THE OCCURRENCE AND METABOLISM OF MELATONIN

A Thesis

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by

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I wish to dedicate this thesis to my children Rebecca and Emma.

CONTENTS

SUMMARY

This thesis concerns a study of the production of melatonin, a putative pineal gland hormone, in man and sheep. In particular it concerns the potential use of measurement of the unique urinary melatonin metabolite, 6-sulphatoxymelatonin (6SM) as an index of melatonin production.

A method for urinary 6SM analysis was developed based on isotope dilution gas chromatography-mass spectrometry. Of 2 mass spectrometers employed, repeated analysis of a control urine gave interbatch coefficients of variation of 9.8% (n=7) and 7.9% (n=17) for the AEIMS-30 and Hewlett-Packard 5992B respectively.

Studies in intact sheep revealed urinary 6SM excretion of 3.0-17.3 μg 24 h^{-1} (n=7), with approximately 80% (n=4) occurring during darkness. An estimate of the urinary production rate (UPR) of melatonin of ewes (39.1-153.5 μg 24 h^{-1}, n=10) was obtained by measurement of the specific incorporation of deuterium unto urinary 6SM following intravenous administration of deuterated melatonin; incorporation of deuterium into urinary 6SM was complete within 24 hours of injection. Two ewes were studied before and after successful pinealectomy. Pre-operatively their melatonin UPRs were 39.1 and 84.1 μg 24 h^{-1} and their urinary 6SM levels 3.4 and 4.7 μg 24 h^{-1}, indicating that only 4.2 and 3.3% respectively of the administered $^{2}H_{3}$-melatonin was excreted as $^{2}H_{3}$-6SM. Post-operatively no endogenous urinary 6SM was detected.

Urinary 6SM excretion for man was 9.5-29.1 μg 24 h^{-1} (n=10) and for menstruating women in the follicular and luteal phases was 5.3-28.9 (n=5) and 9.9-30.9 (n=5) μg 24 h^{-1} respectively. In each case 24 hour urinary 6SM excretion profiles for a man and a menstruating

woman revealed that approximately 80% of the daily output was associated with the nocturnal sleep phase. Over five consecutive days urinary 6SM excretion for a man was 25.7 ± 3.6 μg 24 h^{-1}.

Following intravenous administration of $^{2}H_{3}$-melatonin to a man 99.7% of the $^{2}H_{3}$-6SM was excreted within 24 hours. For that subject melatonin UPR was 23.7-34.5 μg 24 h^{-1} (n=4) while the pre- and post-dosage urinary 6SM excretion was 15.1-22.3 (n=4) and 16.5-22.5 (n=3) μg 24 h^{-1}, respectively. The percentage of $^{2}H_{3}$-melatonin excreted as $^{2}H_{3}$-6SM by that subject was 40.7 ± 2.3 (n=4). For that subject melatonin UPR determined after injection of $^{2}H_{3}$-melatonin at 1300 and 2230 h was 33.0 and 29.8 μg 24 h^{-1}, respectively. In another male melatonin UPR was 29.1 and 28.2 μg 24 h^{-1}.

Urinary 6SM excretion at monthly intervals throughout one year for 4 men at 35°S was 15.9 ± 4.0 (n=13), 28.0 ± 9.6 (n=13), 30.4 ± 6.3 (n=12) and 39.4 ± 7.9 (n=12) μg 24 h^{-1}, with the percentage contribution of the nocturnal sleep component to the daily output at 73.0 ± 7.2 (n=13), 75.6 ± 9.3 (n=13), 72.6 ± 11.4 (n=12) and 74.2 ± 11.6 (n=12), respectively; no clearly defined seasonal changes in excretion pattern were evident.

Daily urinary 6SM excretion for 2 women throughout a menstrual cycle was 19.3 ± 2.2 (n=30) and 24.2 ± 3.1 (n=27) μg 24 h^{-1} with the percentage contribution of the nocturnal sleep component to the daily output at 75.5 ± 5.8 (n=30) and 81.5 ± 4.2 (n=27), respectively; no discernible rhythms in excretion pattern were detected throughout either cycle.

Urinary 6SM excretion for women in the first, second and third trimesters of pregnancy was 20.5 ± 4.5 (n=5), 22.7 ± 7.5 (n=6) and 18.3 ± 6.5 (n=7) μg 24 h^{-1} with the percentage contribution of the

nocturnal sleep component to the daily output at 80.1 ± 7.7 (n=5), 76.1 ± 7.6 (n=4) and 75.2 ± 6.2 (n=7), respectively. No marked changes in excretion pattern of women repeatedly sampled during the course of pregnancy, parturition and early lactation were revealed.

No 6SM was detected in either full term amniotic fluid or neonatal urine in man. Urinary 6SM excretion (approximately 0.3 µg for the nocturnal period) was first observed in a 16 week old boy. In a 12 month old boy urinary 6SM output was 17.4 µg from 1930-0800 h and 0.7 µg from 0830-1000 h. Adult levels and pattern of urinary 6SM excretion were also observed in children aged 2-6 years.

STATEMENT

This thesis contains no material previously submitted for a degree or diploma in any university, and to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference is made in the text.

A. J. FELLENBERG.

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PUBLICATIONS

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Fellenberg, A. J., Phillipou, G. and Seamark, R. F. (1980). Measurement of urinary production rates of melatonin as an index of human pineal function. Endocrine Research Communications, 7, 167-175.

Fellenberg, A. J., Phillipou, G. and Seamark, R. F. (1981). Urinary 6-sulphatoxy melatonin excretion and melatonin production rate: studies in sheep and man. In Pineal Function, pp 143-149. Eds. C. D. Matthews and R. F. Seamark. Amsterdam: Elsevier/North Holland Biomedical Press.

Matthews, C. D., Kennaway, D. J., Fellenberg, A. J., Phillipou, G., Cox, L. W. and Seamark, R. F. (1981). Melatonin in man. Advances in the Biosciences, 29, 371-381.

Seamark, R. F., Kennaway, D. J., Matthews, C. D., Fellenberg, A. J., Phillipou, G., Kotaras, P., McIntosh, J. E. A., Dunstan, E. and Obst, J. M. (1981). The role of the pineal gland in seasonality. Journal of Reproduction and Fertility, Supplement 30, 15-21.

Fellenberg, A. J., Phillipou, G. and Seamark, R. F. (1982). Urinary 6-sulphatoxy melatonin excretion during the human menstrual cycle. Clinical Endocrinology, 17, 71-75.

C H A P T E R 1

INTRODUCTION

CHAPTER 1

INTRODUCTION

SECTION I - GENERAL ASPECTS

HISTORICAL CONSIDERATIONS:

When Kitay and Altschule published their comprehensive review of the pineal literature in 1954, they were able to draw two conclusions; firstly, that the mammalian pineal gland was metabolically active and secondly, that its function was related to that of the gonads (Kitay and Altschule, 1954a). The first insight into how this relationship might be effected stemmed from the studies of Lerner and co-workers, who in 1958 succeeded in isolating from bovine pineal glands a skin lightening factor which had been known for forty years (Lerner et al., 1958). This substance, which they named melatonin because of its structural relationship to serotonin and its effect on melanin pigmentation, was subsequently shown to have anti-gonadotrophic effects (Wurtman et al., 1963).

The nascent pineal science developed rapidly after the explosive impact of the discovery of melatonin, resulting in the evolution of exciting new concepts. In 1965, Wurtman and Axelrod termed the mammalian pineal gland a 'neuroendocrine transducer' - an organ which converts a neural signal concerning environmental photic information, received indirectly from the eyes, into an endocrine signal. Furthermore, they formulated the 'melatonin hypothesis' in which they proposed that melatonin was the hormone secreted by the pineal gland in response to environmental lighting and which was responsible for the

observed gonadal effects (Wurtman and Axelrod, 1965).

These formulations were largely responsible for the bulk of pineal research being concerned with the role of melatonin and related methoxy indoles in pineal function. Despite this, a number of physiologically active 'polypeptide' substances, most of which are incompletely characterised, have been isolated from pineal tissue (Benson, 1977; Wurtman and Moskowitz, 1977).

At the time that work on this project commenced (latter half of 1977), no known pineal compound satisfied all the classical hormonal criteria, i.e., a substance produced exclusively in the pineal gland and having a specific functional influence in a distant target organ(s) (Benson, 1977). At that time plasma and urinary levels of melatonin represented the best available index of mammalian pineal function. In humans, however, such studies were in their infancy (Wurtman and Moskowitz, 1977).

DEVELOPMENT AND ANATOMY:

The epiphysis cerebri of lower vertebrates is a structure with a sensory function containing photoreceptor cells but, in the process of evolution the mammalian pineal has lost its photoreceptive capacity and become a secretory organ (Collin, 1971). Furthermore the pineal has migrated away from the roof of the third ventricle, subsequently losing all connection with the brain except for a thin stalk.

The human pineal gland is a single, small cone shaped organ centrally located deep in the brain (Figure 1). It develops from the roof of the brain in a median region of the diencephalon, at first as a single shallow evagination early in the embryonic phase of human

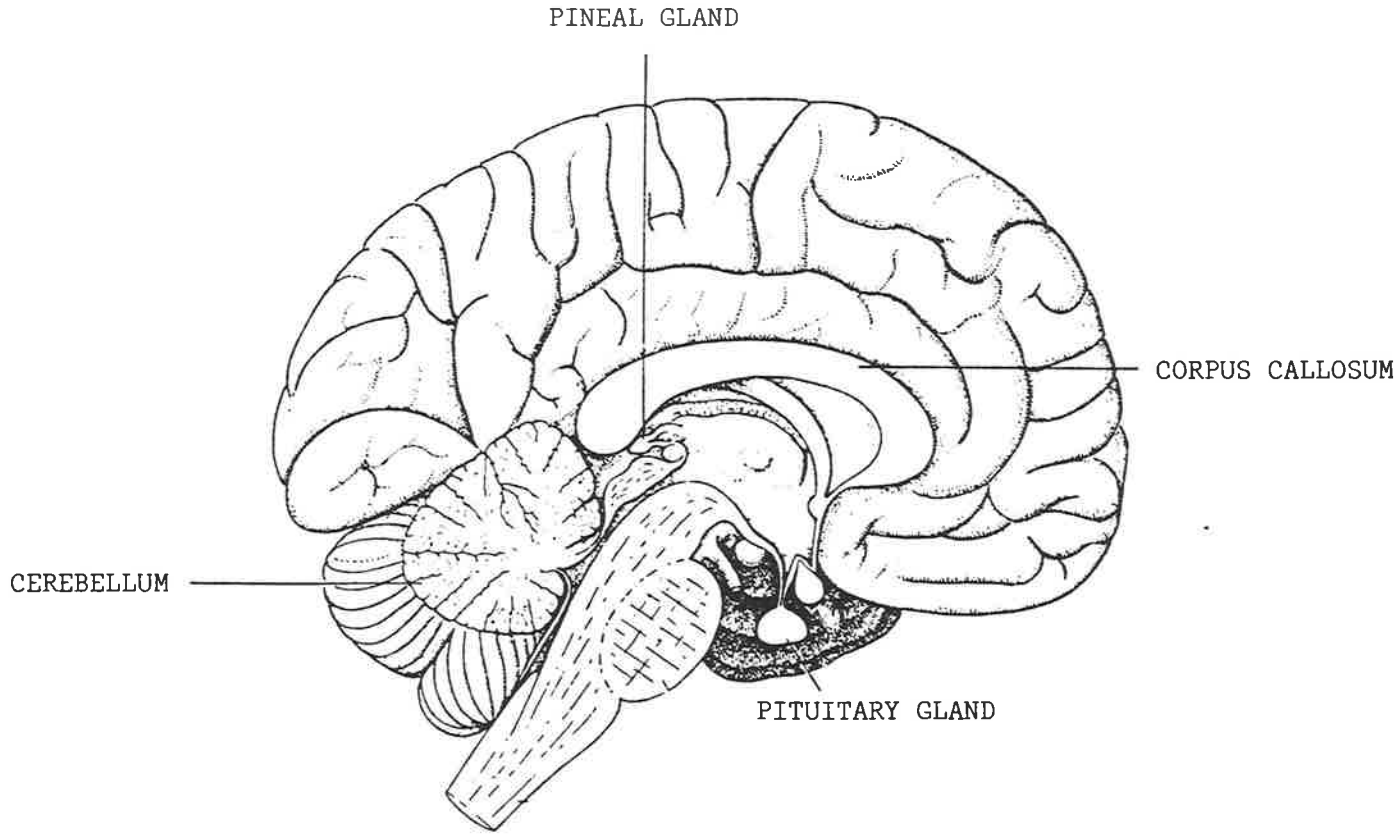


FIGURE 1. Median section of the human brain.

development, at about thirty-three days after ovulation (O'Rahilly, 1968). The neuroepithelial proliferation forming the pineal consists of cords and follicles of cells invested with embryonic mesoderm. The neuroepithelial cells of the pineal give rise to the parenchymal cells and spongioblasts of the organ, and the embryonic mesoderm or meningeal mesenchyme gives rise to the stromal tissue, which consists of connective tissue and contained blood vessels (Quay, 1974a). The pinealocyte or pineal parenchymal cell is the cell-type that is unique to the pineal and usually comprises the majority of the cells within this gland.

Adult form and nearly adult size of the human pineal gland are evident in the newborn. Cytological evidence indicates that pinealocyte differentiation is complete by five to seven years after birth (Quay, 1974a).

Enzymic studies (Wurtman et al., 1964; Otani et al., 1968) and histological investigations (Wildi and Frauchiger, 1965; Tapp and Huxley, 1972) indicate that the human pineal gland is active throughout life despite its tendency to calcify.

The human pineal gland is an extremely well vascularized organ with numerous capillaries and venules (Quay, 1974a).

During ontogenesis histological and cellular changes indicate a development from photoreceptor cell to secretory cell similar to those seen as a result of the evolutionary process. Studies on fetuses from humans, sheep and rabbits revealed a nerve, present during ontogenesis, which can be related phylogenetically to a possible homologous structure present in lower vertebrates (Møller, 1978 and 1979). The nerve

connects the pineal gland with the rostral part of the tectal area and was demonstrated in those human fetuses having a crown rump length of 56 mm. It was still present in fetuses of 169 mm crown rump length but not postnatally. The gestational age in which the nerve degenerates has not yet been determined (Møller, 1979).

Despite its central location deep in between the cerebral hemispheres no sensory or afferent nerve fibres from the mammalian pineal extending to the central nervous system are known at any age after birth. Pineal innervation is thought to be exclusively autonomic and to consist largely of post-ganglionic sympathetic fibres from the superior cervical ganglia (Kappers, 1965; Kenny, 1965; Wurtman et al., 1968a). In rats the central neural pathway whereby information from the eyes reaches the superior cervical ganglia has been partly identified (Moore and Lenn, 1972; Moore and Klein, 1974). Photic input from the eyes traverses the retinohypothalamic tracts to the suprachiasmatic nuclei (SCN). From these nuclei information passes through the lateral hypothalamus and brain stem, in unidentified pathways, to the spinal cord and interomediolateral cell column whose neurons provide preganglionic fibres to the superior cervical ganglia.

The absence of direct neural connections indicates that the pineal gland mediates its effects via hormonal principles. Electron microscope studies affirm the uniqueness and probable primary functional significance of the pinealocyte. This has the modifications and the attributes of a metabolically active endocrine secretory cell (Quay, 1974b).

MELATONIN BIOSYNTHESIS:

Knowledge of the function of the pineal gland in man has lagged considerably behind that of other mammals readily available for

experimentation and by necessity any proposed specific human pineal function has been primarily made on inference. Since the methods of studying the general physiology, and particularly the biochemistry of the pineal gland in animals cannot be easily applied to human subjects in vivo, much of the information about the human pineal is obtained from necropsy specimens. The mammalian species most extensively studied is the rat. Since the discovery of melatonin, attention has been focussed mainly on this and related indole compounds. Mammalian pineal indole biosynthetic pathways are indicated in Figure 2.

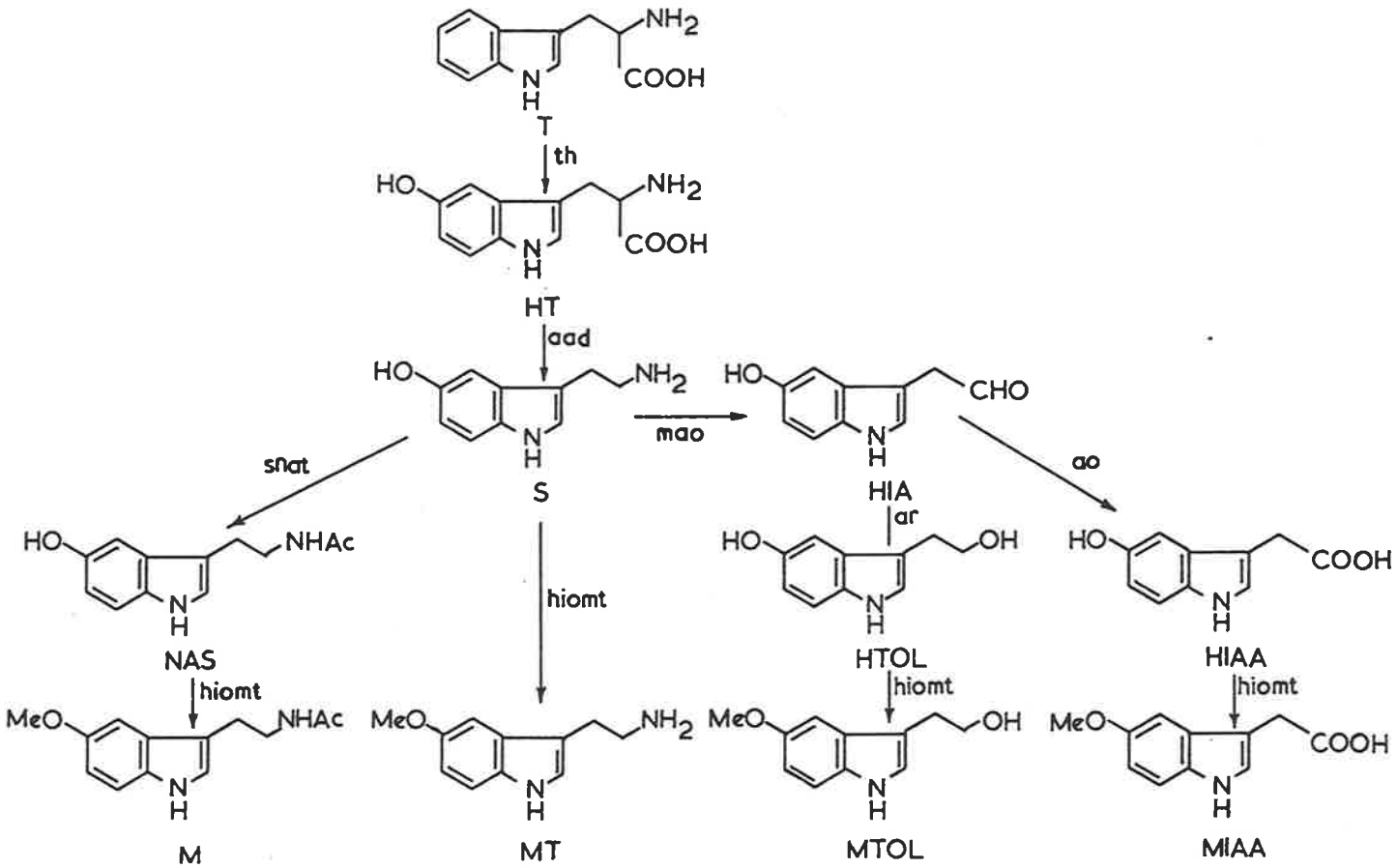
Melatonin biosynthesis is initiated by the uptake of the essential amino acid tryptophan into pineal parenchymal cells (Quay, 1963; Wurtman et al., 1968a). The tryptophan is converted to another amino acid 5-hydroxytryptophan, through the action of the enzyme tryptophan hydroxylase, and then to serotonin. This compound is present in high concentrations in the pineals of most mammals including man (Giarman et al., 1960; Quay, 1963; Wurtman et al., 1968a; Hinterberger and Pickering, 1976). Serotonin is converted to melatonin in a two step process by two enzymes which are characteristic of the pineal: serotonin N-acetyltransferase (SNAT), which converts serotonin to N-acetylserotonin, acetyl CoA serving as donor (Weissbach et al., 1960), and hydroxy indole-O-methyltransferase (HIOMT) which transfers a methyl group from S-adenosylmethionine to the 5-hydroxyl of N-acetylserotonin (Axelrod and Weissbach, 1960). A 24 hour organ culture study established, however, that melatonin is a relatively minor product of serotonin metabolism in the pineal gland (Klein and Notides, 1969).

HIOMT activity and melatonin have been identified in both normal pineal glands and pinealoma tissue obtained at autopsy (Wurtman et al., 1964; Wurtman and Kammer, 1966; Hinterberger and Pickering, 1976). The melatonin content of human pineal glands has been estimated at between 50 and 400 ng g^{-1} (Wurtman and Cardinali, 1974).

FIGURE 2. Mammalian Pineal Indole Biosynthesis.

T	TRYPTOPHAN	th	tryptophan hydroxylase
HT	5-HYDROXYTRYPTOPHAN	aad	aromatic amino-acid decarboxylase
S	SEROTONIN	mao	monoamine oxidase
HIA	5-HYDROXY INDOLE ACETALDEHYDE	ar	aldehyde reductase
HTOL	5-HYDROXYTRYPTOPHOL	ao	aldehyde oxidase
HIAA	5-HYDROXY INDOLE ACETIC ACID	snat	serotonin N-acetyltransferase
NAS	N-ACETYLSEROTONIN	hiomt	hydroxy indole-O-methyltransferase
MIAA	5-METHOXY INDOLE ACETIC ACID		
MTOL	5-METHOXYTRYPTOPHOL		
MT	5-METHOXYTRYPTAMINE		
M	MELATONIN		

A recent finding has been that of O-acetyl-5-methoxytryptophol in rat pineal; a substance that is rapidly hydrolysed on incubation in human plasma (Smith et al., 1980).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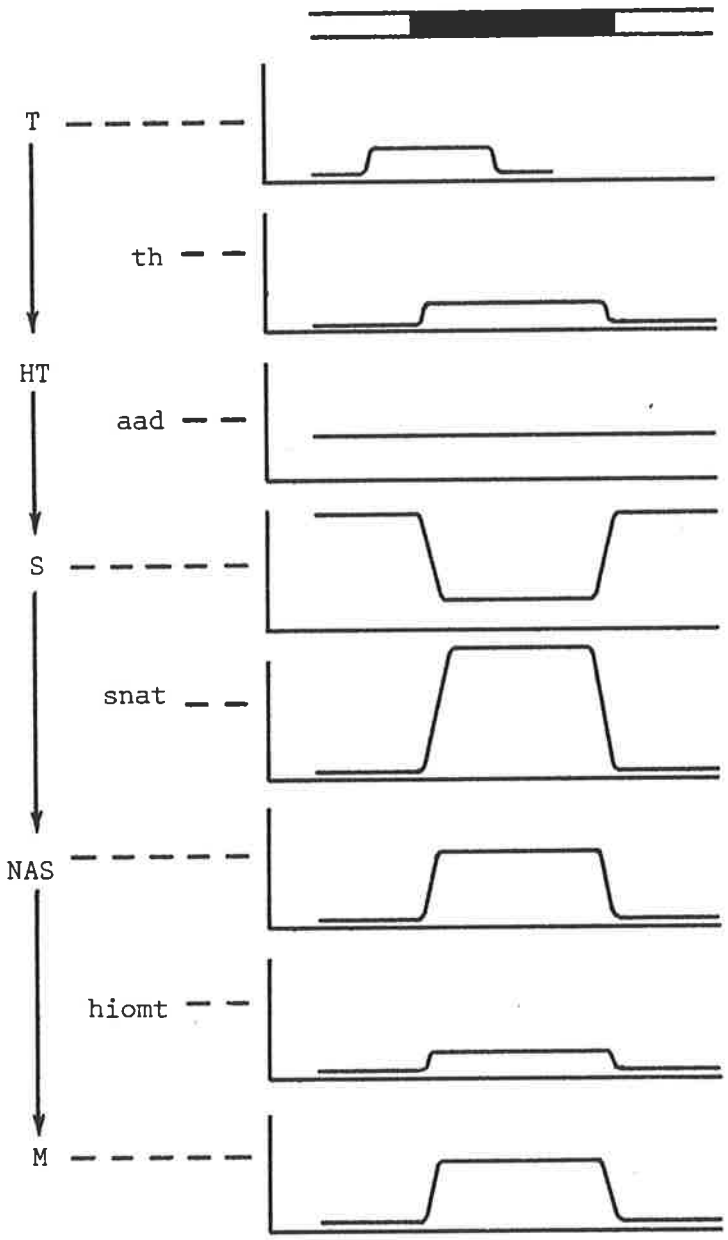
MELATONIN RHYTHMS:

Many of the indoles and enzymes of the mammalian pineal gland are subject to cyclic variations largely dependent on the external lighting environment (Figure 3). Thus pineal serotonin exhibits a marked diurnal variation (Quay, 1963), such that maximal concentrations occur during the daylight hours and, with the onset of darkness falling dramatically as it is converted to melatonin and possibly other 5-methoxy indoles (Wurtman et al., 1968b; Lynch, 1971; Cattabeni et al., 1972). Following the onset of darkness there is an increase in SNAT activity by some 15-60 fold (Klein and Weller, 1970; Binkley, 1983) but only a 2-3 fold increase in HIOMT activity (Axelrod et al, 1965; Binkley, 1983).

In human pineals collected at post-mortem, the SNAT and HIOMT activities were found to be higher in individuals who died between 2400-0400 hours than between 1200 and 1800 hours (Smith et al., 1977a).

Melatonin has been absolutely identified by gas chromatography-mass spectrometry (GCMS) in human plasma and cerebrospinal fluid (CSF) (Smith et al., 1976a). The concentrations of endogenous melatonin in human urine, blood and CSF have been measured by bioassay (Pelham et al., 1973; Lynch et al., 1975a), fluorimetry (Grishchenko et al., 1976; Dudko and Schcherbina, 1977), radioimmunoassay (RIA) (Arendt et al., 1975; Kennaway et al., 1977a; Smith et al., 1977a) and GCMS (Wilson et al., 1977). In all cases urine and blood melatonin levels rose at night in synchrony with the rhythms in pineal melatonin synthesizing enzymes obtained at autopsy.

In both diurnal and nocturnal species, pineal production of melatonin occurs almost exclusively during night time darkness



T	TRYPTOPHAN	th	tryptophan hydroxylase
HT	5-HYDROXY TRYPTOPHAN	aad	aromatic amino-acid decarboxylase
S	SEROTONIN	snat	serotonin N-acetyltransferase
NAS	N-ACETYL SEROTONIN	hiomt	hydroxy indole-O-methyltransferase

FIGURE 3. Mammalian Pineal Circadian Rhythms (Modified after D. Klein and J. Weller, Science 169, 1093 (1970).

(Binkley, 1979). Studies in animals indicate that this pattern is consistent with the suppression of melatonin production by environmental light (Axelrod et al., 1965; Klein and Weller, 1972), but that in constant darkness it persists (Ralph et al., 1971), and is driven by an endogenous pacemaker, thought to be located in the suprachiasmatic nuclei (SCN) of the hypothalamus (Moore and Eichler, 1972; Stephan and Zucker, 1972; Moore and Klein, 1974). The circadian rhythm in the hypothalamic pacemaker is synchronized to the environmental light/dark cycle, through input from the retino hypothalamic track which transmits photic information from the retina to the SCN (Moore and Lenn, 1972). The human pineal appears to be regulated in the same way as is the pineal of other mammalian species, because it was demonstrated that bright artificial light of the intensity of indirect sunlight suppressed nocturnal melatonin secretion in humans (Lewy et al., 1980b). Furthermore, it appears that the same neuroanatomical pathways mediate melatonin secretion in humans as in other mammals (Moore, 1979), as an area in the human brain which is anatomically homologous to the SCN has been described (Lewy et al., 1980b). Moreover, administration of the beta blocking drug propranolol to humans inhibits the nocturnal increase in melatonin secretion (Vaughan et al., 1976; Hansen et al., 1977) indicating that in man, as in rats (Axelrod, 1974), this increase is mediated by sympathetic neurones. Previous attempts to suppress human melatonin secretion (Jimerson et al., 1977; Arendt, 1978b;Lynch et al., 1978; Wetterberg, 1978; Akerstedt et al., 1979; Vaughan et al., 1979a) probably failed because the light was insufficiently intense (Lewy et al., 1980b). Evidence that the light/dark cycle entrains the melatonin circadian secretory rhythm in humans was provided by studies in which this rhythm was forcibly desynchronized by rapid travel across time zones

(Wetterberg, 1978; Fevre-Montange et al., 1981), or by an abrupt phase shift in the light dark cycle (Lynch et al, 1978; Lewy et al., 1980b); under those conditions approximately one week was required for complete re-entrainment of the melatonin circadian secretory rhythm to local time. Further evidence of the importance of light for the proper synchronization of the circadian rhythm of melatonin secretion was provided by the finding of unusual melatonin circadian secretory rhythms in some blind people (Smith et al., 1981b; Lewy and Newsome, 1983). Moreover, abnormal melatonin circadian secretory rhythms have also been associated with psychiatric conditions (Lewy et al., 1979; Mendlewicz et al., 1979; Wetterberg, 1981; Ferrier et al., 1982), and an enhanced inhibition of melatonin by light has been reported in depression (Lewy et al., 1981).

An association between the pineal gland and psychiatric illness has long been suspected (Mullen and Silman, 1977). The nyctohemeral variation in urinary melatonin excretion seemed normal in five drug-free depressed patients and continued normally through one night of sleep/dark deprivation (Jimerson et al., 1977). In another study (Mendelwicz et al., 1979), however, the nocturnal increase in plasma melatonin found in normal subjects was essentially absent in three of four depressed patients and was not affected by subsequent treatment with antidepressant drugs. Wetterberg et al., (1982) found disturbed 0200 h cortisol and melatonin secretion in acutely depressed patients exhibiting an abnormal dexamethasone suppression test but not in those with a normal response. Another report (Lewy et al., 1979) showed higher night time and daytime plasma melatonin in four manic patients compared to four normal, without specification of age, sex, or medication.

An interesting report (Lewy et al., 1981) showed unusual sensitivity of manic-depressive patients to light-induced reduction in night time

plasma melatonin. These workers were apparently able also to terminate a depressive episode in one manic-depressive patient by using strong light for ten days placed at the beginning and the end of the usual light phase (Lewy et al., 1982).

Chlorpromazine administration to psychiatric patients produced high concentrations of plasma melatonin with little rhythm (Smith et al., 1977b and 1979). Previous rat studies demonstrated that this drug slowed the disappearance of labelled and unlabelled melatonin from, and raised endogenous melatonin levels in, the plasma and the pineal (Wurtman and Axelrod, 1966; Ozaki et al., 1976); presumably by inhibition of the hepatic enzymes that catabolize melatonin (Wurtman et al., 1968c). However, psychiatric patients (diagnosis not given) not on chlorpromazine had lower than normal plasma melatonin sampled over 24 h (Smith et al., 1979). Reduced 2400 h plasma melatonin (compared to controls) was also seen in untreated chronic schizophrenic patients (Ferrier et al., 1982), but interpretation is complicated, since lower body weight in the schizophrenics might also have accounted for the lower melatonin.

As well as displaying a circadian rhythm, human circulating and urinary levels of melatonin have also been reported to exhibit menstrual cycle and circannual rhythms (Wetterberg et al., 1976; Dudko and Shcherbina, 1977; Arendt et al., 1977a; Tapp et al., 1980; Birau et al., 1981; Penny, 1982).

The age at which the circadian rhythm in melatonin secretion first appears in man is not defined. In a study of daytime plasma melatonin levels in children, this indole was reported to be present in the youngest subject aged four years (Smith et al., 1976b). A developmental study in neonatal rats indicated that the rhythm in pineal gland SNAT

activity is essentially absent at birth, with the large day-night difference in activity characteristic of the rhythm in adults first appearing during the second week of life (Yuwiler et al., 1977). A sheep study, however, revealed an accumulation of melatonin correlating with a rise in HIOMT activity in the foetal sheep pineal gland during the last few days of gestation (Kennaway et al., 1977b).

SOURCE OF CIRCULATING MELATONIN:

The effect of pinealectomy (Px) on circulating levels of melatonin in man has not been studied.* Evidence for the pineal gland as the sole source of circulating melatonin in animals is conflicting. A sensitive bioassay failed to detect melatonin both in the plasma of Pxd chickens (Pelham et al., 1972) and rats (Pang and Ralph, 1975). Melatonin as measured by RIA, however, has been reported to be present in the plasma of Pxd sheep (Kennaway et al., 1977a) and rats (Ozaki and Lynch, 1976). Furthermore, the presence of HIOMT activity and melatonin has been demonstrated in extra-pineal tissues of a number of animals and man (Barchas and Lerner, 1964; Quay, 1965; Cardinali and Rosner, 1971; Vlahakes and Wurtman, 1972; Raikhlin et al., 1975; Bubenik et al., 1977; Pang et al., 1977).**

* After this thesis was completed and in the preparative stage the first paper on the effect of pinealectomy on human circulating melatonin appeared in the literature (Neuwelt and Lewy, 1983).

** Near completion of this thesis a number of reports appeared in the literature demonstrating that pinealectomy abolished circulating melatonin in a number of animal species (Arendt et al., 1980; Lewy et al., 1980a; Markey and Buell, 1982; Tetsuo et al., 1982a).

MELATONIN METABOLISM:

Studies of exogenous melatonin, radioactively labelled and administered intravenously (i.v.) in man (Jones et al., 1969) and rodents (Kopin et al., 1961; Kveder and McIsaac, 1961; Taborsky et al., 1965; Jones et al., 1969) have indicated that 6-hydroxylation represents the major metabolic pathway for melatonin. A minor metabolite, previously only partly characterised (Kopin et al., 1961), has since been identified and shown to be the major product of melatonin metabolism in rat brain (Hirata et al., 1974). In man the urinary sulphate and glucosiduronate conjugates of 6-hydroxmelatonin (6HM) account for 59-79% and 13-27% respectively of the administered melatonin, with 1-15% unidentified but probably mostly N-acetyl-5-methoxykynurenamine (Hirata et al., 1974), and 1.4% as free 6HM (Jones et al., 1969). Furthermore, over 90% of the administered radioactivity was recovered in the first 24 hour urine sample and the remainder in the next 24 hours (Jones et al., 1969). No unchanged melatonin was detected (Jones et al., 1969).

Rodent studies indicate that within 1 minute of i.v. injection, labelled melatonin was found to be present in all of a large number of tissues examined; the highest concentrations were found in liver and kidney, and the lowest in fat and skin (Kopin et al., 1961). Melatonin was also found in the brain, indicating there is little hindrance to its crossing the blood brain barrier (Kopin et al., 1961). Further experiments indicated a multiphasic disappearance curve for melatonin, with a rapid decrease in the first 10 minutes (approximating to a half life of 2 minutes) and after 40 minutes a much slower rate corresponding to a half life of about 35 minutes (Kopin et al., 1961).

Little information is available about the fate of endogenous melatonin in humans. The route by which melatonin is secreted from the pineal gland is unknown (Wurtman and Moskowitz, 1977). Very low (pg ml^{-1}) levels of melatonin are present in the circulation. Most of this melatonin is bound to serum albumin, although weakly, as this complex is readily dissociable (Cardinali et al., 1972).

In man the very low levels of endogenous circulating melatonin indicate facile metabolism of this indole. As the liver is the major site of the bio-transformation of many endogenous and exogenous compounds in man (Fingl and Woodbury, 1975; Remmer, 1976) and as rat liver preparations have the capacity to hydroxylate melatonin (Kopin et al., 1961), it is probable that this organ is mostly responsible for the efficient metabolism of melatonin. Under these conditions it is likely that the metabolic clearance rate (MCR) of melatonin approximates to that of the hepatic blood flow in man, at about 2000 l 24 h^{-1} (Ganong, 1977). MCR is defined as the volume of blood completely and irreversibly cleared of hormone in unit time (Tait, 1963). MCR, production rate and blood concentration of a hormone are related by the equation:

Production rate = MCR x blood concentration (Tait, 1963)

The enzyme systems concerned in bio-transformation are primarily located in the hepatic smooth endoplasmic reticulum (Fingl and Woodbury, 1975). Aromatic hydroxylation is catalysed by a special enzyme system referred to as mono-oxygenase or mixed function oxidase. This microsomal hydroxylating system is known to consist of at least two catalytic components: a cytochrome of b-type called P-450 and the flavoprotein catalysing the reduction of this cytochrome by NADPH,

termed NADPH cytochrome P-450 reductase (Vainio, 1976).

Glucosiduronate formation is catalysed by various microsomal glucosiduronyl transferases, with uridine diphosphate-glucosiduronic acid as the donor of glucosiduronic acid (Fingl and Woodbury, 1975; Dutton et al., 1977). It is generated from glucose by enzymes in the cytosol. The greater amount of carbohydrate available for conjugation as compared with sulphate or amino acids, and the increased polarity of the conjugate over the aglycone, ensuring more rapid excretion from the organism, are two of the reasons put forward to account for the quantitative importance in general of this conjugation process (Dutton et al., 1977).

Sulpho conjugation requires considerable expenditure of energy, as well as of sulphate itself (DeMeio, 1975; Dodgson, 1977). Two molecules of adenosine triphosphate (ATP) are expended in activation of one sulphate molecule via the enzymes ATP sulphurylase and adenosine 5'-phosphosulphate kinase to 3'-phosphoadenosine-5'-phosphosulphate (PAPS). Finally, transfer of sulphate to a phenol group is catalysed by the enzyme phenolsulphotransferase. The two enzymes involved in the activation of sulphate together with phenosulphotransferase are non-microsomal enzymes located in the cytosol (Fingl and Woodbury, 1975; Remmer, 1976). The primary normal source of sulphate appears to be derived from the oxidation of the amino acid cysteine (Singer, 1975).

The absence of radioactivity in the faeces following i.v. administration of radioactive melatonin (Jones et al., 1969) indicates that neither unchanged melatonin nor its metabolites are excreted via this route in man.

As administration of exogenous tracer revealed the virtual absence of unchanged melatonin in the presence of mainly 6HM metabolites in human urine, the report of very small quantities (pg ml^{-1}) of endogenous urinary melatonin (Lynch et al., 1975a) indicates that the endogenous urinary melatonin metabolites in man are likely to be present at approximately 1000 fold higher concentration. Furthermore, as urinary melatonin appeared to closely reflect circulating levels of melatonin (Lynch et al., 1975a; Jimerson et al., 1977), then the urinary melatonin metabolites too could be expected to behave in a similar fashion.

MELATONIN AND REPRODUCTIVE PHYSIOLOGY:

In mammals the best documented physiological action of pineal extracts related to the inhibition, directly and via the pituitary, of sexual maturation and cyclical sexual activity (Minneman and Wurtman, 1976; Benson, 1977; Reiter and Vaughan, 1977). Because exogenous melatonin produced similar effects, some authors have considered this substance to be responsible for many of the biological activities attributed to this gland (Minneman and Wurtman, 1976). Other authors have considered that it is premature, however, to classify any pineal constituent, be it a polypeptide or an indole, as the anti-gonadotrophic factor of the mammalian pineal gland (Benson, 1977; Reiter and Vaughan, 1977). Furthermore, melatonin when administered under certain conditions has been shown to be pro-gonadotrophic or counter anti-gonadotrophic (Reiter and Vaughan, 1977), as have a number of other polypeptide substances isolated from pineal glands (Benson, 1977; Wurtman and Moskowitz, 1977).

Since the turn of this century endocrine actions have been attributed to the human pineal gland solely on the basis of reproductive disturbances associated with tumours of this structure. Destructive pineal tumours have been associated with precocious puberty, whereas hyperactive tumours have been associated with delayed puberty (Kitay, 1954b). This has led to the suggestion that the human pineal gland may produce a substance that holds sexual maturation in check (Kitay, 1954b). Support for this suggestion came later with reports of the inhibitory action on sexual maturation of both pineal extracts and melatonin in mammals, but disagreement regarding the correlation of pineal neoplasms with hypothalamic damage has persisted (Reichlin, 1974; Wurtman and Cardinali, 1974). The potential use of melatonin in the differential biochemical diagnosis of histologically heterogeneous tumours remains unresolved (Arendt, 1978a; Barber et al., 1978a,b; Tapp, 1978; Kennaway et al., 1979). High levels of melatonin have been observed in histologically confirmed pinealocytoma but also in non parenchymal tumours (Tapp, 1978) bringing the specificity and interpretation of such data into question.

There is now compelling evidence implicating the pineal gland in the mediation of the control by day length (photoperiod) of seasonal reproduction in a number of mammalian animal species (Reiter, 1980; Bittman et al., 1983). Furthermore, the results of recent melatonin feeding experiments strongly suggest that this substance, released from the pineal gland during the hours of darkness, relays the photoperiodic time cues through which seasonal species respond to the annual cycle in day length (Kennaway et al., 1982b; Arendt et al., 1983; Lincoln, 1983). Of the two models that have been proposed to account for the actions of melatonin in photoperiodic time measurement, it is the 'duration' model,

in which it is the total duration of melatonin exposure each day, rather than the 'time of day' model, in which it is the timing of a daily rhythm in sensitivity to melatonin relative to the period of melatonin secretion, that is currently favoured by experimental evidence to dictate the response in mammalian animals (Lincoln, 1983).

Although man has been described as a 'virtually non-seasonal breeder' (Arendt et al., 1979), such a conclusion is almost certainly based on observations made on people living at temperate latitudes. Evidence from studies conducted near the Arctic circle, however, indicates man is a seasonal breeder at those extreme latitudes (Vaughan et al., 1978) where the seasonal changes in photoperiod are much greater (Folk, 1978).

The role, if any, of melatonin in human reproductive function remains to be evaluated (Wurtman and Moskowitz, 1977). Induction of drowsiness is the only clearly demonstrable effect of pharmacological amounts of melatonin administered orally to man (Anton-Tay et al., 1971; Cramer et al., 1974; Nordlund and Lerner, 1977). Three preliminary studies indicate that endogenous melatonin may have a role in human reproductive physiology. In those studies menstrual cycle variations in early morning plasma melatonin levels (Wetterberg et al., 1976) and daily urinary melatonin excretion (Dudko and Shcherbina, 1977) were reported, while the other indicated alterations in daily urinary melatonin output during pregnancy, parturition and early lactation (Grishchenko et al., 1976).

OBJECTIVES:

The aim of this project was to develop methodology based on isotope dilution GCMS to study melatonin production in man. This was to be accomplished by accurate quantitation of the unique melatonin metabolite 6-sulphatoxy melatonin (6SM) in urine. It was anticipated that application of this assay methodology would provide the answers to the following questions:

(a) the manner of metabolism of melatonin to 6SM,

(b) the importance of the pineal gland as a source of melatonin, and

(c) whether melatonin has a role in human reproduction.

SECTION II - ANALYTICAL CONSIDERATIONS

TECHNICAL:

RIA procedures have recently superseded bioassay and fluorometric techniques for the measurement of melatonin in humans because those were too insensitive for clinical studies (Cattabeni et al., 1972; Lynch et al., 1975a; Kennaway et al., 1977) and too laborious for the ready analysis of large numbers of samples (Cattabeni et al., 1972; Arendt et al., 1975; Kennaway et al., 1977a; Wilson et al., 1977). The major problem in RIA of melatonin, however, is that of specificity. Extensive purification of extracts is necessary to improve the specificity of RIA, and validation against accurate techniques is required to demonstrate its specificity (Arendt et al., 1975; Lynch et al., 1975b; Smith et al., 1976a; Kennaway et al., 1977a; Wilson

et al., 1977; Wurtman and Moskowitz, 1977). With RIA techniques, however, it is not possible to be certain that only melatonin is being measured. Only GCMS has been shown to be capable of both identification (Smith et al., 1976a) and quantitation of endogenous melatonin (Wilson et al., 1977). The accuracy of this GCMS procedure, however, could be further improved by using a stable isotope labelled analogue of melatonin as internal standard, i.e. isotope dilution GCMS (Breuer and Siekmann, 1975; Björkhem et al., 1976; Watson, 1976a; Horning et al., 1977), and by conversion of melatonin to a derivative of high molecular weight (Breuer and Siekmann, 1975; Björkhem et al., 1976), not so susceptible to background interference. It is now well accepted that GCMS methodology, particularly isotope GCMS, ranks highest amongst the accurate techniques available in trace analysis (Breuer and Siekmann, 1975; Björkhem et al., 1976; Horning et al., 1977; Siekmann, 1979; Markey, 1981). Its use in quantitation of plasma melatonin levels consistently revealed very low concentrations of melatonin during the day (Lewy and Markey, 1978; Lewy et al., 1980b) in contrast to the much higher daytime levels obtained by the use of other techniques (Arendt et al., 1977b; Weitzman et al., 1978; Wetterberg, 1978; Brown et al., 1979; Weinberg et al., 1979). The use of GCMS, as for any technique, however, is related to the competence of the user, a fact clearly exposed by Markey, 1981.

SAMPLING:

As a result of the marked nocturnal rise in circulating melatonin levels, meaningful description of melatonin secretion obtained from plasma levels would require frequent sampling techniques, which are unacceptable for long term clinical studies. The study implicating

melatonin in the regulation of the human menstrual cycle (Wetterberg et al., 1976) is based on daily measurement of this methoxy indole in single early morning plasma samples. Sampling at a time when the plasma melatonin level is falling (Arendt et al., 1977b) subjects this measurement to two sources of error, firstly, from variations in the timing of collection, and secondly, from alterations in phase of the circadian rhythm of melatonin i.e. alteration of the time at which the nocturnal maximum level of melatonin is achieved. Furthermore, in view of the marked nocturnal rise in melatonin secretion, interpolation from data based on an early morning single point measurement to provide a measure of daily melatonin secretion is limited.

Accurate measurement of the extremely low (pg ml^{-1}) levels of circulating and urinary melatonin has proven difficult to accomplish. The preliminary studies implicating melatonin in human reproduction (Grishchenko et al., 1976; Wetterberg et al., 1976; Dudko and Shcherbina, 1977) and those indicating that it may not derive exclusively from the pineal gland (Lynch et al., 1975b; Ozaki and Lynch, 1976; Kennaway et al., 1977a) need to be re-evaluated with the use of specific techniques, in light of the limitations of some of the preceding methods. As the major urinary melatonin metabolite, 6SM, is expected to closely reflect circulating melatonin levels and to be present in much higher concentration (ng ml^{-1}), then its measurement should be facilitated and provide a convenient and non-invasive means of evaluation of melatonin secretion.

PRODUCTION RATE OF MELATONIN:

It is proposed to obtain a measure of the actual daily production

rate of melatonin. Production rates may be derived several ways from measurements made on either blood or urine following i.v. administration of isotopically labelled hormone (Tait, 1963; Loraine and Bell, 1971a,b). In this project the measurement of urinary production rate (UPR) of melatonin is to be accomplished by determining the specific incorporation of deuterium label into urinary 6SM following a single i.v. injection of deuterated melatonin.

The measurement of UPR has been applied widely in steroid studies and for this measurement to be valid several conditions must be met (Loraine and Bell, 1971a). These are:

1. The isotopically labelled hormone must equilibrate normally throughout the body and be metabolised in the same way as the endogenous hormone.
2. The label must be stable.
3. The metabolite measured must be 'unique' to the hormone measured.
4. The elimination in urine of the isotopic hormone must be virtually complete.
5. The dose of labelled hormone must be too small to exert any physiological effect.

This general procedure may be used for the measurement of secretion rate as well as production rate. It is important to differentiate between these two terms. If the hormone is mainly derived as a result of direct secretion by one of the endocrine glands then the measurement is of the secretion rate. If, on the other hand, the substance is derived from peripheral inter-conversion from precursors as well as from direct secretion, then the estimation is of the

production rate (Loraine and Bell, 1971b).

As it is not known which of the above conditions applies in regard to the metabolism of melatonin in man at present, the term production rate will be used.

SECTION III - GCMS CONSIDERATIONS

GCMS:

During the course of this study two MS were available for use. These were an AEI MS-30 and a Hewlett-Packard 5992B GCMS.

A detailed description of GCMS is outside the scope of this thesis and only the relevant points will be discussed.

GCMS combines the high resolving power of GC with the unique capability of MS to achieve high sensitivity with high selectivity for a specific compound. Thus, as well as high sensitivity, GCMS offers high specificity since the quantitated substance is simultaneously identified by GC retention time and the molecular weight (MW) of an ion. Isotope dilution GCMS is the only adequate technique presently available for the development of definitive reference methods (Breuer and Siekmann, 1975; Björkhem et al., 1976; Watson, 1976a; Horning et al., 1977).

GCMS consists of 3 basic components; a GC operating at 1 atmosphere exit pressure (760 Torr), a pressure reducing interface and an MS at operating pressure of 10^{-5} Torr.

GC

In the GC the components of a mixture are separated by virtue of their differential affinity for a high boiling point liquid (liquid phase) spread as a thin layer, either on finely divided support particles as in packed columns, or on the inside walls of capillary tubing for open tubular columns, and held in an oven at an elevated temperature - usually about 200°C. Thus GC is limited to the analysis of substances which are volatile and thermally stable. Many compounds unsuitable for GC analysis may be converted to suitable compounds by reaction with derivatizing agents (Breuer and Siekmann, 1975; Björkhem et al., 1976; Watson, 1976b; Horning et al., 1977). Derivatization may also be used to substantially increase the MW of the analyte, thereby substantially reducing susceptibility to background interference in GCMS analysis (Breuer and Siekmann, 1975; Björkhem et al., 1976).

In conventional GC analysis quantitation is accomplished by the addition of a known amount of an internal standard (usually a structural analogue of the analyte) to the sample which compensates for losses in the isolation procedure and for variation in the amount of material injected into the GC column (i.e. injection technique), but which has a different GC retention time to that of the analyte. Calculation of peak area or peak height ratios of the analyte and internal standard for the sample and comparison of this value with those values obtained from the construction of a standard curve (i.e. of known amounts of analyte and internal standard) allows quantitation of the amount of analyte in the sample.

In isotope dilution GCMS analysis the internal standard is a stable isotope (usually ^{2}H or ^{13}C) labelled analogue of the analyte. This

substance behaves in identical fashion to that of the analyte during the isolation procedure, and usually has a GC retention time that is virtually identical to that of the analyte. The MS, however, readily distinguishes the analyte from the stable isotope labelled internal standard; the molecular ions of the latter are usually 3 or 4 daltons higher than those of the analyte in order to avoid contribution from the naturally occurring heavy isotopes (particularly ^{13}c) of the analyte (Horning et al., 1977).

For use with the AEI MS-30, a Pye 104 series GC was provided. The GC for use in the other instrument was part of the Hewlett-Packard 5992B integrated GCMS instrumentation. Only packed columns were in use in both GCs.

THE INTERFACE:

A GC normally operates with an outlet pressure of one atmosphere and the MS should be operated at a pressure at least as low as 10^{-5} Torr. Thus, the interface system must reduce the pressure of the carrier gas by eight orders of magnitude and still convey a usable fraction of the sample to the MS, but without increasing the operating pressure in the ion source to levels that promote apparent mass discrimination or peak broadening (i.e. deterioration of resolution in the MS) (McFadden, 1973a; Watson, 1976c). This is accomplished by preferentially removing carrier gas molecules or the sample molecules from the GC effluent. An example of the former type of enrichment device is the jet separator while membrane separators constitute the latter type (McFadden, 1973a; Watson 1976c). It had been necessary to employ separators as the vacuum systems of both MSs were incapable of

efficiently coping with the carrier gas flow rates associated with the use of packed GC columns (McFadden, 1973a; Watson, 1976c). Both the AEI MS-30 and Hewlett Packard MS were interfaced to their respective GCs originally with membrane separators. The membrane separators proved unsuccessful for general use in the department because of the peak broadening and temperature dependence limitations their use imposed (McFadden, 1973a; Watson, 1976c). These deficiencies were subsequently eliminated with their replacement by all glass single stage jet separators (McFadden, 1973a; Watson, 1976c). The jet separators and connecting glass tubing, made as short as possible, were silanized to prevent adverse adsorptive effects (Watson, 1976c). Care was taken to ensure this assembly was able to be evenly heated at a temperature slightly above that of the GC oven and thus avoid 'cold spots'.

MS

A MS may be defined as an instrument that produces ions and then separates them according to their mass to charge (m/z) ratio. Both positive and negative ions are formed in the MS source or ionization chamber, but the majority of instruments are designed for the efficient formation and examination of positive ions only.

The basic components of a MS are an inlet system, ionization chamber, mass analyzer, vacuum system, amplifier and recorder.

ION PRODUCTION:

Many different methods have been devised for the production of

ions in MS (Watson, 1976d), but only electron impact (EI) and chemical ionization (CI) are widely used for analysis in the life sciences. Only these two processes will be discussed.

In an EI source the sample, in the vapour phase, is bombarded with a beam of electrons. Usually, the energy of this beam can be adjusted from about 5 to 80 eV, but by convention it operates normally at 70 eV. Of all the ions formed, only a small proportion are negatively charged. In the case of positive ions, most are singly charged, a few are doubly charged and very occasionally triply charged ions may be formed.

The formation of singly charged positive ions is accomplished as follows:

$$M + e \rightarrow (M)^{+}_{\cdot} + 2e$$

The $^{+}_{\cdot}$ notation for the species $(M)^{+}_{\cdot}$ implies that the latter is an ion radical, i.e. an ion with an odd number of electrons. Ions are considered to be more stable than ion radicals (Watson, 1976d).

The amount of energy necessary to remove an electron from M is called the ionization potential. When the electron beam is operating at 70 eV, an average of several electron volts of energy in excess of the ionization potential is transferred to the molecular ion. Thus in EI ionization these molecular ions are in an unstable, excited state because of excess energy adsorbed during ionization and because of the unpaired electron; it is this excess energy which causes the molecular ion to fragment. Thus typical EI mass spectra exhibit a large number of fragment ions in addition to the molecular ion. In some cases, the molecular ion decomposes so readily that it is not

observed in the spectrum (Watson, 1976d).

The stability of the molecular ion of a cyclic compound is usually much greater than that of an aliphatic compound (Watson, 1976d), and therefore its mass spectrum is likely to feature a much more prominent molecular ion than that of an aliphatic compound.

In CIMS the compound under investigation is ionized by reaction with a set of reagent ions. These reagent ions are formed from the reactant gas by a combination of EI ionization and ion-molecular collisions. The proportion of compound to reactant gas is usually of the order of 1 to 1,000 so that EI ionization of the compound does not occur. The reagent ions react with the sample mainly by proton transfer or hydride abstraction. Since the ions formed $(M + 1)^+$ or $(M - 1)^+$, often described as quasimolecular ions, are even electron species, they are inherently more stable than the $(M)^{+\cdot}$ ion produced by EI ionization. Furthermore, the amount of energy transferred to the quasimolecular ion is much lower than that transferred in an EI source, but is highly dependent upon the reagent gas used (Watson, 1976d). Because of these factors, the amount of fragmentation is usually greatly reduced, and the quasimolecular ion is normally the most intense ion in the CI mass spectrum.

In the two MSs available for use it was possible to examine only positive ions produced by EI ionization.

MASS ANALYZERS:

One of the more characteristic features of a MS is the method used to obtain mass dispersion of the ions produced in the ionization

chamber i.e. the mass analyzer. Although there are many different types of mass analyzers only a few are used in GCMS. Only the two most widely used types, i.e. magnetic and quadrupole designs will be discussed, as these are employed in the AEI MS-30 and Hewlett-Packard 5992B respectively.

A beam of ions of mass-to-charge ratio m/e is deflected on passage through a magnetic field according to the equation

$$m/e = H^2R^2/2V$$

where H is the strength of the magnetic field, R is the radius of the circular path into which the ions are deflected, and V is the voltage used to accelerate the ions out of the source (McFadden, 1973b; Watson, 1976e).

A mass spectrum can be produced by having a collector placed behind a narrow slit through which the ions must pass, in which case the magnetic field may be scanned downwards or upwards, in order that successively decreasing or increasing m/e values achieve the correct path to pass through the collector slit, the accelerating voltage being kept constant. Alternatively, the magnetic field can be held constant while the accelerating voltage is scanned upwards or downwards, allowing ions of successively increasing or decreasing m/e values to arrive at the slit.

To cover a wide range of m/e values magnetic scanning is used in most instruments since electric scanning can pose a number of problems (McFadden, 1973b; Watson, 1976e). Electric scanning, however, may be used satisfactorily over a narrow range of m/e values, and it has the advantages of being easier to control than magnetic scanning and of

having a linear relationship between the m/e value and the accelerating voltage. The technique of selected ion monitoring (SIM) in magnetic instruments almost always depends upon switching the accelerating voltage between the values necessary to collect selected ions while the magnetic field is kept constant (Watson, 1976a). For magnetic instruments the effective mass range for SIM is limited by the extent at which the accelerating voltage may be attenuated while sensitivity is maintained (McFadden, 1973b; Watson, 1976e). The magnetic mass spectrometer is prone to problems in stability of the mass scale in SIM work (Björkhem et al., 1976; Watson, 1976a; Aldercreutz, 1977).

Resolving power or resolution R is defined by:

$$R = M/\triangle M$$

where M is the mean m/e value of two adjacent ions and $\triangle M$ is the difference in m/e value between those ions (McFadden, 1973b).

The equation for ion separation given for single focussing magnetic instruments makes no provision for the fact that ions of the same m/e value can have a range of kinetic energies. In order to improve resolution, these ions should have a narrower band of kinetic energy. This state of affairs can be approached by passing the beam of ions through a radial electrostatic field, usually before the ion beam passes through the magnetic sector (McFadden, 1973b; Watson, 1976e).

Double focussing MSs are capable of static resolving powers greater than 10,000 (McFadden, 1973b). In the scanning mode, however, dynamic resolving powers are somewhat less than these, and are dependent upon scan speed and sample level, amongst other considerations. This limitation is imposed by the necessity of obtaining a reasonable

statistical representation of the peak shape at a given m/e value in the spectrum (McFadden, 1973b).

The AEI MS-30 is a double focussing, double beam MS (McFadden, 1973b). With the instrument employed, however, only one beam was utilized and resolving power was limited to only 1000 and 3000. Adjustment of the mass scale was done manually by introduction of the calibration compound perfluorokerosene via a reservoir inlet and adjusting instrumental parameters to focus ions of the m/e values of interest onto the detector.

The quadrupole mass filter consists of a quadrant of four parallel hyperbolic or circular rods. Opposite rods are connected to radio-frequency (rf) and direct current (dc) voltages, one pair of rods being 180° out of phase with the other. Only ions of one particular m/e value can pass down the centre of the assembly for a particular value of rf and dc voltages. A mass spectrum is obtained by sweeping the voltages from a low value to a high value at a constant rf/dc ratio (McFadden, 1973b; Watson, 1976e).

Quadrupole MSs are capable of scanning the mass spectrum in a few milliseconds if necessary, and, not requiring high voltage acceleration, can tolerate high source pressures, making them particularly suitable for GCMS work and CI. The quadrupole is very well suited for SIM because selected ions from any region of the mass spectrum can be monitored without altering the optimum conditions in the ion source or mass analyzer. Furthermore, the parameters (superimposed rf and dc fields) which control the mass scale can be changed rapidly with good response and are well stabilized throughout the mass range (Watson, 1976e). The resolving power of most commercial quadrupole instruments

is limited to unit resolution (Watson, 1976e).

Quadrupole MSs exhibit high mass discrimination i.e. high mass ions will be observed to have lower relative abundance (McFadden, 1973b).

ION DETECTION:

For ion detection, the electron multiplier is used in the vast majority of MS in current use. The fast response (negligible time constant) and high sensitivity (Typical gain of 10^5 or greater) make the electron multiplier indispensable in GCMS. Drawbacks of the electron multiplier are (a) the gain is variable depending on usage and operating conditions, and (b) it exhibits some mass discrimination i.e. a given number of ions of m/e 500 will not generate an output signal as great as an equal number of ions of m/e 50 (Watson, 1976f).

SENSITIVITY:

Many factors influence the sensitivity of MS analysis but, in essence, there are two aspects of MS that must be considered in defining sensitivity. One is the basic instrument sensitivity that gives the amount of current arriving at the collector for a specified sample consumption rate. The second is the method of handling the sample which thus determines the efficiency of sample utilization (McFadden, 1973b). The amount of sample consumed per unit time is a critical factor in evaluating MS performance. Ideally, sample should enter the ion chamber in a square wave fashion for a period of time that is only slightly longer than the scan time. In practice, this

is difficult, but in many ways GCMS offers the closest approach to ideal sample introduction (McFadden, 1973b). Actually, the percent of total GC effluent that can be put into the MS may vary widely depending on the carrier gas flow rate, the interface enrichment, and the MS pumping system. The true sample utilization is thus reduced by the sample introduction split or yield factor (McFadden, 1973b). Sensitivity is enhanced with improved GC column efficiency, as in capillary GC, because the sample enters the MS ionizing chamber contained in a sharper and more symmetrical peak.

Sensitivity is influenced by events taking place in the ionization chamber. The effectiveness of EI ionization increases rapidly with electron energy from 10 eV to approximately 20 eV for most organic compounds. Most reference spectra are obtained and reported at 70 eV, because at this level perturbations in electron energy have negligible effect on ion production. The efficiency of EI ionization is also related to the effectiveness of interaction or collision between sample molecules and energetic electrons. The interaction could be effectively increased by (1) increasing the trap current, (2) increasing the cross section of the electron beam, or (3) increasing the sample pressure in the ion source. All of these suggestions, however, have practical limitations. The limitation in the case of (1) is the life time of the filament used to generate the electron beam. The other two suggestions for increasing ionization efficiency are limited by the resolution desired from the MS (Watson, 1976d).

It was recently observed that negative ion CIMS can afford sample ion currents for electron capturing compounds that exceed ion currents in the positive ion mode by two or three orders of magnitude (Hunt,

1976). Although enhanced sensitivity in the electron capture negative ion mode will only be realised for sample molecules which possess a positive electron affinity and a large cross section for electron capture, many compounds may be analyzed by this technique by conversion to derivatives that impart to them these properties (Hunt, 1976).

The sensitivity of a MS bears an inverse relationship to the resolving power used (McFadden, 1973b). For this reason most GCMS systems are operated at a resolution of about 1000 for quantitation at high sensitivity.

The m/e value of an ion may influence the sensitivity of its detection. Quadrupole MSs, generally exhibit mass discrimination at high m/e values. The electron multipliers used in most MS also show some mass discrimination at high m/e values.

QUANTITATIVE GCMS:

Two methods are at present used to quantitate compounds introduced into the MS via a GC. The first and most popular one is to minitor the intensity of a limited number of ions which are characteristic of the compounds being determined, by switching the MS analyzer rapidly between these ions (Watson, 1976a). This is called SIM. The second method is to scan the spectrum repetitively, with the data from these scans being acquired by a computer. The varying intensity of selected ions can be retrieved from the scans, and the output presented in a similar fashion to the real time output of SIM (Watson, 1976a).

Selected ion retrieval (SIR) is one term used to describe this technique.

SIM is much more sensitive than SIR by up to a factor of several thousand, depending on the sampling time, but requires a foreknowledge of which ions are required for analysis. The reason for extra-sensitivity in SIM is that the detection system is dedicated to monitoring only a few ions (maximum sensitivity can be attained by monitoring the ion current at only a single m/e value), whereas in SIR, the data acquisition system spends the majority of the analysis time sampling the barren region of the mass spectrum in between the maxima of individual ion beams (Watson, 1976a). SIR while less sensitive, has the advantage that any ions of choice can be retrieved from the scan data at the termination of the experiment, so that the most useful ions for the analysis can be determined without the need for further injection of sample.

The Hewlett-Packard 5992B GCMS package was supplied ready for use in the SIM mode, but the AEI MS-30 required the addition of a multiple ion monitoring device. By means of this device the ions of interest (albeit over a limited mass range) are monitored by alternative switching of the accelerating voltage.

In GC the problem of high bleed from columns can be overcome by the use of a dual column system that balances out the simultaneous bleed signal from a control column and the analytical column. Unfortunately this system cannot be used in a single source GCMS system. Vapours from the GC liquid phase or the column septa, or both, enter the ion source, at which point they undergo continuous ionization and fragmentation; thus, their characteristic fragmentation pattern will be present in each scan of the mass spectrum. The GC background

spectrum can interfere with the recognition of the mass spectra of minor components in a sample mixture; it is often background of this sort that limit the useful sensitivity of GCMS (Watson, 1976a).

For quantitative GCMS work a prior knowledge of the GC background spectrum can be used to advantage, in selecting a derivative of the compound of interest which yields ions of m/e values that do not coincide with those of the GC background. For example, in scans from the widely used silicone GC phases, prominent peaks can usually be found at m/e 207, 281, 355, 429 and 481 (Watson, 1976g). Ions of high m/e values (i.e. in excess of 500) are particularly valuable in quantitative GCMS analysis as they are much less likely to result from ionization of the GC bleed or other substances in the sample extract.

C H A P T E R 2

EXPERIMENTAL METHODS

CHAPTER 2

EXPERIMENTAL METHODS

GENERAL CONSIDERATIONS:

An isotope dilution GCMS procedure employing SIM has been developed for the determination of the unique urinary melatonin metabolite 6SM, which, when coupled with the administration of deuterium labelled melatonin (Kennaway et al., 1977a) (Figure 4), also allowed determination of the urinary production rate (UPR) of melatonin, after the method of Pinkus et al. (1971).

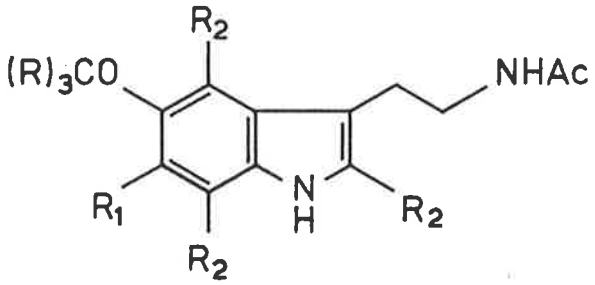
For the determination of absolute levels of 6SM, known amounts (500 ng) of a stable isotope labelled analogue internal standard (Figure 4) were added to the urine prior to analysis, and quantitation was achieved by determining the ratio of unlabelled and labelled 6SM in the sample, and relating this to a standard curve (Figure 5).

When determining melatonin UPR no internal standard was added. Melatonin UPR was obtained by determining the specific incorporation of deuterium label into the 6SM of urine, collected following i.v. administration of deuterated melatonin, and substitution in the equation of Pinkus et al. (1971), shown in Figure 6.

EXPERIMENTAL:

1. Materials

6 HM was purchased from Sigma (St. Louis, U.S.A.). Deuterium oxide,



Melatonin	$R_2 = R_1 = R = H$
$^2H_3$-Melatonin	$R = {}^2H$, $R_1 = R_2 = H$
6-Hydroxy melatonin	$R_1 = OH$, $R_2 = R = H$
$^2H_3$-6-Hydroxy melatonin	$R_1 = OH$, $R_2 = {}^2H$, $R = H$
6-Sulphatoxy melatonin	$R_1 = OSO_2OH$, $R_2 = R = H$
$^2H_3$-6-Sulphatoxy melatonin	$R_1 = OSO_2OH$, $R_2 = {}^2H$, $R = H$

FIGURE 4. Structure of Melatonin and its Analogues

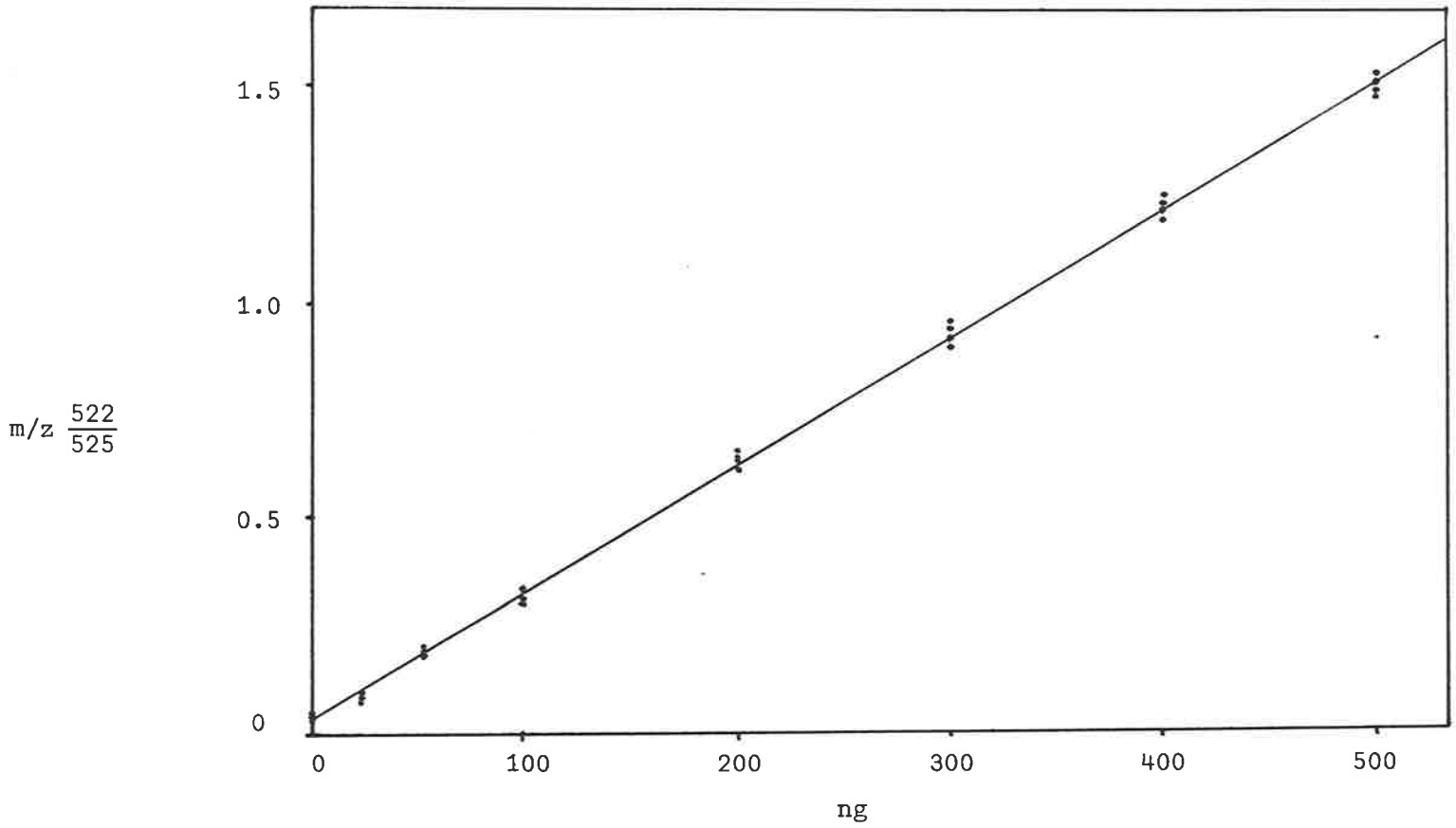


FIGURE 5. Plot of the ratio of m/z 522 and 525 for various quantities of 6SM analysed with 500 ng of $^{2}H_{3}$-6SM

$$\text{PRODUCTION RATE} = \frac{a \cdot m}{ef \cdot t} - \frac{m}{t}$$

a = isotopic purity (%),

m = mass (μg) of $^2H_3$ melatonin administered

ef = enrichment factor = $^2H_3(^2H_3 + ^2H_0)^{-1} \cdot 100$

t = number of days

FIGURE 6. Melatonin Urinary Production Rate Equation.

deutero-sulphuric acid and deutero-methanol were all obtained from R/M Isotopes Co. (Maynard, U.S.A.). Pentafluoropropionic anhydride (PFPA) was a product of Pierce Chemical Co. (Rockford, U.S.A.). All solvents were the highest purity grades available and were redistilled before use.

Amberlite XAD-2 resin was purchased from BDH Chemicals (Australia). Florisil was obtained from Supelco Inc. (Pennsylvania, U.S.A.). Sephadex LH-20 (Pharmacia) was allowed to swell in chloroform-methanol (1:1) containing sodium chloride (10 mmol l^{-1}) prior to use. Silica gel (100-200 mesh) was from Koch-Light Laboratories Ltd. (Buckinghamshire, England). Lipidex-5000 was obtained from Packard Instrument Co. Downers Grove, U.S.A.).

2. Synthesis of 6SM

This was prepared from 6HM (Figure 4) according to the procedure reported by Baillie et al., (1975a) for steroid sulphates except that a shorter reaction time (0,25 hour) was employed. Particular care was taken to ensure that only anhydrous reagents (dimethylformamide, N, N-dicyclohexyl carbodiimide and sulphuric acid) were used. The reaction was monitored by thin layer chromatography (TLC) using 5 cm x 20 cm glass plates coated with 0.25 mm layer silica gel 60 F_{254} (Merck AG, Darmstadt, Germany) in the solvent system, ethanol : ethylacetate : 25% aqueous ammonium hydroxide (5:5:1). Under these conditions the R_f of 6SM was 0.75 and that for 6HM 0.91; compound visualization was by Ehrlich's reagent (Dawson et al., 1969). The reaction products were purified on a column (28 cm x 1 cm) of Sephadex LH-20 (5g) and the overall yield (70-100 ml fraction) estimated by reaction with Ehrlich's reagent (2 hours, room temperature, absorbance at 620 mm) was 46%. The sulphate was stored as

an ethanol solution in the dark at -15°C.

Purity was assessed by TLC analysis as described previously. The coloured oxidation products were well separated from both 6SM and 6HM in both LH-20 column chromatography and TLC systems. 6SM and 6HM were the only Ehrlich reactive substances in those column fractions as assessed by TLC.

The stability of 6HM and 6SM at concentrations of 10-50 µg/ml in both 0.1 mol/l phosphate buffers of PH 1 through 13 and in ethanol was monitored by ultra violet spectrophotometry and by TLC as described previously.

3. Synthesis of $^{2}H_{3}$-6SM

A solution of 6HM (15 mg) in 5 ml deutero-methanol (CH_3 OD, 99.5 atoms % D) containing one drop of 10% deutero-sulphuric acid (D_2 SO4, 99.5 atoms % D) in deuterium oxide (D_2O, 99.8 atoms % D) was heated (70°C) under a nitrogen atmosphere for 48 hours. The mixture was then cooled, neutralized and taken to dryness in vacuo at ambient temperature. The residue was dissolved in chloroform/methanol (1:1) and chromatographed on Sephadex LH-20 (5g). Collection of the 20-30 ml fraction and removal of the solvent gave a pure sample of $^{2}H_{3}$-6HM (70% yield), most of which was converted to its respective sulphate as described previously. GCMS analysis showed it to have an isotopic purity of 0.7% $^{2}H_{0}$, 0.3%, $^{2}H_{1}$, 27% $^{2}H_{2}$ and 72% $^{2}H_{3}$.

4. Analytical Procedure

A 10 ml portion of the urine, to which had been added $^{2}H_{3}$-6SM

(500 ng) was diluted to 15 ml with distilled water and transferred to a XAD-2 column (25 g, 10 mm diam.). Alternative washes with water (30 ml), methanol (50 ml), water (60 ml), triethylammonium sulphate solution (10 ml) and water 30 ml) removed all non-conjugated materials (Bradlow, 1977). The absorbed 6 SM was then eluted with methanol (50 ml) and the solvent removed on a rotary evaporator at 40°. The residue was transferred in methanol (0.25 ml) to a Florisil column (0.5 g, 4 mm diam.) and the first 2.0 ml of methanol eluant collected. The solvent was removed and the samples dehydrated with anhydrous benzene (2 x 0.5 ml) evaporation.

Derivatization was accomplished by reaction with a mixture of ethyl acetate and pentafluoropropionic anhydride (PFPA) (0.2 ml, 1:1) for 1 hour at room temperature. The solvent was removed and the residue was transferred in anhydrous benzene (0.2 ml) to a silica gel column (0.2 g, 4 mm diam.) from which the spiro derivatives were eluted with cyclohexane (0.4 ml). Removal of solvent was accomplished and the spiro derivatives were dissolved in anhydrous benzene (0.2 ml), transferred to a LIPIDEX-5000 column (50 mm x 4 mm diam.) and eluted with cyclohexane (2.0 ml). The solvent was removed and the residue dissolved in ethyl acetate (0.05 ml).

Quantitative GCMS was performed on 2 instruments operated under the following conditions.

An AEI MS-30 double focussing Ms was fitted with a multiple ion monitor and was interfaced to a Pye 104 GC via an SGE single stage glass jet separator. GC conditions: column, 3 m x 2 mm i.d. glass coiled packed with 1.6% OV-101 on 100/120 mesh Gas Chrom Q; oven temperature

200°; injector, 220°; helium flow, 30 ml min^{-1} or column, 3 m x 2 mm i.d. glass coiled packed with 3% OV-17 on 100/120 mesh Gas Chrom Q; oven temperature 235°; injector 240°; helium flow, 30 ml min^{-1}. MS conditions: Resolution 1000; ionising current 100 microamps; electron voltage, 40 eV; source temperature, 200°; separator temperature, 250°; ions monitored m/z 522.06 and 525.08; dwell time, 150 milliseconds.

A Hewlett-Packard 5992B GCMS was fitted with a single stage jet separator. Conditions: injection port 220°; 3 m x 2 mm i.d. glass coiled column packed with 2% OV-101 at 200°; helium flow, 25 ml min^{-1}; selected ion monitoring, m/z 522.1 and 525.1; dwell time, 500 milliseconds, mass window, 0.4 a.m.u.

RESULTS AND DISCUSSION:

Initial experiments with 6HM indicated the labile nature of this phenolic indole. It was found to be unstable in aqueous solutions at both acidic and alkaline pH values, particularly above pH 9. This decomposition was accelerated by the presence of air and light. It was found to be reasonably stable in the dark in ethanol at -15° under an atmosphere of nitrogen. 6SM on the other hand was found to be infinitely more stable in ethanol and in aqueous alkaline solution (but not acid) in the presence of both air and light.

This project did not gain momentum until a way of derivatizing 6SM directly was found. Direct derivatization of phenolic sulphates with perfluoro acid anhydrides rapidly and in quantitative yields under mild conditions had been reported (Touchstone and Dobbins, 1975; Murray et al., 1977). Direct conversion of 6SM into a suitable GCMS

derivative, a procedure shown to be generally applicable to phenolic sulphates (Murray and Baillie, 1979), eliminated the necessity of isolating 6HM liberated from conjugation by hydrolytic means. The labile nature of 6HM had frustrated one attempt at its detection in urine (Green and Williams, 1967), and necessitated the rapid work up of a large urine sample for its identification in another (Sisak et al., 1979). Furthermore, quantitation of urinary conjugated 6HM by a hydrolytic procedure (Tetsuo et al., 1981a) necessitated the use of the very sensitive technique of GC negative chemical ionization MS, because of poor recovery (16%) of 6HM from urine. In contrast, the infinitely more stable 6SM has allowed use of much less sophisticated MS for its quantitation.

The selection of the perfluoro propionyl derivative was based on two important criteria from a quantitative GCMS point of view. Firstly, the spiro derivative of 6HM (and 6SM), being non-indolic (Blau et al., 1977) had excellent GC properties, and secondly, this derivative yielded an EI ionization spectrum with a high mass molecular ion that carried a high proportion of the total ion current.

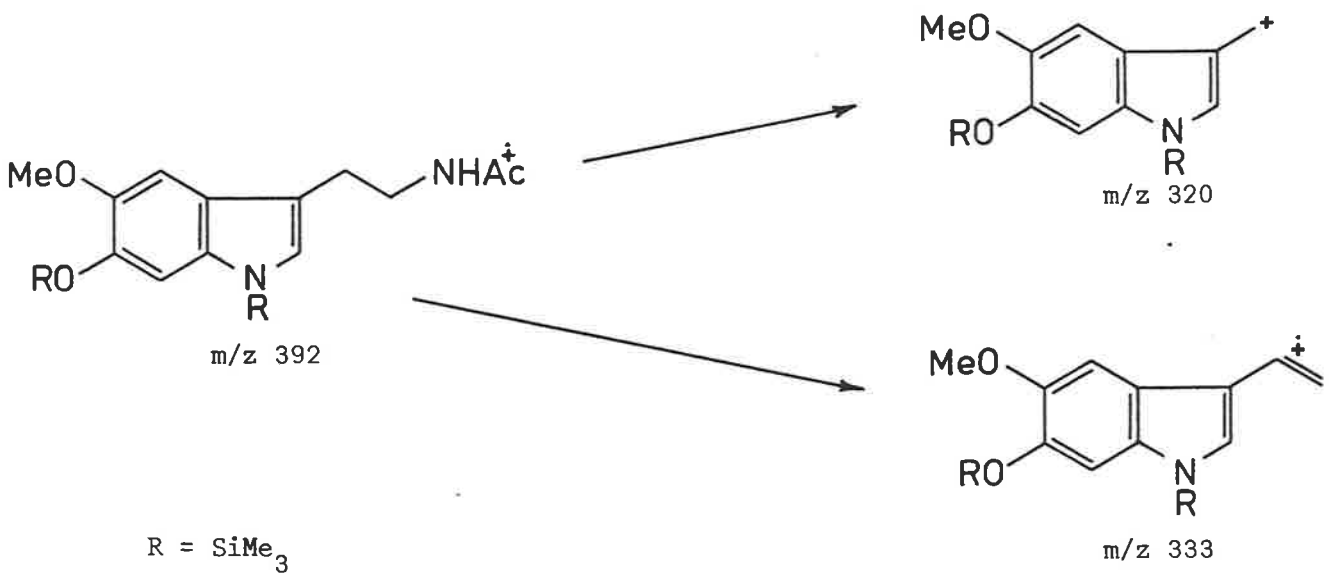
Preparation of $^{2}H_{3}$-6SM was an important aspect of the project since as an internal standard it allowed an accurate estimation of endogenous urinary 6SM. While the yield of formation was less than reported for steroid sulphates (Baillie et al., 1975a), this was expected since 6HM was found to be particularly labile.

Deuterium labelling of 6HM was performed using an acid catalysed enolization method developed for steroid labelling (Tökes and Throop, 1972). Examination of the mass spectrum of the trimethylsilyl

derivative of the labelled 6HM showed prominent peaks at m/z 395, 336 and 323, indicating a mass shift in each peak of 3 daltons over unlabelled 6HM (Scheme 1 and Figure 7). This established that three deuterium atoms were incorporated at positions 2, 4 and 7. Shaw (1978) has reported the synthesis of $^{2}H_{4}$-6HM in high isotopic purity. Although the present procedure is more facile than that reported by Shaw, it suffers from the label being located in potentially enolizable positions. Such losses, however, were not noted in the assay.

When $^{2}H_{3}$-6SM was derivatized to its corresponding dipentafluoropropionate derivative, it was observed that no loss of label occurred. This confirms reports that such derivatization procedures form the spiro derivatives (Blau et al., 1977) and not carbolines as originally proposed (Cattabeni et al., 1972) (Scheme 2 and Figure 8).

In 1968 Bradlow described a general procedure for the isolation of steroids and steroid conjugates from aqueous media by adsorption on columns of Amberlite XAD-2 resin and their subsequent elution with methanol (Bradlow, 1968). More recently, Bradlow reported problems with the elution of polar steroid conjugates from later batches of XAD-2; neutral steroids were readily eluted while the recovery of conjugates was quite poor (Bradlow, 1977). Initial experiments with 6HM and 6SM indicated that 6HM was readily eluted from XAD-2 with methanol but that the recovery of 6SM was extremely poor (<5%) by this route. The use of Bradlow's modified procedure (Bradlow, 1977), however, in which the ion pairing agent tri-ethyl ammonium sulphate was used, resulted in acceptable recovery (85%) of 6SM from urine. Thus, use of this procedure allowed elution of 6HM in the initial methanol wash and, after ion pair formation, elution of 6SM in the second methanol wash.



SCHEME 1

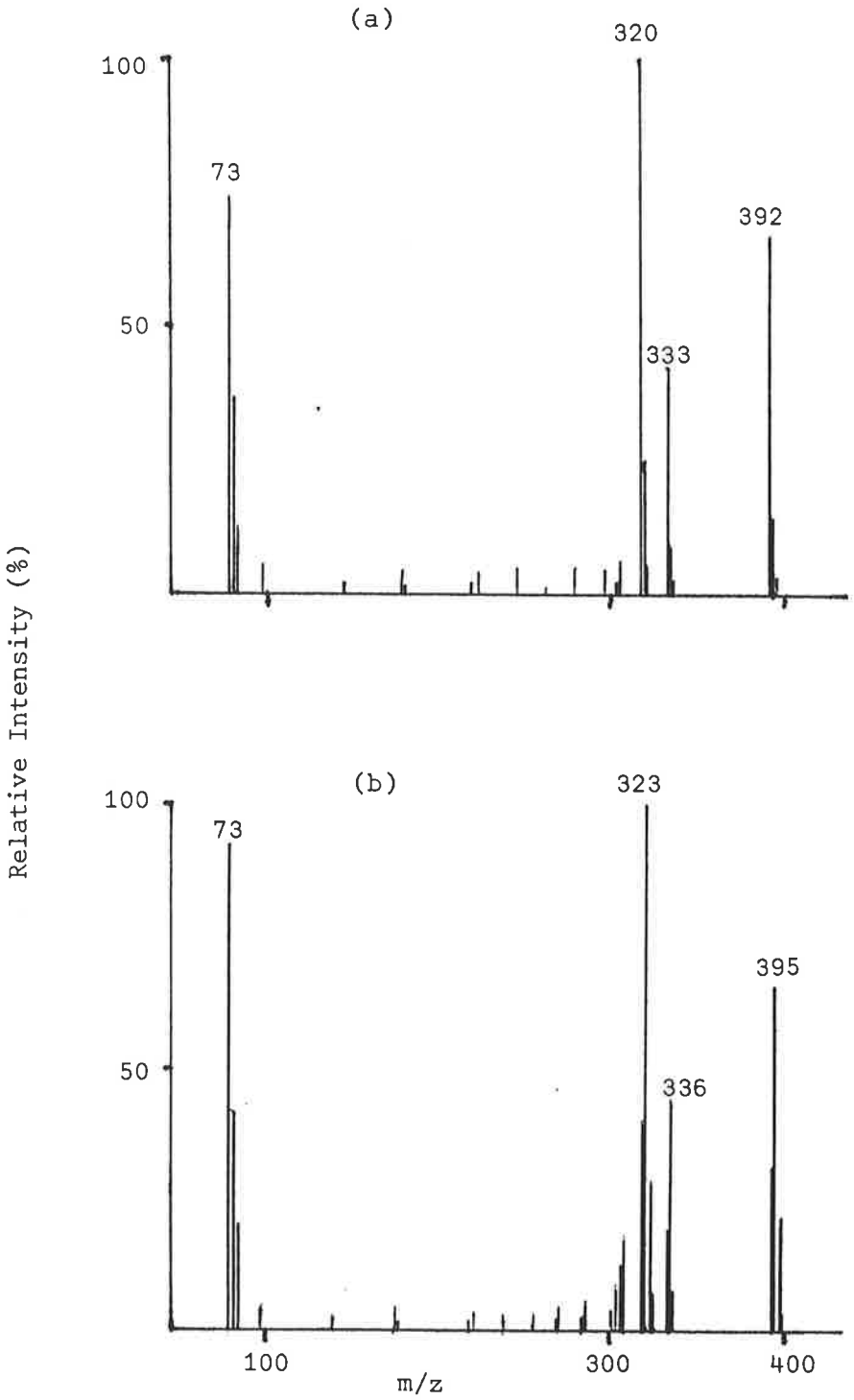
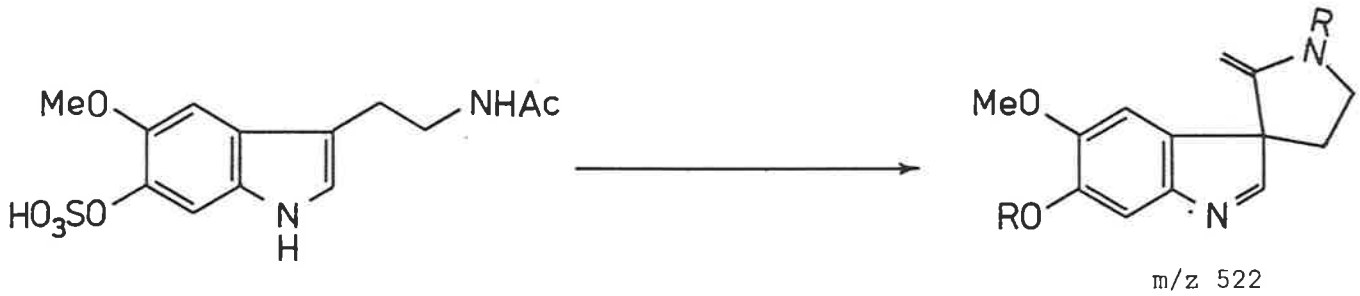


FIGURE 7. El Mass spectrum of ditrimethylsilyl derivative of

(a) 6-hydroxy melatonin and

(b) $^{2}H_{3}$-6-hydroxy melatonin



m/z 522

R = COC_2F_5

SCHEME 2

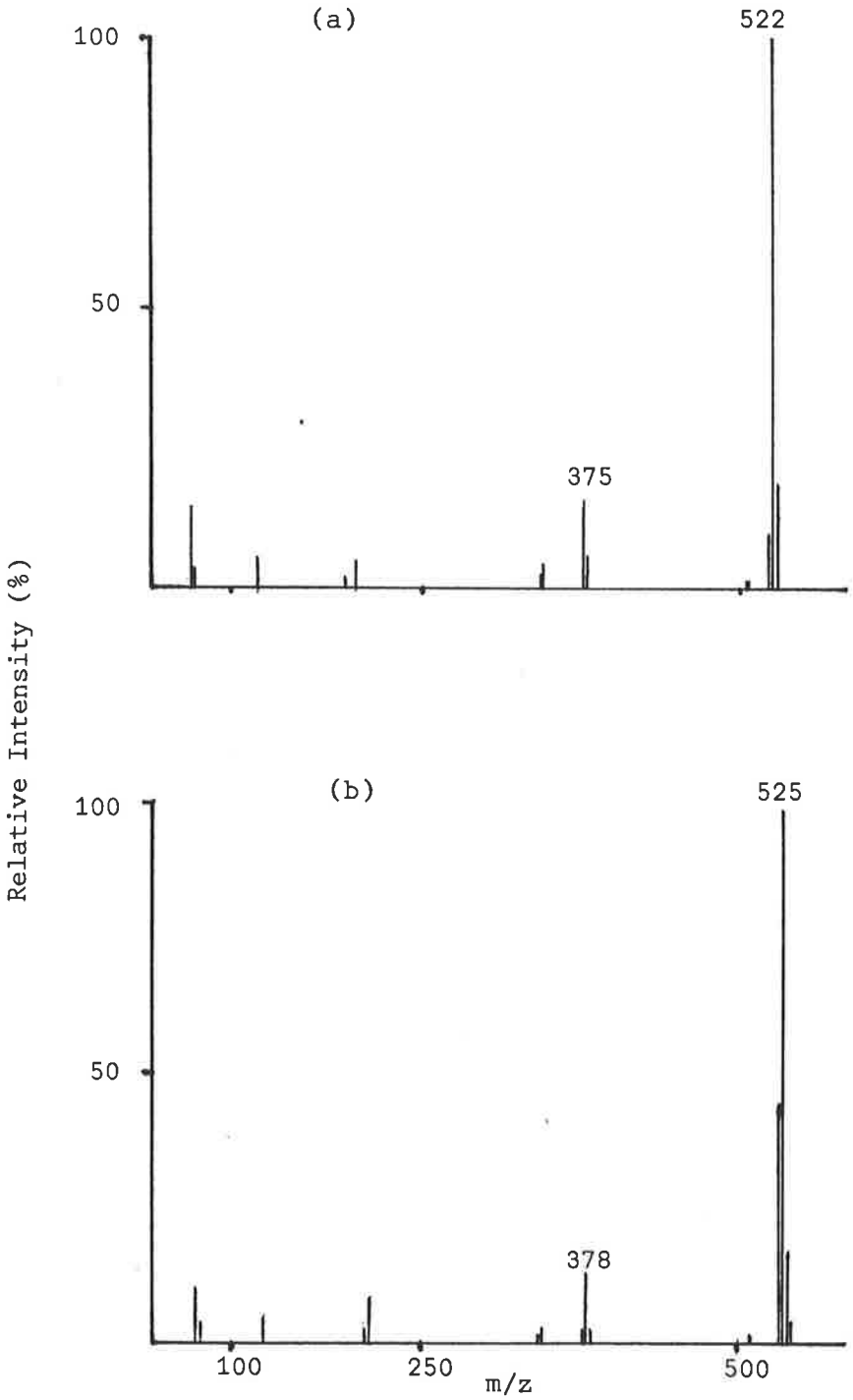


FIGURE 8. El Mass spectrum of dipentafluoropropionate derivative of

(a) 6-sulphatoxy melatonin and

(b) $^{2}H_{3}$-6-sulphatoxy melatonin.

Derivatization at this stage was not possible due to formation of interfering compounds. Accordingly a second chromatography step was added using Florisil microcolumns. Overall recovery at this stage was estimated at approximately 65%. Derivatization at this point resulted in a GCMS profile which allows ready determination of both 6SM and $^{2}H_{3}$-6SM (Figure 9). The double column chromatographic clean-up step of the spiro derivatives was necessary in order to extend the period of satisfactory operation of the quadrupole MS. The spiro derivative recovery for this purification step was approximately 90%.

Losses of 8, 29 and 71% of the original 6SM content occurred when fresh human urine was kept for 1 week at -15, 4 and 21°C, respectively. Subsequently it was found that boric acid at a final concentration of 1-2% completely prevented losses of 6SM from human urine samples stored at least 1 week, 3 months and 15 months at 21, 4 and -15°C, respectively.

Analyses were performed using 2 different MSs. The AEI MS-30 was used initially for only a limited period with most of the data being acquired by use of the Hewlett-Packard instrument.

Errors affecting precision are introduced in SIM analysis at two stages, first, in the sample manipulation steps, and second, in the GCMS analysis.

Instrument reproducibility was evaluated by repeated injection of the same test solution comprising approximately equal amounts of the spiro derivatives of unlabelled and trideuterated 6SM, into both instruments on separate occasions, twice for the AEI MS-30 and thrice for the quadrupole instrument. These data are shown in Table 1. The overall analytical precision for the determination of urinary 6SM was

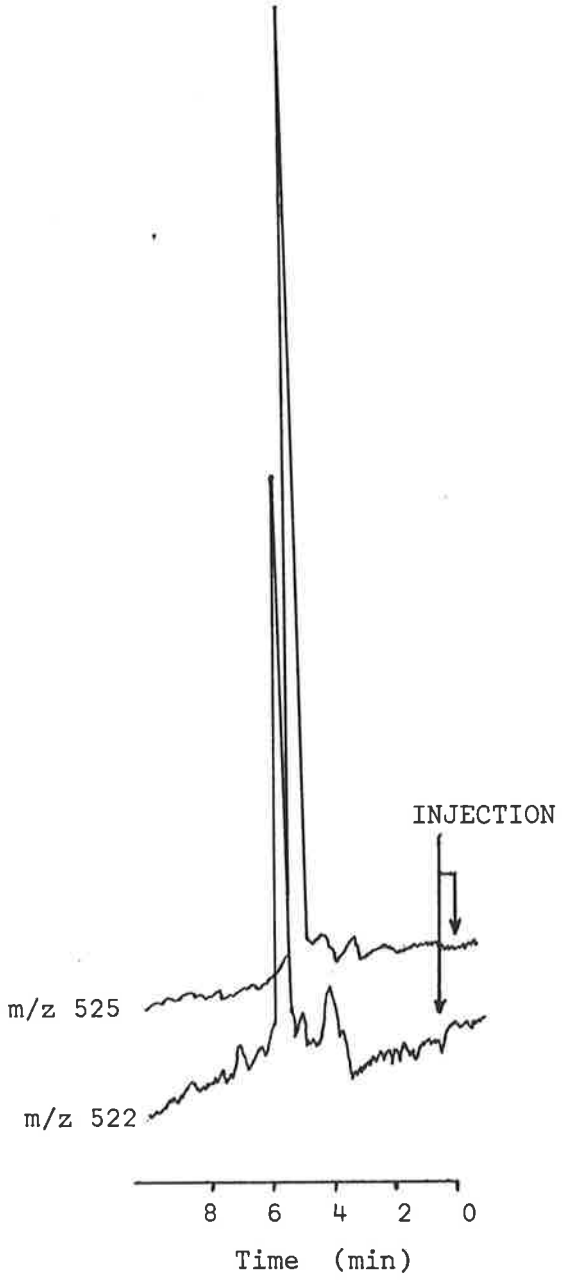


FIGURE 9. Selected ion chromatogram for the measurement of 6-sulphatoxy melatonin in urine as its dipentafluoropropionate derivative.

TABLE 1

Instrument reproducibility for GCMS analysis of test solution

	m/z 522/525		
Instrument	Mean	Coefficient of variation (%)	n
AEI MS-30	0.987	5.1	5
AEI MS-30	0.972	3.8	5
HP 5992B	0.959	1.6	5
HP 5992B	0.975	1.7	5
HP 5992B	0.964	2.0	5

evaluated by the repetitive analysis of aliquots of a control urine. Both instruments were used and the results are indicated in Table 2.

Reference to both Tables 1 and 2 indicate that a major contribution to the less consistent intrabatch and poorer interbatch precision provided by the use of the AEI MS-30 was the inferior stability of that instrument. The procedure used to minimize this problem of magnetic field instability was to allow the magnet to equilibrate thermally for several hours, and then to confirm the focus every hour or so by bleeding in perfluoro kerosene and if necessary making the adjustments required to bring the calibration ion (m/z 517) back into focus at its maximum (Björkhem et al., 1976; Watson, 1976a; Aldercreutz, 1977). Nevertheless, the mean value for the control urine obtained by the procedure employing the magnetic MS was within 4% of that for the quadrupole instrument.

A typical standard curve over the range of 0-500 ng 6SM showed a linear regression equation of $Y = 2.95 \times 10^{-3} + 0.03$ (standard error of estimate = 0.0177, r = 0.998) (Figure 5). This curve was found to be linear for at least 2000 ng 6SM.

The limits of detection (2 ng) and quantitation (5 ng) set for this procedure result not from a lack of sensitivity but from the poor precision with which the small amount of unlabelled 6SM (0.7%) in the internal standard was differentiated from the small quantity of sample 6SM. Adequate sensitivity is available for this procedure as only 5-10% of the final extract is usually required for the actual quantitative GCMS. The high sensitivity is due to the stability of 6SM and the excellent GC and MS properties of the spiro derivatives used for quantitation (Figures 8 and 9).

TABLE 2

Assay reproducibility for GCMS determination of urinary 6-sulphatoxy melatonin

Instrument	Reproducibility	Mean[a]	Coefficient of variation (%)	n
AEI MS-30	Intra Assay	19.6	7.2	3
		18.5	5.0	3
		19.5	1.4	3
AEI MS-30	Inter Assay	18.7	9.8	7
HP 5992B	Intra Assay	19.5	3.3	5
		18.6	3.5	5
		19.0	4.0	5
HP 5992B	Inter Assay	19.3	7.0	17

[a] $\mu g\ 24\ h^{-1}$

Accuracy may be defined as the nearness with which a given analytical result approaches the "true" result. The only adequate technique presently available for the development of definitive reference methods is isotope dilution GCMS (Breuer and Siekmann, 1975; Björkhem et al., 1976; Watson, 1976a; Horning et al., 1977; Siekmann, 1979).

A procedure is considered accurate if it satisfies the criteria of (a) recovery and (b) specificity. One aspect of recovery is the major advance brought to quantitative analyses by isotope labelled internal standards in correcting for procedural losses during the entire procedure. $^{2}H_{3}$-6SM has been shown to be an ideal internal standard for quantitative GCMS in accurately compensating for manipulative losses and having a spiro derivative with identical GC behaviour to that of the corresponding derivative of 6SM. Use of a deuterium labelled analogue internal standard has, as well as meeting the criteria of an ideal internal standard (Breuer and Siekmann, 1975; Björkhem et al., 1976; Watson, 1976a; Horning et al., 1977), the added advantage of allowing examination of the chromatographic peaks for symmetry, and the absence of 'shoulders' as an additional assurance of specificity.

Specificity may be defined as the ability of a technique to absolutely identify a compound to the exclusion of all others. GCMS techniques are specific since the analyte must be simultaneously characterised by both its GC mobility and mass. Furthermore, the higher the m/z value to be recorded the less probable it is that the accompanying impurities from the biological matrix or from the continuous bleeding of the stationary phase of the GC column interfere with the determination (Breuer and Siekmann, 1975; Siekmann, 1979).

The high MW of the spiro derivatives employed has resulted in a GCMS profile virtually free of background (Figure 9). Furthermore, examination of the GCMS profiles from urine samples with and without added $^{2}H_{3}$-6SM, at both resolution 1000 and 3000 (AEI MS-30), revealed interference of only 1-2%.

The high sensitivity and high specificity of negative CIMS has been exploited to accomplish the accurate quantitation of the extremely low (pg ml^{-1}) circulating levels of melatonin in man (Lewy and Markey, 1978). Although conventional GCMS instrumentation (i.e. for positive ions) used in the SIM mode offers very high sensitivity for the detection of trace organic compounds, however, when used for quantitation of endogenous compounds in a biological matrix the practical sensitivity of these methods is limited to nanogram amounts of those compounds (Watson, 1976a; Lewy and Markey, 1978). Problems of increased chemical background and irreversible adsorption phenomena are major factors affecting sensitivity. Sources of background include the biological sample, solvents, derivatizing agents, plastic vials and caps, GC septa, GC stationary phase, and vacuum pump oil. Thus reduction of background to a minimum is an important requirement in exploiting the full quantitative potential of SIM.

The high specificity of negative CIMS is a result of this technique being limited only to those compounds with a positive electron affinity and a large cross section for electron capture (Hunt et al., 1976). Thus, virtually all of the potentially interfering compounds that comprise the usual background by forming positive ions in EI ionization and CI modes, will be incapable of forming negative ions and providing interference. The choice of derivative for electron capture negative CIMS is critical to the attainment of high sensitivity

and structural specificity. Cyclic derivatives or polycyclic compounds will generally yield structurally specific anions, whereas acyclic derivatives with good anion leaving groups will generate only reagent specific ions. Suitably derivatized organic compounds are efficiently ionised by the resonance capture of thermal energy electrons in an exothermic process, the excess energy being dissipated either by fragmentation or collisional stabilization (Lewy and Markey, 1978).

The most prominent ion in the negative CI mass spectrum of the spiro cyclic derivative of melatonin was 150 times more intense than the corresponding ion by positive CI (Lewy and Markey, 1978). It has been estimated that at 70 eV in the most efficient EI sources available only about one molecule in a thousand is ionized (Millard, 1978). As the overall sensitivity of positive CI is quite comparable to that obtained by conventional EI, then the ionization efficiency for positive CI would be expected to be similar at about 0.1%. Thus in the CIMS analysis of melatonin, the efficiency of ionization of the spiro cyclic derivative of melatonin could be expected to be about 0.1% in the positive mode, and about 15% in the negative mode.

As well as providing a very intense and structurally specific anion by negative CIMS, this non indolic spiro cyclic derivative of melatonin (Blau et al., 1977) has much improved GC properties over the indole from which it derives, thus markedly decreasing the degree of adsorptive sample less. To further minimise this phenomena, which is of critical importance in quantitation of picogram and femtogram amounts of biological compounds, glassware was acid washed and silanized. Chemical background was minimized by utilizing reagents of high purity and by extensive washing of the spiro cyclic derivative.

Interference from the continuous bleeding of the GC stationary phase was eliminated by selecting a silicone phase (OV-225) which was virtually incapable of forming negative ions. The maintenance of high sensitivity was ensured by stringent and frequent cleaning of the ion source and/or quadrupole rods (Lewy and Markey, 1978).

"- Accurate measurement is not a democratic process with all laboratories and methods equally weighted" (Markey, 1981). This situation apparently applies in the measurement of melatonin in biological fluids. The high specificity of the negative CIMS procedure is exmplified by the finding of much lower day time values and lower night time values, together with a considerably smaller dynamic range of human melatonin levels than those reported previously (Lewy and Markey, 1978; Markey, 1981). The development of this accurate procedure has provided a reference method for melatonin measurement, and has demonstrated the grossly inaccurate character of those other methods. As a result, data obtained with the use of those other procedures must be interpreted cautiously and any previous hypotheses based on those data should now be reviewed.

Sound hypotheses can be developed only if they are based on accurate analytical data. It is confidently expected that the isotope dilution GCMS procedure developed for the determination of urinary 6SM and melatonin UPR will provide such data.

C H A P T E R 3

SHEEP STUDIES

CHAPTER 3

SHEEP STUDIES

The effect of Px was studied in sheep as this was not available in humans.

STUDIES IN INTACT AND PX'D SHEEP:

The effect of Px was initially explored in 11 merino cross-bred sheep aged 4-6 years and weighing 35-45 Kg. These sheep, which had been sham operated or Px'd 4-5 years earlier, were studied in December 1979 (Summer) when they were sexually quiescent.

A second study was performed during May-September, 1980 (Winter) on 10 reproductively active 4-5 year old merino cross-bred ewes weighing 40-45 Kg. Both sets of sheep were obtained from University flocks maintained at Mortlock experimental station, Clare, South Australia.

During the studies the sheep were enclosed in individual pens (2 m x 3 m) in an animal house with the ambient temperature maintained at 21°C and the photoperiod conditions set to simulate the natural prevailing lighting periods at latitude 35°S. Light was provided by fluorescent tubes which provided 600 Lux at the sheep's head. Alfalfa chaff and oaten hay were provided at 1100 h and 1600 h daily. A period of at least 14 days adjustment was allowed between the sheep entering the animal house and the urinary studies. For urine collection the sheep were confined without other constraint to a smaller section (1 m x 3 m) of the cage, and urine, collected via a bladder catheter

(Folotex FG14) into containers containing boric acid (final concentration 1-2%), was stored at -15°C until analysis.

The results of the initial study are shown in Table 3. The 24 hour urinary excretion values indicated a significant difference ($P<0.01$, Mann Whitney test) between the sham operated and Px'd animals. Furthermore, a marked circadian rhythm in urinary 6SM excretion was shown in 4 animals from each group; 88.5 $\pm$ 6.4 and 87.9 $\pm$ 9.3% of the 24 hour output occurred during the hours of darkness in the sham and Px'd animals respectively.

As no control was exercised over the initial surgery and as its efficiency was not definitely established after the collection of urine, the results of this study were inconclusive.

In the second study urinary 6SM excretion and melatonin UPR were determined in pre- and post-Px'd merino cross-bred ewes. For injection crystalline $^{2}H_{3}$-melatonin, prepared as previously described (Kennaway et al., 1977), was formulated as a sterile solution in propylene glycol: water (3:1). For the metabolism studies, 2 mg $^{2}H_{3}$-melatonin was slowly injected into the external jugular vein of the conscious unanaesthetised ewe and urine collections instituted 1 day prior to, and 2 days post administration of the tracer. Px was carried out according to the procedure of Roche et al. (1970) and at the conclusion of the investigation it was assessed to be histologically complete by the negative results of a melatonin assay (Kennaway et al., 1977a) performed on homogenates of tissue obtained from the pineal area of the brain.

The melatonin UPR data are summarised in Table 4. Incorporation of deuterium into urinary 6SM was evident in the 0-24 hour post injection but absent in the 24-48 hour samples. The melatonin UPR values in the 10 intact animals were 107.9$\pm$ 38.6 µg 24 h^{-1}. Two ewes (Nos. 157 and

TABLE 3

Urinary excretion of 6-sulphatoxy melatonin (μg 24 h^{-1})

in pinealectomised (Px) and sham operated sheep*

	Ewes	Rams	Castrated-Rams	Mean ± S.E.	(n)
Sham	4.6, 9.0, 11.3, 14.0, 17.3	13.1	3.0	10.3^{a} ± 5.1	(7)
Px	2.4, 3.0	2.3	1.9	2.4^{a} ± 0.5	(4)

*Animals were Px or sham operated in 1974-75 and urinary collections made on individual animals shown in December, 1979.

$^{a}P<0.01$.

TABLE 4

Estimates of the amount of melatonin produced by sheep based on the measurement of melatonin urinary production rate (UPR)

Ewe identification number	34	277	316	333	370	393	288	32	157	460
Melatonin UPR $\mu g\ 24\ h^{-1}$	101.8	153.3	153.5	143.7	99.1	113.8	128.8	61.5	39.1 (<1)*	84.1 (<1)*

*Values in parenthesis were obtained in repeated assays after successful & histologically confirmed Px (see text)

460) were studied before and after successful Px. Pre-operatively their melatonin UPRs were 39.1 and 84.1 µg 24 h^{-1} and their 24 hour urinary 6SM excretion levels were 3.4 and 4.7 µg respectively. From UPR data for those 2 animals, and based on the assumption that the endogenous 6SM levels in the 0-24 h urine samples following the dose of tracer were very similar to the levels in the pre-dose 24 hour urines, it was estimated that only 4.2% (ewe No. 157) and 3.3% (ewe No. 460) of the administered $^{2}H_{3}$-melatonin was excreted as $^{2}H_{3}$-6SM.

Post-operative assessment of both animals at fortnightly intervals on 4 occasions revealed no endogenous 6SM (<0.2 ug 24 h^{-1}) in the pre-injection urines and no dilution of administered label indicating a melatonin UPR of <1 µg 24 h^{-1}. Thus Px had removed virtually all (>97.5%) of endogenous circulating melatonin.

SHEEP PINEAL 6HM and 6SM:

Pineal glands (average weight 75 mg) were obtained within 20 minutes of slaughter (0800-1000 h) from ewes of mixed age at the local metropolitan abbatoir (SAMCOR). Tissue was placed on ice and maintained at 0.4°C until analysis (1-2 hours).

For tissue studies the chilled pineals were homogenized in ice cold water (20 ml per gram tissue) and centrifuged at 4°C. Aliquots of the supernatant equivalent to 10 glands were taken for 6HM and 6SM analysis.

For tissue 6SM the procedure used was identical to that described for urine. For 6HM determinations, 500 ng of $^{2}H_{3}$-6HM was added to the homogenate followed by extraction with ethylacetate (2 x 5 volumes).

Thereafter the extract was derivatized, purified and the 6HM content quantitated by GCMS as in the 6SM analysis.

Acceptable recovery of both 6HM and 6SM for pineal gland homogenate was experienced in the analyses.* Neither 6HM (<200 pg per gland) nor 6SM (<200 pg per gland) was detected in sheep pineal tissue.

DISCUSSION:

This study represents the first quantitative estimation of both melatonin UPR and an endogenous urinary melatonin metabolite in sheep.

The evidence provided by the second study and other studies in Px'd rats (Pang and Ralph, 1975; Lewy et al., 1980a; Markey and Buell, 1982), chickens (Pelham et al., 1972), sheep (Arendt et al., 1980) and monkeys (Tetsuo et al., 1982a) indicate that the pineal gland is virtually the sole source of circulating melatonin. These findings cast doubt on the specificity of the RIA procedures used in those studies in which melatonin was reported to be present in the plasma of Px'd rats (Ozaki and Lynch, 1976; Yu et al., 1981) and sheep (Kennaway et al., 1977a) and thus some of the inferences drawn. They do not exclude the possibility that melatonin can be formed in tissue other than the pineal (for recent reviews see: Cardinali, 1981, Ralph, 1981). They do, however, set a limit on the amount of melatonin which may be released

* By this is meant that if 500 ng of $^{2}H_{3}$-6SM (or $^{2}H_{3}$-6HM) and 100 ng of 6SM (or 6HM) was added to separate aliquots of pineal homogenate and this was put through the 6SM procedure (or in the case of 6HM extracted with ethylacetate) and a comparison made of the strength of the GCMS signals for the processed and unprocessed 6SM (or 6HM) then it was found that for 6SM and 6HM the strength of the processed signals were approximately 60% and 80% respectively those of the unprocessed signals.

from those sources to contribute to circulating melatonin levels and subsequently be cleared into the urine as 6SM.

Quantitation of urinary 6SM or of circulating melatonin in Px'd animals may give a measure of the efficiency of Px. This was convincingly demonstrated in sheep whereby an animal in which residual pineal tissue was present after surgery was identified by means of the presence of circulating melatonin (Arendt et al., 1980). Furthermore, this experience attests to the difficulty in performing efficient Px in this species. In the initial study of sham operated and Px'd sheep a marked circadian rhythm in urinary 6SM was demonstrated in both groups, although the levels of the Px'd animals were significantly lower than those of the sham operated group. Thus, it is highly likely in those 'Px'd' sheep that residual pineal tissue was the source of the urinary 6SM measured.

Measurements in ewes indicate that the sheep pineal produces 39-154 μg 24 h^{-1} with over 80% of this production occurring during the dark phase. Thus these animals were producing 2-9 μg h^{-1} during the dark phase of about 14 hours duration. These values are considerably lower than those derived by Rollag et al. (1978) from their studies of MCR of melatonin in sheep. Their estimates of production rates during the dark period of 107-357 μg h^{-1}, however, were dependent on precise measurement of plasma melatonin levels. In view of recently obtained values for melatonin plasma levels in normal sheep (Arendt et al., 1980; Kennaway et al., 1982a) these were certainly overestimated. Recalculation of their data, but using the more recent values, provide estimates more in accord with the amount indicated in this study.

The absence of both 6HM and 6SM in sheep pineal glands, collected

at a time when each pineal is known to contain approximately 100 ng melatonin (Kennaway et al., 1977a), indicated that urinary 6SM stemmed from hepatic, and possibly other organ metabolism of melatonin. Studies in rats (Kopin et al., 1961; Wurtman and Axelrod, 1966; Wurtman et al., 1968c; Ozaka et al., 1976; Reppert and Klein, 1978; Weinberg et al., 1981) and humans (Smith et al., 1979; Iguchi et al., 1982a) implicate the liver as the major site of formation of 6HM and its conjugates in mammals.

For the calculation of the percentage of labelled melatonin excreted as labelled 6SM in 2 sheep it was assumed that the endogenous 6SM levels in the 24 hour urine samples following the dose of label were very similar to the levels in the pre-dose 24 hour urines. This was a valid assumption since it was recently shown in this species that there is no negative feedback of melatonin upon its own synthesis and release (Kennaway et al., 1982a). Furthermore, the same study revealed that Px'd sheep implanted with capsules containing melatonin maintained very stable circulating melatonin levels throughout both light and dark periods, indicating virtually no circadian change in the rate of metabolism of melatonin. Thus the usual noctural rise in plasma melatonin and urinary 6SM levels observed in sheep may be directly attributed to increased melatonin production, and not to a decrease in the melatonin MCR.

The fate of intravenously administered melatonin in sheep has not been studied before. The percentage of melatonin excreted as urinary 6SM in sheep is low compared with man (Jones et al., 1969) and the rat (Kopin et al., 1961; Kveder and McIsaac, 1961; Jones et al., 1969; Reppert and Klein, 1978). Those studies also demonstrated the facile

and virtually complete metabolism of melatonin, with the metabolites being rapidly cleared, predominantly via the urine. This study indicated that in this regard the sheep was no exception; the urinary clearance of $^{2}H_{3}$-6SM derived from intravenously administered $^{2}H_{3}$-melatonin was complete within 24 hours. Therefore in the sheep, the measurement of urinary 6SM may be expected to closely reflect circulating melatonin levels.

In mammals, the sulphate required for conjugation is obtained from cysteine (Singer, 1975), or from the essential amino acid methionine, following its conversion to cysteine via the transulphuration pathway (Radcliff and Egan, 1974; Greenberg, 1975). Methionine may also serve as a source for both S-adenosyl methionine, the primary methyl group donor of mammalian metabolism, and for cysteine for incorporation into proteins, including keratin (Radcliffe and Egan, 1972). Since cysteine is a major constituent of wool and because methionine is frequently an amino acid limiting for efficient nitrogen-retention in sheep, the metabolism of the sulphur containing amino acids are of special importance in this species (Armstrong, 1973; Radcliffe and Egan, 1978). Although the sulphur amino acid status of the sheep used in this study was not determined, it was almost certainly adequate as their diets were similar to those previously shown to be adequate for wool growth in this species (Armstrong, 1973). Thus a limited supply of sulphate would <u>not</u> appear to be the reason why less than 5% of administered deuterated melatonin was excreted as deuterated 6SM in the urine of sheep. Furthermore, other studies have demonstrated that phenolic sulphate and glucosiduronate conjugation are both very active in this species (Miyazaki et al., 1972; Kao et al., 1979).

In sheep and a large number of other mammalian species, phenolic compounds are excreted in urine predominantly as mixed sulphate/glucosiduronate pairs, the relative percentages of which show wide interspecies variation, and which may also show wide intraspecies variation, dependent on the nature of the phenol (Miyazaki et al., 1972; Davies, 1977; Hirom et al., 1977; Smith and Caldwell, 1977; Kao et al., 1979). Therefore, the findings of a low percentage of 6HM excreted as 6SM in sheep urine meant that the glucosiduronate conjugate of 6HM was likely to be the major urinary excretory product of melatonin in this species. Furthermore, as phenol has been found to be conjugated with phosphate to a limited extent in sheep (Kao et al., 1979), it is possible that phosphate conjugation of 6HM too, may occur in this species. Another possible urinary melatonin metabolite may be N-acetyl-5-methoxy kynurenamine found in both rat (Kopin et al., 1961; Hirata et al., 1974; Reppert and Klein, 1978) and human (Jones et al., 1969) studies of the metabolism of this indole.

On the other hand, the low percentage of melatonin excreted as urinary 6SM in sheep may reflect the importance of the biliary route for excretion of melatonin metabolites in this species. Urine and bile are complementary pathways for the excretion of organic compounds, a major determinant being MW (Hirom et al., 1976). Evidence from a study of estriol metabolism suggested that the MW threshold factor in sheep was somewhat less than the MW of oestriol-3-glucosiduronate at 464, as most of this substance is excreted in the bile whereas oestriol-3-sulphate (MW 368) is excreted in varying amounts in the urine or the bile (Miyazaki et al., 1972). Although 6HM has a MW 40 daltons less than that of oestriol it is possible that in the sheep some 6HM-glucosiduronate and 6SM is excreted via the bile and undergoes enterohepatic circulation.

The rapid elimination of $^{2}H_{3}$-6SM via the urine within 24 hours following administration of $^{2}H_{3}$-melatonin, indicates that, in the sheep, prolonged enterohepatic circulation of 6HM conjugates with their ultimate excretion via the urine as 6SM does not occur, particularly in view of the large doses of $^{2}H_{3}$-melatonin used.

Thus, whether the low percentage of melatonin excreted as urinary 6SM in sheep reflects the presence of an alternative pathway of melatonin metabolism, or the pre-eminence of the biliary route for excretion of melatonin metabolites, in this species, remains to be resolved.

It is concluded that the sheep pineal gland is virtually the sole source of circulating melatonin and of its urinary metabolite 6SM,* and that the measurement of either substance with an accurate technique may provide a reliable index of pineal function. This statement may be extended to include humans, as the results of a very recent study indicate that the pineal gland is the sole source of circulating melatonin in man (Neuwelt and Lewy, 1983).

*Near completion of this thesis it was reported that pinealectomy abolished circulating melatonin in sheep (Arendt et al., 1980) but no reports were available on the effect of this procedure on 6SM excretion.

CHAPTER 4

STUDIES IN MEN

CHAPTER 4

STUDIES IN MEN

24 HOUR URINARY 6SM EXCRETION:

The men studied were 10 healthy subjects aged 20-45 years. Their urinary 6SM excretion was 22.5 ± 5.4 μg 24 h^{-1} (range 9.5-29.1).

24 HOUR PROFILE OF URINARY 6SM EXCRETION:

The excretion profile of urinary 6SM over 24 hours was assessed in a healthy 35 year old man by means of consecutive random urine sample collection. The results of this in relation to the hours of light and darkness and the sleep phase are shown in Figure 10. The marked nocturnal sleep phase rise in urinary 6SM was associated with 80.4% of the 24 hour output.

URINARY 6SM EXCRETION OVER 120 HOURS:

The variation in urinary 6SM output over 5 consecutive days was assessed in a healthy 35 year old male subject. He slept for a constant 8 hours from 2200 hours each day with urine collected in 2 parts, the first for the sleep period and the other, for the remainder of the 24 hour period. A highly significant difference ($P<0.005$, paired t-test) was observed between the nocturnal sleep phase (18.2 ± 2.0 μg) and the daytime activity phase (7.5 ± 1.7 μg) excretion levels. The urinary 6SM excretion for this subject over this period was observed to be quite stable (25.7 ± 3.6 μg 24 h^{-1}), particularly when the nocturnal sleep

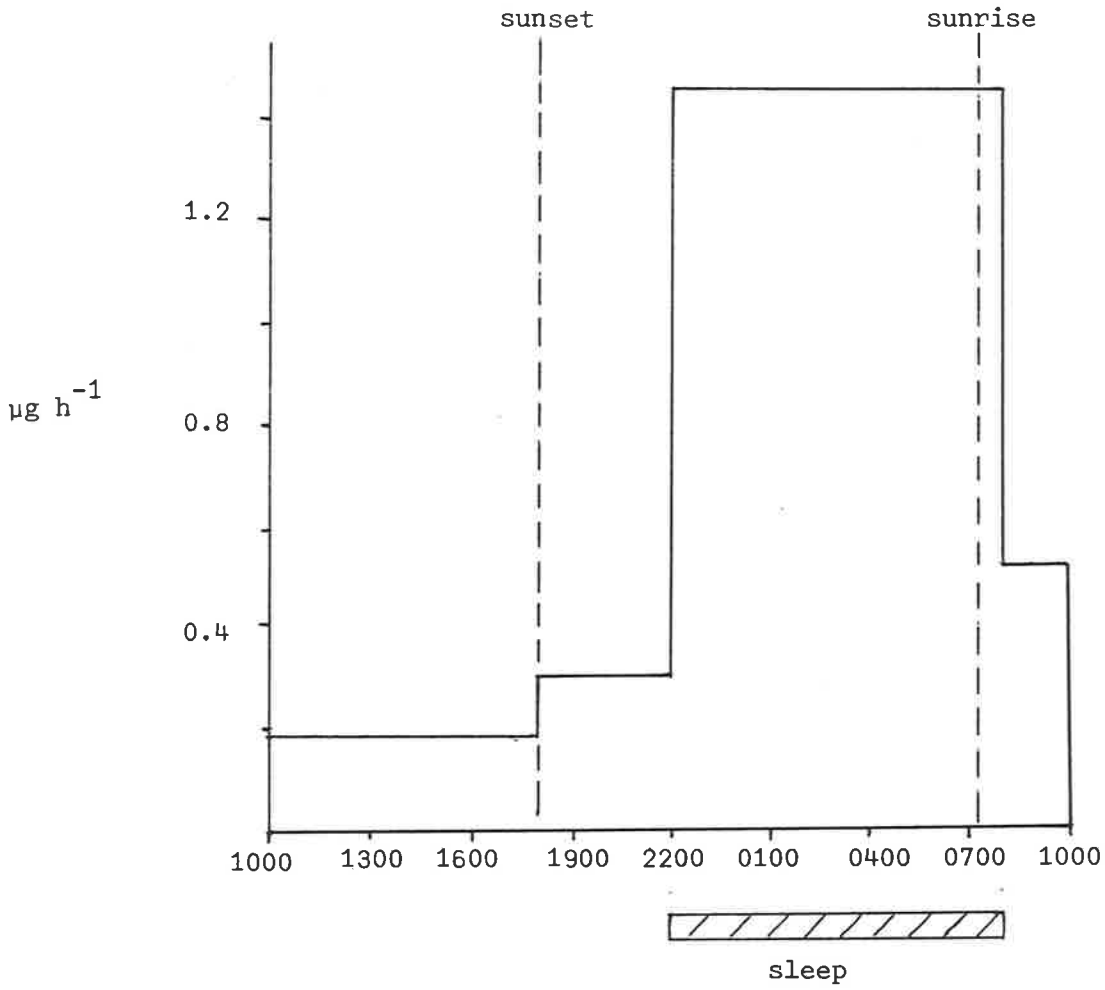


FIGURE 10. The 24 hour excretion pattern of urinary 6-sulphatoxy melatonin in a male subject.

phase component output was expressed as a percentage of the total 24 hour output (71.2 ± 2.5).

MELATONIN UPR STUDIES:

Crystalline $^{2}H_{3}$-melatonin (Kennaway et al., 1977a) was formulated as a sterile solution for injection in propylene glycol:water (3:1). For UPR assessment, injection of $^{2}H_{3}$-melatonin was made into the antecubital vein of informed male volunteer subjects, and urine collection instituted for 1 day prior to and 2 days post administration of dose.

Data depicting enrichment factors (efs) of UPRs at different dose levels for the two male subjects (I, 42 year; II, 40 year) is summarised in Table 5. These calculations were based on the assumption that the response of 6SM and $^{2}H_{3}$-6SM were equal. Such an assumption is justified, since previous work in this laboratory (Kennaway et al., 1977a) on the GCMS analysis of melatonin and $^{2}H_{3}$-melatonin established an almost identical response for either compound, reflecting both the high chemical and high isotopic purity of the synthesized tracer. The coefficient of variation (interassay, n=3) in the measurement of the $^{2}H_{3}$-6SM:6SM ratio used to estimate the ef and hence UPR ranged from 4.1 and 4.9% for dose 50 µg, through 4.5 and 6.2% for 100 µg, to 7.9 and 10.1% for 1000 µg.

Following i.v. administration of a 1000 µg dose of $^{2}H_{3}$-melatonin to subject I, 99.7% of the $^{2}H_{3}$-6SM was excreted within the first 24 hour period. Only trace amounts of $^{2}H_{3}$-6SM were evident in the 24-48 hour specimen.

Urinary 6SM levels in the 24 hour samples taken immediately prior

TABLE 5

Melatonin urinary production rate (UPR) in 2 males determined using $^{2}H_{3}$-melatonin as tracer

	DOSE (µg)	ef (%)	UPR (µg 24 h^{-1})
SUBJECT I	1000	96.8	23.7
	100	73.7	34.5
	50*	59.7	33.0
	50**	62.1	29.8
SUBJECT II	1000	96.3	29.1
	100	77.3	28.2

*Administered 1300 hours

**Administered 2230 hours

to giving the $^{2}H_{3}$-melatonin were not significantly different from those in the post (i.e. 24-48 hour) collections (Table 6).

The determination of urinary 6SM requires the addition of $^{2}H_{3}$-6SM and cannot therefore be extended to those samples already containing $^{2}H_{3}$-6SM derived from administration of $^{2}H_{3}$-melatonin (i.e. the 1-24 hour post-dosage collections). Endogenous 6SM in those samples was estimated by 'spiking' 10 ml aliquots of urine with 0, 50, 100 and 150 ng 6SM and extrapolation of the obtained curve to the X-axis. To gauge the accuracy of the 'spiking' technique, 24 hour urinary 6SM was determined in 2 samples by both this method and the conventional procedure. The 24 hour 6SM levels for the 2 urine samples from Subject I determined by both methods, with the value for the 'spiking' method listed second, were 21.6, 25.1 and 16.5, 13.8 ug. The endogenous 6SM level for Subject I (dose 100 µg) in the 0-24 hour urine collection obtained by the 'spiking' technique was 25.8 µg. This 6SM value, although obtained by the less accurate 'spiking' technique, exhibited no significant difference with respect to the pre- and post-collection values (Table 6).

For Subject I the marked stability in melatonin UPR (30.3 ± 4.8 µg $24\ h^{-1}$, n=4) was reflected by similar stability in urinary 6SM excretion (18.9 ± 2.1 µg $24\ h^{-1}$, n=7). Using the latter value it was estimated, from a knowledge of the dose of tracer and of the resulting $^{2}H_{3}$-6SM:6SM ratios used to calculate the data in Table 5, that approximately 40% of the administered $^{2}H_{3}$-melatonin was excreted as $^{2}H_{3}$-6SM in this subject. Furthermore, this percentage remained virtually constant (40.7 ± 2.3, n=4) for this subject despite tracer dosages of 50-1000 µg.

TABLE 6

Pre- and post-dosage urinary 6SM excretion

($\mu g\ 24\ h^{-1}$) for Subject I

DOSE (µg)	PRE-DOSAGE	POST-DOSAGE
1000	21.6	17.9
100	22.3	22.5
50*	15.1	16.5
50**	16.1	-

*Administered 1300 hours

**Administered 2230 hours

URINARY 6SM EXCRETION THROUGHOUT ONE YEAR:

To assess long term urinary 6SM output, 4 healthy male subjects (aged 25-40 years) collected a 24 hour sample of urine at monthly intervals throughout the year in Adelaide (35°S) during 1979 and 1980. Collection was divided into 2 parts; a night time sample to include the period from bedtime to that of the first voiding in the morning after awakening and the other, the remainder of the day until the ensuing bedtime. Overall details and results are summarized in Figure 11 and Table 7. The early morning collection period was fixed at 0800-0830 hours for subjects AF and FA and at 0830-0900 hours for subjects AB and KP in an attempt to detect any marked phase shifts in the nocturnal acrophase. No clearly defined seasonal changes in urinary 6SM excretion were evident for the 4 subjects studied.

DISCUSSION:

In this study both the quantitation of a unique urinary metabolite of melatonin and measurement of the UPR of melatonin in man were achieved for the first time. The necessity of i.v. injection of label for melatonin UPR estimation, however, has limited its use in human studies.

The excretion profile of urinary 6SM over 24 hours for the subject in this study exhibited a marked circadian variation, similar to that recently observed for plasma melatonin (Lewy and Markey, 1978), urinary melatonin (Lynch et al., 1978; Wetterberg, 1978) and for urinary conjugated 6HM (Tetsuo et al., 1980). Furthermore, the two recent reports of a high correlation between plasma and urinary melatonin (Lynch et al., 1978; Wetterberg, 1978) represents further evidence

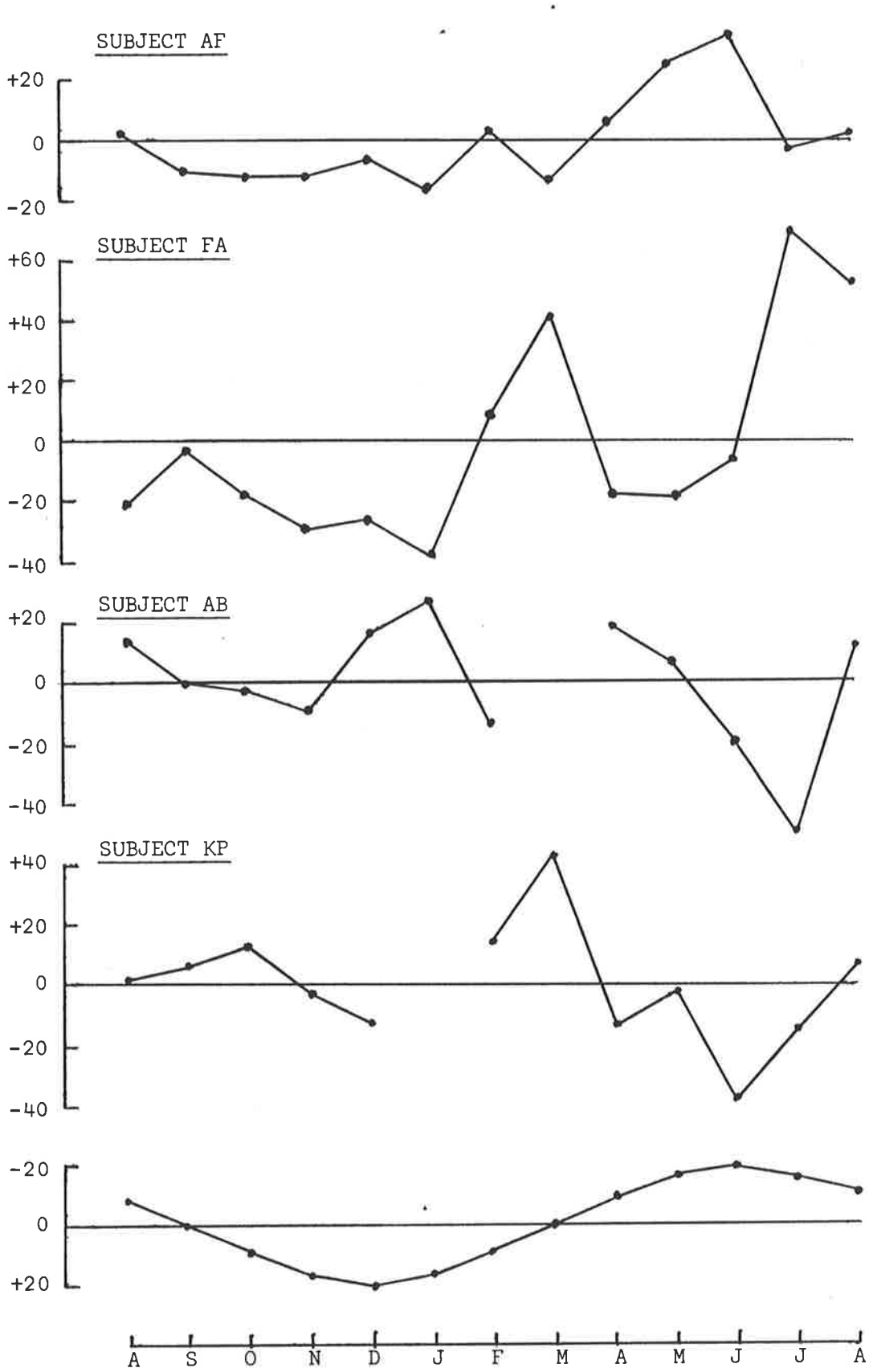


FIGURE 11. Monthly percentage difference from mean for 24 hour urinary 6-sulphatoxy melatonin excretion (upper 4 panels) and for dark phase length.

TABLE 7

Male urinary 6SM excretion at monthly intervals throughout one year*

	SUBJECT AF	SUBJECT FA	SUBJECT AB	SUBJECT KP
24 hour (µg)	15.9 ± 4.0 (13)	28.0 ± 9.6 (13)	30.4 ± 6.3 (12)	39.4 ± 7.9 (12)
Night time (µg)	11.6[a] ± 1.8 (13)	21.0[b] ± 8.3 (13)	22.1[c] ± 5.7 (12)	28.6[d] ± 3.5 (12)
Day time (µg)	4.4[a] ± 1.5 (13)	7.0[b] ± 3.9 (13)	8.3[c] ± 3.8 (12)	10.8[d] ± 7.1 (12)
$\frac{\text{Night time}}{\text{24 hour}} \times 100(\%)$	73.0 ± 7.2 (13)	75.6 ± 9.3 (13)	72.6 ± 11.4 (12)	74.2 ± 11.6 (12)
Sleep length (hours)§	10.06 ± 0.34 (13)	9.23 ± 0.44 (13)	11.42 ± 0.67 (12)	9.83 ± 0.62 (12)

*Values quoted are mean ± S.D., n in parenthesis

a, b, c, d all $P<0.001$

§No direct relationship could be established between sleep length and 6SM excretion during the night phase

indicating that urinary 6SM closely reflects circulating levels of melatonin. Moreover, accurate measurement of urinary 6SM has been facilitated as a result of its high (ng ml^{-1}) concentration. Thus, a ready assessment of nocturnal sleep phase melatonin secretion, as well as the 24 hour output may be accurately and simply obtained by collection of urine for 6SM determination in 2 parts, the first to cover the nocturnal sleep phase, and the other the remainder of the 24 hour period. To establish the circadian rhythm of 6SM excretion, however, at least three equal time length samples of urine would be necessary.

The 24 hour urinary excretion levels of 6SM found in the subjects in this study are in close agreement with daily urinary levels of conjugated 6HM in male volunteers of similar age reported recently (Tetsuo et al., 1980).

The use of radioactive tracers has long been the method of choice in investigations of hormone production rates, but their use has the disadvantage that uncertainties may arise in associating mass with radioactivity in material isolated from urine (Loraine and Bell, 1971a; Baillie et al., 1975b). In contrast, the use of stable isotope labelled compounds allows repeated clinical studies in patients which otherwise, for ethical reasons, would be impossible to perform (Pinkus et al., 1971; Baillie et al., 1975b).

From the results in Table 5, it is apparent that the value of the melatonin UPR is largely unaffected at the different doses, suggesting no major alterations in clearance or metabolism. More accurate estimations however, were possible at a smaller dose (50 µg) because of the improved precision in measuring the $^{2}H_{3}$-6SM: 6SM ratio. The stability of endogenous daily urinary 6SM excretion exhibited by Subject I before,

during and after administration of the tracer again implies that no pharmacological effect was manifested.

The lack of pharmacological effect at the doses employed of i.v. administered melatonin, labelled in the 5-methoxy group and which is known not to be involved in melatonin metabolism (Kopin et al., 1961; Jones et al., 1969), together with that proportion of the dose excreted as the unique melatonin metabolite 6SM virtually complete within 24 hours, indicate that the conditions necessary for the estimation of melatonin UPR to be valid have been met. Furthermore, the use of doses as low as 50 μg $^{2}H_{3}$-melatonin, available in high isotopic purity, together with the simple GCMS determination of the specific incorporation of deuterium into urinary 6SM in a single 24 hour specimen, represents a facile and accurate procedure for melatonin UPR estimation.

It has been established that a marked nocturnal rise in urinary 6SM excretion is concordant with the rise in plasma melatonin concentration. Interpretation of these data has been limited, however, due to lack of information concerning the relationship between plasma melatonin levels, melatonin production rate and the MCR of this putative hormone. This nocturnal rise may now be directly attributed to increased melatonin production at night as no obvious change in UPR was evident in night time versus day time administered melatonin (Table 5).

A compelling argument has been made to indict the pineal gland as the sole source of circulating melatonin and that the nocturnal rise is due to increased pineal activity. Based on the present melatonin UPR estimates, it may be calculated that during the nocturnal period the human pineal gland is producing approximately 3 μg h^{-1} and about $^{1}/_{10}$th

this amount during the day.

Although repeated measurement of both urinary 6SM excretion and melatonin UPR were made in only one subject, the results of this study prove unequivocally that urinary 6SM excretion is a reliable index of melatonin production in man. This reliability is due not only to the provision of a close integrated representation of plasma events but also to the very consistent percentage of circulating melatonin excreted as 6SM. In this study this percentage was found to be approximately 40%, thus confirming the significance of this route as a major excretory pathway. It is likely that the excretion of the remainder of the circulating melatonin was mostly shared between the urinary metabolites 6HM glucosiduronate and N-acetylkynurenamine, as indicated by Jones et al. (1969).

The results of this study differ from those of a previous investigation in humans (Jones et al., 1969), in two ways. Firstly, the percentage of labelled melatonin excreted as labelled urinary 6SM in that study was higher at between 59 and 79%, and secondly, some of the labelled urinary 6SM (less than 10%) was excreted in the 24-48 hour sample.

The reason for the 1.5-2 fold lower percentage of labelled melatonin excreted as labelled 6SM found in this study, compared with that of Jones et al. (1969), is not immediately apparent. This difference may simply be a reflection of the variation of this parameter in males or even in the general population, or it may be a consequence of the presence of disease.

The reason for the delayed clearance of labelled urinary melatonin metabolites in the earlier study of Jones et al. (1969) is also not

immediately apparent. The dose of labelled melatonin (2.1 µg) was considerably smaller than that used in this study, and is likely to amount to less than 10% of the expected melatonin UPR. Therefore, it is not likely to be a major contributing factor to the delayed clearance of urinary melatonin metabolite in those subjects.

The five male subjects in the study of Jones et al. (1969), were not healthy individuals as three were suffering from schizophrenia, one had Huntington's chorea and the other brain damage. It is not known what effects, if any, those disorders have on the metabolism and/or clearance of melatonin in man, although reduced nocturnal secretion of melatonin has been described in chronic schizophrenics (Ferrier et al., 1982).

The schizophrenics were not taking any medication but the case of Huntington's chorea was being prescribed dilantin (100 mg b.i.d.), methylphenidate (5 mg b.i.d.), phenobarbitone (30 mg t.i.d.) and diazepam (5 mg at night) and the brain damaged subject was receiving diazepam (5 mg t.i.d.) and chlorpromazine (50 mg at night) Jones et al., 1969). Chlorpromazine administration (100-600 mg) daily was shown to result in an increase in serum melatonin levels, but left CSF melatonin unaffected (Smith et al., 1979). Those authors concluded that chlorpromazine reduces the rate of metabolism of melatonin in the liver, rather than increasing the rate of synthesis in the pineal gland, which would be expected to lead to an elevation of both serum and CSF melatonin concentration. The dose of chlorpromazine being prescribed for the brain damaged subject was much lower than that used in the later study of Smith et al. (1979), and there was no evidence that the chloropromazine inhibited either 6-hydroxylation of melatonin or excretion of radioactivity by this patient (Jones et al., 1969). The effects of the

other drugs on the metabolism and/or clearance of melatonin in man is not known, but those appear not to have had any effect on the excretion of radioactivity (Jones et al., 1969).

The ages of the five male subjects of the earlier study were not given (Jones et al., 1969). If they were much older than that of the male subject in the present study, however, then this would offer a likely explanation for the delayed clearance of the metabolites of melatonin via the urine observed in that study, as both glomerular filtration rate and the function of the hepatic microsomal enzymes responsible for hydroxylation are known to decline with advancing age (Greenblatt et al., 1980).

Another reason for the delayed clearance observed in the earlier study (Jones et al., 1969) may have been the participation of some of the melatonin metabolites in biliary excretion and subsequent enterohepatic circulation (Slaunwhite et al., 1973). The virtually complete excretion of labelled melatonin as labelled urinary 6SM within 24 hours of i.v. administration in the present study indicates that conjugated 6HM did not undergo prolonged enterohepatic circulation. While it is likely that most, if not all, of the 6SM was cleared directly by the renal route, it is possible that some of the 6HM conjugated with sulphate, or more likely, with the higher MW glucosiduronate, did undergo a limited number of cycles of the enterohepatic circulation, before being ultimately excreted via the urine as 6SM within that 24 hour period.

The results of this study of 4 male subjects sampled at monthly intervals throughout one year indicated no clearly defined seasonal changes in both the amount and pattern of urinary 6SM excretion, which is interpreted to reflect a lack of a clearly defined seasonal pattern

of melatonin UPR. This finding contrasts with other studies which suggest annual or seasonal rhythms in human circulating melatonin levels (Arendt et al., 1979; Birau et al., 1981) and in human pineal HIOMT activity (Smith et al., 1981). Furthermore, the bimodal annual variation reported for human circulating melatonin levels at 0800 and 2400 hours (Arendt et al., 1979), and for human post-mortem pineal enzyme activity (Smith et al., 1981a) was not confirmed by the average monthly melatonin serum concentration (Birau et al., 1981).

The marked discripancy between the present results and those of the circulating melatonin level studies may be largely attributed to a lack of specificity in the RIA procedures used. The argument may be substantiated on several grounds. Firstly the circulating levels reported by either Arendt et al. (1979) or Birau et al. (1981), would require daily melatonin production rates > 100 µg, whereas estimates of the present study suggest an upper limit to this output of approximately 35 µg 24 h^{-1}. Since the clearance rate for melatonin is rapid and therefore represents minimal extrahepatic metabolism a MCR of 2000 l 24 h^{-1} can be expected. Accepting these latter values (MCR = 2000 l 24 h^{-1}, production rate = 35 µg 24 h^{-1}) the average daily circulating level of melatonin should be 17.5 pg ml^{-1}. This figure is in agreement with the findings of Lewy and Markey (1978), who quantified plasma melatonin by an accurate GCMS method, establishing a nocturnal maximum of 42 pg ml^{-1} and a nadir of 1.5 pg ml^{-1} during the day. This contrasts with the much higher values reported by Arendt et al. (1979), and Birau et al. 1981). A recent inter-laboratory evaluation of melatonin RIAs (Wetterberg and Eriksson, 1981) attests to the real problems of specificity and operator bias.

Although an annual bimodal rhythm in human pineal HIOMT activity was demonstrated, this rhythm was not very substantial, as the difference between the maximal values in January and July and the minimum activities in March and October (unpaired t-test), barely reached significance at the P=0.05 level (Smith et al., 1981a).

The present study was an attempt to determine whether the change in scotophase length that occurs in gradual fashion from approximately 14 hours per day in winter to 10 h in summer at 35°S was reflected by similar changes in urinary 6SM and presumably melatonin UPR. The result of this study, indicating no clear seasonal variation in urinary 6SM excretion in men at 35°S, was conducted in an environment where the seasonal changes in scotophase length are rather subtle and any influence that this parameter may have had on melatonin production could possibly have been masked by the usual day to day variation in urinary 6SM excretion. Thus it may be necessary to conduct a similar study at a location close to the polar regions where the seasonal changes in scotophase length are much greater in order to demonstrate the existence of a relationship between melatonin production and this parameter in man.

In conclusion, the urinary excretion of 6SM has been unequivocally proven to be a reliable index of melatonin production in man, providing a close integrated representation of plasma events. The variation in the amount and pattern of urinary 6SM excretion, and presumably melatonin UPR in the four males studied at 35°S, indicates the absence of a clearly defined annual rhythm in this parameter in man at this latitude. It is apparent that the previously postulated altered annual patterns of melatonin production were based on erroneous data, generated by the use of poorly specific techniques.

C H A P T E R 5

MENSTRUAL CYCLE STUDIES

CHAPTER 5

MENSTRUAL CYCLE STUDIES

FOLLICULAR AND LUTEAL PHASE 24 HOUR URINARY 6SM EXCRETION:

Twenty-four hour urinary 6SM excretion was determined in 10 healthy women aged 20-45 years. Five were studied during the follicular phase and the others in the luteal phase. The results of this initial study are shown in Table 8, indicating no significant difference in 6SM excretion between those phases of the menstrual cycle and also between that for 10 healthy adult males aged 20-45 years reported elsewhere ($P>0.05$ Mann-Whitney test; t-test).

FOLLICULAR PHASE 24 HOUR URINARY 6SM EXCRETION PROFILE:

The 24 hour urinary 6SM excretion profile during the follicular phase for a 27 year old healthy female was obtained by collection of consecutive random urine samples. This profile in relation to the hours of sleep and light and darkness is shown in Figure 12. A significant difference ($P< 0.02$, Mann-Whitney test) was found between the nocturnal sleep phase and the day time activity phase rates of 6SM excretion. This marked nocturnal rise in urinary 6SM excretion was associated with 83.5% of the 24 hour output. Interruption of the nocturnal sleep phase and exposure to 5 minutes of low intensity artificial light (small bedside lamp of less than 500 Lux) on two occasions did not markedly suppress urinary 6SM excretion.

TABLE 8

Values of urinary 6SM (µg 24 h^{-1}) in 20-45 year old female subjects

SUBJECTS	MEAN ± S.D.	RANGE
Follicular phase (n=5)	15.3 ± 9.0	5.3-28.9
Luteal phase (n=5)	19.0 ± 8.0	9.9-30.9

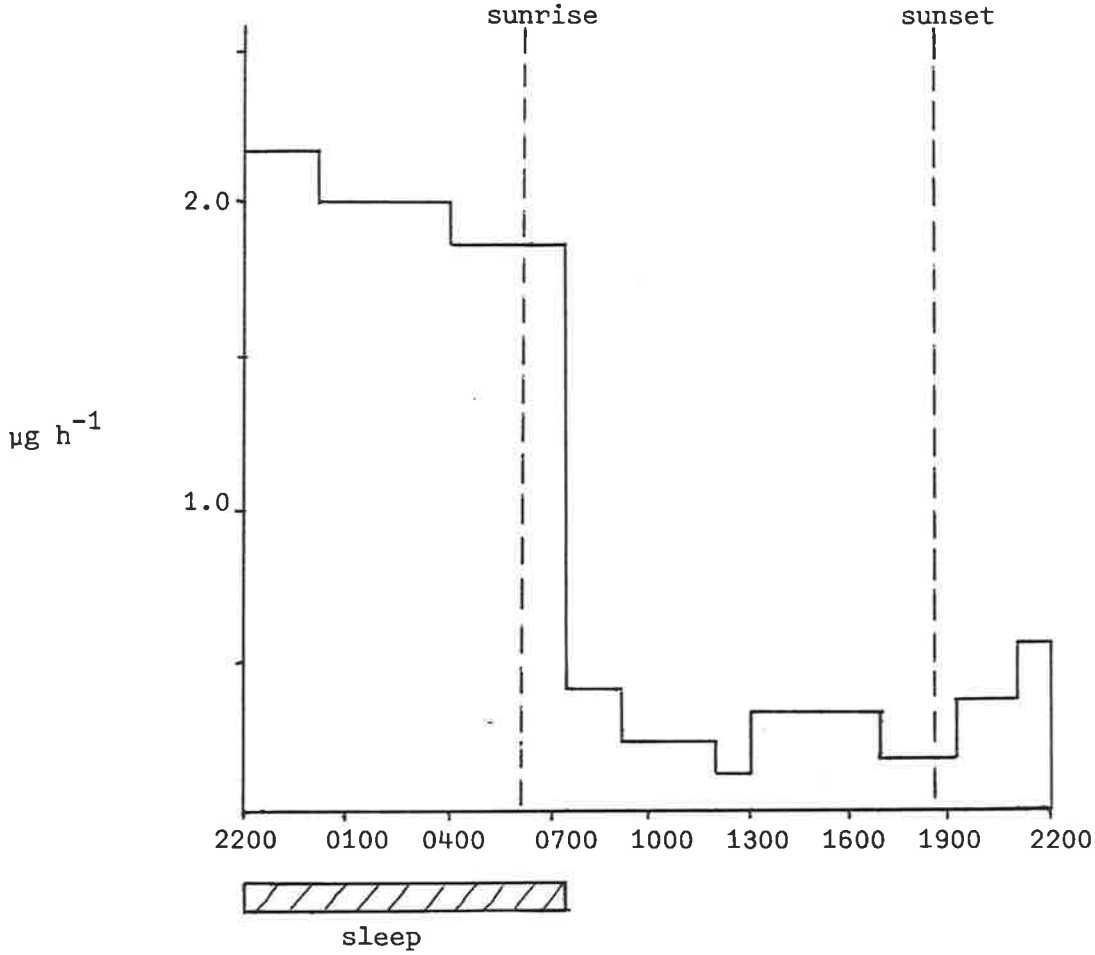


FIGURE 12. The 24 hour excretion pattern of urinary 6-sulphatoxy melatonin in a female subject.

URINARY 6SM THROUGHOUT THE MENSTRUAL CYCLE:

Urinary 6SM was used to evaluate in greater detail melatonin production daily in 2 normal ovulating women throughout a menstrual cycle. The subjects (RM and BG) were both aged 36 years and were not taking oral contraceptives or other drugs. Daily urine collection was in 2 parts; one to include the period from bedtime to that of the first voiding in the morning after awakening, and the other the remainder of the day until the ensuing bedtime. The early morning collection period was fixed at 0730-0800 hours for RM and 0830-0900 hours for BG, in an attempt to detect any marked phase shifts in the nocturnal maximum.

To obtain a measure of ovarian function, the 24 hour urinary levels of pregnanediol and oestrone were quantitated by gas chromatography (Barrett and Brown, 1970) and fluorimetry (Cox, unpublished) respectively.

Overall details and results for the two subjects are summarized in Figures 13 and 14 and Table 9. Statistical analysis (t-test; Mann-Whitney test) of the follicular, ovulatory and luteal phases of the cycle with respect to the absolute levels of 6SM revealed no significant differences ($P > 0.05$). The patterns and levels of excretion for urinary pregnanediol and oestrone confirmed that both women had normal ovulatory cycles.

DISCUSSION:

Several recent studies implicate melatonin in the regulation of the human menstrual cycle but the results are contradictory. The investigation based on single daily measurements of serum melatonin

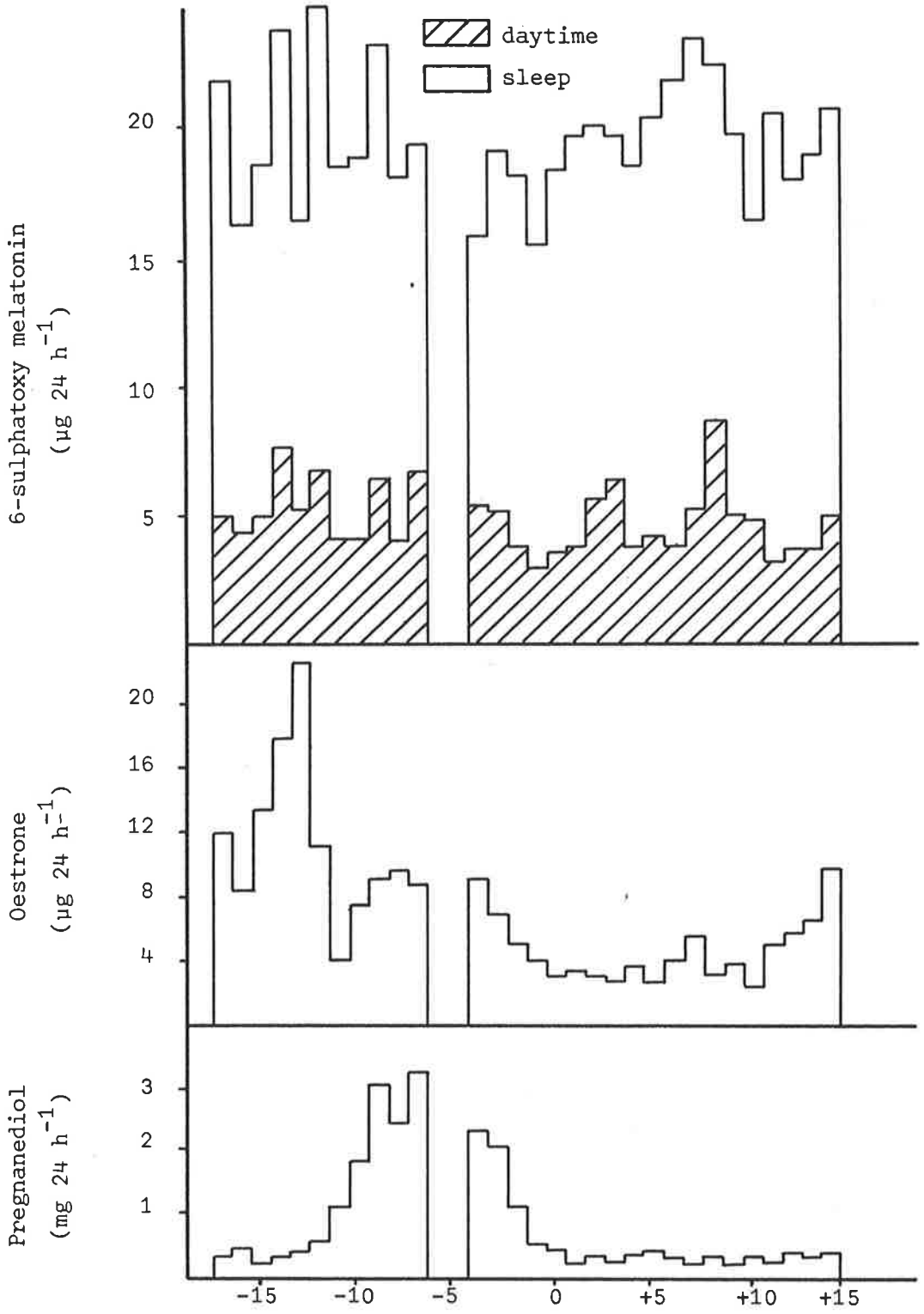


FIGURE 13. Menstrual cycle daily urinary excretion of 6-sulphatoxy melatonin, oestrone and pregnanediol for Subject RM.

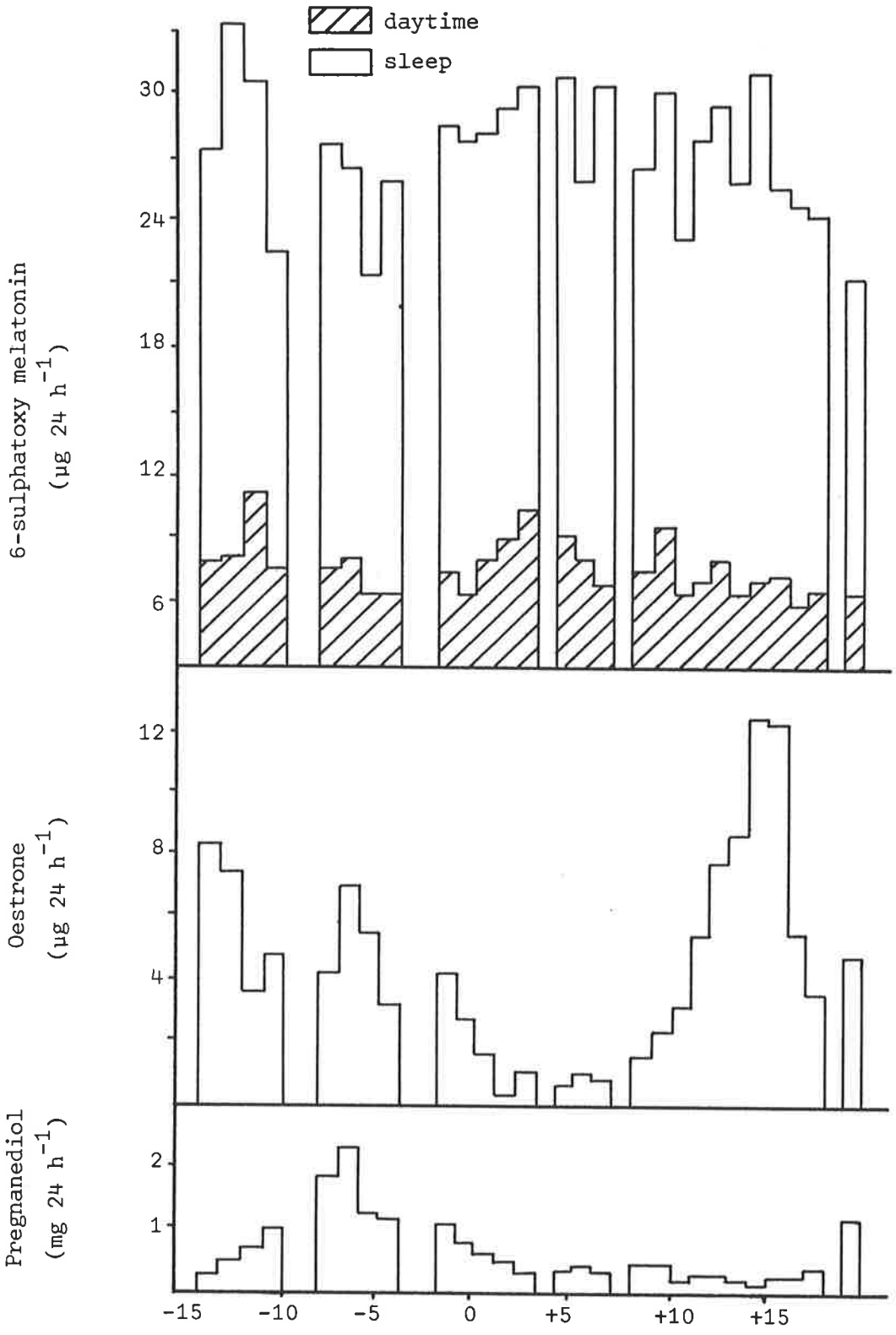


FIGURE 14. Menstrual cycle daily urinary excretion of 6-sulphatoxy melatonin, pregnanediol and oestrone of Subject BG.

TABLE 9

Daily experimental data for menstrual cycle subjects*

	SUBJECT RM	SUBJECT BG
6SM ($\mu g\ 24\ h^{-1}$)	19.3 ± 2.2 (30)	24.2 ± 3.1 (27)
6SM night (%)/total	75.5 ± 5.8 (30)	81.5 ± 4.2 (27)
Sleep length (h)§	8.6 ± 0.8 (30)	9.7 ± 0.8 (27)

*Values quoted are Mean ± S.D., n in parenthesis

§No direct relationship could be established between sleep length and excretion of 6SM during the night phase

Wetterberg et al., 1976; Tapp et al., 1980), or averaged daily serum values (Birau et al., 1980), revealed a nadir at ovulation, and in two cases (Wetterberg et al., 1976; Birau et al., 1980), exceptionally elevated values on the first day of menstruation, whereas an investigation of overnight urinary melatonin levels (Penny, 1982) revealed a maximum near the mid-cycle gonadotrøpin surge.

In contrast serial daily measurements of urinary melatonin during the menstrual cycle showed a general but inconsistent small decrease in levels from the follicular phase through to the luteal phase (Dudko and Shcherbina, 1977).

The measurement of daily urinary 6SM output for two ovulating women reveal a remarkable consistency in both the amount and pattern of excretion (Figures 13 and 14, Table 9), which are interpreted to reflect a stable melatonin UPR. These data are therefore at variance with changes in serum melatonin levels reported by Wetterberg et al. (1976), and Birau et al. (1981), and the marked urinary melatonin level variations reported by Penny (1982).

The discrepancy between the present and previous results may be largely attributed to a lack of specificity in the RIA procedures used. Previous compelling argument on several grounds has substantiated this claim with regard to circulating melatonin RIA procedures. Direct RIA for urinary melatonin, such as that used by Penny (1982), lack specificity to the extent that their extracts require chromatographic purification (Lynch et al., 1978; Arendt, 1981).

Although the results of Dudko and Shcherbina (1977) agree with the lack of significant menstrual cycle variation in melatonin production

indicated by this study, their procedure is grossly inaccurate, as the levels quoted are two orders of magnitude higher than those obtained by bioassay (Lynch et al., 1975a) and RIA (Lynch et al., 1978; Wetterberg, 1978; Lang et al., 1981; Lemaitre et al., 1981; Penny, 1982).

The finding that exposure of an adult female to short periods of artificial light of low intensity during the nocturnal sleep phase failed to suppress urinary 6SM excretion (Figure 12) is consistent with the findings of other similar studies in man (Vaughan et al., 1976; Jimerson et al., 1977; Arendt, 1978; Wetterberg, 1978; Akerstedt et al., 1979; Lynch et al., 1979; Vaughan et al., 1979a,b).

The failure of low intensity artificial light to suppress the nocturnal increase in melatonin secretion in man, as opposed to the ease with which such suppression is accomplished in experimental animals, has led some investigators to propose that the regulation of melatonin production is substantially different from that of all other mammals (Perlow et al., 1980), despite the apparent mediation of this nocturnal increase in melatonin secretion by sympathetic neurons (Vaughan et al., 1976; Hanssen et al., 1977; Kneisley et al., 1978; Tetsuo et al., 1981b). Furthermore, they have speculated that escape from direct control by the environmental light-dark cycle has conferred on humans an evolutionary advantage.

The demonstration that the nocturnal increase in human plasma melatonin could be suppressed by light of the intensity of indirect sunlight, i.e. light of higher intensity than that used in previous studies, (Lewy et al., 1980b), has led to the suggestion that in man, melatonin secretion may be profoundly influenced by the natural light/dark cycle, but not by ordinary artificial light (Lewy, 1981;

Lewy et al., 1982).

In conclusion it is apparent that interference by unknown substances during different stages of the menstrual cycle has led previous investigators to erroneously interpret their results and postulate that the pattern of melatonin production is altered. The stability of 6SM excretion and presumably melatonin production in the two females studied, provokes the thesis that any influence exerted by the pineal on the menstrual cycle is not mediated through changes in melatonin production.

C H A P T E R 6

PREGNANCY AND PERIPARTUM PERIOD STUDIES

CHAPTER 6

PREGNANCY AND PERIPARTUM PERIOD STUDIES

SUBJECTS:

The pregnant subjects were healthy volunteers aged 18-40 years who were not taking any drugs.

GENERAL PREGNANCY STUDY:

Pineal function was evaluated during the course of pregnancy by measurement of urinary 6SM excretion from women in the first, second and third trimesters (12-14 week periods) of pregnancy. The urine samples were collected in 2 parts: one to include the period from bedtime until one hour after awakening and the other to include the remainder of the day until the ensuing bedtime. Data obtained from this study is shown in Table 10. As indicated in all women studied, the amount of urinary 6SM excreted during the nocturnal sleep phase was significantly greater than that excreted during the day time activity period (paired t-test). There was no significant differences ($P>0.05$, Kruskal-Wallis test), however, between groups in either the 24 hour urinary 6SM excretion, or in the amount of 6SM excreted during the nocturnal sleep phase, indicative of any marked changes in melatonin output as pregnancy progressed.

INDIVIDUAL CASE STUDIES:

To examine for pregnancy and perinatal period related changes in

TABLE 10

Urinary 6-sulphatoxy melatonin excretion in pregnancy*

STAGE	TOTAL (µg 24 h-1)	SLEEP PHASE (µg)	DAY TIME (µg)	$\frac{\text{SLEEP}}{\text{TOTAL}}$ x 100 (%)
1st Trimester	20.5 ± 4.7 (5)	16.4[a] ± 4.2 (5)	4.1[a] ± 1.7 (5)	80.1 ± 7.7 (5)
2nd Trimester	22.2 ± 7.5 (6)	17.4[b] ± 5.9 (4)	5.6[b] ± 2.8 (4)	76.1 ± 7.6 (4)
3rd Trimester	18.3 ± 6.5 (7)	13.7[c] ± 4.7 (7)	4.6[c] ± 2.3 (7)	75.2 ± 6.2 (7)

*Values quoted are mean ± S.D., n in parenthesis

[a] $P<0.005$

[b] $P<0.025$

[c] $P<0.002$

greater detail, repeated measurements of urinary 6SM were made in other women at various stages, and the results are shown in Table 11. For subjects MK and DW, the overnight collection period was standardized to finish at 0830 hours and 0800 hours respectively, to detect any marked phase shifts in the nocturnal acrophase.

For subject MK Spearman regression analysis revealed no significant correlation between 24 h urinary 6SM excretion and the week of gestation. Furthermore, the amount of urinary 6SM excretion at 3 weeks post-partum (23.4 μg 24 h^{-1}) was not significantly different from the amount excreted during pregnancy (24.8 ± 1.6 μg 24 h^{-1}, n=9). The mean sleep period for this subject during the study was 9.6 ± 1.6 h, and the urinary excretion of 6SM during the nocturnal sleep phase (19.2 ± 2.5 μg, n=7) and day time activity phase (5.9 ± 2.0 μg, n=7), together with the percentage contribution of the nocturnal sleep phase component to the 24 h output (76.4 ± 7.6, n=9), were remarkably consistent.

For subject DW, the daily excretion of 6SM recorded on 5 (12.7 μg) and 15 (15.7 μg) days post-partum were marginally greater ($P<0.05$, Mann-Whitney test) than the mean daily output (10.5 ± 1.0 μg, n=5) recorded during the 4 week period prior to delivery, but overall there was little variation in the amount or pattern of 6SM excretion. The mean recorded sleep period for DW during the course of the studies was 10.2 ± 0.5 h (n=7) with 79.9 ± 7.3% (n=7) of the total 24 h urinary 6SM excreted during this time.

The other subjects studied also showed a surprising consistency in daily 6SM excretion.

TABLE 11

Urinary 6-sulphatoxy melatonin excretion in the perinatal period.

The day of collection relative (days prior, -n; days post +n) to the day of birth (day 0) and the total amount of 6SM excreted (μg 24 h^{-1}) are shown for individual subjects. The subjects age and parity are indicated in parenthesis (p.g., primi gravida).

SUBJECT	DAYS FROM BIRTH	6SM
MK (21 y, p.g.)	-147	25.2
	-133	28.2
	-119	24.3
	-118	24.4
	-117	22.7
	-105	23.8
	- 91	25.9
	- 70	24.5
	- 42	23.9
	+ 20	23.4
DW (32 y, para 3)	- 25	10.6
	- 18	8.9
	- 7	10.9
	- 2	11.7
	- 1	10.4
	+ 5	12.7
	+ 15	15.7
KB (28 y, para 3)	- 7	15.2
	+ 1	15.6
DR (28 y, p.g.)	- 20	19.8
	+ 1	18.5
EP (33 y, p.g.)	+ 6	9.7
	+ 21	8.0
EC (28 y, para 2)	+ 3	7.2
	+ 21	9.4

LABOUR STUDY:

Consecutive random urine sample collection was made during the labour period and during a similar period 1-3 weeks earlier in two women to evaluate possible labour related changes in urinary 6SM excretion. Data for this study is indicated in Figure 15. No significant difference ($P>0.0.5$, Mann-Whitney and Wilcoxon tests) between the urinary 6SM excretion for the labour period, and for that over a similar period 1-3 weeks earlier, was detected for subjects KB and DR.

During the course of these studies, one subject (HT 32 years, primi gravida) was found to have extremely low urinary 6SM values. This apparently healthy woman, experienced a normal pregnancy and delivery, but recorded low 24 h urinary 6SM output, both when repeatedly sampled during the 8 to 20 weeks of gestation (2.1 ± 0.6 µg, n=11), and again 2 days prior to (2.2 µg) and 5 days post partum (1.2 µg). Despite the low overall level, this subject appeared to maintain a circadian rhythm in excretion, as the urinary 6SM output was significantly higher ($P < 0.02$, paired t-test) during the nocturnal sleep period (1.3 ± 0.5 µg, n=8) than during the day time activity phase (0.7 ± 0.3 µg, n=8), with the mean percentage contribution of the nocturnal sleep phase component to the 24 h output (65.9 ± 10.1, n=8) similar to that found in other subjects studied.

DISCUSSION:

Assessment of human pineal function in pregnancy through melatonin measurement has led to conflicting results.

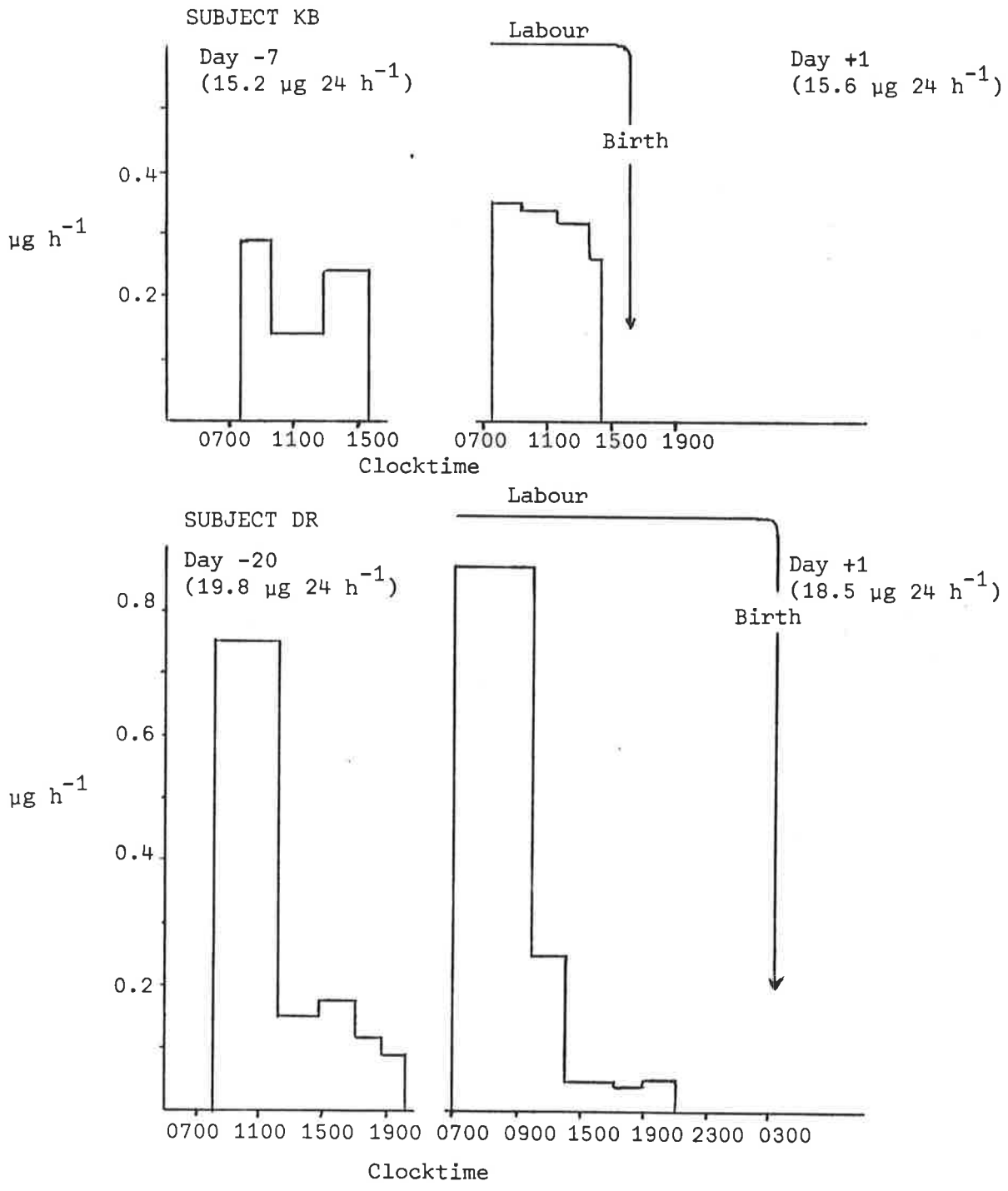


FIGURE 15. Urinary 6-sulphatoxy melatonin excretion rate (µg h^{-1}) for individual case study subjects during parturition and the prenatal period.

An early study by Grishchenko et al (1976) found a sudden marked increase in the putative urinary melatonin content in late prenancy, whereas other workers reported an unchanged (Mitchell et al., 1979), enhanced (Kennaway et al., 1981) or diminished (Evans et al., 1979) circadian rhythm in plasma melatonin.

In the perinatal period Grishchenko et al. (1976) found the increased output of melatonin in the urine during late pregnancy declined markedly on the day preceding labour, but rose immediately after birth to levels higher than those found in non-pregnant subjects. These higher levels of output were then maintained for at least 6 days. By contrast Mitchell et al. (1979) noted a small increase in the mean plasma melatonin content during labour, which was significant in term labour.

In the present study, measurement of urinary 6SM throughout pregnancy and the peripartum period has revealed a remarkable consistency in both the amount and daily pattern of excretion, indicating a stable pattern of melatonin production. Similar stable patterns of melatonin production have previously been observed in females throughout the menstrual cycle and in adult males. Taken overall, these results raise doubts concerning the previous claims, that purport to show changes in melatonin output which relate to the endocrine changes associated with human reproductive function. The apparent discrepancy between the results of this study and those of previous studies may relate to a lack of specificity of assay procedures previously employed. The fluorimetric procedure of Grishchenko et al. (1976) for the determination of melatonin in urine gave levels 2 orders of magnitude higher than those obtained by bioassay (Lynch et al., 1975a) or RIA techniques (Lynch et al., 1978; Wetterberg,

1978; Lang et al., 1981; Lemaitre et al., 1981; Penny, 1982).

We have previously argued that many of the reported levels of both circulating and urinary melatonin determined by RIA are overestimates. This problem has prompted recent suggestions of (a) the use of a highly specific technique such as GCMS to validate the RIA procedures, and to occasionally check the degree of accuracy of the RIA data (Arendt, 1981), and of (b) the employment of high performance liquid chromatography (HPLC) to remove interfering substances from sample extracts (Arendt, 1981). Recently, both suggestions have been implemented (Lang et al., 1981; Tamarkin et al., 1982).

The apparent stability of melatonin production during pregnancy and parturition is remarkable, particularly so in view of the many metabolic changes which take place as the mother adjusts to the demands of the developing conceptus (Simpson and MacDonald, 1981; Llewellyn-Jones, 1982a). Maternal plasma levels of melatonin would be expected to decrease in proportion to the marked plasma volume increase that occurs as pregnancy progressed.

In view of studies in sheep indicating that the foetal pineal may contribute to melatonin output near term (Kennaway et al., 1977b; Kennaway et al., 1981), it was anticipated that there might have been changes in maternal metabolite levels due to a contribution made by the foetal melatonin production. If the human foetus is producing melatonin at term, however, the amounts were not discernible in the maternal urines of this present study.

It is concluded that melatonin output is largely unperturbed by pregnancy and that previous reports of pregnancy related changes based

on blood or urinary studies are misleading, probably due to unreliable assay methods.

CHAPTER 7

STUDIES IN NEONATES AND PREPUBERTAL CHILDREN

CHAPTER 7

STUDIES IN NEONATES AND PREPUBERTAL CHILDREN

NEONATAL STUDY:

Six full term amniotic fluid samples were obtained, 4 between 1015 and 1315 hours and 2 from 2315 to 0100 hours No 6SM (<200 pg ml^{-1}) was detected in either the day time or night time samples.

Random urine samples were collected with the aid of pediatric collection bags, between 0600 and 1800 hours, and from 1800 to 0600 hours at 2-3 days, and again at 12 days of age from a healthy bottle fed boy and girl. No 6SM (<200 pg ml^{-1}) was detected in any of those samples.

Using preweighed disposable nappies, urine was collected from a breast fed boy over 24 hour periods at 2, 3, 4, 5, 6, 7, 8, 9, 11 and 16 weeks of age. Nappies soiled with faeces were rejected and not included in this study. After the addition of a known volume of water and mixing, the diluted urine was recovered by compression. The recovery of urinary 6SM from nappies by this procedure was 95% i.e. virtually quantitative. No 6SM (<200 pg ml^{-1}) was detected in any of the urines taken from age 2-11 weeks. At 10 weeks of age, however, 6SM was present in the night time urines. Data from the 16 week urine samples are shown in Table 12.

PREPUBERTAL CHILDREN'S STUDY:

Initial attempts at urine collection from a 15 month old boy

TABLE 12

Twenty four hour urinary 6SM excretion from a 16 week old male subject

COLLECTION PERIOD (h)	6SM (ng)
1055-1315	<2
*1455-1705	<2
1705-1900	<2
1900-2105	29
*2105-0725	311
0725-0905	17
*0905-1055	7

*Sleep periods

yielded only unsatisfactory small random samples at 1500 and 2300 hours which contained 20 and 390 ng 6SM respectively. Using pre-weighed disposable nappies as previously described, urine was collected from a 12 month old boy. For the sleep period from 1930-0800 hours, and the waking period from 0830-1000 hours, the 6SM output for this subject was 17.4 and 0.7 µg respectively.

Urine was collected by voiding into a pot under close supervision from each of 4 boys aged 2-6 years, and a 6 year old girl for the nocturnal sleep period, and again for a portion of the day time activity period. Urinary 6SM excretion data are indicated in Table 13.

DISCUSSION:

Amniotic fluid at term in the human is the product of numerous exchanges with the foetal and, indirectly the maternal compartments (Seeds, 1980; Llewellyn-Jones, 1982b), and it is generally appreciated that alterations of this fluid compartment in many instances parallel changes in the foetus (Seeds, 1980). At term foetal urine forms the main source of the amniotic fluid (Llewellyn-Jones, 1982b). It is generally accepted that foetal urine makes an important contribution to the steroid composition in term amniotic fluid (Peltonen and Laatikainen, 1975). The steroids in amniotic fluid are mainly in the conjugated form (Peltonen et al., 1979) and quantitatively, the foetal urine is in all likelihood the major source of corticosteroid sulphates in this fluid at term (Fench et al., 1980). Thus at term, if the human foetus was able to produce melatonin, convert it to 6SM and excrete it in the foetal urine, then this would be expected to be reflected in the amniotic fluid.

TABLE 13

Urinary 6SM excretion in prepubertal children

SUBJECT	COLLECTION PERIOD (h)	6SM (µg)
Boy, 2 y	0100-0730	7.7
	0930-1200	1.4
Boy, 3 y	2130-0230	16.3
	1230-1330	2.3
Boy, 4 y	1930-0710	11.4
	1215-1600	0.4
Boy, 6 y	2100-0820	24.5
	0820-2100	4.1
Girl, 6 y	1950-0720	24.5
	0720-1950	3.0

The human foetal liver and adrenal has been shown to possess the necessary components of the microsomal mono-oxygenase system in amounts of the same magnitude as in adult liver (Eriksson and Yaffe, 1973; Short et al., 1976). Furthermore, the capacity of those tissues to hydroxylate both endogenous and exogenous compounds is significant, the usual capacity being about one third that of adults. The capacity of the human foetus to produce glucosiduronates, however, is quite low (Eriksson and Yaffe, 1973; Dutton, 1978). In contrast to this, sulphation is much more active during intra-uterine life; in some tissues actually exceeding that of adults (Wengle, 1966; Bostrom and Wengle, 1967).

Glucosiduronate conjugation in particular, and hydroxylation to a lesser extent, appear to be the two metabolic pathways which are most defective at birth (Eriksson and Yaffe, 1973; Morselli, 1976; Short et al., 1976; Dutton, 1978; Turner, 1978), while sulphate conjugation activities are close to the adult pattern (Morselli, 1976; Yaffe and Danish, 1978). Those deficiencies in drug metabolizing enzymes may persist for at least a month post-natally (Eriksson and Yaffe, 1973; Turner, 1978), and only approach adult levels by about eight weeks after birth (Creasey, 1979).

Evidence of active sulphoconjugation during intra-uterine life and immediately after birth is provided by the finding of many sulphated derivatives in body fluids in the human organism during foetal and early neonatal life (Peltonen et al., 1979; Shackleton et al., 1979; Fencl et al., 1980). There have been indications that the availability of sulphate for sulphoconjugation processes may be relatively limited and can be readily exhausted (Dodgson, 1977; Dutton, 1978; Jakoby et al.,

1980). Several lines of evidence, however, indicate that this does not normally occur in the neonatal period. In a study of pharmacokinetics of acetaminophen in the human neonate, the average rate constant for acetaminophen sulphate formation was larger than in adults (Levy et al., 1975). At birth the levels of the amino acids cysteine and methionine in the circulation of the neonate were both significantly higher than the corresponding maternal levels, and, although the levels of both amino acids fell shortly thereafter, they remained relatively constant throughout infancy and childhood (Rosenberg and Scriver, 1980). Circulating levels of inorganic sulphate were found to be higher in neonates and infants than in older children and adults (Cole and Scriver, 1980).

Thus it is likely that the human foetus, neonate and infant has the capacity to convert melatonin to 6SM. Furthermore, the reason for the absence of 6SM in term amniotic fluid and urine for the first three months of life in the human, as indicated by this very limited study, is most likely that melatonin production does not occur during this period. Further support for this claim is provided by the absence of urinary 6SM in eight week old infant urine, the age at which both hydroxylation and sulphation capacity are almost certainly at adult levels.

Although the ontogeny of the development of melatonin production in the human was limited to one subject in this study, it was noted that when melatonin production was first detected, it occurred during the hours of darkness, and at the age at which circadian rhythm of sleep and wakefulness was fully established and completely synchronised with the day and night regimen (Meier-Koll et al., 1978; Minors and Waterhouse, 1981). The adult levels of urinary 6SM, shown by this

study to be excreted by a one year old child, are thus achieved between the age of 16 weeks and 1 year.

The presence of 6SM in urine samples taken after the overnight sleep period from the 16 week old male subject (Table 12), is probably due to its delayed renal clearance as a result of the inefficient active tubular secretion and decreased renal blood flow which characterises renal function in the first 6-12 months of extra-uterine life (Morselli, 1976; Yaffe and Danish, 1978).

Other neonatal studies, performed using RIA techniques, have yielded conflicting results. The results of the present study are supported by those of another in which melatonin was reported to be absent from the following; full-term amniotic fluid, umbilical venous and arterial blood, and 6 day old neonatal day time and night time blood and urine samples (Sizonenko et al., 1980). In contrast to this, melatonin was reported to be present in human amniotic fluid during late pregnancy at greater concentrations in labour than before the onset of labour (Mitchell et al., 1978). Furthermore, melatonin was reported to be present in cord blood in three studies. In one a marked circadian rhythm with higher night time levels than those during the day time was reported (Evans et al., 1979), in another levels were found to be raised above those of the maternal circulation (Mitchell et al., 1979), while in the third melatonin appeared to be present in some samples at levels similar to those in the maternal circulation (Kennaway et al., 1981). Moreover, melatonin was reported to be present in both plasma and urine of male infants from age one day to one year (Hartmann et al., 1982).

Compelling argument has been used elsewhere to demonstrate the

generally poor specificity of melatonin RIA procedures. The lack of agreement between the present and previous results is therefore probably largely attributed to a lack of specificity in the RIA procedures used. While the evidence provided by this study indicates that the human neonate does not produce melatonin, it is based on the assumption that the neonate has the capacity to convert melatonin to 6SM and then to excrete it in the urine. Ethical considerations, however, have so far precluded the testing of the presence of this capacity in the human neonate by us.

From the study of human pineal neoplasms has evolved the idea that the human pineal gland produced a substance that held sexual maturation in check (Kitay, 1954b). This speculation has been fueled by the evidence that melatonin inhibits gonadal development in experimental animals (Minneman and Wurtman, 1976). The report by Silman et al. (1979), of dramatically higher day time plasma levels in prepubertal boys, strengthened the pineal-gonadal interaction hypothesis.

Since the report by Silman et al. (1979) other studies have yielded conflicting results. The results of the present study, showing the levels and pattern of urinary 6SM excretion in prepubertal children to be similar to those observed in adults, indicate that melatonin production rate appears to be maintained at the same level from the age of 1 year through puberty into adulthood. One consequence of a stable melatonin production rate over this range would be a gradual decrease with increasing age of circulating melatonin levels, resulting from the expanded circulation volume associated with increased body size. Support for this was suggested by reports of age dependent reductions in day time levels of both serum and CSF melatonin in

human subjects (Brown et al., 1979; Iguchi et al., 1982b). The results of a much more extensive study of urinary conjugated 6HM excretion in 3-16 year old children of both sexes and adults were similar to those of the present urinary 6SM study; an unexpected finding, however, was that of increased nocturnal urinary conjugated 6HM excretion at the time of the onset of breast development (Tanner stage II) in girls (Tetsuo et al., 1982b). For the urinary melatonin studies employing RIA, no significant changes between different pubertal stages for both sexes was reported in one study (Sizonenko et al., 1980); in contrast, others reported an increase throughout childhood into adulthood and an increase during puberty above those levels found in adults were reported (lemaitre et al., 1981; Penny, 1982). In the circulating melatonin studies employing RIA, a lack of variation of day time melatonin concentration within different pubertal stages for both sexes was suggested (Lenko et al., 1982), while the 24 hour profile of plasma melatonin was reported not to vary as a function of pubertal status or whether the puberty was precocious or delayed (Ehrenkranz et al., 1982; Tamarkin et al., 1982).

The generally poor specificity of melatonin RIA procedures has been established elsewhere, and is probably largely responsible for the discrepancy between the results of the present study and those of some of the studies that employed RIA. The reason for the difference between the results of Silman et al. (1979) and those of the two melatonin metabolite studies is almost certainly methodological. The GCMS procedure employed by Silman et al. (1979), and described elsewhere (Wilson et al., 1977; Leone et al., 1979), has been severely criticised on a number of grounds to the extent that both the specificity and precision of this procedure appear to be seriously deficient, the

major reasons being the use of a structural analogue as internal standard, rather than a stable isotope labelled analogue, in amounts three orders of magnitude higher than the reported amounts of melatonin (Markey, 1981).

Other evidence has been presented that does not support the presumption that melatonin suppresses gonadal function in the human. A coincident nyctohemeral rise in plasma melatonin and LH in pubertal boys conflicts with the concept of a melatonin anti-gonadotropin influence (Fevre et al., 1978), and an acute constant infusion of a pharmacologic amount of melatonin did not inhibit pituitary response to LHRH in men (Weinberg et al., 1980).

CONCLUDING REMARKS

CONCLUDING REMARKS

The isotope dilution GCMS procedure described allows for accurate quantitation of the unique melatonin metabolite 6SM, and can, when coupled with the i.v. administration of deuterium labelled melatonin, be used to provide a reliable estimation of melatonin UPR. This has allowed the first assessment of these parameters in man and sheep, thus forming a sound analytical basis for investigations of the occurrence of this hormone.

In man and sheep urinary 6SM excretion follows the circadian rhythm found in plasma melatonin determinations, with the major part being produced during the hours of darkness.

Pineal ablation in sheep removed virtually all urinary 6SM, indicating that extrapineal synthesis of melatonin does not contribute significantly to circulating melatonin and confirming the use of this substance as an index of pineal function.

Urinary 6SM excretion has been established as a reliable index of melatonin production in man providing a useful integrated representation of known plasma events. Approximately 40% of deuterium labelled melatonin entering the blood is excreted as urinary deuterated 6SM within 24 hours in man, confirming the significance of this route as a major secretory pathway. By contrast, in the sheep only about 5% of the melatonin produced is excreted as urinary 6SM, qualifying its use as an index of pineal function in this species.

The pattern of urinary 6SM excretion in the males studies at 35°S indicates the absence of a clearly defined annual rhythm in melatonin

production in man at this latitude.

Judged by measurement of 6SM the human foetus and neonate do not appear to produce melatonin. Urinary 6SM excretion was first evident at 16 weeks but had achieved an adult circadian rhythm and levels of production at the end of the first year of life.

No marked changes in diurnal pattern or amount of urinary 6SM excretion were evident during puberty, the menstrual cycle, pregnancy, parturition or early lactation. This contrasts with several previous claims but it is argued that the assay methodology used by those workers was suspected to have led them to erroneously interpret their results.

The stability in the amount of melatonin produced each day, and in the circadian pattern of its production, clearly indicates that if the pineal is exerting an influence through melatonin, it does so through subtle adjustments to the timing of melatonin output, rather than through quantitative changes. Future research into abnormalities of pineal function needs therefore to be designed to take this fully into account. The method developed by us to provide an accurate and reliable measure of urinary 6SM can provide important base line date for these studies, as it has the advantage that it can be used in conjunction with heavy isotope labelled tracer to provide an accurate measure of melatonin production rate. However, for the potential usefulness of urinary 6SM measurement to be fully realized, more practical procedures based on RIA or HPLC techniques need to be developed.

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