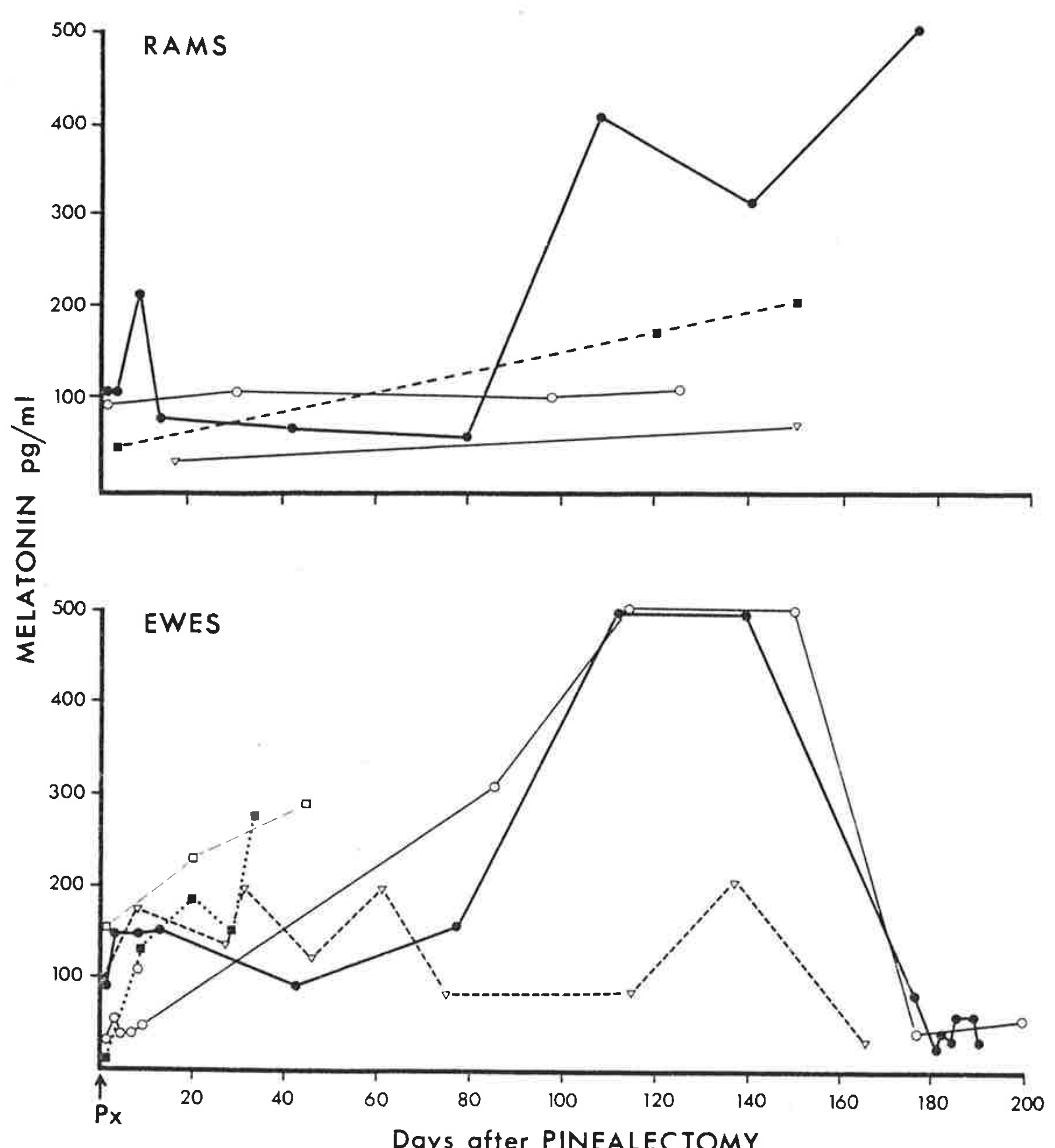
6 rams and 6 ewes which had been pinealectomised in the animal house facility had blood samples taken during daylight at irregular intervals after operation. Following good post-operative recovery (usually 14 days) the sheep were returned to Mortlock Experimental Station. Blood samples were taken every month under natural conditions. Figure 29 indicates that melatonin as measured by radioimmunoassay was present in all sheep following pineal removal. Levels of melatonin ranging from 200-500 pg/ml were common. The fluctuations in plasma melatonin could not be consistently associated with the time of year or any other parameter such as sex, age at pinealectomy or reproductive state.

4) Diurnal Rhythm of Plasma Melatonin Following Pinealectomy

Four pinealectomised ewes were brought to the animal house for intensive blood sampling over a 24 hour period using the automated sampling procedure (configuration A). All ewes were acclimatised in the animal house for at least 14 days. Two ewes (022 and 288) had been pinealectomised 3 months earlier

Figure 29

Plasma melatonin concentrations in pinealectomised rams and ewes. The data is of individual animals normalised to the day of pinealectomy (Px). Samples were taken during daylight.


EFFECT of PINEALECTOMY on DAYTIME LEVELS of
MELATONIN in RAMS and EWES
RAMS
EWES
MELATONIN pg/ml
500
400
300
200
100
Px
20
40
60
80
100
120
140
160
180
200
Days after PINEALECTOMY

and the others 6 months prior to study. One sham operated ewe 018 was also studied. The 24 hour plasma melatonin profile for the pinealectomised ewes is shown in figure 30. The pinealectomised ewes elicited no diurnal rhythm in plasma melatonin. In 3 out of the 4 animals, however, melatonin was present in concentrations comparable to, or above normal night time concentrations. Plasma cortisol levels in these four sheep were comparable to intact sheep sampled under similar conditions (figure 31). The mean daytime levels of melatonin in the sham operated ewe were 57 $\pm$ 7.3 pg/ml compared with mean nighttime levels of 148 $\pm$ 20 pg/ml. This difference was significant ($p < 0.05$).

5) Plasma Melatonin Levels in Pregnant Sheep After Pinealectomy

4 sham operated and 4 pinealectomised pregnant ewes were brought to the animal house for daily blood sampling during the last 14 days of pregnancy. Samples were obtained by venepuncture at 0930h and 1530h. Table 14 shows the individual plasma melatonin levels in these groups around the time of parturition. Melatonin was consistently low in both groups throughout the period of study.

6) Discussion

Axelrod and Weissbach (1960) identified an enzyme in the pineal gland of monkey, cow and cat which synthesised melatonin from N-acetyl serotonin. It was reported that no enzyme activity was present in brain, in areas surrounding the pineal gland (habenula, fornix, stria medullaris), liver, kidney, heart, lung, adrenal gland, pancreas, salivary gland or skin.

Figure 30

24 hour profiles of plasma melatonin in four pinealectomised ewes. The open boxes represent melatonin concentrations from a 30 minute collection period. The vertical broken line indicates lights out (2050h) and lights on (0650h).

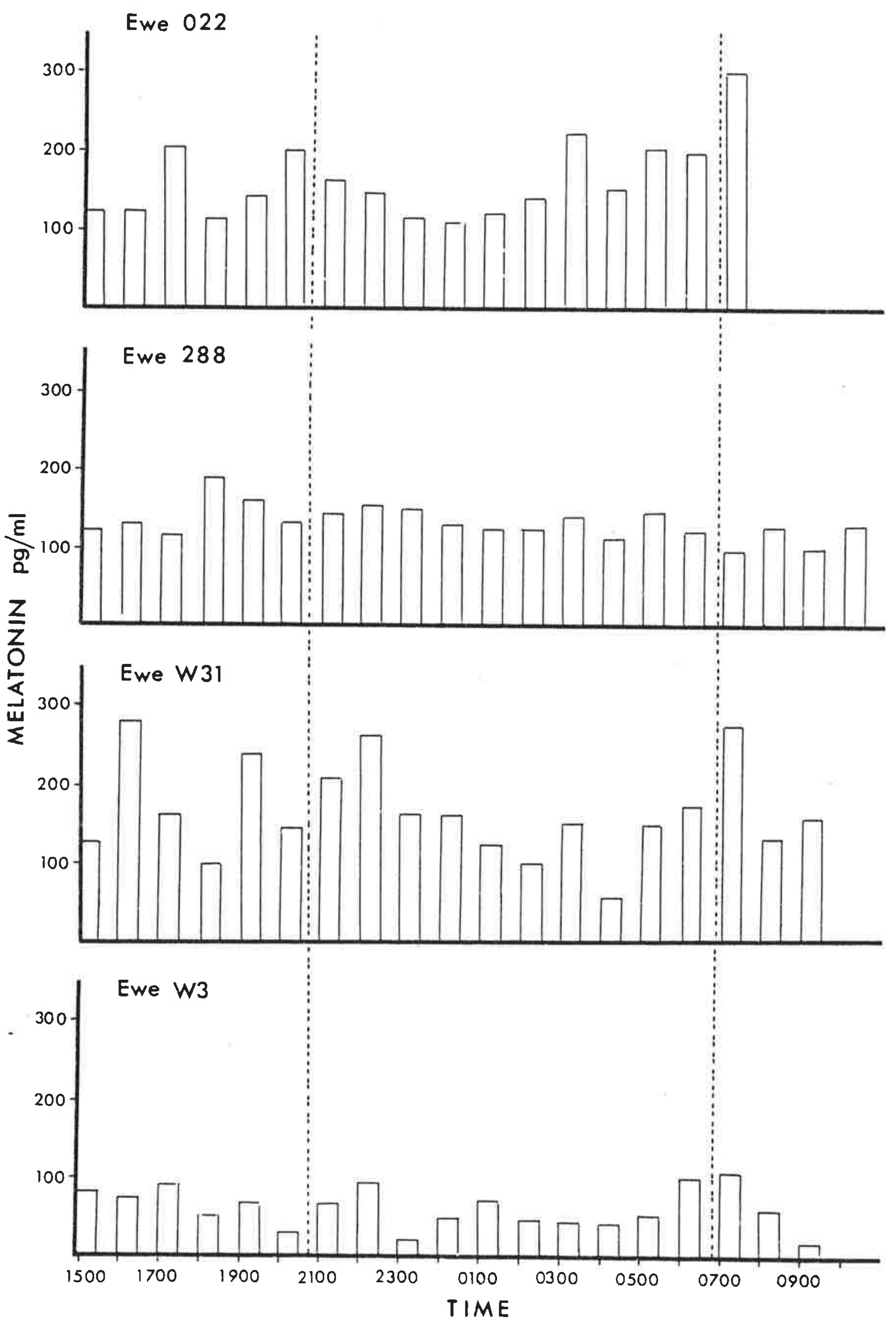
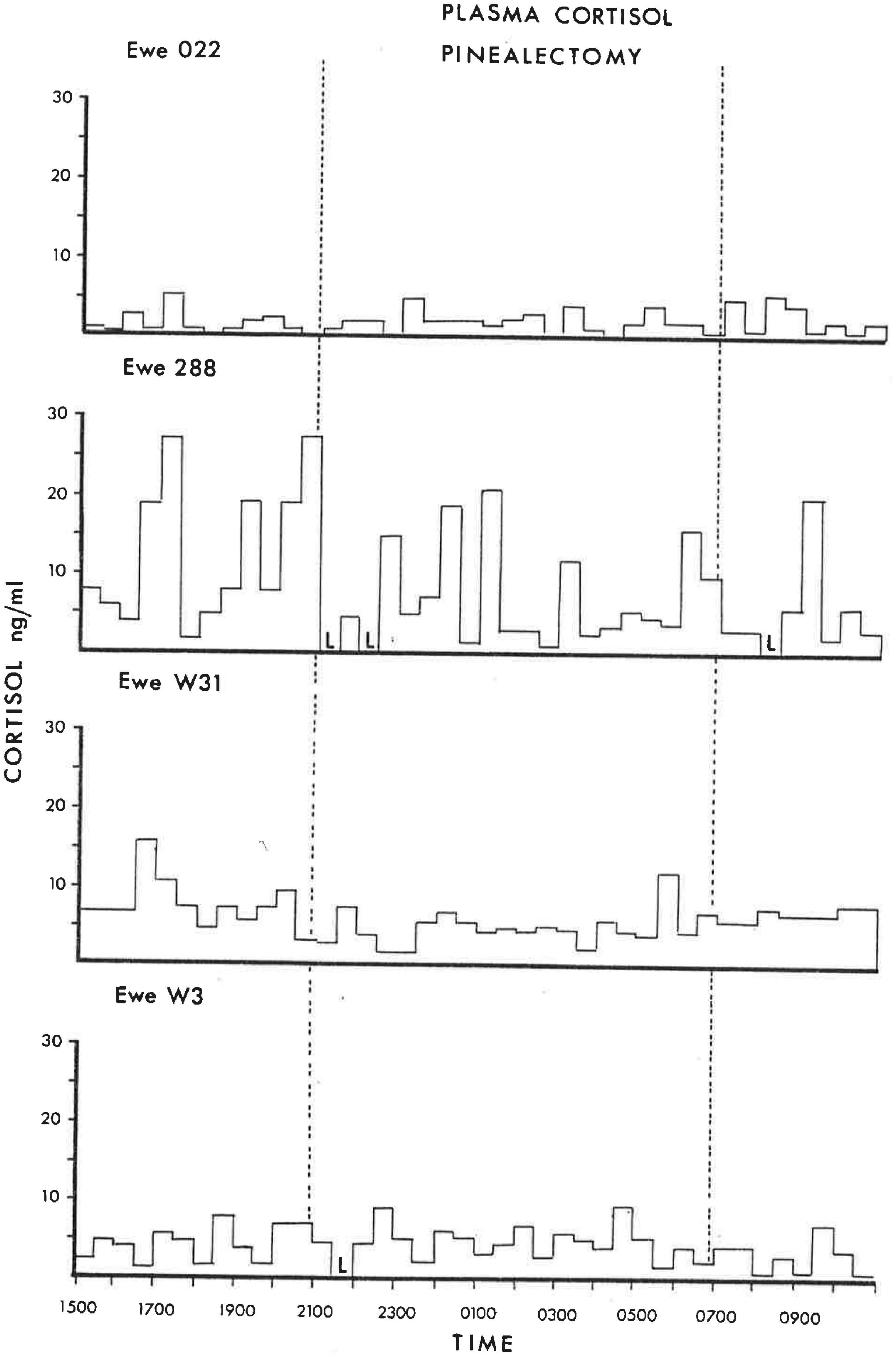

PLASMA MELATONIN
PINEALECTOMY
Ewe 022
Ewe 288
Ewe W31
Ewe W3
MELATONIN pg/ml
300
200
100
1500
1700
1900
2100
2300
0100
0300
0500
0700
0900
TIME

Figure 31

24-hour profiles of plasma cortisol in four pinealectomised ewes. The open boxes represent cortisol concentrations from a 30 minute collection period. The vertical broken line indicates lights out (2050h) and lights on (0650h). L designates sample lost.



PLASMA CORTISOL
PINEALECTOMY
Ewe 022
Ewe 288
Ewe W31
Ewe W3
30
20
10
L
CORTISOL ng/ml
1500
1700
1900
2100
2300
0100
0300
0500
0700
0900
TIME

TABLE 14

PLASMA MELATONIN CONCENTRATION AROUND THE TIME OF PARTURITION IN PINEALECTOMISED AND SHAM PINEALECTOMISED EWES

Days prior to parturition		(Melatonin pg/ml) Pinealectomised				Sham Pinealectomised			
	Ewe No.	W1	W3	W4	W13	Y14	Y4	Y20	Y23
- 8½		-	30	-	-	-	-	-	-
- 8		-	49	-	-	-	-	-	-
- 7½		-	30	-	-	13	-	-	-
- 7		-	47	-	-	66	-	-	-
- 6½		-	37	-	-	14	-	-	14
- 6		37	45	24	-	34	-	-	-
- 5½		-	40	-	-	86	-	-	25
- 5		ND	52	14	14	71	-	-	-
- 4½		7	30	-	-	54	-	-	-
- 4		23	-	ND	23	-	13	5	-
- 3½		-	-	2	-	-	-	-	-
- 3		ND	-	10	34	-	28	12	21
- 2½		-	-	2	-	-	28	5	-
- 2		37	13	-	55	111	22	5	-
- 1½		33	19	-	-	110	41	10	12
- 1		-	17	-	50	86	-	-	18
- ½		-	29	-	34	71	-	-	17
0		-	21	32	64	71	-	-	7
+ ½		-	10	9	-	-	-	-	-
+ 1		-	22	5	70	83	22	20	27
+ 1½		-	12	-	-	-	-	-	-
+ 2		-	-	37	-	-	18	31	-
+ 2½		-	-	-	-	-	12	-	-
+ 3		31	-	-	-	-	18	33	-

Subsequent analyses have indicated that the retina (Cardinali and Rosner, 1971) and harderian gland (Vlahakes and Wurtman, 1972) and red blood cells (Rosengarten *et al.*, 1972) can synthesise melatonin. Koslow (1974) has identified melatonin in the hypothalamus of rats following pinealectomy. Bubenik *et al.*, (1977) have demonstrated immunohistochemically that melatonin is present in the rat digestive system. Using both radioimmunoassay and bioassay methods Ozaki and Lynch (1976) demonstrated that pinealectomy in rats removed the diurnal rhythm of melatonin excretion during fasting but melatonin was still present in detectable amounts.

The results of the present study confirm the findings of Koslow (1974) and Ozaki and Lynch (1976) in that pineal removal failed to eliminate circulating melatonin in the sheep. Only the diurnal rhythm of melatonin in plasma is removed. While Ozaki and Lynch (1976) have implicated a dietary source of melatonin in pinealectomised rats fed *ad libitum*, the indole is still present after fasting. A dietary source of melatonin in the sheep cannot be eliminated nor can it be adequately tested because of the bacterial flora present in the rumen.

The daily fluctuations in plasma melatonin in rams and ewes following pinealectomy showed no apparent pattern. High ($>$100 pg/ml) levels of melatonin were apparent in some animals within a week of operation. Two ewes had melatonin concentrations in excess of 500 pg/ml on 2 successive occasions and then had levels less than 100 pg/ml for an extended period. At this time there is no explanation for this degree of variation. It

is of some interest to note that some normal ewes exhibited similar bizarre fluctuations in daytime melatonin levels (e.g. ewe 024 in Figure 16).

Plasma melatonin levels in pregnant pinealectomised and sham operated sheep were consistently low during the last 14 days of gestation. Since foetal derived melatonin can cross to the maternal circulation it is possible that an increase in maternal melatonin could occur during the period when the foetal pineal is active. No such rise was seen in either group. The progress and outcome of pregnancy in the pinealectomised ewes was not different from the sham operated controls (C. D. Matthews, personal communication).

The results obtained in this study indicate that melatonin is synthesised in areas outside the pineal gland of the sheep. The pineal gland may simply impart a dark induced increase in plasma levels of melatonin over a baseline secretion. It remains to be established whether circulating daytime levels of melatonin are exclusively of pineal origin or are derived from the extra-pineal site.

While the radioimmunoassay for melatonin has been extensively validated, a possibility does exist that pinealectomy has upset the sheep's metabolism of tryptophan so that increased amounts of N-acetyltryptamine are formed. This lingering doubt can be eliminated by mass-spectral analysis of the plasma from pinealectomised ewes. If this immunoreactive substance which is suspected to be melatonin is finally proved by mass spectrometry to be authentic melatonin, then the useful-

ness of melatonin as a marker of pineal function must be questioned.

CHAPTER 6

PINEAL FUNCTION IN MALE AND FEMALE HUMANS

1) Introduction

Until the development of radioimmunoassay for melatonin, there could be only limited investigations of the pineal gland in humans. It has been necessary, therefore, to extrapolate results obtained from animal studies to humans in order to explain the clinical findings of patients with pineal tumours. The development of the radioimmunoassay reported in this thesis facilitated preliminary studies of pineal function in normal subjects, hospitalised pregnant and non-pregnant patients and post-menopausal women.

2) Subjects

Three female and six male laboratory personnel volunteered to have 20 ml blood samples taken every four hours for a complete day. This necessitated sleeping overnight in a hospital ward. Seven volunteers were sampled from an arm vein and two from an indwelling butterfly infusion set inserted into a vein in the back of a hand. Sampling at night was performed with the room lights switched off. (A small torch was used to illuminate the site of venepuncture). Lights in the ward were switched off from 2200 h until 0600 h. All subjects slept from about 2300 h until 0600 h and reported only minor disturbances during the night sampling.

In the studies of hospitalised patients, informed consent was obtained from both the patients and their physicians to

undergo blood sampling by venepuncture every 4 hours for 24 hours. Attention was paid to the particular drug therapy each subject was exposed to as well as the reason for admission. Non-pregnant subjects had non-endocrine disorders. The pregnant women were admitted for various reasons including enclampsia and hypertension.

Plasma samples were made available from a separate study of post-menopausal serum gonadotrophins by Dr. M. Wellby, Department of Clinical Chemistry, Queen Elizabeth Hospital. These samples were obtained during daylight and had previously been assayed for luteinising hormone, follicle stimulating hormone and prolactin by standard radioimmunoassay procedures.

3) Radioimmunoassay of Human Plasma Melatonin

Because the high lipid content of human plasma resulted in a very thick emulsion during normal chloroform extraction, the procedure was altered slightly. Four ml plasma was mixed with 4 ml borate buffer (0.5M, pH10) and extracted with 20 ml petroleum spirit (60 - 80 b.p.) followed by extraction with 20 ml chloroform. After centrifugation a band of emulsified lipid remained between the plasma and chloroform. The amount of chloroform remaining after aspiration of the plasma and emulsified lipid varied between 15 ml - 17 ml. The recovery of chloroform was quantitated for each tube and the appropriate corrections made when calculating the melatonin concentration. The radioimmunoassay procedure outlined in chapter 3 was then followed.

4) Diurnal Rhythm of Plasma Melatonin in normal Volunteers

Figure 32 shows the mean ± SEM plasma melatonin levels in 3 female and 6 male volunteers over a period of 24 hours during October 1976. Highest melatonin levels occurred at 0200 h (56 ± 6.1 pg/ml). On the same graph the mean ± SEM plasma corticoid concentrations are plotted. The highest plasma corticoid concentrations occurred at 0600 h (96 ± 11 ng/ml) while the lowest occurred at 0200 h (27 ± 48 ng/ml).

5) Diurnal Rhythm of Plasma Melatonin in Hospitalised Patients

Table 15 indicates the plasma melatonin levels in male and female patients. Reason for admission and drug therapy are as indicated. No sex difference could be determined. No significant effect of drug therapy could be deduced, and so data from hospitalised subjects was combined. The mean melatonin levels of this group do not differ significantly from non-hospitalised healthy volunteers ($p<0.05$).

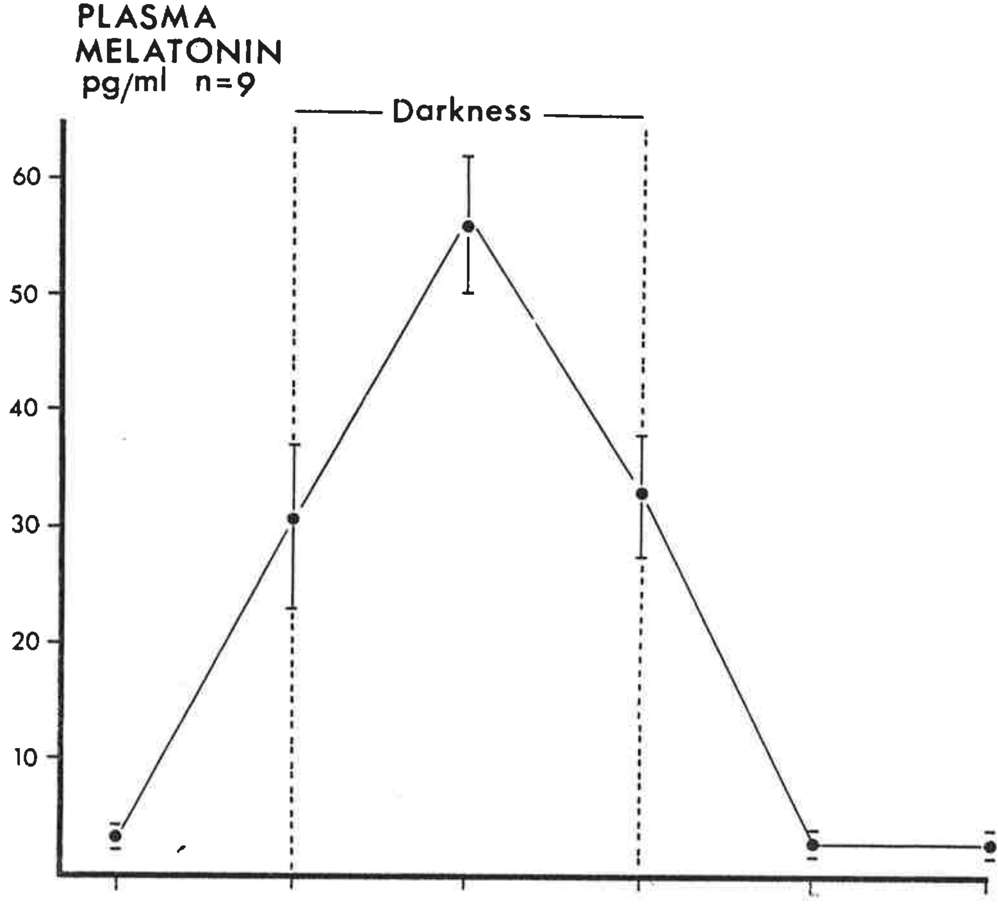
6) Diurnal Rhythm of Plasma Melatonin During Pregnancy

Table 16 shows the plasma melatonin levels in 29 women in the third trimester of pregnancy. The patients had been admitted towards the end of normal pregnancy or for various pregnancy disorders such as hypertension, eclampsia and placental abruption. There was no suggestion that a particular disorder of pregnancy or that a particular drug consistently affected melatonin levels. It was apparent that melatonin was present in pregnant human plasma and that a diurnal rhythm existed. The 0200h concentration of melatonin in the last 5 weeks of

Figure 32

24-hour profiles of plasma melatonin and corticoid in normal humans. Each value represents mean $\pm$ SEM. Vertical broken line designates the approximate time ward lights were switched off (2200h) and switched on (0600h).

NORMAL HUMANS



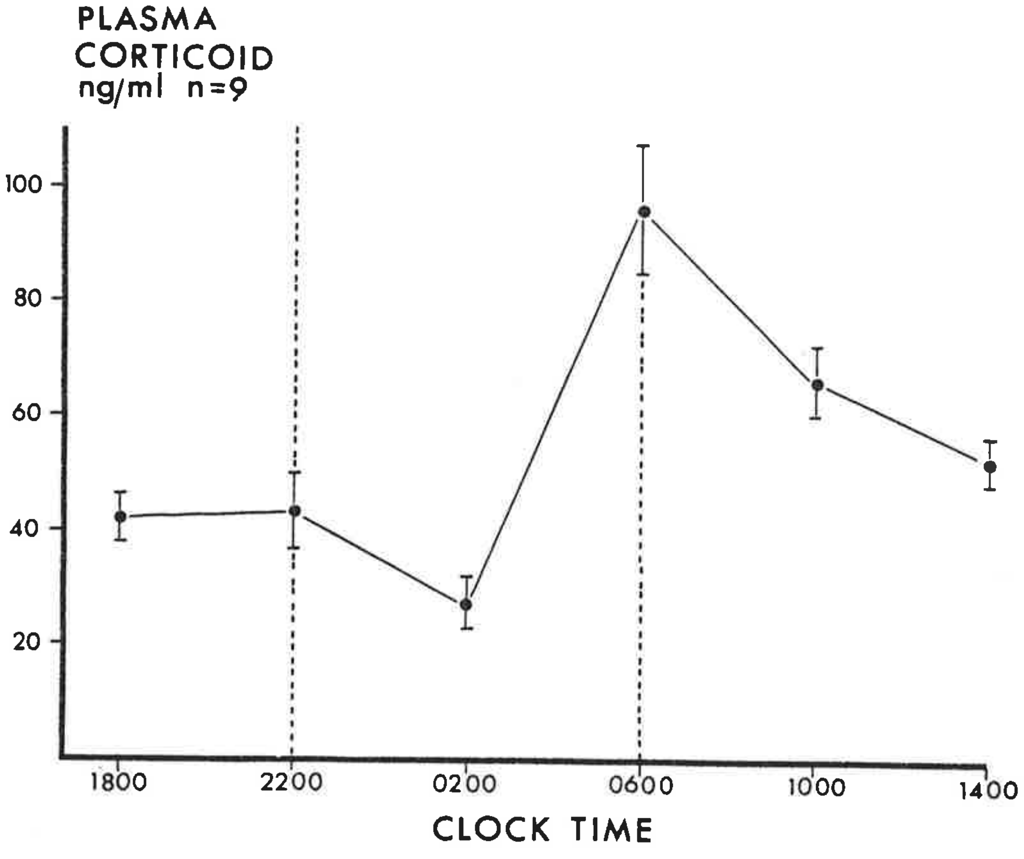


TABLE 15

PLASMA MELATONIN IN HOSPITALISED PATIENTS

Subject	Sex	Age	Illness	Drug	1800h	2200h	0200h	0600h	1000h	1400h
A.G.	M	27	Haematuria	-	6	12	68	33	6	6
Di	F	40	Lupus Erythematosus	-	3	6	18	1	4	3
L.T.	F	30	Tubal Reanastomosis	-	10	-	45	92	10	5
I.D.	M	53	Ulcer	Heparin	8	12	55	12	9	33
D.G.	F	28	Observ	-	10	75	61	27	9	17
W.	F	?	Observ	-	5	8	13	16	16	17
Cr	F	29	Fibroadenoma of breast	-	8	13	19	9	8	5
C.P.	F	21	Pelvic Infectn.	Antibiotic	2	13	113	63	10	2
E.T.	F	65	Cholecystect.	Mogadon	9	13	21	10	6	12
E.S.	F	15	Pelvic Inflam.	Mogadon	3	4	27	51	8	3
E.F.	F	28	Vaginal Repair	Mogadon	16	18	29	17	18	13
E.D.	F	58	Incontinence	Mogadon	7	5	37	52	-	14
A.B.	M	14	Perf. Jejunum	Pethidine	16	18	29	17	18	13
D.A.	M	15	Fracture	Pethidine	20	20	43	25	9	20

TABLE 16

PLASMA MELATONIN DURING HUMAN PREGNANCY

Subject	Gestation	Drug	1800h	2200h	0200h	0600h	1000h	1400h
E.C.	16 wk	Pethidine	14	14	34	16	16	44
P.R.	24	-	11	31	37	35	13	13
P.D.	28	Antibiotic	6	17	41	48	17	4
K.D.	28	-	15	11	14	17	9	30
E.H.	29	-	-	14	23	30	12	11
Ga	29	-	4	11	45	23	14	9
Kr	29	Methyl Dopa	2	22	62	43	5	4
B.D.	32	-	8	44	139	69	20	0
E.R.	32	-	27	28	171	-	49	32
P.P.	32	-	6	10	20	57	9	2
V.H.	35	Urolucosil	8	8	25	16	9	10
V.G.	33	-	9	28	49	41	16	13
L.C.	36	-	9	1	92	94	13	15
V.L.	36	-	6	4	50	40	3	1
S.M.	37	-	5	7	30	35	7	18
J.S.	37	-	7	40	160	60	13	0
J.P.	37	Mogadon	4	46	62	-	15	2
K.C.	37	Methyl Dopa	6	15	152	27	30	8
V.O.	37	-	4	40	68	44	7	2
H.K.	38	Insulin	19	26	126	118	27	66
L.C.	38	-	7	0	61	90	7	12
Br	38	-	3	5	73	103	17	6
D.H.	39	Heparin	9	13	14	10	7	9

TABLE 16

(cont'd)

Subject	Gestation	Drug	1800h	2200h	0200h	0600h	1000h	1400h
H.D.	39	-	20	54	64	52	5	15
T.D.	39	-	10	52	53	28	15	11
Pa	39	-	4	15	153	130	26	7
B.W.	39	Valium	7	22	32	29	3	10
Fu	39	Methyl Dopa	1	13	92	116	16	1
A.P.	Labour	Pethidine	20	54	64	51	5	15
Mean	16-33 wk		10 ± 2	21 ± 3	58 ± 15	38 ± 5	16 ± 3.5	14 ± 4
Mean	35-39 wk		8 ± 1	23 ± 4	76 ± 10	61 ± 9	12 ± 2	11 ± 3
Non-Pregnant (Hosp)			8 ± 1	16 ± 5	42 ± 7	32 ± 7	10 ± 1	11 ± 2
Normal			3 ± 0.7	31 ± 7	56 ± 6	33 ± 6	2 ± 0.6	3 ± 0.7

pregnancy was slightly elevated while the 0600h level was significantly higher ($p < 0.05$) than either normal, hospitalised or 16-33 week pregnant patients.

7) Daytime Plasma Melatonin Levels in Post Menopausal Women

Plasma samples from post menopausal women were assayed for melatonin. The mean age of these women was 63 ± 2 years. Plasma luteinising hormone, follicle stimulating hormone and prolactin concentrations were 35.3 ± 3.5 mU/ml, 85.5 ± 11 mU/ml and 10.4 ± 2 ng/ml respectively. Plasma melatonin was detectable i.e. ($>$10 pg/ml) in only one woman (62 pg/ml). Plasma gonadotrophins in this subject were close to the mean concentrations.

8) Discussion

Modern interest in the endocrinology of the pineal gland extends as far back to 1898 when Heubner reported a case of precocious puberty in a child with pinealoma. Studies of the relationship between disorders of the pineal gland and its reproductive consequences have continued (see next chapter) despite the fact that no markers of pineal function were available. The discovery of the pineal enzyme hydroxyindole-O-methyltransferase in human pineal tissue (Wurtman et al., 1964 and Otani et al., 1968) offered some justification for adapting the so called "melatonin hypothesis" of animal pineal function to the human. These two studies failed to establish a relationship between the pineal enzyme activity, time of death, age or cause of death. Enzyme activity was present even in heavily calcified pineal glands, thus questioning the

time honoured adage that calcification of the pineal was indicative of atrophy and loss of function.

Arendt et al., (1975) developed a radioimmunoassay for melatonin and applied it to the measurement of plasma melatonin in daylight and darkness. Melatonin was higher at night than during the day. This result corroborated the earlier bioassay work of Pelham et al., (1973). It thus had become possible to easily measure melatonin in small volumes of blood taken under natural or altered experimental conditions. Arendt et al., (1977) have further extended knowledge of melatonin secretion using more frequent sampling. Plasma melatonin displays a highly significant diurnal rhythm in both males and females with the initial rise occurring before darkness and peak levels attained at 0200h. J. Smith et al., (1977) have also reported peak plasma melatonin levels at 0200h. These workers also measured the pineal enzymes hydroxyindole-O-methyltransferase and serotonin-N-acetyltransferase in post mortem specimens. In contrast to the results of Wurtman et al., (1964) and Otani et al., (1968) they found highest enzyme activity in pineals from people who died during darkness. Several investigators have attempted to measure melatonin in body fluids other than blood. Arendt et al., (1977) and I. Smith et al., (1976) detected melatonin in cerebrospinal fluid and Lynch et al., (1975a,b) detected the indole in urine.

The above initial reports have provided a basis for further investigations of the possible reproductive functions of the pineal gland. Such work is in its infancy, however,

Wetterberg et al., (1976) have presented evidence suggesting that "early morning" plasma melatonin concentrations were elevated at the time of menstrual bleeding and depressed at the time of ovulation.

The results of the present study confirm the existence of a diurnal rhythm in plasma melatonin in males and females. Peak melatonin concentrations occur at 0200h. There is a significant elevation of plasma melatonin at 2200h i.e., before both darkness and sleep. A melatonin rhythm persists in males and females confined to hospital beds. This result is at variance with the report by Lynch et al. (1975a) who concluded that the rhythm of melatonin excretion was absent in bedfast patients. This disparity is difficult to explain.

The therapeutic drugs on which some of the patients in this study were maintained had little apparent effect. Interpatient variation was so great even in normal people that even if some drugs did depress pineal function it could not be shown statistically. To overcome this problem it would be necessary to use each subject as his own control. There is some evidence that the β adrenergic antagonist Propranolol can suppress the nighttime melatonin levels, (Vaughan et al., 1976b). Other adrenergic agonists or antagonists may have similar effects. Closer scrunity of Methyldopa and the benzodiazepine tranquilisers is warranted.

The diurnal rhythm of plasma melatonin persists during human pregnancy unlike the sheep. During the later stages of pregnancy in the human the amplitude of the rhythm is greater

than that observed in normal subjects. It has recently been reported by Grischenko et al., (1976) that melatonin excretion into urine during pregnancy is highest during the night. The amplitude of this rhythm increased during labour and decreased in the post-partum period. Grischenko et al., (1976) used a fluorometric method to detect the melatonin. Experimental details including specificity of the method were not stated and so confirmation of these results is required using either radioimmunoassay or mass spectrometry techniques.

The study of plasma melatonin concentrations in post menopausal women was instituted to test whether the high circulating gonadotrophin and low circulating prolactin levels acted as a stimulus for melatonin secretion during the day. Melatonin was undetectable (<10 pg/ml) in 21 out of 22 women studied. Post-menopausal gonadotrophins, therefore, do not induce secretion of melatonin during the day. The effect of menopause on the diurnal rhythm has not been investigated; however, one of the hospitalised women was post menopausal (age 65) and yet she had normal melatonin concentrations at 0200h.

CHAPTER 7

REPRODUCTIVE FUNCTION IN TWO PATIENTS WITH PINEAL TUMOURS

1) Introduction

The effects of pineal tumours on the timing of puberty in humans have been known for many years. Heubner (1898) described a boy with a pinealoma with symptoms of premature puberty. Marburg (1913) advanced the proposition that this "pubertas praecox" was caused by inadequate function of the pineal gland due to pineal degeneration or to destruction of the specific pineal parenchyma. Early German authors also maintained that hyper-pinealism could occur, resulting in delayed and incomplete development of the gonads. Kitay and Altschule (1954) reviewed the literature to that time and concluded that tumours of pineal-ocytes (pinealocytomas) delayed the onset of puberty while tumours destroying the gland advanced it. The hormone(s) responsible for these effects are unknown but suspected to be melatonin. There have been few thorough studies of the endocrinology of pineal tumours. This section covers limited investigations on pituitary, adrenal, testicular and pineal hormones in two males with pinealomas. I am grateful to Dr. G. McCulloch and the patients for permission to complete this investigation.

2) Case History 1. S.D.

This 15-year old white boy was admitted from Mount Gambier on 5/9/76 with a short (3-4 weeks) history of double vision. He complained of bifrontal headaches for about five weeks.

These headaches usually occurred late in the afternoon after exercise and vomiting was associated with some of these episodes. The patient reported mild polydipsia but no polyuria.

Neurological examination indicated paralysis of upward gaze. There was no papilloedema. The clinical endocrinology of this patient was not explored. Serum electrolytes, enzymes, glucose, urea, creatinine etc., were all within normal limits. Complete blood count was normal. Cerebro-spinal fluid globulin was elevated indicating possible presence of a cranial tumour.

A Tc^{99} brain scan indicated a space-occupying lesion in the pineal region with associated hydrocephalus. Electroencephalography produced an abnormal record with posteriorly situated slow wave dysrhythmia but without any definite focal or lateralising features. Skull X-ray, right carotid angiogram and right vertebral angiography indicated mild hydrocephalus and asymmetry but failed to localise the lesion. A ventriculogram showed a tumour bulging into the posterior half of the third ventricle. The tumour contained calcium and its appearance was that of a pinealoma.

On 13/9/76 six blood samples were taken at four-hourly intervals for hormone analysis. On 15/9/76 a ventriculo-cisterna/magna shunt was implanted (Torkildsen's procedure). This relieved the symptoms of the hydrocephalus (headache). Biopsy of the tumour was not feasible. A course of radiotherapy was instituted (5000 rads) and the patient discharged on 8/10/76. When re-examined on 4/2/77 the patient was well and had had no further episodes of headaches although the diplopia was still present.

3) Case History 2. E.S.

Patient E.S. was a 43-year-old white male who was first admitted on 3/10/76. He presented with a 10 week history of intermittent headache, confusion and diplopia. He had been diagnosed as a manic depressive for 20 years but responded well to mensyndol and high doses of lithium. On admission the patient was observed to have intermittent gait disturbance, confusion and urinary incontinence. Blood count, serum electrolytes and enzymes etc. were normal. The clinical endocrinology of this patient was not explored.

Skull X-rays, indicated raised intracranial pressure. Right carotid angiogram indicated marked ventricular dilatation. A ventriculogram indicated a space filling lesion of the posterior portion of the third ventricle in the region of the pineal gland. A ventriculo-atrial shunt was implanted to relieve the intra-cranial pressure on 10/10/76. Blood was sampled for hormone analysis on 12/10/76 after a good recovery.

The diplopia was minimal by 13/10/76. A course of radiotherapy (5700 rads for 38 days) was instituted at the patient discharged on 16/10/77.

The patient was readmitted on 20/6/77 after complaining of severe headache. Bilateral carotid and vertebral angiograms and a ventriculogram (28/6/77) indicated right thalamic and hypothalamic invasion. The patient was discharged on 29/6/77 and died 19/8/77. The post-mortem report appears in the appendix.

4) Blood Collection and Hormone Assays

Twenty ml blood was collected from an arm vein by venepuncture every 4 hours from S.D. on 13/9/76 and from E.S. on 13/10/76 and 22/6/77. In addition blood was sampled from S.D. at 11 a.m. on 18/2/77. S.D. had blood sampled 30 minutes and 1 minute prior to injection with 50 mg intramuscular chlorpromazine on 27/9/76. Blood was sampled 30, 60, 90, 120, and 180 minutes after injection.

Plasma luteinising hormone, follicle stimulating hormone and prolactin were assayed by standard radioimmunoassay methods in the Department of Clinical Chemistry at The Queen Elizabeth Hospital. Plasma melatonin was assayed by radioimmunoassay. Plasma corticoid was assayed by competitive protein binding. A sample of urine collected from S.D. between 2200h and 0700h on 27/9/76 was assayed for androgenic steroids by gas liquid chromatography by Dr. G. Phillipou, Department of Obstetrics and Gynaecology, The Queen Elizabeth Hospital.

5) Hormone Levels in Patient S.D.

Table 17 shows the 24-hour profile of circulating hormones in patient S.D. Plasma concentrations of luteinising hormone and follicle stimulating hormone were below the established normal ranges at each sampling period. Prolactin levels were grossly elevated throughout the day and night, and there was no evidence of a diurnal rhythm. Plasma corticoid levels were within 2 standard deviations of the mean of levels obtained from 9 healthy volunteers (figure 32). Melatonin could not be detected at any time during the day or night. By 18/2/77

TABLE 17

HORMONE LEVELS IN PATIENT WITH A PINEALOMA

Case 1 S.D. date 13/9/76

	1400h	1800h	2200h	0200h	0600h	1000h
Melatonin (pg/ml)	ND	ND	ND	ND	ND	ND
Luteinising Hormone (mU/ml) (normal range 8.7-24)	3.3	5.1	4.0	6.0	5.1	4.6
Follicle Stimulating Hormone (mU/ml) (normal range 5-20)	0.9	1.2	1.8	1.4	1.1	1.2
Prolactin (ng/ml) (normal range 5-15)	27	36	30	43	41	30
Corticoid (ng/ml)	22	26	20	42	53	36

luteinising hormone and follicle stimulating hormone concentrations were low at 8.3 mU/ml and 6mU/ml respectively while plasma prolactin was still very high (520 IU/ml, normal range 70-400 IU/ml). Qualitative analysis of urinary androgenic steroid excretion (2200h - 0700h) indicated apparently low testicular activity. Injection of chlorpromazine (50 mg, 27/9/76) had no effect on plasma melatonin or plasma prolactin. Pre-injection prolactin levels were 32.5 and 29.1 ng/ml; post injection levels 26, 29.1, 24.2, 25.1 and 27 ng/ml. Plasma melatonin was not detected.

6) Hormone Levels in Patient E.S.

Table 18 shows the 24-hour profiles of circulating hormones in patient E.S. 2 days and 8 months after implantation of the ventriculo-atrial shunt. Luteinising hormone and follicle stimulating hormone concentrations throughout both sampling periods were within the normal range. Prolactin levels were very low, especially on 13/10/76. Plasma corticoids appeared elevated (i.e., outside 2 standard deviations of the mean of healthy volunteers) on 13/10/76, but superimposed on these high levels was an early morning rise in levels. During the 24-hour sampling on 21/6/77, plasma corticoids were apparently normal and an early morning rise still appeared. Melatonin was not detectable at any time during the study.

7) Discussion

Tumour of the pineal gland is an uncommon lesion accounting for 0.4%-2% of all intracranial neoplasms (Puschett and Goldberg, 1968). Abnormalities in the pineal gland have long

TABLE 18

HORMONE LEVELS IN PATIENT WITH A PINEALOMA

Case 2 E.S.

1) 13/10/76

	TIME					
	1400h	1800h	2200h	0200h	0600h	1000h
Melatonin (pg/ml)	ND	ND	ND	ND	ND	ND
Luteinising Hormone (mU/ml) (normal range 8.7 - 24)	-	7.8	8.3	7.4	6.9	11.6
Follicle stimulating Hormone (mU/ml) (normal range 5-20)	-	14	12	13	13	14
Prolactin (ng/ml) (normal range 5-15)	2.2	1.6	1.5	2	ND	2.8
Corticoid (ng/ml)	77	105	81	108	180	122

2) 21/6/77	1200h	1600h	2000h	2400h	0400h	0800h
Melatonin	ND	ND	ND	ND	ND	ND
Luteinising Hormone (mU/ml) (normal range 8.7-24)	8.8	13	11	11	11	9
Follicle stimulating Hormone (mU/ml) (normal range 5-20)	11.6	9	12	12	12.5	13
Prolactin (IU/ml) (normal range 70-400)	70	50	70	80	60	ND
Corticoid (ng/ml)	83	28	29	22	44	81

been associated with aberrations in the timing of puberty in humans (Kitay and Altschule, 1954). Without the adequate methodology, investigations of pineal hormone imbalance arising from tumour development have been impossible. The precocious puberty which often follows pineal destruction in children has been attributed to melatonin on the basis of animal research. Thus it is believed that a destructive pineal tumour removes sites of melatonin synthesis and secretion and results in an early onset of puberty. The corollary also applies i.e., parenchymatous tumours secrete excess amounts of melatonin and thus delay puberty. Partial support for this concept was advanced by Wurtman and colleagues (Wurtman et al., 1964c, Wurtman and Kammer, 1967) who demonstrated hydroxyindole-O-methyltransferase activity and melatonin in parenchymatous metastatic tumours. Evidence against the melatonin hypothesis is the report that melatonin injections into normal adult males and post-menopausal women failed to affect either basal or stimulated gonadotrophins (Fideleff et al., 1976). While comparable experiments have not been performed in children there is a possibility that melatonin is not antigonadotrophic in humans.

Patients with pineal tumours present usually with three symptoms, diabetes insipidus, pituitary dysfunction and visual disturbances (Puschett and Goldberg, 1968). Despite the general interest in the pineal, tumours in this region have tended to interest mainly neurologists and neurosurgeons. The result is that despite the hypotheses of Wurtman et al. (1964),

the paper by Puschett and Goldberg (1968) remains the only significant study of the endocrinology of pineal tumours. In their 5 cases there was evidence of adrenal insufficiency and pituitary dysfunction. There were abnormalities in adrenocorticotrophin, thyroid stimulating hormone, luteinising hormone and follicle stimulating hormone as well as diabetes insipidus.

The two patients with pineal tumours who have been the subjects of the present study appear to differ slightly from the usual clinical picture. Diabetes insipidus was absent in both although S.D. did have mild polydipsia. Neurological disturbances were consistent with a pineal tumour which was blocking cerebrospinal fluid flow and thus raising intracranial pressure. The two patients did, however, have quite different pituitary hormone profiles and a marked difference in radiosensitivity of the tumours.

Patient S.D. had plasma gonadotrophin levels which indicated poor testicular activity. Low excretion of androgenic steroids substantiates this. Unfortunately, clinical data concerning the pubertal development of this patient were unavailable. If puberty in this patient was delayed, the failure to detect melatonin at any time during the day is surprising - the "melatonin hypothesis" would suggest that hypersecretion of melatonin should have been observed. In various types of precocious puberty (excluding pineal tumours) there are a sleep associated increases in plasma luteinising Hormone and follicle Stimulating Hormone levels with the pattern of secretion no different from normal pubertal children (Boyar et al., 1973).

While the sampling times in patient S.D. were not ideally suited to detecting sleep associated secretory episodes of gonadotrophins it is obvious that he had some pituitary/testicular dysfunction, and possibly delayed puberty.

The chlorpromazine injection was given to patient S.D. for two reasons. Firstly chlorpromazine can increase circulating melatonin levels in rats (Ozaki et al., 1976). The raised melatonin levels are a result of either decreased liver metabolism or increased secretion or both. Secondly, Schwinn et al., (1975) demonstrated differential effects of chlorpromazine and thyrotrophin releasing hormone on prolactin secretion. Chlorpromazine stimulates prolactin secretion within 60 minutes in subjects with an intact hypothalamo-pituitary axis, but not in subjects with an impaired hypothalamus. Thyrotrophin releasing hormone has a direct effect on pituitary secretion of prolactin. Chlorpromazine (50 mg intra-muscular) had no effect on plasma melatonin levels. This treatment has not been given to any other subjects and so it is not known at this stage whether it was a normal response. Of more significance was the failure of the drug to raise the plasma prolactin concentration. Thus it would appear that S.D. had hypothalamic failure resulting from pressure of the tumour (or the hydrocephalus) or the elaboration of a non-melatonin "anti-hypothalamic hormone". The restoration of normal plasma gonadotrophin concentrations and the persistence of elevated prolactin levels following ventricular shunting and radiotherapy is difficult to explain. While a pressure phenomenon cannot be eliminated neither can be hype-

secretion of a prolactin releasing hormone from the tumour be ignored. Blask et al. (1976) have shown normal human pineal glands do possess potent prolactin releasing activity.

The second patient (E.S.) showed a quite different hormone pattern. Plasma gonadotrophins were normal at every sampling time but plasma prolactin was grossly low at every sampling time. Since blood sampling was performed 3 days after the ventriculo-atrial shunt procedure the low prolactin is unlikely to result from hydrocephalus although trauma to the hypothalamic area cannot be eliminated. The patient was, however, showing signs of improvement even at this early stage. Unfortunately, it was not possible to test the responsiveness of the hypothalamus with chlorpromazine in this patient. On the second occasion that blood was sampled a similar plasma hormone profile was obtained. Some thalamic and hypothalamic invasion had occurred and yet prolactin levels were still very low. Melatonin was undetectable in this patient at all times. The pattern of circulating pituitary hormones could be explained by suggesting hypothalamic damage. Such damage, however, would result in a decrease in Prolactin Inhibiting Hormone output and a rise in plasma prolactin. An alternative hypothesis is that the tumour was elaborating high levels of a prolactin inhibiting factor.

In summarising the available data on these two patients several observations are important. The two patients complimented the very marked sex bias of pinealoma occurrence (Puschett and Goldberg, 1968). Both patients had visual disturbances, hydrocephalus, apparent pituitary dysfunction and

no apparent melatonin. The diurnal rhythm of the pituitary/ adrenal axis was maintained. Diabetes/inspidus was absent in both patients. S.D. has apparently responded well to radiotherapy, a common finding with pineocytomas while radiotherapy failed to suppress tumour growth in E.S.

While it is possible to hypothesise that a prolactin releasing factor and a prolactin inhibiting factor were elaborated from these tumours, the evidence is only circumstantial. More important is the failure to detect melatonin since some pineal tumours had been expected to produce excessive amounts of melatonin. If the tumours have destroyed the capacity of the gland to produce melatonin why doesn't an extra site of melatonin production appear as happens following pinealectomy in sheep? Thus either both of these tumours are atypical or the "melatonin hypothesis" of pineal tumour function should be reassessed.

CHAPTER 8

PINEAL FUNCTION IN THE SCINCID LIZARD (*Tiliqua rugosa*)

1) Introduction

In the course of evolution the lateral eyes have become the only photo-receptive organs in mammals. Associated with this change from extra-ocular photoreception, there has been a shift in the site of hydroxyindole-O-methyltransferase activity from retinal and brain sites to the pineal gland. Amphibians, for example, have comparable retinal and pineal enzyme activity, but in reptiles and higher animals the pineal enzyme predominates (Quay, 1974). Many lizards possess a secondary photoreceptor, the parietal organ, which has intimate connections with the pineal gland and the rest of the brain (Figure 1). There is considerable evidence that the parietal organ is involved in thermal adaptation to the environment and Firth and Heatwole (1976) further implicated the pineal gland and melatonin in this phenomenon. The new radioimmunoassay for melatonin permitted analysis of the lateral eye-parietal organ-pineal gland interactions. This work was performed in close collaboration with Dr. B. Firth, Department of Anatomy, University of Adelaide.

2) Animals - Surgical Techniques

Male and female scincid lizards (*Tiliqua rugosa*) were captured at either Mannum or Morgan in the Murray Valley of South Australia and kept under natural photoperiod and temperature conditions at the Waite Agricultural Research Institute.

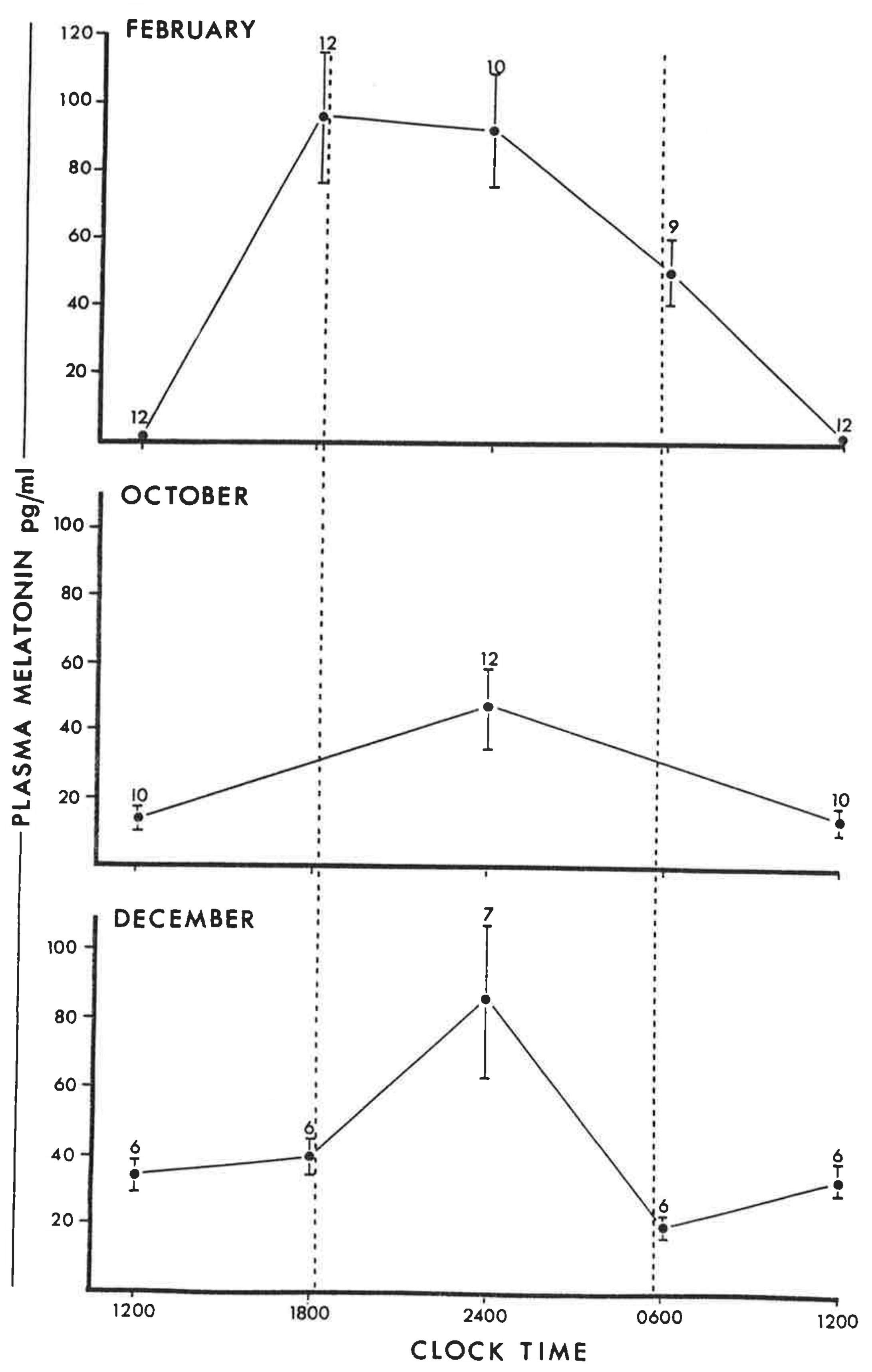
Animals were acclimatised for at least 14 days to a photoperiod of 13h light: 11h dark (lights on from 0530h - 1830h) and a temperature of 24-25.5 C. Parietalectomy was performed using the method of Firth and Heatwole (1976), with only minor modifications. Eye shielding was achieved by adhering strips of sticking plaster overlain with aluminium foil over the eyes and sealing the edges with contact cement. Blood was obtained by cardiac puncture and transfered to heparinised tubes. Plasma was stored at -10 C until assayed. Melatonin was assayed using 2 ml plasma and borate buffer/chloroform extraction. Insufficient plasma was available to completely evaluate the assay in this species, however, the chromatography step in the procedure is expected to eliminate all other indoles except N-acetyltryptamine.

3) Diurnal Rhythm in Plasma Melatonin

There was a highly significant ($p<0.05$) diurnal rhythm in plasma melatonin at all times of the year (Figure 33). Melatonin concentrations were highest during darkness. There is some suggestion that there is an anticipatory rise in plasma melatonin at 1800h (i.e., 30 min. prior to lights off) in this experiment and in a subsequent experiment (Figure 35). Whether this anticipatory effect is a true physiological response or due to sampling bias (i.e., order of sampling of animals) remains to be established. There was a suggestion that the peak melatonin levels were lowest during October (47 ± 12 pg/ml) cf. February (92 ± 17 pg/ml) and December (86 ± 22 pg/ml).

Figure 33

Plasma melatonin at 3 times of the year in *Tiliqua rugosa*. Values represent means ± SEM. Numbers above each bar are the sample size. The vertical broken line designates lights out (1830h) and lights on (0530h).


TILIQUA RUGOSA: SEASONALITY
FEBRUARY
OCTOBER
DECEMBER
PLASMA MELATONIN pg/ml
120
100
80
60
40
20
12
12
10
9
12
12
10
10
7
6
6
6
6
1200
1800
2400
0600
1200
CLOCK TIME

4) Effect of 12 Hour Phase Shifts, Constant Light and Constant Dark

The 12 hour phase shift experiments were performed in May - June. Sham parietalectomised and parietalectomised animals were acclimatised to 13h light: 11h dark for ten days, followed by a reversed photoperiod of 13h L : 11h D. After 12 days in the reversed photoperiod blood samples were taken. The animals remained in the reversed photoperiod for another 16 days and then the photoperiod was returned to the original phasing. Blood samples were taken 15 days after this procedure.

The constant light and constant dark experiments were performed in early October. Samples were taken 9 days after the initiation of the constant conditions.

Exposing the lizards to a 12 hour phase shift inverted the plasma melatonin profile after 12 days. Highest plasma melatonin levels occurred in both sham operated and parietalectomised animals at 1200h (121 $\pm$ 26.8 pg/ml vs. 109 $\pm$ 18.8 pg/ml). At 2400h both groups were low (13.3 $\pm$ 4.6 pg/ml vs. 31.2 $\pm$ 8.3 pg/ml) See Figure 34. Returning the animals to the original phasing did not restore the previously significant day-night differences in either group.

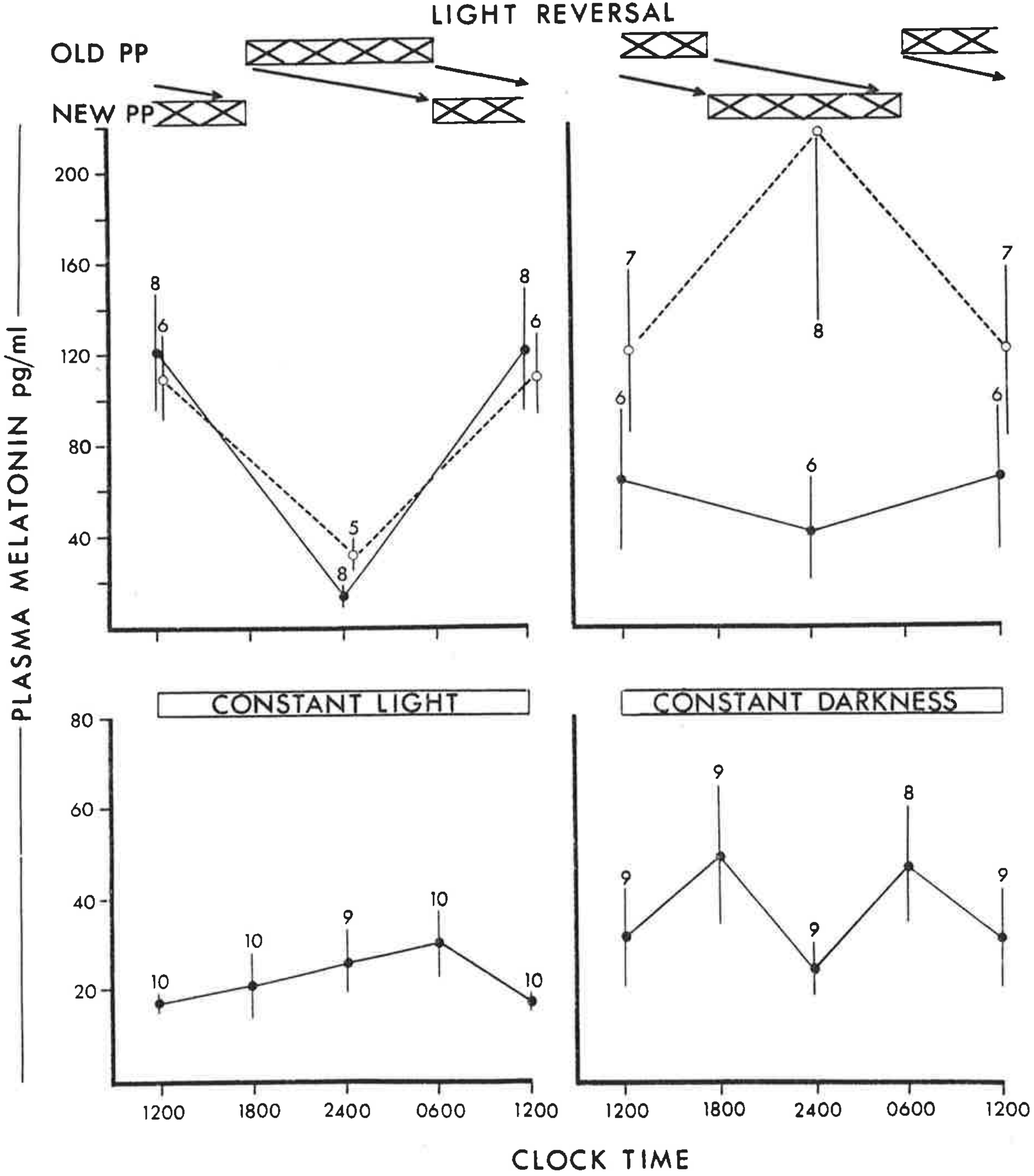
Constant light or constant dark removed the diurnal rhythm of plasma melatonin. These experiments were performed during a period when the normal amplitude was low.

5) Effect of a 6-Hour Phase Shift, Parietalectomy and Eye-Shielding

Unoperated and eye-shielded lizards were exposed to a 6 hour phase shift in photoperiod in late October. Lights were

Figure 34

Effect of a 12 hour phase shift and constant light and dark on plasma melatonin in *Tiliqua rugosa*. The box denoted old PP (old photoperiod) was abruptly changed to a new photoperiod (new PP). In the top right panel the animals were returned to their original phasing. Values are means $\pm$ SEM. Sample size is indicated above each point. Closed circles are sham operated animals. Open circles are parietalectomised animals.


LIGHT REVERSAL
OLD PP
NEW PP
PLASMA MELATONIN pg/ml
CONSTANT LIGHT
CONSTANT DARKNESS
CLOCK TIME
1200
1800
2400
0600
1200
200
160
120
80
40
60
20

turned on from 1130h - 0030h. Ten days later blood samples were taken from groups of animals at four different times. Following blood sampling both experimental groups were parietal-ectomised, and further blood samples taken 21 days later. A large number of postoperative deaths occurred which forced new parietalectomised animals to be included. This precluded within-animal comparisons.

There was no significant difference between control or eye-shielded lizard plasma melatonin levels. Both groups exhibited significant diurnal rhythms. Parietalectomy resulted in a non-significant increase in melatonin from 2400h - 0600h. The mid-dark levels were not significantly different from the control levels obtained at a similar time. When eye-shielding was accompanied by parietalectomy not only was there no significant diurnal rhythm in melatonin, but also a significant difference between this treatment and eye-shielding at mid-dark (0600h), (Figure 35).

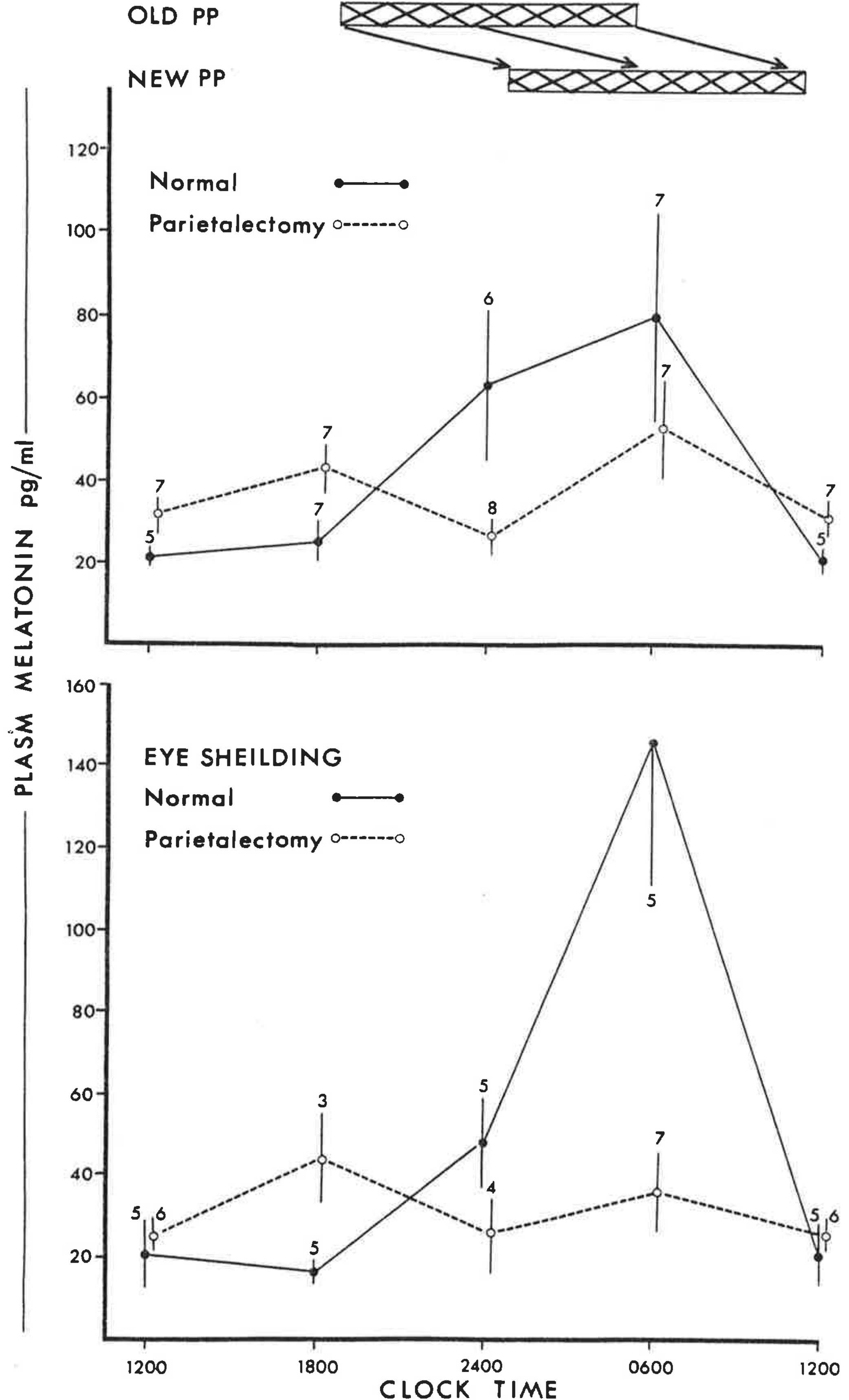
6) Discussion

Investigations of pineal function in reptiles have, like most studies in mammals, been hampered by a lack of suitable assays for the gland's hormone(s). There is some evidence that the reptilian pineal contains an enzyme which synthesises melatonin (Quay et al., 1971). The aim of this study was to characterise melatonin secretion in response to various photoperiod changes and also to complement earlier work by Firth and Heatwole (1976).

Melatonin was detected in the plasma of *T. rugosa* and was

Figure 35

Effect of a 6-hour phase shift on melatonin levels in *Tiliqua rugosa*. The normal photoperiod (old PP) was abruptly changed to a new photoperiod (New PP) the lights being switched off 6 hours later. Values are means ± SEM. The number above each bar is the sample size. Closed circles are normal animals, open circles are parietalectomised animals.



OLD PP
NEW PP
PLASM MELATONIN pg/ml
Normal
Parietalectomy
EYE SHEILDING
Normal
Parietalectomy
120
100
80
60
40
20
160
140
120
100
80
60
40
20
1200
1800
2400
0600
1200
CLOCK TIME

shown to exhibit a diurnal rhythm. Highest plasma melatonin levels occurred during darkness even after 6-hour and 12-hour shifts in the photoperiod. It was apparent that the onset of darkness was an important component in this rhythm, however, the influence of an endogenous rhythm centre associated with an anticipatory response to darkness cannot be ignored. Continuous light and continuous darkness suppressed the rhythm, which is in accord with a dark mediated phenomenon. Plasma melatonin levels at mid-dark tended to be lower during October than at other times, but the large standard deviations in the various time groups prevented the difference from reaching significance. It is of some interest that the lower melatonin levels during spring occur during this animal's breeding season (Bourne et al., 1971). Seasonality of pineal melatonin synthesis and secretion has been proposed before (Reiter, 1974b), but there is currently evidence available only for the house sparrow (Barfuss and Ellis, 1971).

The involvement of the parietal organ with pineal melatonin synthesis has been proposed by Quay et al, (1971). These workers reported reductions in pineal hydroxyindole-O-methyltransferase activity of *Sceloporus occidentalis* following removal of the parietal organ. In the present study using *T. rugosa*, plasma melatonin levels were no different from either unoperated or sham operated controls.

This particular finding prompts a re-evaluation of the hypothesis presented by Firth and Heatwole (1976) purporting to implicate the pineal gland of *Amphibolurus muricatus* in the

daily and seasonal changes in panting theshold. A. muricatus has a circadian rhythm in the panting threshold higher than the nighttime. This rhythm can be abolished by parietalectomy, eye-shielding and injection of serotonin and melatonin (Firth and Heatwole, 1976). Parietalectomy significantly lowers the panting threshold in spring coincident with an endogenous increase usually seen at that time of year. The combination of parietalectomy and eye-shielding results in a cooperative depression of the threshold.

It was hypothesised by Firth and Heatwole (1976) that parietalectomy raises the peripheral melatonin levels during the day while having no effect on the maximal secretion during the night (i.e., the parietal organ suppresses the pineal secretion of melatonin during the day). Parietalectomy of a similar scincid lizard T.rugosa (A. muricatus is an agamid lizard) does not result in increased daytime or nighttime levels of melatonin nor does lateral eye-shielding. Both of these procedures do, however, act cooperatively to suppress the diurnal rhythm.

There thus seems to be a dual control of pineal melatonin secretion in T. rugosa - absence of light can be detected by either the lateral eyes or the parietal organ. It is unlikely that the changes in panting threshold of the agamid lizards observed during the daylight in response to parietalectomy or eye-shielding are due to changes in circulating melatonin unless there is a marked species difference. What is perhaps more likely is that the pineal via melatonin is responsible for the diurnal rhythm and the seasonality of the panting threshold.

CHAPTER 9

PINEAL FUNCTION IN OTHER SPECIES

1) The Role of the Pineal During the Period of Blastocyst Activation in the Tammar Wallaby (*Macropus eugenii*)

The tammar wallaby is a precise seasonal breeding animal indigenous to certain parts of southern Australia. After conceiving in January the embryo stops developing at a relatively undifferentiated stage. Unless there is a loss of suckling neonate during the next few months, the embryo is held in diapause until an appropriate photoperiodic signal occurs during the summer months (Tyndole-Biscoe et al., 1974). The activation of the dormant blastocyst occurs during a very precise period of time around the summer equinox (Sadleir and Tyndale-Biscoe, 1977). It was of some interest to see whether the maternal pineal gland had any role in this event.

In December 1974, Dr. C. H. Tyndale-Biscoe collected pineal glands from adult females on Kangaroo Island, South Australia each night for 8 nights during the period when blastocyst activation was expected to take place. The pineals were frozen on solid carbon dioxide and sent to the laboratory for analysis. At that time a melatonin assay was not available and so hydroxyindole-O-methyltransferase and monoamine oxidase assays were used as indices of pineal function. The glands were randomised and assayed over five assays. The results are shown in Table 19. There was a precipitous drop in hydroxyindole-O-methyltransferase activity on the night of December 24, 1974. Unfortunately attempts to correlate the changes in the enzyme

TABLE 19

TAMMAR WALLABY PINEAL ENZYMES

Date	Pineal Weight (mg)	Body Weight (kg)	HIOMT Total Activity (Units/gland)	Monoamine Oxidase Total Activity (Units/gland)
21/12	6.9 ± 0.7	4.9 ± 0.5	627 ± 166	2311 ± 164
22/12	7.7 ± 0.7	5.0 ± 0.5	566 ± 98	2515 ± 248
23/12	8.4 ± 1.4	5.5 ± 0.3	799 ± 164	2684 ± 413
24/12	7.0 ± 0.7	5.8 ± 0.2	275 ± 42 *	2839 ± 358
25/12	5.6 ± 0.5	5.4 ± 0.7	602 ± 50 *	2750 ± 239
26/12	7.3 ± 0.7	5.2 ± 0.7	810 ± 112	3190 ± 334
27/12	6.7 ± 1.7	5.6 ± 0.4	426 ± 117	3057 ± 717
28/12	8.0 ± 0.6	5.3 ± 0.5	673 ± 155	3312 ± 375

* Designates significantly different from preceding group ($p < 0.05$).

and changes in the blastocyst failed to show a temporal association. This was due in part to the crude semi-quantitative assay of RNA polymerase activity which was used to estimate whether activation occurred. Throughout this period pineal monoamine oxidase activity was quite constant.

These results demonstrate that hydroxyindole-O-methyltransferase and monoamine oxidase are present in the Tammar Wallaby pineal gland. While it is obviously premature to suggest that the pineal gland is involved in the blastocyst activation following diapause in this species, further investigations are warranted to study this fascinating photoperiod dependent phenomenon.

2) Studies on Pineal Function in the Rat

In collaboration with Dr. W. G. Breed, Dept. Anatomy, University of Adelaide, some preliminary observations were made on plasma melatonin levels in rats under various experimental conditions.

The initial experiment involved sacrificing female Wistar rats at mid-light and mid-dark of a 13h light: 11h dark lighting regime. Trunk blood was collected and serum removed for melatonin assay. There was a significant day/night difference. The mid-light serum melatonin levels were 6.3 $\pm$ 3 pg/ml (Mean $\pm$ SEM, n = 5) while at mid-dark the levels were 75 $\pm$ 27 pg/ml (n = 8).

The next series of experiments involved preliminary observations on the effects of castration and steroid replacement in female and male rats on serum melatonin. The results

of these experiments are given in Tables 20 and 21. No consistent conclusions can be drawn from any of these experiments. Not only are there no obvious differences between treatment groups, but the melatonin levels are extremely high in most cases. The only explanation of these rather bizarre results is that the animals were unduly stressed during the injection regimes or at the time of blood sampling. Lynch et al., (1977) have shown that the rat pineal gland does respond to stressful stimuli.

This hypothesis is supported to some extent in the final experiment involving injections of Human Chorionic Gonadotrophin (HCG) into rats. More care was taken not to stress the animals unduly in this short term experiment. The treatment which is expected to raise endogenous steroid concentrations in blood had no significant effects. In all cases the serum melatonin concentrations were relatively low (range - not detectable - 133 pg/ml) (Table 22).

Thus this preliminary study has demonstrated a day-night difference in melatonin levels which is comparable to those reported by others (Pang and Ralph, 1975, Wilkinson et al., 1977 and Ozaki et al., 1976). Acute injections of HCG into intact male and female rats has no effect on serum melatonin 6 hours later. The study further suggests that due consideration be given to the effects of stress in ovariectomised and castrated rats on serum melatonin measurements.

TABLE 20

RAT OVARIECTOMY EXPERIMENTS - HORMONE REPLACEMENT

Experiment 1

Treatment	Plasma Melatonin (pg/ml)
Intact (saline)	135, 500, 168, 747
Ovariectomised (saline) (sacrificed 5 days post op.)	72, 461, 632
Ovariectomised (10 mg Progesterone day 1 and 2, 5 mg Progesterone days 3,4,5)	211, 95, 155, 164
Ovariectomised (10 ug Oestradiol 17β days 1-5)	210, 84, 69, 432

Experiment 2

Treatment	Plasma Melatonin (pg/ml)
Ovariectomised (saline)	500, 267, 700, 1000
Ovariectomised (days 7 & 8, 0.01 ug Oestradiol 17β days 10,11,12, 5 mg progesterone days 13,14, 5 mg Prog. + 0.1 ug Oest. days 15, 16, 5 mg prog day 17 sacrificed)	232, 610, 192, 295
Ovariectomised (as above, except no oest on days 13, 14)	221, 138, 255, 47
Ovariectomised (0.1 ug Oestradiol 17β days 13-16)	84, 147, 35

TABLE 21

RAT OVARIECTOMY AND CASTRATION EXPERIMENTS - HORMONE REPLACEMENT

Experiment 3

Treatment	Plasma Melatonin (pg/ml)
Ovariectomy (saline) (sacrificed 13 days post op.)	93, 689, 198
Ovariectomy (Nothing days 1-5, 0.05 ug or 0.1 ug Oestradiol 17β days 6-12)	76, 78, 1000, 730 650, 87
Ovariectomy (5 ug Oest)	320, 158 120, 270
Ovariectomy (50 ug Oest)	757
Ovariectomy (5 mg Progesterone)	64, 43, 43

Experiment 4

Treatment	Plasma Melatonin (pg/ml)
Castrated male rats- no hormones for 6 days. Then injected for next 7 days and killed on the 14th day.	
Intact (saline)	169, 187, 306, 138
Castrate (saline)	27, 24
Castrate (0.1 ug testosterone)	25, 700
Castrate (1 ug testosterone)	55, 92, 80
Castrate (10 ug testosterone)	15, 47
Castrate (1 mg Dehydroepiandro-sterone)	487, 29

TABLE 22

RAT EXPERIMENT - INJECTION OF HUMAN CHORIONIC GONADOTROPHIN

Treatment	Plasma Melatonin (pg/ml)	Mean ± SEM
Control Males (saline)	29, 6, 34	23 ± 8.6
Males + 50 IU HCG (killed 3½ h later)	7, 1, 22	10 ± 6.2
Control Females (saline)	19, 47, 26	30 ± 8.4
Females + 50 IU HCG (killed 3½ h later)	45, 39, 12	32 ± 10.1
Control Males (saline)	4, 31, 49, 3	22 ± 11.1
Males + 50 IU HCG (killed 6h later)	47, 0, 17, 35	25 ± 10.2
Control Females (saline)	19, 34, 133, 26	53 ± 26
Females + 50 IU HCG (killed 6h later)	54, 47, 36, 43	45 ± 3.7

3) Plasma Melatonin in Cattle, Donkeys, Chickens, Pig and Camel

These studies were performed in collaboration with Dr. J. Obst, and Prof. W. V. McFarlane. The object of the study was to further investigate the universality of the occurrence of melatonin.

Three cows (Brahman and Jersey breed) were maintained in open pens at the Struan Research Centre, South Australia. In December 1974, jugular blood samples were obtained very hour for 24 hours while the animals were exposed to natural photoperiod changes (sunrise 0530h, sunset 2000h). Blood was chilled in ice and later centrifuged and plasma stored at -20 C until assayed. Table 23 gives the individual and mean plasma melatonin levels. In each cow nighttime melatonin concentrations were significantly higher than daytime. These results agree favourably with the results obtained recently by Hedlund et al., (1977).

Jugular vein blood was obtained from two donkeys, a pig and a camel at mid-light and mid-dark while the animals were grazing natural pasture. Chicken blood was obtained via wing vein puncture from 3 animals at the same times. Table 23 shows the results obtained for these animals. This is the first report of donkey, pig and camel melatonin and indicate that this hormone was higher at night. The day-night difference in chicken melatonin levels agree with the reports of Pelham (1975) and Pang and Ralph, 1975b).

TABLE 23A

PLASMA MELATONIN IN CATTLE

Time	Animal C1193	C1283	C1112	Mean ± SEM
		Melatonin pg/ml		
1500h	79	13	39	43 ± 19
1600h	80	18	60	52 ± 18
1700h	92	15	55	54 ± 22
1800h	79	15	34	37 ± 22
1900h	60	22	26	36 ± 12
2000h	51	9	21	27 ± 12
2100h	141	65	110	105 ± 22
2200h	90	117	185	130 ± 28
2300h	234	97	252	194 ± 49
2400h	80	53	280	137 ± 71
0100h	83	84	399	188 ± 105
0200h	44	22	100	55 ± 23
0300h	110	57	80	82 ± 15
0400h	55	45	269	123 ± 73
0500h	137	21	102	86 ± 34
0600h	29	11	102	48 ± 27
0700h	25	14	47	28 ± 10
0800h	61	19	45	41 ± 21
0900h	-	11	73	42 ± 31
1000h	-	-	-	-
1100h	-	-	-	-
1200h	86	-	-	-
1300h	97	-	-	-
1400h	79	ND	34	37 ± 22

TABLE 23B

PLASMA MELATONIN IN DONKEYS CHICKENS, PIGS AND CAMELS

Donkeys	(2)	midlight	24 pg/ml	mid-dark	108 pg/ml
Chickens	(3)		50		200
Pig	(1)		22		76
Camel	(1)		29		221

4) <u>Melatonin in the Brush-Tailed Possum (Trichosurus vulpecula)</u>

As part of an ongoing project on secretions from the subcommissural organ and pineal gland in brush tailed possums by Dr. R. S. Tulsi, a preliminary investigation into the occurrence of melatonin in pineals and plasma was initiated.

Possums were obtained from suburban Adelaide in January, February and July, 1977. The animals were maintained in an animal house for up to 3 weeks prior to the experiments under a photoperiod regime of 13h light and 11h dark. Possums were killed by cervical dislocation and blood samples obtained by cardiac puncture. Pineal glands and subcommissural organs were dissected, placed in plastic vials containing 0.1 ml saline and frozen until assayed. Blood was immediately centrifuged and plasma removed and stored frozen at - 10 C. The gonads were dissected, fixed with buffered formalin, sectioned and stained with haematoxylin and eosin.

Pineals and subcommissural organs were homogenised in 0.5 - 1.0 ml, 0.5 M borate buffer pH10 and 1 ml 0.5M sodium hydroxide and then extracted with chloroform. Plasma (2 ml) was extracted with borate buffer and chloroform. Extracts were then chromatographed on lipidex 5000.

Of the 4 possums killed at midlight in January, 1977, melatonin content of both pineals and subcommissural organs was less than 150 pg/gland. One animal killed at mid-dark (adult male) had a pineal melatonin content of 954 pg/gland but undetectable (less than 150 pg/gland) in the subcommissural organ. The melatonin content of organs from the other 3 animals killed

at this time was less than 150 pg/gland. Gonadal histology was not performed on this group.

In February, 3 more animals were killed at mid-light and 3 at mid-dark and pineal glands and plasma analysed for melatonin. Table 24 indicates the melatonin contents of pineals and plasma related to the time of death, sex and gonadal status. Inactive gonads were defined as having no evidence of spermatogenesis, follicles or corpora lutea. Intermediate stage included animals with large follicles but no apparent corpora lutea. Active gonads had spermatogenesis in testes and corpora lutea in ovaries. 5 out of 6 animals appeared to have functioning gonads, but pineal and plasma melatonin content was low and showed no consistent relationship with the state of the gonads or time of death.

A further 6 animals were killed at mid-light and mid-dark in July and pineal glands and plasma assayed for melatonin. Again melatonin was very low at both times of day although two animals had substantial pineal melatonin contents of 232 pg and 545 pg/gland (Table 24). The females in this series were characterised by apparently active gonads while there was little evidence of active spermatogenesis in the males. There was no consistent relationship between pineal gland melatonin, plasma melatonin, gonadal function or time of death.

The results of this study indicate that melatonin is present in some brush-tailed possum pineals but at relatively low levels. The occurrence of melatonin in either plasma or pineal glands cannot be associated with time of day or gonadal status. The physiological meaning of this apparent divergence

TABLE 24

BRUSH-TAILED POSSUM (*Trichosurus vulpecula*)
PINEAL AND PLASMA MELATONIN

Animal Number	Sex	Time	Pineal Melatonin (pg/gland)	Plasma Melatonin (pg/ml)	Gonads
February					
129	F	Mid-Light	64	<15	Intermediate
130	F	Mid-Light	<30	<15	Intermediate
131	M	Mid-Light	156	<15	Intermediate
132	M	Mid-Dark	30	60	Inactive
133	F	Mid-Dark	65	20	Active
134	F	Mid-Dark	137	<15	Active
July					
145	F	Mid-Light	88	25	Active
146	M	Mid-Light	95	27	Inactive
147	F	Mid-Light	232	<15	Active
148	F	Mid-Light	96	<15	Intermediate
149	M	Mid-Light	545	27	Inactive
150	M	Mid-Light	125	39	Inactive
151	M	Mid-Dark	<30	40	Inactive
152	F	Mid-Dark	48	39	Active
153	M	Mid-Dark	74	41	Inactive
154	F	Mid-Dark	35	24	Active
155	M	Mid-Dark	67	<15	Intermediate
156	F	Mid-Dark	<30	<15	Active

from other animals studied in this thesis and those recorded by Quay (1974) remains unclear. Melatonin has been identified in other marsupials, the red and grey kangaroos (Quay and Baker, 1965) and is implicated in the functioning of the Tammar wallaby reproductive cycle. Normally the brush-tailed possum is a seasonally breeding animal, conceiving during April - May. While the tests of gonadal function mentioned here were rather crude it would appear that the population of possums captured in Adelaide are not precise seasonal breeders. Ready accessability of food probably ensures neonatal survival year round in this population.

The tentative conclusion which can be drawn from the above work is that the pineal gland does not play a major role in the reproductive physiology of the possum. If the pineal gland is involved in the control of reproduction in this species it may do so via other indoles such as 5-methoxytryptophol or pineal peptides.

CONCLUSION

Since the days of the early Greeks and Romans, the pineal gland has commanded attention because of its central position in the brain. It was not until the 1960's, however, that specific functions were attributed to this gland. 1974 heralded a new era in pineal physiology with the development of a radioimmunoassay for one of the pineal's probable hormones, melatonin (Arendt, et al., 1974). From that time it became feasible to investigate actual levels of the indole in the circulation of animals.

Thus the old measures of pineal function, changes in pineal weight and pineal enzymes have now been superceded. There is, however, reason for some caution before plasma melatonin is accepted as an index of pineal function. While this present thesis has documented plasma levels in various animals under various conditions, a number of anomalies have arisen. Probably the most serious was the indentification of immunoreactive "melatonin" in the plasma of pinealectomised sheep. While considerable effort was expended in validating the radioimmunoassay used here for normal sheep, definitive evidence for the identity of the immunoreactivity in pinealectomised sheep is unavailable. On the basis of cross reaction studies and chromatography it is likely that the compound is melatonin, although N-acetyltryptamine cannot be eliminated. Confirmation that the material is melatonin is available in the report of Ozaki and Lynch (1976) who identified melatonin in pinealectomised rat plasma. It is obvious then that if plasma melatonin is to be

used as an index of pineal function then the site of the residual melatonin must be identified and proved not to fluctuate in response to experimental stimuli.

The results from the preliminary study of pineal function in *Trichosurus vulpecula* question to some extent the universal nature of melatonin as a pineal hormone. Not only were pineal and plasma melatonin levels low, but they showed no differences at different times of day.

If plasma melatonin levels can be used as an index of pineal function the current thesis has indicated a number of things. Darkness is a powerful stimulant for melatonin secretion in sheep, humans and *Tiliqua rugosa*. Shifting the phasing of darkness can also shift the melatonin secretion pattern in *T. rugosa*. In all animals studied except *T-vulpecula* melatonin levels were high during the night and lower during the day. Investigations into the reproductive consequences of melatonin secretion were inconclusive. For instance no alterations in daytime (basal?) secretion of melatonin during the sheep oestrous cycle were detected. Nor did removal of the ovaries interfere with melatonin secretion. In pregnancy, however, an interesting species difference arose. In maternal ewes there was a tendency for a suppression of melatonin secretion during pregnancy while in humans there was a tendency towards elevated levels in the last five weeks of pregnancy. This discrepancy requires further investigation.

It is hoped that melatonin radioimmunoassays such as the one described in this thesis will prove useful in unravelling

the true nature of that mysterious organ of the brain, the pineal gland.

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APPENDIX

1) Preparation of 4-(N,N,-Dimethylaminomethyl)-N-Acetylserotonin.

This work was performed by Mr. G. Frith and Dr. G. Phillipou. Paraformaldehyde (4.2 mg, 0.14 mmol) and dimethylamine (16 ul, 0.14 mmol, 40% aqueous) in ethanol (5 ml) were stirred at room temperature for 15 min. N-acetyl serotonin (30 mg, 0.14 mmol) was then added and the whole mixture stirred overnight at room temperature under nitrogen. The solvent was then removed *in vacuo* and the residue chromatographed on Sorbsil. Elution with methanol: chloroform (1:19) gave the major component (17 mg) shown to be pure by thin layer chromatography and identified by proton magnetic resonance (PMR) spectroscopy (Varian T-60 instrument, samples dissolved in deutered acetonitrile, with tetra methyl silane as internal standard) as 4-(N,N-dimethylamino methyl)-N-acetylserotonin.

This model system for the Mannich reaction indicated that substitution occurred exclusively at the 4 position even though it is more sterically hindered than the corresponding 6 position.

2) Preparation of $^{2}H_{3}$ Melatonin

This work was performed by Dr. G. Phillipou and Mr. G. Frith.

N-acetylserotonin (50 mg, 0.23 mmol) and sodium hydroxide (18 mg, 0.45 mmol) in 1,2 dimethoxy ethane (5 ml) were stirred at room temperature for 5 min. in the dark. Deuterated methyl iodide (100 mg, 0.74 mmol) was then added and the reaction

mixture stirred for a further 4 hours at which time the excess base was neutralised (5% dilute hydrochloric acid) and the solvent removed on a rotary evaporator. The remaining residue was preabsorbed and chromatographed on neutral alumina grade II (5g), care being taken to exclude sunlight. The column was developed stepwise with increasing amounts of chloroform in benzene (1-50%). It was then eluted with chloroform to give $^2H_3$ melatonin (43 mg, 81%), which was recrystalised from benzene to yield pale yellow crystals mp 115.5-117C. Thin layer chromatography and gas chromatography (GC) showed the product to be homogeneous; its isotopic composition as determined by mass spectrometry was 99.1% $^2H_3$.

3) Selected Ion Monitoring

This work was performed by Dr. G. Phillipou.

For analysis of melatonin by gas chromatography mass spectrometry, the penta fluoro-propionyl (PFP) ester was formed (Koslow and Green, 1973) and the product subjected to selected ion monitoring. An AEIMS-30 mass spectrometer, interfaced to a Pye GC (column, 1.0m x 2 mm id; 1% OV-225 on 100/120 mesh support; temp., 230C; helium flow, 25 ml/min) via a single stage jet separator (S.G.E. Melbourne, Australia). Quantitation was achieved by selective ion monitoring (SIM) of the molecular ions of the corresponding PFP derivatives of melatonin and $^2H_3$ melatonin (M/Z 360 and 363, respectively; dwell time per ion, 200 m sec; resolution, 1000; ionising current, 300 u A).

4) Post-Mortem Report of Patient E. S.

This report was compiled by Dr. P. C. Blumbergs.

Macroscopic

The brain weighed 1535g. The attached dura mater and superior sagital sinus were normal. The leptomeninges were normal. The cerebral hemispheres externally were symmetrical. There was a ventricular-atrial shunt present in the posterior part of the right temporal lobe, the ventricular 2 cm being occluded by whitish flocculent material. There was symmetrical equilateral tonsilar grooving and symmetrical mild uncal notching. The vessels of the circle of Willis were of normal anatomical configuration and virtually free of atheroma. The cerebral hemispheres were sectioned serially in the coronal plane and the cerebral cortex measured 4 mm in the frontal regions. The posterior one-half of the third ventricle was obliterated by grey-white tumour tissue with small areas of yellow necrosis and small areas of punctate haemorrhage. The pineal gland could not be identified with certainty. The tumour extended laterally to involve both thalamic nucleii, more so on the right. The posterior part of the right forniceal system was expanded by grey-white granular tumour and the tumour extended symmetrically down the mid-brain with obliteration of the aqueduct of sylvius and replacement of most of the tectal region, leaving only the substantia nigra and cerebral peduncles intact. The basal ganglia were macroscopically normal. The mamillary bodies were normal. The right lateral ventricle was partially collapsed

and the septum pellucidum was deviated towards the right. The right half of the corpus callosum was expanded and there were small, diffuse, ill defined irregular areas of greyish discolouration suggestive of tumour involvement in this structure. Sections of the pons and medulla were normal. Sections of the cerebellum were normal.

Conclusion

1) Tumour (? pinealoma) obstructing third ventricle and aqueduct of Sylvius with extension bilaterally into the thalamic nucleii and caudally into the rostral brainstem.
2) Ventricular atrial shunt.

Microscopic

Vermis, pons, dentate nucleus and medulla - normal.

Tumour

The lesion is a highly cellular neoplasm composed of primitive cells rich in chromatin and with an ill-defined eosinophillic cytoplasm. The predominant pattern is that of irregular sheets of cells with the formation of numerous rosettes of the Homer-Wright-type. Many of the rosettes are ill-defined and poorly formed but the best preserved rosettes show central eosinophillic material within which delicate fibrillary tangles and club-shaped expansions are readily visible with oil immersion. Many of the cells have fibrillar eosinophilic cytoplasmic processes closely resembling fibrous astrocytes but these, fail to stain with the PTAH preparations, whereas the surrounding reactive fibrous astrocytes in the periventricular

white matter and forniceal system stain positively. The tumour is predominantly confined to the third ventricle although there is superficial invasion of the surrounding periventricular white matter which shows a well developed reactive fibrous gliosis.

Multiple scattered areas of necrosis are present but mitoses are relatively infrequent.

The features are those of a malignant pineoblastoma. The irradiation is very likely to have contributed to the tumour necrosis.

Comment

These tumours are rare, highly malignant and behave in a similar fashion to medulloblastomas.