

**CHARACTERISATION OF A LOW-AFFINITY MELATONIN
BINDING SITE IN SYRIAN HAMSTER BRAIN**

By

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McMaster University

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SITE IN SYRIAN HAMSTER BRAIN**

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(Medical Sciences: Neuroscience)

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in Syrian hamster brain

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ABSTRACT

The pharmacology of 2-[¹²⁵I]iodomelatonin ([¹²⁵I]MEL) binding was investigated in Syrian hamster brain regions. Both kinetic and saturation binding analysis revealed a site with an affinity of = 2 nM in the hypothalamus. Correlation of the pharmacological profiles in different brain regions indicated this site also to be present in the cerebral cortex and hippocampus. This binding site appeared to be present throughout the CNS and peripheral tissues of the hamster and is distinct from the picomolar-affinity melatonin receptor. Using the technique of radiation inactivation, the molecular sizes of the chick retinal picomolar-affinity melatonin receptor ($M_r = 44$ kDa) and hamster hypothalamic low (nanomolar)-affinity binding site ($M_r = 30$ kDa) were found to be significantly ($p < 0.04$) different. This, along with the pharmacological differences, supports the notion of two separate melatonin binding sites.

Binding studies indicated that the continuous cell line RPMI 1846 (Syrian hamster melanoma) possesses a melatonin binding site similar to that found in the hamster CNS. A high correlation was observed for a series of compounds between the K_i in hamster hypothalamic membranes vs RPMI 1846 membranes. Scatchard-Rosenthal analysis of saturation binding of [¹²⁵I]MEL to membranes indicated a site of similar affinity to that in brain. This cell line was used to study potential signal transduction mechanisms for the site. No effect of melatonin was seen on basal or forskolin-stimulated membrane adenylate cyclase activity. Using [³H]adenine and [³H]myo-inositol prelabel-

ling of cells, melatonin was found to be without effect on in situ basal or stimulated cAMP or basal IP_3 levels. In ^{45}Ca -flux experiments, melatonin was without effect on basal or K^+ -stimulated uptake in hamster brain synaptosomes or basal uptake in RPMI 1846 cells. This nanomolar-affinity melatonin binding site therefore does not seem to be coupled to the adenylate cyclase or inositol phosphate second messenger systems and its biochemical and physiological function(s) remain(s) unknown.

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ABBREVIATIONS

5-HT	serotonin: 5-hydroxytryptamine
5-MT	5-methoxytryptamine
8-OH-DPAT	(±)-8-hydroxydipropylaminotetralin·HBr
6-CCE	6-carboline 3-carboxylate ethyl ester
AcCoA	acetyl coenzyme A
ANOVA	analysis of variance (one-way)
ANB-NOS	N-(5-azido-2-nitrobenzoyl)oxysuccinimide
ATP	adenosine 5'-triphosphate
b	y-intercept of a linear equation
B _{max}	maximal binding site density
BOC-ON	N-t-butoxycarbonyloxymino-2-phenylacetonitrile
BSA	bovine serum albumin
cAMP	cyclic adenosine 3',5'-monophosphate
CAPS	3-(N-cyclohexylamino)propanesulfonic acid
cDNA	complementary deoxyribonucleic acid
cGMP	cyclic guanosine 3',5'-monophosphate
CHES	2-(N-cyclohexylamino)ethanesulfonic acid
CHO	chinese hamster ovary cells
CNS	central nervous system
CPM	counts per minute
CSF	cerebral spinal fluid

BSA	bovine serum albumin
\bar{D}_{37}	apparent radiation dose reducing control biological activity by 63%
DAG	diacylglycerol
df	degrees of freedom
DMEM	Dulbecco's modified Eagle medium
DMSO	dimethylsulfoxide
DNase	deoxyribonuclease
DOI	(±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane-HCl
DPM	disintegrations per minute
DTT	D,L-dithiothreitol
EC ₅₀	concentration of compound producing 50% of the maximal effect
EDTA	(ethylenedinitrilo)tetraacetic acid (disodium salt)
EGTA	ethyleneglycolbis(6-aminoethyl ether)-N,N,N',N'-tetraacetic acid
F-12	Ham's nutrient mixture, F-12
FNPA	4-fluoro-3-nitrophenylazide
FSH	follicle-stimulating hormone
FURA-2	1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2'-amino-5'-methylphenoxy)-ethane-N,N,N',N'-tetraacetate
G protein	guanine nucleotide-binding protein
G _i (α)	G protein coupled to inhibition of adenylate cyclase; (the α subunit of this protein)
G _o (α)	G (other) protein; (the α subunit of this protein)
GABA	γ-aminobutyric acid
GDP	guanosine 5'-diphosphate

GppNHp	6- γ -imidoguanosine 5'-triphosphate
GTP	guanosine 5'-triphosphate
GTP γ S	guanosine-5'-O-(3-thiotriphosphate)
HBSS	Hanks' balanced salt solution
HCOOH	formic acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
H/H	HBSS + 30 mM HEPES buffer, pH 7.4 (with NaOH)
HIOMT	hydroxyindole-O-methyltransferase
HK/HCa	high potassium + high calcium buffer
HPLC	high pressure liquid chromatography
IBMX	isobutylmethylxanthine
IC ₅₀	inhibitor concentration reducing radioligand binding by 50% or functional activity by 50%
[¹²⁵ I]CYP	[¹²⁵ I]iodocyanopindolol
IMEL	2-[¹²⁷ I]iodomelatonin (non-radioactive)
[¹²⁵ I]MEL	2-[¹²⁵ I]iodomelatonin; 2-[¹²⁵ I]iodo-5-methoxy-N-acetyltryptamine
IP ₁₋₃	Inositol mono, bis, or trisphosphate
K _d	receptor-ligand dissociation constant
K _B	receptor-antagonist dissociation constant
K _i	receptor-inhibitor dissociation constant
LDH	lactate dehydrogenase
LET	linear energy transfer (keV/ μ m)
LH	luteinizing hormone
LHRH	luteinizing hormone releasing hormone
LK/LCa	low potassium + low calcium buffer

m	slope of a line
mCPP	1-(3-chlorophenyl)piperazine-HCl
ME	median eminence
MEL	melatonin; N-acetyl-5-methoxytryptamine
MSH	melanocyte-stimulating hormone
N _o	Avogadro's number (6.02 x 10 ²³ particles/mole)
NAD	6-nicotinamide-adenine dinucleotide
NADH	6-nicotinamide-adenine dinucleotide, reduced
NAS	N-acetylserotonin; N-acetyl-5-hydroxytryptamine
NE	norepinephrine; noradrenaline
NMR	nuclear magnetic resonance
NPP	1-(1-naphthyl)piperazine-HCl
ODS	octadecylsilane
p	probability level
PAGE	polyacrylamide gel electrophoresis
PBS	(Dulbecco's) phosphate buffered saline
PDE	phosphodiesterase
PEI	polyethylenimine
PI	phosphoinositide
PIF	prolactin inhibiting factor
PKC	protein kinase C
ppm	parts per million
PT	pars tuberalis
r	correlation coefficient
RIA	radioimmunoassay

RNA	ribonucleic acid
RNase	bovine pancreatic ribonuclease (E.C. 3.1.4.22)
SAD	seasonal affective disorder
SAM	S-adenosylmethionine
SANPAH	N-succinimidyl-6-(4'-azido-2'-nitrophenylamino)hexanoate
SCG	superior cervical ganglion
SCN	suprachiasmatic nuclei
SD	standard deviation
SEM	standard error of the mean
SDS	sodium dodecyl sulfate
SNAT	serotonin N-acetyltransferase
TCA	trichloroacetic acid
TDCB	3-tropanyl-3,5-dichlorobenzoate
TEA	triethylamine
TLC	thin layer chromatography
Tris	tris(hydroxymethyl)aminomethane
TX-100	triton-X 100 detergent
UV	ultraviolet
\bar{v}	partial specific volume (cm^3/g)
VIP	vasoactive intestinal peptide

CHAPTER ONE

GENERAL INTRODUCTION

1.1 The Beginnings of Pineal Research

Until the turn of this century, the mysticism surrounding the pineal body was in a more advanced state than the understanding of pineal physiology or biochemistry. (For an interesting, brief review of the earliest [at least the earliest recorded] years of 'pineal musings', the reader is referred to Kappers [1979]). The first evidence for a physiological function of the pineal came when McCord and Allen (1917) and Huxley and Hogben (1922) found that bovine pineal gland extracts contained a substance which caused a lightening of the colour of frog skin. The nature of this substance remained unknown for the next four decades until, Lerner and coworkers (1958, 1959) isolated and purified the active compound, obtaining ≈ 0.1 mg from 100 g of lyophilised bovine pineals (= 2,500 glands). The chemical structure of the factor was determined to be N-acetyl-5-methoxytryptamine and was given the name melatonin (from the Greek *melanos*, black) to reflect its indole nature and its ability to blanch skin pigment cells. Since that time, the physiology of the pineal and melatonin has been extensively investigated in an extraordinarily wide variety of species (from Armadillo to Xenopus). In the last few years a virtual explosion of research has led to an increased understanding of the biochemical mechanisms controlling the production of melatonin and the generation of its intracellular signal in target tissues.

1.2 Anatomy and Physiology of the Pineal

1.2.1 Location and Structure

The mammalian pineal gland (also called the epiphysis cerebri) develops from the roof of the brain in a median region at the posterior end of the diencephalon, anterior to the posterior commissure. The gland is not part of the brain proper and actually lies outside of the blood-brain barrier. The mammalian pineal develops postnatally in terms of its specific biochemical functions [see: Kappers (1960) for a comprehensive study of the development and maturation of the rat pineal; Hewing (1979) and Sheridan and Rollag (1983) for that of the Syrian hamster]. In the rat, the pineal body becomes drawn out from the diencephalon during development and is observed to be attached to the meninges just under the skull, being connected to the habenular and posterior commissures by a few nerve fibres (pinealopetal nerves), parenchymal cells and some vasculature which all comprise the pineal stalk.

In some rodents, specifically the rat and hamster, the pineal body (pineal complex) consists of two parts, one proximal and one distal which are connected by the pineal stalk (Vollrath, 1979; Sheridan and Rollag, 1983). The so-called 'superficial' pineal (distal) lies under the skull as related above and the smaller 'deep' pineal (proximal) is located at the base of the pineal stalk, between the habenular and posterior commissures in the vicinity of the third ventricle. Some aspects of the morphology and biochemistry of the deep pineal appear to be different from that of the superficial pineal (Vollrath 1979; Dombrowski and McNulty 1984) while others, such as the diurnal rhythm in melatonin biosynthesis [vide infra], are similar (Sheridan and Rollag, 1983). In the hamster, the deep pineal does seem to have a blood-pineal barrier (Chen

et al., 1989; Hewing and Bergmann, 1985). Studies of the deep pineal in rodents are difficult due to its location and small size and much further investigation needs to be done to determine its physiological role.

1.2.2 Pinealectomy

The deep pineal remains intact after pinealectomy (superficial) however its small size (1 - 3% of the superficial pineal [Boeckmann, 1980]) and the observation that in the rat it undergoes post-pinealectomy atrophy (Heidbüchel and Vollrath, 1983) suggests that it is not an important source of circulating melatonin in the pinealectomised rat. The sympathetic innervation of the deep pineal arrives via the superficial pineal and pineal stalk so that superficial pinealectomy deprives the deep pineal of its sympathetic neural input (Reiter and Hedlund, 1976) most likely leading to its atrophy. In addition, studies in the intact Syrian hamster revealed that the deep pineal produces only ≈ 5% as much melatonin as the superficial pineal (Sheridan and Rollag, 1983) again implying a minor role in the production of the circulating levels of the hormone.

1.2.3 The Pinealocyte

The functional unit of the pineal gland is the pinealocyte which represents the majority (≈ 90%) of cells present in the gland. Immature astrocytes, connective tissue cells, peripheral sympathetic nerves fibres, central commissural nerve fibres and blood vessels comprise the remainder of the pineal body (Karasek, 1983). Two different classes of pinealocyte are present in some mammals, the distinction being made on ultrastructural bases (Pévet, 1981). Functional differences between the two classes have not been rigorously demonstrated. The rat and hamster pineal contain only a single population of pinealocytes (type I).

The pineal gland of lower vertebrates (e.g. birds) is capable of being directly stimulated by light penetrating the skull (Dodt, 1973). In fish and amphibia the pinealocytes are, both ultrastructurally and functionally, true photoreceptors; however in mammals they have evolved and lost the ability of direct photoreception (Oksche, 1983). Biochemical and immunocytochemical studies have shown that pinealocytes across the phyla share many structural and functional molecular markers typical of retinal photoreceptor cells, e.g. rhodopsin- and opsin-like immunoreactivity (Flight, 1979; Korf and Oksche, 1986) and that mammalian pinealocytes have phylogenetically evolved from photoreceptor cells (Pévet et al., 1977; Oksche and Hartwig, 1979). Perhaps one of the most striking similarities between the retina and the pineal is the ability of both to synthesise melatonin (Binkley, 1985; Wiechmann, 1986). Mammalian pinealocytes have evolved from photoreceptor cells into secretory cells which are indirectly influenced by light stimuli via a complex neural pathway [vide infra] (Figure. 1). The photic stimuli are then transduced into the production and secretion (by passive diffusion into the blood) of a hormonal signal, melatonin (Wurtman and Antón-Tay, 1969).

1.2.4 Pinealopetal Innervation

Non-sympathetic, central innervation of the pineal also occurs. The existence of such innervation was revealed by studies where pineal responses to light were measured in the rat following bilateral superior cervical gangli-onectomy (e.g. Martin and Meissl, 1990), implying that the pineal receives projections from the visual system via central pinealopetal nerves. VIP has been identified in nerve endings in the rat pineal (Uddman et al., 1980) and VIP receptors have been characterised on pinealocytes (Kaku et al., 1983). VIP is

able to induce the melatonin biosynthetic pathway by stimulation of adenylate cyclase, increasing intracellular cAMP and activity of the melatonin biosynthetic enzyme serotonin N-acetyltransferase as does norepinephrine [vide infra] (Kaneko et al., 1980; Yuwiler, 1983; Morgan et al., 1988). GABA receptors have been detected in the bovine pineal gland (Chan and Ebadi, 1980) and apparently mediate an inhibition of melatonin biosynthesis. Peripheral type benzodiazepine receptors have also been reported in rat pinealocytes (Matthew et al., 1984) where, in vitro, they enhanced noradrenergic stimulation of melatonin biosynthesis. Thus, though pinealopetal innervation is documented for a variety of neurotransmitters, its precise origin(s) and function(s) remain obscure. For general reviews of pinealopetal innervation see: Korf and Møller (1984); Ebadi and Govitrapong (1986) and Cardinali and Vacas (1987).

1.2.5 The Photoneuroendocrine Pineal

A pineal-gonadal axis had been hypothesised since the turn of the century when Heubner (1898) and Marburg (1907) observed a relationship between human pinealoma (pineal tumour) and precocious puberty in children. Pineal-ectomy of the immature female rat was shown to cause an increase in gonadal weight (Izawa, 1926; Kitay, 1954) while administration of pineal extracts to intact immature females produced ovarian atrophy (Kitay and Altschule, 1954). Following the isolation and identification of melatonin by Lerner and coworkers (1958, 1959) as the active pineal factor, Wurtman et al. (1963a) demonstrated that it had the same effects on ovarian weight in the rat as pineal extracts. It was also shown that an antagonism existed between melatonin and light exposure on the reproductive competence of the rat. This was the first demonstration of the hormonal status of melatonin and soon thereafter a photo-

neuroendocrine role was proposed for melatonin and the pineal (Wurtman and Axelrod, 1965; Wurtman and Antón-Tay, 1969). The Syrian hamster is a long-day breeder and the photoneuroendocrine effects of melatonin on the reproductive physiology of this species have been extensively studied [vide infra, Section 1.6.1.1] (see: Reiter (1980) for a review).

1.3. Retina-Pineal Neural Pathway

Retinohypothalamic projections, the first neural component in the transmission of photic stimuli from the retina to the pineal, were first demonstrated in the rat by Hendrickson et al. (1972) and Moore and Lenn (1972). Anterograde transport of intraocularly injected radioactive amino acids and electron-microscopic examination of degenerating nerve fibres both indicated a projection from the retina to the suprachiasmatic nucleus (SCN) of the mammalian hypothalamus. Stimulatory signals reaching the SCN then travel via efferent projections to the periventricular [paraventricular nuclei (Picard and Turek, 1983; Lehman et al., 1984)] and ventral tuberal areas of the hypothalamus (Swanson and Cowan, 1975). Neural connections exist between the ventral tuberal area and the lateral part of the hypothalamus (Szentágothai et al., 1960) and a direct projection from the lateral hypothalamus, via the medial forebrain bundle and the midbrain reticular formation, to the upper thoracic intermediolateral cell column has been described (Saper et al., 1976; Swanson and Sawchenko, 1983). Neurons of the thoracic intermediolateral cell column provide preganglionic input to the superior cervical ganglia (SCG) which are part of the sympathetic nervous system. Postganglionic sympathetic fibres from the SCG travelling along the tentorium cerebelli form the nervi conarii

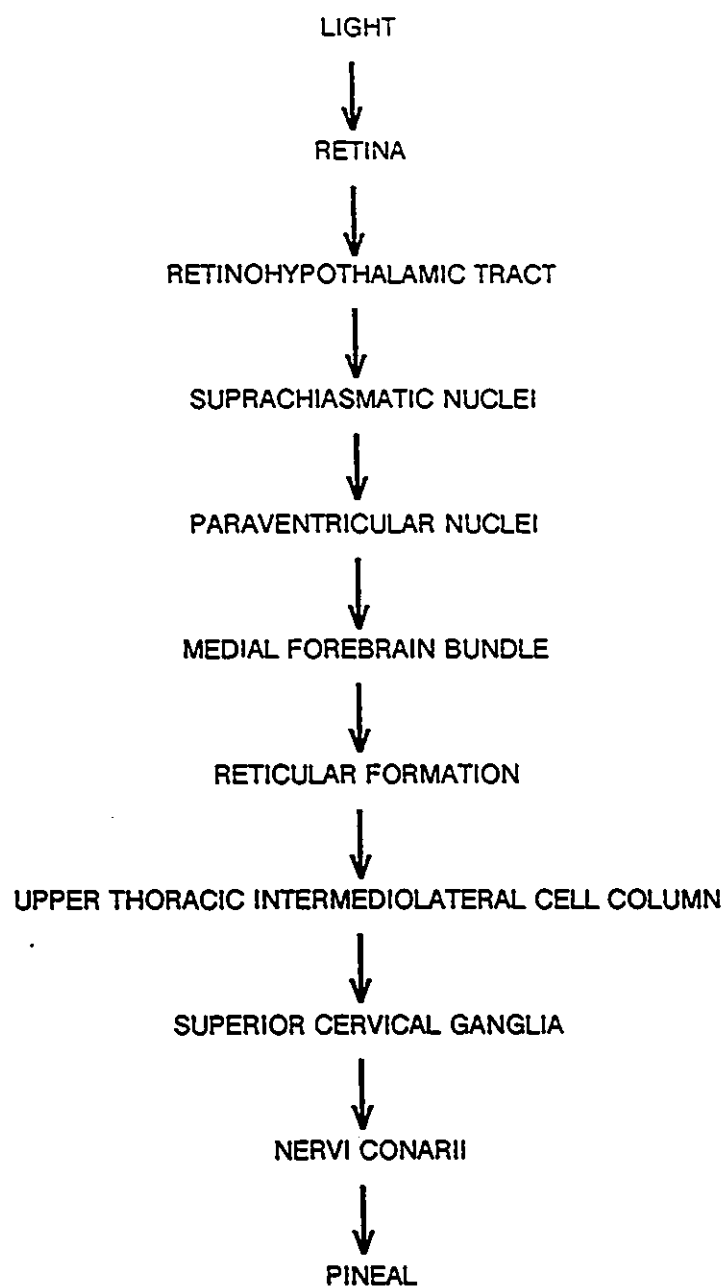


Figure 1. A summary of the retinal-pineal neural pathway.

See Moore (1978) for a review.

which directly innervate the pineal body. Additionally, in mammals sympathetic innervation can reach the pineal by way of the small pial blood vessels (Kappers, 1960).

1.4 Pineal Melatonin Biosynthesis

The biosynthetic pathway of melatonin in the pineal and its regulation by the sympathetic nervous system have been extensively studied topics (see: Sugden, 1990; Reiter, 1991). The initial precursor of all indole biosynthesis in man and rat is the essential amino acid tryptophan. Since man and rat are unable to produce L-tryptophan de novo, dietary tryptophan must be utilised as the source of indole biosynthesis. While cultured pinealocytes will accumulate radiolabelled tryptophan from the growth medium (e.g. Wurtman et al., 1968; Morton, 1990) and the levels of pineal tryptophan have been quantified (Young and Anderson, 1982; Mefford et al., 1983), the mechanism of tryptophan uptake has never been rigorously studied in pinealocytes. It is presumed that a neutral amino acid transport system similar to that found in the brain (for example in serotonergic neurons especially) (Grahame-Smith and Parfitt, 1970) is similarly present in pinealocytes. This is perhaps an important, overlooked aspect of pineal physiology. Since the pineal is outside the blood-brain barrier, it is conceivable to design drugs which could somewhat selectively target tryptophan uptake by pinealocytes and consequently modulate pineal indole biosynthesis. Intracellularly, tryptophan undergoes hydroxylation by tryptophan hydroxylase [L-tryptophan, tetrahydropteridine: oxygen oxidoreductase (5-hydroxylating) E.C. 1.14.16.4; tryptophan monooxygenase) to give 5-hydroxytryptophan (Lovenberg et al., 1967). Decarboxylation of this by aroma-

tic amino acid decarboxylase (5-hydroxytryptophan decarboxylase E.C. 4.1.1.28 and/or DOPA decarboxylase E.C. 4.1.1.26) yields 5-hydroxytryptamine (serotonin, 5-HT) (Clark et al., 1954; Shein et al., 1967). Pineal serotonin can be oxidised by monoamine oxidase B (E.C. 1.4.3.4) to 5-hydroxyindoleacetaldehyde which is further oxidised to 5-hydroxyindoleacetic acid or is reduced to 5-hydroxytryptophol (Håkanson and Owman, 1965, 1966; Håkanson et al., 1967; Masson-Pévet and Pévet, 1989). Alternatively, serotonin can be released to be taken up by sympathetic nerve terminals (Owman, 1965; Håkanson and Owman, 1966; Aloyo and Walker, 1987, 1988) or it can be acetylated by serotonin N-acetyltransferase (SNAT) (arylalkylamine N-acetyltransferase; E.C. 2.3.1.87) to produce N-acetylserotonin (NAS) (Weissbach et al., 1960). O-methylation of NAS by hydroxyindole-O-methyltransferase (HIOMT; E.C. 2.1.1.4) gives N-acetyl-5-methoxytryptamine (melatonin) (Axelrod and Weissbach, 1960, 1961). Tryptophan hydroxylase has been purified and characterised from bovine pineal (Nukiwa et al., 1974; Ichiyama and Hasegawa, 1983) and rat brain (Cash et al., 1985) and a cDNA clone of the rat pineal enzyme has been isolated (Darmon et al., 1986). Pineal HIOMT (a 76 kDa homodimer) has been purified from several species and its kinetic and physical properties studied (Axelrod and Weissbach, 1961; Kuwano et al., 1978; Nakane et al., 1983; Sugden et al., 1986; Sugden and Klein, 1987).

Relatively recently, the cDNA of bovine pineal HIOMT has been cloned and functionally expressed in a Chinese hamster ovary (CHO) cell line (Ishida et al., 1987a, 1987b). SNAT has been partially purified and characterised (Namboodiri et al., 1987a,b). The presence of N-acetyltransferases in liver led one group to attempt, unsuccessfully, to use cDNA's of the liver enzymes to probe a pineal cDNA library for SNAT (Ohtomi et al., 1989). This failure

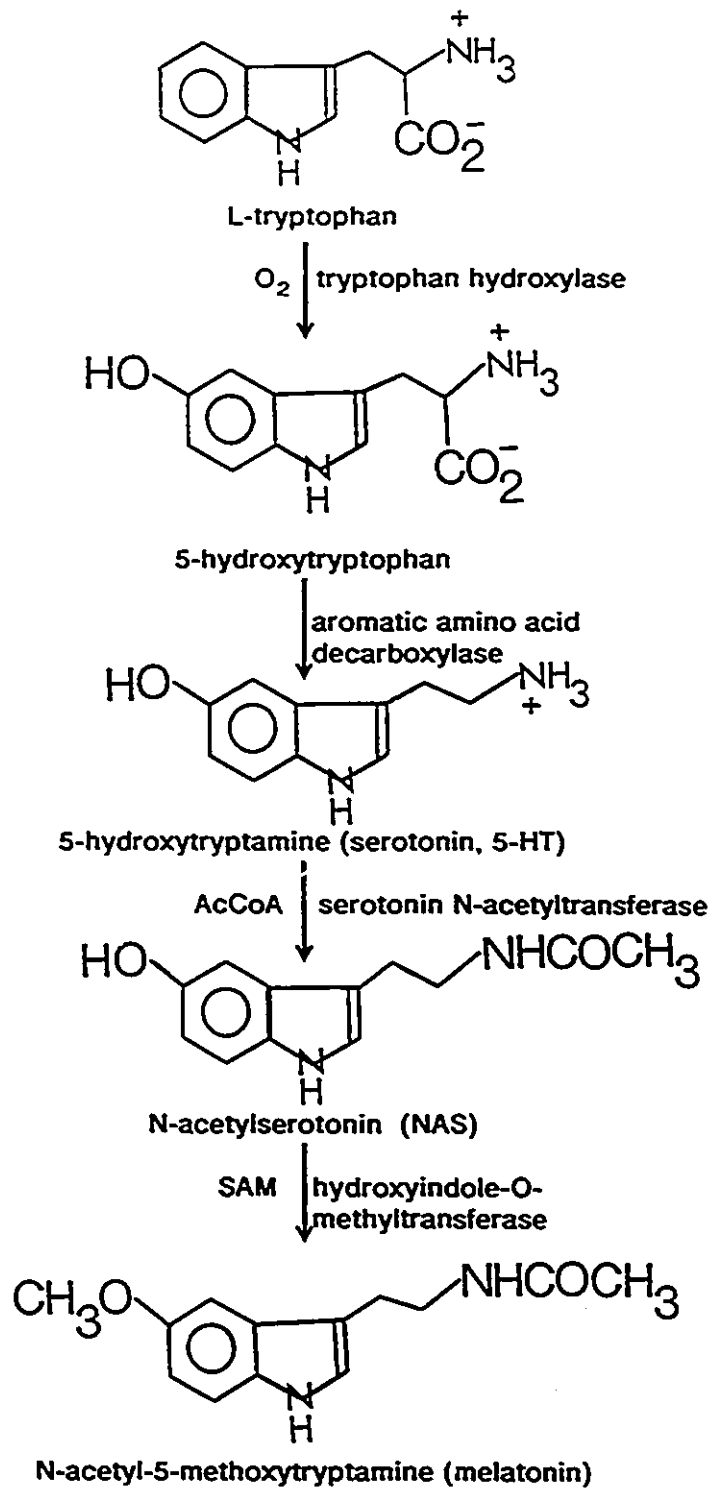


Figure 2. A summary of the pineal biosynthetic pathway. (AcCoA, acetyl coenzyme A; SAM, S-adenosylmethionine).

is rather unfortunate since regulation of SNAT activity is the focal point in the control of melatonin biosynthesis in the pineal and the precise mechanism of SNAT regulation is yet unknown. Obtaining the SNAT cDNA would be a boon to this area of research.

1.5 Regulation of Melatonin Biosynthesis

1.5.1 The Suprachiasmatic Nuclei

The suprachiasmatic nuclei (SCN) in the anterior hypothalamus are considered to contain a 'biological clock' which gives mammals (including humans) an endogenous circadian rhythm controlling many biological processes (Meijer and Rietveld, 1989; Rusak and Bina, 1990). The SCN are thought to be primarily responsible for the generation of the circadian oscillation of norepinephrine release in the pineal that stimulates melatonin biosynthesis [vide infra] and which continues even in blinded animals (Reppert et al., 1981). Light stimuli act upon the retinohypothalamic neural tract [vide supra] to entrain (synchronise) this endogenous rhythm to environmental lighting cycles (Klein et al., 1981). The endogenous nature of the pineal melatonin rhythm is evidenced by its persistence in animals kept in constant dark or in blinded animals (Ralph et al., 1975; Tamarkin et al., 1980; Yellon et al., 1982).

1.5.2 Light and the Pineal

The photoneuroendocrine theory of pineal gland function, as proposed by Wurtman and Axelrod (1965) [vide supra] was based mainly upon the physiological data accumulated to that time regarding the antigonadotropic effects exerted by the pineal in the rat. Important biochemical evidence in support of the theory had come with the demonstration of the effects of light exposure (and

darkness) upon pineal melatonin biosynthesis (Wurtman et al., 1963b). Light was found to suppress HIOMT activity and a diurnal rhythm in rat pineal melatonin concentration, with the peak in the subjective night, was shown by fluorometric analysis (Quay, 1964), bioassay (Lynch, 1971) and more conclusively by radio-immunoassay (RIA) (Pang et al., 1977). A circadian rhythm in rat pineal HIOMT activity was seen, where dark phase levels were 2 - 3 times higher than those of the light phase (Axelrod et al., 1965; Balemans et al., 1979). In addition, the HIOMT rhythm was disrupted by treatments of constant light, constant dark or blinding. Constant light depressed HIOMT activity while continuous darkness caused an increase in activity over a period of several days attaining a sustained, high level (Wurtman et al., 1963b; Moore and Rapport, 1971). This increase in HIOMT activity in long term darkness was eventually shown by immunotitration to be due to increased amounts of enzyme in the pineal (Yang and Neff, 1976) where a 2 - 3 fold increase in activity was noted after 2 - 3 weeks of darkness.

1.5.3 The Sympathetic Nervous System

The involvement of the sympathetic nervous system in transmission of light stimuli to the pineal (via the SCG) was first demonstrated by Wurtman et al. (1964a). Within a decade, the neural pathway from the retina to the pineal had been delineated [vide supra]. The pineal content of norepinephrine (NE) was found to undergo a circadian rhythm with peak amounts occurring in darkness (Wurtman et al., 1967), while blinding or decentralisation could abolish this rhythm. Superior cervical ganglionectomy of rats also abolished the pineal HIOMT rhythm (Axelrod et al., 1965; Nagle et al., 1973). Pre- and postganglionic nervous activity of the rat SCG was recorded in vivo and these electrophysio-

logical experiments demonstrated that light suppressed sympathetic neurotransmission in the pineal (Taylor and Wilson, 1970). Direct biochemical evidence for the effects of NE on pineal function came when NE was shown to stimulate melatonin biosynthesis from [^{14}C]tryptophan in rat pineal organ culture (Axelrod et al., 1969).

Although it was originally thought that control of HIOMT activity was the important regulatory point in melatonin biosynthesis, Klein and Weller (1970) provided evidence that, in the rat pineal, melatonin biosynthesis was regulated in vivo by a noradrenergic control of SNAT activity. A greater than 15- fold increase in dark phase SNAT activity was observed as compared to the modest 2- 3-fold increase in HIOMT activity during darkness. In one study a 60- fold difference was seen in the dark:light SNAT activity ratio of rat pineal (Rudeen et al., 1975). The diurnal rhythms in rat pineal SNAT activity, pineal melatonin and serum melatonin have been shown to be coincidental (Wilkinson et al., 1977). In addition, the high dark phase levels of N-acetylserotonin measured in the rat pineal by RIA supported the idea that activation of SNAT activity could be the rate-limiting step in the stimulation of melatonin biosynthesis (Pang et al., 1977; Young and Anderson, 1982). A clear dose-response relationship was then shown between light intensity and inhibition of SNAT activity in the rat pineal in vivo (Minneman et al., 1974) and in the Syrian hamster pineal (Brainard et al., 1983) where the changes in melatonin levels paralleled SNAT activity. Direct electrical stimulation of the pre- or post-ganglionic nerves of the SCG were shown to cause increases in rat pineal SNAT activity (Bowers and Zigmond, 1980). It therefore seems that while diurnal changes in HIOMT activity may be of minor importance in generating the diurnal

melatonin rhythm, the large changes in SNAT activity undoubtedly are of major importance.

1.5.4 Species Differences

Most studies on pineal function have been carried out in the rat, however some species differences seem to occur if we examine the hamster (Rudeen et al., 1975; Steinlechner et al., 1985; Pangerl et al., 1990). A circadian rhythm in Syrian hamster pineal melatonin levels, with a dark phase peak, have been documented by RIA (Panke et al., 1978, 1979; Tamarkin et al., 1979; Rollag et al., 1980) and by gas chromatography-mass spectrometry (Beck and Pévet, 1984). This rhythm was abolished by superior cervical ganglionectomy (Panke et al., 1979), as is the case in the rat. No diurnal rhythm in HIOMT activity was seen in the Syrian hamster pineal (Tamarkin et al., 1979; Steinlechner et al., 1984). While the former study did not find a rhythm in SNAT activity, the latter study found an SNAT rhythm paralleling the pineal melatonin rhythm. In contrast, Pévet et al. (1980) found a modest diurnal HIOMT rhythm in the Syrian hamster pineal which peaked in the dark phase. SNAT activity in the Syrian (Rudeen et al., 1975; Vaněček and Ilnerová, 1982) and Djungarian (Hoffman et al., 1981) hamster was found to be elevated in the dark phase. In the Djungarian hamster SNAT activity remained elevated throughout the dark phase regardless of the length of the phase. The rhythm was very sensitive to light suppression since a one minute light pulse in mid dark phase caused a rapid decline in SNAT activity to normal daytime levels (Hoffman et al., 1981). The Syrian hamster displays slightly different patterns of SNAT activation and suppression (Tamarkin et al., 1985; Steinlechner et al., 1985) indicating that variations in SNAT regulation can occur within very closely related species. This may be part of

the explanation of the differing levels of sensitivity of Djungarian and Syrian hamsters to photoperiod regulation of sexual maturation (Hoffman, 1978; Tamarkin et al., 1985; Underwood and Goldman, 1987; Bartness and Goldman, 1989). Thus, while HIOMT rhythms may be even more modest (or non-existent) in the hamster than the rat pineal, SNAT rhythms are robust in each species and subtle dissimilarities in the onset and phasing of the rhythms are likely to be of importance in considering the physiology of pineal function in the two species.

1.5.5 Molecular Control of Melatonin Biosynthesis

Norepinephrine released from the sympathetic synapses within the pineal acts upon δ_1 - and α_1 -adrenoceptors on the plasma membrane of pinealocytes (Dickinson et al., 1986). δ_1 -adrenoceptor stimulation causes activation of adenylate cyclase and increases in intracellular cAMP levels, leading to an increase in SNAT activity (Klein and Weller, 1970; Klein et al., 1970). Since bilateral superior cervical ganglionectomy, decentralisation or constant light exposure leads to a slow decrease in rat pineal HIOMT activity over several days to \approx 30% of control values (Sugden and Klein, 1983a) it is thought that tonic control of pineal HIOMT activity is also mediated by the rhythmic NE stimulation of δ_1 -adrenoceptors on the pinealocytes (Sugden and Klein, 1983b). δ_1 -adrenoceptor activation alone produces a 7- 10-fold increase in cAMP in pinealocytes and a 2- 4-fold increase in cGMP (O'Dea and Zatz, 1976; Vaněček et al., 1985). Selective stimulation of the pinealocyte α_1 -adrenoceptor alone has no effect upon cyclic nucleotide accumulation (Vaněček et al., 1985). However, simultaneous activation of both the δ_1 - and α_1 -adrenoceptors, as is the case with the endogenous release of NE, produces 60-fold increases in cAMP and 400-

fold increases in cGMP (Vaněček et al., 1985). Increases in these cyclic nucleotides are required for SNAT activation. An accounting of the molecular mechanism of interaction between α_1 - and δ_1 -adrenoceptor activation, cAMP and cGMP elevation, PKC activation, etc. can be obtained from the following reviews: Sugden (1989a), Ho and Chik (1990), Reiter, (1991). One recent interesting finding is that the c-fos protein has been shown to be induced in rat and Syrian hamster pineals during darkness (Koistinaho and Yang, 1990). Therefore, it seems probable that transcriptional activation of the SNAT gene, mediated by the AP-1 transcriptional activator (Morgan and Curran, 1991), may be the mechanism whereby dark phase melatonin biosynthesis is induced.

1.5.6 Non-Sympathetic Regulation

In addition to the sympathetic regulation of pinealocyte activity, central (pinealopetal) and hormonal regulation occurs [vide supra] (Sugden, 1989a; Cardinali and Vacas, 1987). Recently, in cultured rat pinealocytes serotonin has been shown to amplify the δ_1 -adrenergic response, potentiating SNAT activity a further 10-fold (Sugden, 1990). VIP stimulation of cAMP levels and SNAT activity is also well established in the rat pinealocyte and these can be potentiated by α_1 -adrenoceptor activation (Chik and Ho, 1991). Circadian rhythms in α_1 - and δ_1 -adrenoceptor numbers have been shown in the pineal, with species differences being apparent (Pangerl et al., 1990). These findings obviously add a further complexity to the study of the regulation of pineal activity.

1.6 Melatonin and Reproduction

1.6.1 Reproductive Physiology

Wurtman et al. (1963a) were the first to demonstrate a direct effect of melatonin on reproductive physiology (in the rat). To date, the best characterised hormonal role of melatonin in mammals is its antigonadotropic role in the hamster (Reiter, 1980; Stetson and Watson-Whitmyre, 1986; Bartness and Goldman, 1989; Ebling and Foster, 1989) and the rat (Rivest et al., 1986) and progonadotropic role in the sheep (Lincoln et al., 1982; Karsch, 1986; Ebling and Foster, 1989). Because the pineal (and melatonin) can mediate antigonadotropic effects in long-day breeding species, such as the hamster, and progonadotropic effects in short-day breeders, such as the sheep, it is likely that the melatonin message merely conveys information about the environmental photoperiod. It is the target structure(s) of the given species which translate(s) the message as anti- or progonadotropic.

1.6.1.1 The Hamster

The albino rat is not a strongly photoperiodic species and sheep are not an animal most laboratories can accommodate, hence most data on the reproductive physiology of melatonin have been obtained from hamsters (Syrian [or Golden] and Djungarian [or Siberian], usually). Hamsters are seasonally breeding rodents classified as 'long-day' breeders. This is to say that during the spring and summer months, when the daylength is long, the animals will be reproductively competent. In the opposite circumstance, during short days and long nights of winter, the animals are infertile. This breeding strategy ensures that the pups are born during the season most favourable to their survival, summer. While other factors such as environmental temperature, food

supply, etc. contribute to the coordination of the reproductive cycle with the seasons. In the hamster it is the daylength which is the most important signal perceived by the animal.

Short days cause testicular regression in the male hamster and anestrus in the female, while long days induce or maintain the fertile state (Reiter, 1968; Gaston and Menaker, 1967; Tamarkin et al., 1976, 1978). The pineal gland was demonstrated to mediate the gonadal atrophy induced by short photoperiods (Hoffman and Reiter, 1965). Constant darkness has been shown to cause a gradual decline in circulating luteinizing hormone (LH), follicle-stimulating hormone (FSH) and testosterone in male Syrian hamsters (Berndtson and Desjardins, 1974) which paralleled their testicular regression. Upon return to L:D = 14:10 (long-days), LH and FSH levels returned to normal within ten days. Testicular regrowth and testosterone production increased more slowly, reaching normal values by 50 days. Chronic melatonin injection of male Syrian hamsters housed in a long-day photoperiod (i.e. in a reproductively competent state) was demonstrated to cause gonadal atrophy (i.e. mimicking the effects of a short-day photoperiod) [Reiter et al., 1976]. Interestingly, this result is dependent upon the time of injection, being effective only near the end of the light phase, and also upon the presence of an intact, sympathetically innervated pineal. These observations suggested a time-dependent sensitivity of the reproductive system to the effects of melatonin and that exogenous melatonin acted in concert with endogenous melatonin to produce these effects. The interaction(s) of the circadian rhythm of melatonin biosynthesis with possible rhythmic target systems is reviewed by Reiter (1987). In brief, he discusses a duration hypothesis vs a coincidence hypothesis as possible mechanisms by which the melatonin message

is read by the animal. In the former hypothesis it is the duration of the dark phase elevated melatonin peak which encodes the photoperiod message. In the latter hypothesis, the sensitivity to melatonin of the target system(s) is proposed to undergo rhythmic changes. When the rhythm of sensitivity of a target system is synchronised with the rhythm of elevated melatonin, the appropriate response is obtained. If the two rhythms are out of phase with each other, then no response is obtained to the elevated levels of melatonin.

1.6.2 Hormonal Interactions

1.6.2.1 The Rat

Low doses (1- 10 μg) of melatonin administered by intraventricular injection in adult male rats was reported to decrease serum LH and FSH levels and increase prolactin release while hypophyseal portal vein perfusion with melatonin did not affect these hormones (Kamberi et al., 1970, 1971). This was interpreted to mean that melatonin did not act directly on the anterior pituitary to inhibit LH release but likely acted centrally by inhibition of luteinizing hormone-releasing hormone (LHRH) and prolactin inhibiting factor (PIF) release in the hypothalamus.

In vitro studies have clearly shown that low concentrations (1 nM) of melatonin inhibit the LHRH-induced release of LH and FSH from cultured *neonatal* rat anterior pituitaries (Martin and Klein, 1976; Martin and Sattler, 1979). The parallelism observed between LH and FSH suppression by melatonin suggested that melatonin specifically, functionally antagonised LHRH receptor stimulation of gonadotropin release (Martin and Sattler, 1979). The relative potencies of a series of indoles revealed that this effect is selective for melatonin (Martin et al., 1977, 1980b). These data would seem to contradict the studies

of Kamberi et al. in that they definitely show an effect of melatonin directly on the anterior pituitary. However, whereas Kamberi et al. measured changes in basal LH release in adults, these in vitro studies measured changes in LHRH-stimulated LH (and FSH) release in neonates. In addition, it was confirmed that the inhibitory effect of melatonin was lost between 15 - 20 days of age in the rat (Martin et al., 1977; Martin and Sattler, 1979). In vivo studies in neonatal male and female rats corroborated the in vitro data. Low doses of melatonin (1 μ g) injected peripherally could inhibit the LH release induced by a peripheral injection of LHRH (Martin et al., 1980a). In adult rats, melatonin (\leq 100 μ g) had no effect on the induced LH or FSH release.

Interestingly, the ontogeny of melatonin receptors in the rat anterior pituitary indicated that the high fetal and neonatal receptor density had decreased by 90% in 29 day old rats (Vaněček, 1988a; Williams et al., 1991). This is most likely the reason for the developmental loss of the inhibitory effect of melatonin on LHRH-induced LH and FSH release. Vaněček and Vollrath (1990a) recently reported that low nanomolar concentrations of melatonin inhibit the LHRH-stimulated increase in cAMP and cGMP levels in rat anterior pituitary in vitro. Again, the selectivity of melatonin was indicated by the 1,000-fold lower potency of N-acetylserotonin and 5-methoxytryptamine. The ontogeny of this effect correlated well with the developmental changes in melatonin receptors in the anterior pituitary and with the effectiveness of melatonin to inhibit LHRH-induced LH and FSH release.

The loss of melatonin sensitivity of the anterior pituitary in 2- 3 week old rats relieves the inhibition of LHRH responsiveness leading to increased LH and FSH secretion. This increase in serum gonadotropins could then promote

the development of the reproductive system leading to puberty. In the adult rat perhaps melatonin exerts its effects by acting on other sites in the hypothalamus such as the suprachiasmatic nuclei and paraventricular nuclei. This may explain the results of Kamberi et al. (and others) in adult rats.

1.6.2.2. The Hamster

As noted above (Section 1.5.4), species differences are apparent in the role of melatonin in reproductive physiology. These differences are also evident at the level of the hormonal responses to melatonin. Melatonin was found to be without effect on the LHRH-induced LH release from neonatal or adult Syrian hamster anterior pituitary cells in vitro (Bacon et al., 1981). This same study also examined the effect of a subcutaneous injection of melatonin in adult male hamsters upon the increase in serum LH caused by a subcutaneous LHRH injection. No effect was seen on serum LH levels by melatonin. This investigation of the acute effects of melatonin injection contrasts with earlier studies showing that chronic melatonin injections in the afternoon in male Syrian hamsters housed on L:D = 14:10 caused testicular regression and decreases in serum LH and FSH over a period of several weeks (Tamarkin et al., 1976).

Exposure of male Syrian hamsters to short photoperiods induces testicular regression and a slight decline in serum prolactin levels (Goldman et al., 1981). After = 20 weeks, prolactin levels have increased to normal values and testicular recrudescence has occurred (Matt and Stetson, 1979). Therefore, in the hamster, changes in serum prolactin concentration may play some role in the inhibition of testes function during short photoperiods (Bex et al., 1978). LH and FSH levels decrease as well in male Syrian hamsters exposed to short photoperiods or complete darkness for several weeks (Berndtson

and Desjardins, 1974; Picard and Silverman, 1979). The hypothalamic content of LHRH has been reported to increase in adult male Syrian hamsters exposed to short photoperiods, treated with melatonin injections or by blinding (Picard and Silverman, 1979; Jackson et al., 1984). The plasma levels of LH and FSH and testes weight all decreased during the same time frame as hypothalamic LHRH increased. It has been demonstrated that the in vivo pituitary responsiveness to LHRH injections in castrated Syrian hamsters was independent of the photoperiodic history of the animal (Turek and Menaker, 1977). This indicated that the anterior pituitary does not change in its sensitivity to LHRH in a manner dependent upon the photoperiod. Bonnefond et al. (1989) recently found that in pinealectomised, castrated Syrian hamsters having lesions of the anterior hypothalamus, melatonin infusions could no longer suppress the rise in serum FSH caused by the pinealectomy. This indicated that an intact anterior hypothalamus is needed for the response to the melatonin signal. In this case, melatonin did not seem to act on the anterior pituitary to inhibit FSH release. The targets for melatonin action in the hamster are most likely the suprachiasmatic nuclei and/or paraventricular nuclei of the hypothalamus where melatonin receptors have been identified in the hamster (Duncan et al., 1989; Carlson et al., 1991; Weaver et al., 1991).

Thus, the prevailing data indicate that in the *neonatal* rat, melatonin can modulate reproductive function at the level of the anterior pituitary to functionally antagonise the LHRH receptor. Whether melatonin may also act at the hypothalamus in the neonatal rat to modulate LHRH synthesis and/or release is unknown. In the hamster (and perhaps in the adult rat) however, melatonin may act at the level of the hypothalamus to regulate the synthesis and/or release

of LHRH or PIF. While hamster ME/PT explants contain melatonin receptors coupled to inhibition of adenylate cyclase, the physiological response regulated by this is unclear.

1.7 Extra-Pineal Melatonin Biosynthesis

The pineal was originally thought to be the sole source of melatonin synthesis in vertebrates. However, it was found that following pinealectomy, blood melatonin levels were greatly diminished but not eliminated (Osaki and Lynch, 1976; Yu et al., 1981a). The clear implication of these findings were that non-pineal sources of melatonin must be present which can contribute to the circulating levels of the hormone. This spurred endeavours to find such tissues. Subsequent to this, analysis of plasma using the highly specific and sensitive method of gas chromatography-mass spectrometry revealed that melatonin levels were not detectable (i.e. < 1 pg/mL) in pinealectomised rats (Lewy et al., 1980). This indicated that circulating melatonin actually was derived virtually entirely from the pineal (at least in the rat) and that extra-pineal sources of melatonin did not contribute substantially to circulating hormone levels.

1.7.1 Retina

The known phylogenetic relationship between the photoreceptor cells of the retina and pinealocytes (Wiechmann, 1986 [vide supra]) led Quay (1965) to the discovery of HIOMT activity in the retinae of lower vertebrates. Cardinali and Rosner (1971) were first in describing the ontogenic development of retinal HIOMT activity in a mammal (the rat). It was found to precede that of pineal HIOMT activity which was not detected until the 12th postnatal day. The

kinetic properties of the rat retinal and pineal enzymes were found to be comparable, suggesting that the two enzymes are similar (Cardinali and Wurtman, 1972). Interestingly enough, the recent cloning of bovine pineal HIOMT cDNA allowed examination of bovine retinal tissue for HIOMT mRNA and no hybridisation was detected (Ishida et al., 1987a). This suggests that the pineal and retinal enzymes are distinct (at least in the cow). Unfortunately, low-stringency conditions, which could have detected the presence of an HIOMT isozyme, were not tried. Polyclonal antibodies prepared against bovine pineal extracts have shown the existence in the retina of HIOMT having a different molecular weight, by SDS-PAGE, than the pineal enzyme (25 vs 39 kDa, respectively) (Wiechmann et al., 1985).

HIOMT was immunocytochemically localised to the retinal photoreceptor cells in the rat, cow and human (Wiechmann et al., 1985) and to human (Wiechmann and Hollyfield, 1987, 1989) and rat (Wiechmann and O'Steen, 1990) cone bipolar cells. HIOMT activity has recently been shown to be present in the Y-79 human retinoblastoma cell line which produces melatonin in cell culture (Kyritsis et al., 1987) and promises to be a useful *in vitro* model of retinal melatonin biosynthesis. SNAT activity has also been demonstrated in rat and hamster retina (Binkley et al., 1979; Miller et al., 1980) and gerbil retina (Olcese and Møller, 1989). Melatonin itself has been identified immunohistochemically in the human retina (Osol and Schwartz, 1984), rat retina (even post-pinelectomy) (Bubenik et al., 1974, 1976b, 1978; Vivien-Roels et al., 1981) and guinea pig retina (Yu et al., 1982), by gas chromatography-mass spectrometry in the Syrian hamster retina (Beck and Pévet, 1984) and by HPLC with electrochemical detection in bovine retina (Hall et al., 1985).

1.7.2 Harderian Gland

The Harderian glands are large, bilobed, tubuloalveolar glands situated in the posterior region of the orbital cavities in all vertebrates examined with the exception of the higher primates which do not appear to possess these glands. Its proximity to the eye and content of porphyrins (in rodents at least) that fluctuate depending upon environmental light exposure led Wetterberg et al. (1970a,b) to examine the relationship between the Harderian gland and the pineal gland. Following these studies, HIOMT activity was demonstrated in the rat Harderian gland (Vlahakes and Wurtman, 1972). The enzymatic properties of HIOMT in the pineal, retina and Harderian gland were compared and it was concluded that the pineal and retinal enzymes were different from that of the Harderian gland (Cardinali and Wurtman, 1972). It would be interesting to test whether the bovine pineal HIOMT cDNA probe (Ishida, 1987a) would be able to hybridise to poly(A)⁺RNA from bovine Harderian gland, under both low and high stringency conditions. This could help to resolve the issue of multiple forms (isozymes) of HIOMT.

Several reports on the immunohistochemical localisation of melatonin in the Harderian gland have been published (Bubenik et al., 1976a,b, 1978; Pang et al., 1977; Reiter et al., 1983; Vivien-Roels et al., 1981). Melatonin was localised to the secretory cells (Bubenik et al., 1976b) where a correlation was observed between melatonin content and porphyrin content.

1.7.3 Gut

The biosynthesis of melatonin in various regions of the gastrointestinal system has been hypothesised since a frog skin blanching substance was identified in rabbit gastrointestinal tract extracts (Raikhlin et al., 1975;

Raikhlin and Kvetnoy 1976). Subsequently, HIOMT activity was demonstrated in rabbit intestinal preparations (Quay and Ma, 1976). Immunohistochemical techniques have localised melatonin in several regions of the rat gastrointestinal tract, the colon seeming to have relatively high levels (Bubenik et al., 1977; Holloway et al., 1980; Bubenik, 1980a).

The presence of gastrointestinal melatonin is still not firmly established in that the number of reports are few and that only melatonin-like immunoreactivity has been measured. Analysis of gut tissue extracts by gas chromatography-mass spectrometry should be done to provide more convincing evidence for the localisation of melatonin in this tissue.

1.7.4 Other Areas

Melatonin synthesis has also been reported in rabbit platelets in vitro (Lemaitre et al., 1981; Launay et al., 1982) as well as in human erythrocytes in vitro (Rosengarten et al., 1972). Using immunohistochemical methods, melatonin-like immunoreactivity has been observed in rat salivary glands (Bubenik, 1980b). Melatonin-like immunoreactivity and melatonin biosynthesis were identified in the guinea pig cochlea (Biesalski et al., 1988) and Syrian hamster extraorbital lacrimal gland (Mhatre et al., 1988). A melatonin-like substance, identified only by bioassay, was found in human appendix mucosal extracts (Raikhlin et al., 1975; Raikhlin and Kvetnoy, 1976). The significance of these putative sources of melatonin is unknown.

1.8 Function of Extra-Pineal Melatonin

Of the three best established extra-pineal sources of melatonin (retina, Harderian gland and gut), the local actions of the hormone are best understood in the retina and are speculative in the other two tissues.

1.8.1 Retina

In the Syrian hamster no apparent diurnal rhythm in retinal melatonin levels was observed (Binkley et al., 1979; Pévet et al., 1980; Beck and Pévet, 1984) which is in contrast to that seen in the rat where high darktime levels of the hormone occur as in the pineal (Bubenik et al., 1978; Pang et al., 1980a; Yu et al., 1981a). Diurnal rhythms of retinal melatonin were still present in pinealectomised rats indicating an endogenous origin (Yu et al., 1981b; Reiter et al., 1983). Rat retinal HIOMT activity similarly undergoes a diurnal rhythm, even in constant darkness, which is abolished by constant light (Nagle et al., 1972, 1973) and pinealectomy (Nagle et al., 1973). In the pinealectomised animals HIOMT activity was constantly high. In the Syrian hamster, no significant retinal HIOMT rhythm was observed (Pévet et al., 1980). Therefore, species differences seemingly exist between the rat and hamster with regards to retinal melatonin synthesis. The significance of these differences is unclear at present.

It appears that, just as in the pineal, the diurnal synthesis of melatonin in the retina of lower vertebrates (i.e. fish, chickens, frogs) is controlled by regulation of SNAT activity (Hamm and Menaker, 1980; Hamm et al., 1983; Iuvone and Besharse, 1983) which is suppressed by light and increases in the dark. It has been shown that increases in cAMP which occur in the darkness, or by pharmacological manipulations in the light, are responsible for the

increase in SNAT activity (Iuvone and Besharse, 1983, 1986a; Besharse et al., 1984; Pierce et al., 1989; Wiechmann et al., 1990). These effects require de novo protein synthesis (Iuvone and Besharse, 1983), suggesting an increase in the amount of SNAT enzyme during the dark phase. Until SNAT cDNA is cloned or an antibody to SNAT prepared, the exact mechanism of regulation of retinal SNAT activity (i.e. transcriptional, translational or posttranslational) is likely to remain unanswered.

The dark phase increase in SNAT activity is mediated by a process involving Ca^{2+} influx through voltage-dependent calcium channels (Iuvone and Besharse, 1986b) suggesting that neurotransmitter release may be involved in stimulating SNAT activity, as is the case with the pineal gland [vide supra]. It has been shown that dopamine acting via D2 dopamine receptors inhibits the dark phase increases in cAMP and SNAT activity and so inhibits melatonin synthesis (Iuvone, 1986; Zawilska and Iuvone, 1989; Iuvone et al., 1990). Conversely, melatonin has been demonstrated to suppress dopaminergic neurotransmission in the rabbit retina by potently inhibiting the release of dopamine (Dubocovich, 1983). This effect of melatonin is mediated by high-affinity receptors present in the retina (Dubocovich, 1985a,b) [vide infra]. It has therefore been suggested that melatonin and dopamine are important components of a regulatory mechanism controlling rhythmic photoreceptor metabolism and that dopamine represents a signal for light while melatonin represents a signal for darkness (Pierce and Besharse, 1985).

1.8.1.1 Physiological Roles for Retinal Melatonin

Melatonin has been shown to cause aggregation of pigment granules within the retinal pigmented epithelium (RPE) in the guinea pig in vivo (Pang and Yew,

1979). Hypothetically, during the night high melatonin levels would cause aggregation of the granules and thereby increase the reflectivity of the epithelium, increasing the sensitivity of the retina. During the day, low melatonin levels would allow dispersion of the granules and decrease the reflectivity of the pigmented epithelium, perhaps helping to protect the photoreceptors from light damage. Melatonin has also been implicated in the regulation of photoreceptor membrane turnover: (Besharse and Dunis, 1983; White and Fisher, 1989) and cone photoreceptor movements (Pierce and Besharse, 1985). [See Pierce and Besharse (1986) for a discussion of melatonin and its potential role(s) in photoreceptor metabolism; see also: Dubocovich (1988a)].

The functional antagonism between melatonin and dopamine observed in the retina is interesting because it suggests that a similar relationship may be present centrally. Dopaminergic neurotransmission has been proposed to be responsible (at least in part) for regulation of LHRH release in the hypothalamus (Almeida et al., 1988; Kuljis and Advis, 1989). Perhaps melatonin also modulates dopaminergic activity in the hypothalamus to suppress LHRH release [vide supra].

In summary, a local paracrine role for retinal melatonin is suggested by the following observations: (1) the retina contains the enzymes necessary for melatonin synthesis, (2) in pinealectomised animals a diurnal retinal melatonin rhythm is still present, (3) retinal dopamine inhibits cAMP accumulation, SNAT activity and melatonin synthesis in photoreceptor cells via D2 receptors, (4) melatonin, in turn, inhibits dopamine release in the retina via an high-affinity melatonin receptor [vide infra], (5) melatonin and dopamine are involved in the regulation of some rhythmic retinal events such as cone

elongation and contraction (Pierce and Besharse, 1986). (6) the retina is capable of metabolising endogenous melatonin (Cahill and Besharse, 1989).

1.8.2 Harderian Gland

The Harderian gland was originally thought to lubricate the cornea due to its secretion of a lipoidal fluid (Cohn et al., 1955). Evidence then accumulated suggesting an extra-retinal photoreceptor role for the Harderian gland since Harderianectomy of blinded neonatal rats abolished the response of pineal serotonin levels and HIOMT activity to dark phase light exposure (Wetterberg et al., 1970a,b). These authors proposed that melatonin may act as a substance affecting the visual process by modifying the porphyrin content of the Harderian gland.

Diurnal variations in Harderian gland melatonin have been demonstrated in the rat (Pang et al., 1977; Bubenik et al., 1978; Reiter et al., 1983) and Syrian hamster (Pévet et al., 1980; Balemans et al., 1983), with peak levels late in the dark phase. The presence of a diurnal melatonin rhythm in the Harderian gland of the pinealectomised rat supported the idea of melatonin biosynthesis in this gland (Reiter et al., 1983). Continuous light treatment (1 week) was found to depress pineal melatonin levels but not those in the Harderian gland, indicating a difference in the regulation of the biosynthesis of the hormone in the two glands (Reiter et al., 1983).

Rat Harderian gland SNAT activity was reported not to undergo diurnal rhythms (Brammer et al., 1978); while SNAT activity in vitro was not stimulated by isoproterenol nor dibutyryl-cAMP indicating that the mechanism of regulation of melatonin biosynthesis in this tissue is different from that of the pineal gland. However, this diurnal study was not detailed and the in vitro assay

employed still left the question of SNAT rhythms open. Menéndez-Pelaez et al. (1989b) provide a mini-review regarding SNAT regulation in the Syrian hamster Harderian gland, where clear diurnal rhythms are present.

The morphological and biochemical differences seen in the Harderian glands of male vs female Syrian hamsters are also reflected in the characteristics of Harderian gland melatonin biosynthesis in this species (Hoffman et al., 1985). The differential levels and rhythms of Harderian gland melatonin between the sexes implicate an involvement of the Harderian gland in the pineal-gonadal endocrine axis. Castration converts the male Harderian gland to that of the female while testosterone treatment reverses this process (Hoffmann et al., 1985, 1989a; Menéndez-Pelaez et al., 1987, 1988a,b, 1989a,b; Buzzell et al., 1989b). An effect of photoperiod and melatonin treatment on cytosolic androgen receptors in the Syrian hamster Harderian gland has been reported (Stankov et al., 1989). Hence, a function for the Harderian gland in the modulation of reproductive physiology and sexual behaviours in the hamster is established (Hoffman, 1971; Clabough and Norvell, 1973, Thiessen, 1986) but the precise role that melatonin plays is still uncertain. Finally, some studies suggest an interaction between the Harderian gland and thyroid gland in the Syrian hamster (Hoffman, 1971; Hoffman et al., 1989b; Buzzell et al., 1989a).

1.8.3 Gut

A few reports present findings of the effects of pharmacological doses of melatonin (i.e. micro- to millimolar) on the gastrointestinal system of experimental animals (rat and mouse). The main effect observed was a functional antagonism by melatonin of serotonin-mediated contractions (Quastel and Rahamimoff, 1965; Hertz-Eshel and Rahamimoff, 1965; Harlow and Weekley, 1986;

Bubenik and Dhanvantari, 1986, 1989; Cho et al., 1989). Interestingly, Harlow and Weekley (1986) found that in the rat gut, areas which exhibited the greatest responsiveness to melatonin in vitro (colon, duodenum) were those which had been shown to contain the highest melatonin-like immunoreactivity (Bubenik et al., 1977). The fact that large doses of melatonin are required to produce these effects indicates that it is likely a non-physiological action of melatonin. However, the potential clinical implications of these studies (e.g. the application of melatonin or an analogue in the treatment of ileitis/colitis) warrant further examination of the mechanism(s) involved.

1.9 Role of Melatonin in Humans¹

Although the biochemistry and physiology of melatonin synthesis and action are becoming clear in some species, the role of melatonin in the human is still speculative. It is known that the human and rat pineal calcifies with age (Krstić, 1986) and that this process seems to have no deleterious effects on many of the measurable pineal processes since it remains functional in old age (De Martino et al., 1964; Wurtman et al., 1964b; Tapp and Huxley, 1971). Therefore, the potential exists for the pineal to have a physiological role in humans throughout life. Recently, high-affinity melatonin receptors have been identified in human adult and fetal brain tissue (Reppert et al., 1988) in the suprachiasmatic nuclei (SCN), a region of the brain thought to contain the 'biological clock' controlling our circadian rhythms (Armstrong, 1989).

¹ For further information on this topic see Vaughan (1984) and Wurtman and Waldhauser (1985).

1.9.1 Reproduction

Following along the early clinical observations of Heubner (1898) and Marburg (1907) [vide supra] in relation to the documented effects of melatonin on the reproductive physiology of non-human mammals (Reiter, 1980), it has been thought by many that the pineal is involved in some manner in human reproductive physiology. Several studies have tried to correlate human pineal function (as assessed by plasma melatonin and/or urinary metabolite levels) to the onset of puberty (reviewed by Ebling and Foster [1989]). However, the results of these studies are equivocal. A study along a similar vein has examined male infants from birth to one year of age, an age when hormonal changes occur related to sexual maturation (Hartmann et al., 1982). Low plasma melatonin levels were found during the first three months, a time when plasma testosterone and urinary luteinizing hormone are high due to elevated testicular activity. While a cause and effect relationship cannot be presumed from this and other studies, the general inference is that there is some involvement of the pineal in human sexual development, the exact role being unknown. For a review of the involvement of the pineal in human reproductive physiology see Brzezinski and Wurtman (1988).

1.9.2 Affective Disorders

Abnormalities in circadian rhythms (Hallonquist et al., 1986) and the melatonergic neuroendocrine system (Lewy et al., 1987; Arendt, 1989; Brown, 1989) have been proposed to be involved in certain forms of depression, such as seasonal affective disorder (SAD). While changes in a patient's diurnal melatonin rhythm are sometimes observed under a successful phototherapy regimen (Lewy et al., 1987; Terman et al., 1988; Winton et al., 1989) a cause and effect

relationship between pineal function and SAD cannot be drawn. Administration of melatonin to SAD patients, in the hope of modifying their circadian rhythms, was found to be ineffective (Wirz-Justice, 1990). The evidence to date would seem to indicate an indirect relationship, if any, between SAD and the pineal.

Thus, though much speculation exists over the role of the pineal in human physiology, it remains almost as mysterious to us in the present day as it was to the anatomist Herophilos of Alexandria (325-280 B.C.) [see: Kappers, 1979].

1.10 Melatonin Receptors and Binding Sites

An abundance of information has been compiled since the turn of this century regarding virtually all aspects of the pineal and melatonin except for one important area, the molecular target(s) mediating the response to melatonin. It is only relatively recently that melatonin receptors have been characterised by functional assays and radioligand binding techniques. Information about the G proteins involved in the signal transduction coupling of melatonin receptors has been obtained only within the last few years. Research related to the biochemical mechanism of action of melatonin is currently in an explosive growth phase and slowly a bridge between the physiology and biochemistry is being built. For recent reviews of melatonin receptors and their signal transduction mechanism see: Vaněček (1988b); Morgan and Williams (1989); Stankov and Reiter (1990); Krause and Dubocovich (1991); Reiter, (1991).

1.10.1 Functional Studies

1.10.1.1 Melanophore Blanching

The first quantifiable functional response attributable to melatonin was the blanching effect the hormone was found to have on fish and amphibian

melanophores (McCord and Allen, 1917; Huxley and Hogben, 1922). This formed the basis of the first sensitive bioassay of melatonin activity (Lerner and Case, 1959, 1960; Quay and Bagnara, 1964; Kastin and Schally, 1966; Quay, 1968; Reed, 1968; Ruffin et al., 1969; Reed et al., 1969). Structure-activity comparisons revealed that melatonin was the most potent endogenous indole tested. 6-Hydroxymelatonin, the major in vivo metabolite of melatonin, was several orders of magnitude less potent. Minor chemical modifications of the melatonin structure such as 6-halogenation or increasing the N-acyl chain length up to four carbons were well tolerated and could even increase the potency somewhat (Heward and Hadley, 1975; Frohn et al., 1980; Sugden, 1989b). N-acetylserotonin proved to be a partial agonist in the frog skin blanching bioassay while N-acetyltryptamine was an antagonist (Heward and Hadley, 1975). A semi-quantitative bioassay of the ability of melatonin and analogues to inhibit ovulation in rats produced similar results (Flaugh et al., 1979). The general conclusions drawn from these studies were that the 5-methoxy group is necessary for full agonist activity (efficacy) while N-acylation is required for affinity.

1.10.1.2 Retinal Dopamine Release

As discussed above (Section 1.8.1), the retina not only produces melatonin but also responds to melatonin (i.e. melatonin has a paracrine role in this tissue). The best characterised function of melatonin in the retina is its inhibition of dopamine release. In fact, in the retina dopamine was hypothesised to represent a neurotransmitter signal for light stimuli while melatonin was proposed to be a signal for darkness [vide supra].

Dubocovich (1983) discovered the potent inhibitory effects of melatonin

upon the release of preloaded [^3H]dopamine from electrically stimulated rabbit retinal tissue. Melatonin could inhibit dopamine release with an IC_{50} value of 9 pM and was 1,000-fold more potent than its biosynthetic precursor, N-acetylserotonin. In agreement with the amphibian melanophore bioassay, N-acetyltryptamine was found to be an antagonist of melatonin's effects on dopamine release in chicken retina while it was a partial agonist in rabbit retina (Dubocovich, 1984). Detailed structure-activity analysis indicated the specificity of the effect for melatonin as other endogenous indoles had IC_{50} values 100-fold or more greater than melatonin (Dubocovich, 1985b). In addition, similar conclusions were made as those arrived at using the melanophore bioassay. Namely, the 5-methoxy group is essential for agonist efficacy (and affinity) while N-acylation seems necessary for receptor binding affinity. Halogenation or methylation at positions 2 or 6 on the indole ring was found to increase the potency of melatonin (Dubocovich, 1988b). This could have been due to decreased metabolism of the substituted melatonin by the retinal tissue or due to the increased hydrophobicity of the compounds which could potentially increase the affinity for the receptor. (From our current knowledge of the affinities of these compounds in radioligand binding competition experiments [vide infra], the latter explanation seems correct). Based on these functional studies, the receptor site in fish and amphibia melanophores seems to be similar to that of the retina.

The use of this functional assay to characterise retinal melatonin receptors led to the development of the full antagonist 2-benzyl-N-acetyltryptamine (luzindole) [Dubocovich, 1988b]. 5-Methoxyluzindole proved to be a partial agonist ($\text{IC}_{50} = 1.3 \text{ nM}$) with high affinity ($K_B = 0.063 \text{ nM}$). Compared

to luzindole itself ($IC_{50} > 10,000$ nM; $K_B = 20$ nM), it can be deduced that the 5-methoxy group confers agonist efficacy as well as contributing substantially to binding affinity (Krause and Dubocovich, 1991). Luzindole has been reported to antagonise some effects of melatonin *in vivo* (Fang and Dubocovich, 1988; Dubocovich et al., 1990a). However, since luzindole did not antagonise melatonin-induced gonadal regression in the adult Siberian hamster (Duncan et al., 1990) its antagonist properties are questionable.

1.10.2 Receptor Binding Studies

1.10.2.1 [³H]Melatonin

Early attempts to characterise the nature of melatonin receptors using radioligand binding methodologies employed tritiated melatonin as the radio-label. Cohen et al. (1978) were first in reporting the identification of putative melatonin receptors in cytosolic fractions of hamster, rat and human ovarian tissues. Detection of [³H]melatonin binding sites in brain tissue membrane and cytosolic fractions was subsequently reported (Cardinali et al., 1978, 1979; Vacas and Cardinali, 1979,1980; Niles et al., 1979; Niles, 1987). One study has reported on the characterisation of melatonin binding sites in the frog retina using [³H]melatonin for receptor binding and autoradiography (Wiechmann et al., 1986). The affinity of melatonin in all of these studies was relatively low and nonspecific binding high. The low specific activity of [³H]melatonin (≤ 85 Ci/mmol) and low receptor density in many of these tissues combined to create a low signal-to-noise ratio in these studies. The result of this was a large degree of interexperiment variability and inconsistency in the literature. In retrospect it can be seen that these preliminary descriptions of melatonin 'receptors' likely were wrought with artifacts of nonspecific binding.

1.10.2.2 2-[¹²⁵I]iodomelatonin

The synthesis of 2-[¹²⁵I]iodomelatonin ([¹²⁵I]MEL) by Vakkuri et al. (1984a,b) was originally for the purpose of developing a sensitive melatonin RIA. However, this radioligand proved to be an extremely useful probe for the characterisation of melatonin receptors. The high specific activity of the ligand (= 2,000 Ci/mmol) provided an approximately 25-fold increase in sensitivity over tritiated melatonin. In addition, time-consuming scintillation counting could be avoided. The detection of the high-affinity, low capacity melatonin receptors was facilitated by this high specific activity, high-affinity radioligand and the majority of our knowledge of melatonin receptors has come since the advent of this radiolabel.

High-affinity binding sites for [¹²⁵I]MEL have been detected in a wide variety of species and tissues using both radioreceptor filtration and quantitative autoradiographic techniques. The pars tuberalis (part of the pituitary stalk connecting the anterior pituitary and median eminence) has been found to contain the highest density of these sites in all the mammalian species so far examined. The pars tuberalis/median eminence of the rat (Vaněček et al., 1987; Williams and Morgan, 1988), Syrian hamster (Williams et al., 1989; Vaněček and Janský, 1989), Djungarian (also called Siberian) hamster (Duncan et al., 1989; Carlson et al., 1989; Weaver et al., 1991), sheep (Morgan et al., 1989c), human (Reppert et al., 1988), ferret (Weaver and Reppert, 1990), mouse (Weaver et al., 1990) and rabbit and horse (Stankov et al., 1991) all contain a single population of high-affinity ($K_d = 20-100$ pM) [¹²⁵I]MEL binding sites. In some of these species, GTP regulation of site affinity and/or density has been shown [vide infra]. High-affinity binding has been found also in chicken brain and retina

(Dubocovich and Takahashi, 1987; Dubocovich et al., 1989; Rivkees et al., 1989b; Laitinen and Saavedra, 1990b; Ying and Niles, 1991), rabbit retina (Blazynski and Dubocovich, 1991), quail brain (Yuan and Pang, 1990) and lizard brain (Rivkees et al., 1989b, 1990). The binding pharmacologies reveal that melatonin and its halogenated analogues have the highest affinity for this site while N-acetylserotonin and 5-methoxytryptamine have affinities several orders of magnitude lower and serotonin has negligible affinity. These results parallel the findings of the functional studies [vide supra] and second messenger studies [vide infra].

Interspecies comparisons of the pharmacological profiles reveal an apparently identical site across the species in the brain (chicken, quail, lizard) or retina (rabbit, chicken) or mammalian pars tuberalis/median eminence (Dubocovich et al., 1989; Sugden and Chong, 1991; Stankov et al., 1991). All the criteria necessary to classify this site as a melatonin receptor (i.e. saturability, reversibility, selectivity, specificity, localisation, hormone affinity, functionality, etc.) have been met only in some of these species and tissues [vide infra]. The pharmacological identity of these remaining sites to those classified as receptors implies that they also are melatonin receptors even though second messenger coupling has not yet been demonstrated. Preliminary attempts have been made at solubilisation and purification of functional receptors from the chicken brain (Ying and Niles, 1991) and lizard brain (Rivkees et al., 1989b, 1990).

1.10.3 Signal Transduction

1.10.3.1 Early Studies

Efforts to elucidate the molecular mechanism of action of melatonin predate radioligand binding and autoradiographic studies of the receptors. The ability of melatonin to blanch frog skin had been known for many decades when Abe et al. (1969) discovered that melatonin potently antagonised the skin darkening effect of melanocyte stimulating hormone (MSH) by suppressing intracellular cAMP concentrations. Inhibition of adenylate cyclase was the proposed mechanism. The effect was pharmacologically distinct from the similar action of norepinephrine acting through α -adrenoceptors.

Several reports have found effects of melatonin on cAMP and/or cGMP levels in a variety of tissues. Melatonin increased cGMP levels in human mononuclear leukocytes (Sandler et al., 1975) and in rat testicular slices in vitro (Kano and Miyachi, 1976) while cAMP levels were unaffected. Melatonin inhibited the norepinephrine-induced increases, but not the basal levels, of intracellular cAMP in rat astroglial cell cultures (Vacas et al., 1984). In the rat brain, melatonin increased cAMP and decreased cGMP levels in medial basal hypothalamic slices in vitro (Vacas et al., 1981). Niles (1985) reported that the inhibitory effect of melatonin on rat brain membrane homogenate adenylate cyclase activity was dependent upon GTP.

In vivo, intracisternal injection of melatonin was found to increase cerebrospinal fluid (CSF) cGMP levels \approx 20- fold in the rabbit while cAMP levels were not changed (Rudman et al., 1976). Oral administration of melatonin to humans also increased CSF cGMP concentrations (Young et al., 1984). The difficulty with all of these studies is that the concentrations of melatonin used

were high, causing one to question the physiological relevance of these findings. Since the peak circulating concentration of melatonin is generally ≈ 0.5 nM in the rat (Wilkinson et al., 1977) or ≈ 0.15 nM in the Syrian hamster (Vaughan et al., 1986), these effects of melatonin are likely of a pharmacological nature as opposed to a physiological nature. The relevance of measurements of extracellular (i.e. CSF) cAMP and cGMP is also vague. One study, using *physiological* concentrations (0.1 pM - 1 nM) of melatonin, found an enhancement in guanylate cyclase activity in assorted rat tissues (Vesely, 1980).

1.10.3.2 Recent Studies

Following the identification of putative melatonin receptors using the high-affinity radiolabel [125 I]MEL in radioligand binding and autoradiographic assays [vide supra], efforts were focused upon studying signal transduction via these receptors in these tissues.

1.10.3.2.1 The Rat

Vaněček and Vollrath (1989, 1990a) demonstrated that picomolar concentrations of melatonin could inhibit both LHRH-stimulated cAMP and cGMP accumulation in neonatal rat anterior pituitary explants *in vitro*. This effect was specific for melatonin since N-acetylserotonin and 5-methoxytryptamine were 1,000-fold less potent. These effects of melatonin correlated well with the hormonal antagonism between melatonin and LHRH in the rat anterior pituitary, the ontogeny of the hormonal responses and the ontogeny of [125 I]MEL binding sites in this tissue [vide supra]. The LHRH receptor is coupled to phosphoinositide turnover (Dan-Cohen et al., 1990) while the melatonin receptor is coupled negatively to adenylate cyclase [vide infra]. Therefore, the antagonism between LHRH and melatonin on gonadotropin release speaks of a complex

interaction between the two signal transduction pathways (so-called second messenger 'crosstalk'). Inhibitory effects of melatonin upon LHRH-induced diacylglycerol (DAG) formation, and arachidonic acid release were similarly found in neonatal rat anterior pituitary in vitro (Vaněček and Vollrath, 1990b).

High-affinity [125 I]MEL binding sites have also been identified by quantitative autoradiography in rat suprachiasmatic nuclei (Laitinen and Saavedra, 1990a) and rat area postrema (Laitinen et al., 1990) which exhibit guanine nucleotide sensitivity and binding characteristics similar to that of the rat pars tuberalis/median eminence (PT/ME). Second messenger investigations in these regions have not yet been reported but it is presumed that these binding sites are functional melatonin receptors. Electrophysiological studies have demonstrated dose-dependent inhibition of SCN neuronal activity by melatonin, supporting the presence of functional melatonin receptors (Mason and Brooks, 1988).

1.10.3.2.2 The Hamster

In Djungarian hamster brain slices, high-affinity [125 I]MEL binding sites identified by autoradiography in the ME/PT proved to be sensitive to guanine nucleotides (Carlson et al., 1989). GTP, GTP γ S and GDP potently inhibited radioligand binding in a dose-dependent fashion. Scatchard-Rosenthal analysis of saturation data revealed that the addition of GTP γ S reduced receptor affinity and site density. GTP sensitivity of a picomolar-affinity [125 I]MEL binding site in the Syrian hamster hypothalamus has also been reported using radioreceptor filtration techniques (Niles, 1990). The detection of this high-affinity site seems to be dependent upon assay temperature (30°C) since at a

lower temperature (4°C) only a lower-affinity (nanomolar) site is observed. Inhibition of adenylate cyclase activity by picomolar concentrations of melatonin in membrane homogenates of this tissue (Niles and Hashemi, 1990) suggest the presence of the melatonin receptor found in the ME/PT of mammals.

Melatonin and analogues inhibited forskolin-stimulated cAMP levels in ME/PT explants in a dose-dependent manner; an effect which was blocked by treatment with pertussis toxin (Carlson et al., 1989). These results are consistent with the observations made in the rat (Vaněček and Vollrath, 1989, 1990a) and mouse (Weaver et al. 1990) pars tuberalis/anterior pituitary. However they conflict with the physiological data [vide supra] where no effect of melatonin was seen on the LHRH-induced gonadotropin release in the hamster anterior pituitary (Bacon et al., 1981). Using [³²P]NAD, pertussis toxin labelled a ≈ 40 kDa protein in ME/PT explants which could correspond to either G_iα or G_oα (Carlson et al., 1989).

1.10.3.2.3 The Sheep

Similar results have been obtained in studies using sheep pars tuberalis where high-affinity [¹²⁵I]MEL binding has been observed [vide supra]. Melatonin potently inhibited forskolin-stimulated cAMP increases in ovine pars tuberalis cells in vitro (Morgan et al., 1989a). Guanine nucleotides inhibited binding of [¹²⁵I]MEL and GTPγS was observed to decrease receptor density without affecting receptor affinity (Morgan et al., 1989b). Preliminary investigations of the possible involvement of inositol phosphate turnover and calcium influx in ovine pars tuberalis melatonergic signal transduction have yielded negative results (Thompson et al., 1991). In the ovine pars tuberalis, a recent report claims the existence of multiple inhibitory G-proteins coupled to the melatonin receptor,

based on pertussis toxin and GTP sensitivities of [125 I]MEL binding (Morgan et al., 1991). A 41 kDa protein was labelled in this tissue by pertussis toxin and [32 P]NAD which is proposed to be the α subunit of a G protein coupled to the melatonin receptor. One very recent report claims to have expressed functional melatonin receptors (using ovine pars tuberalis poly (A)⁺ RNA) in *Xenopus* oocytes (Fraser et al., 1991). However, the response in these cells was only seen at a concentration of 1 mM melatonin, making it dubious that a true melatonin receptor was being functionally expressed.

1.10.3.2.4 Non-Mammals

Guanine nucleotide-sensitive high-affinity [125 I]MEL binding sites have been detected in lizard brain (Rivkees et al., 1989a) chicken retina (Laitinen and Saavedra, 1990b) and chicken brain (Dubocovich et al., 1990b; Ying and Niles, 1991) suggesting that they are true receptors. GTP γ S was seen to alter the association and dissociation rate constants of the ligand-receptor complex in both the lizard and chicken brain. Melatonin was found to inhibit adenylate cyclase activity in semi-purified membrane preparations of chick brain and retina (Niles et al., 1991). These results strongly suggest that the picomolar-affinity [125 I]MEL binding site found in the chicken is coupled to inhibition of adenylate cyclase by an inhibitory G protein, as is the case in the mammalian pars tuberalis.

Pertussis toxin ($EC_{50} \approx 0.13$ nM) was found to block the effects of melatonin on melanophore pigment aggregation in *Xenopus laevis* tadpole explants in vitro (White et al., 1987). Blockade of melatonin responsiveness correlated with increased labelling of a 41 kDa protein by pertussis toxin and [32 P]NAD, similar to the results seen in the hamster and sheep.

A lack of agreement in these various studies upon the effects of guanine nucleotides on receptor binding parameters (i.e. K_d and B_{max}) can be partially explained by the fact that most of these reports did not use saturating concentrations of radioligand. Additionally, species and tissue differences in receptor densities could add to this problem. GTP sensitivity generally is taken to imply a receptor which can exist in a high- and low-affinity state, GTP converting the receptor to the low-affinity state by uncoupling the hormone-receptor-G protein complex (Gilman, 1987). GTP sensitivity therefore supports the hypothesis that the melatonin receptor is coupled to a G_i or G_o protein. In addition, the labelling of a 40- 41 kDa protein by pertussis toxin in several different species, which correlated with a loss of melatonin suppression of cAMP levels, strongly suggests that the melatonin receptor is coupled to inhibition of adenylate cyclase via an inhibitory G protein.

1.11 Thesis Objectives

In contrast to the high-affinity (picomolar) melatonin receptor found in the mammalian pars tuberalis, suprachiasmatic nuclei, retina and other tissues [vide supra], a lower-affinity (nanomolar) melatonin binding site also exists. It was this lower-affinity site which was in fact first identified, by our group and others, in hamster brain using the radioligand 2-[^{125}I]iodomelatonin (Duncan et al., 1986; Niles et al, 1987). Studies of this site have lagged behind those of the high-affinity receptor.

This thesis presents a characterisation of this low-affinity melatonin binding site. The radioligand binding pharmacology and regional distribution of this binding site in Syrian hamster brain is described in Chapter 2. In

Chapter 3, the molecular characteristics of the low-affinity binding site are compared to the high-affinity receptor using the technique of target-size analysis. In Chapter 4 possible second messenger systems which could be coupled to the low-affinity site are examined.

CHAPTER TWO

PHARMACOLOGICAL CHARACTERISATION OF A
LOW-AFFINITY BINDING SITE IN HAMSTER BRAIN

2.1 Introduction²

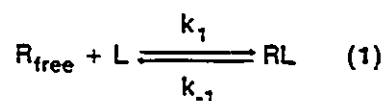
In the mid 1970's an important technique was introduced for receptor studies, that of radioligand binding (Holienberg and Cuatrecasas, 1978). (Some of the pitfalls and practical aspects of radioligand binding are discussed by Williams and Wood [1986]; Munson [1984]; Enna [1984]). The interaction between a radiolabelled agonist or antagonist with a receptor can be measured in terms of the affinity of the receptor for the ligand or the kinetics of ligand-receptor interaction. Information on the receptor density also can be obtained. In addition, one can indirectly measure the affinity of unlabelled drugs, hormones, neurotransmitters, etc. for the receptor and determine its specificity and selectivity requirements.

Biochemical definition of receptors requires that a molecule recognised by the receptor can be appropriately labelled. The ability to bind a radio-labelled compound to a receptor (either reversibly or irreversibly) with specificity allows the design of experiments to measure biochemical properties of the receptor (e.g. molecular mass, association with other proteins, etc.) as well as changes in these properties under various experimental conditions. The sensitivity of detection for radioactivity makes it possible to use biological samples which may have the receptor of interest present as only a very small percentage of the total protein. Most commonly, ³H or ¹²⁵I labelling is used and each has its advantages and disadvantages. Tritium labelling leaves the structure of the compound chemically unchanged (as far as the receptor is

² For more detailed information on the mathematical models involved in receptor binding studies the reader is referred to: Weiland and Molinoff (1981); Bennett and Yamamura (1985); Hrdina (1986); Bylund and Yamamura (1990).

concerned) while iodination produces very high specific activity radioligands, though at the expense of a change in structure which may or may not be well tolerated by the receptor.

Some biochemical properties of receptors can be determined using equilibrium analysis (saturation studies of the equilibrium dissociation constant) or kinetic analysis (rate constants of association and dissociation). The interactions between a labelled ligand and various unlabelled ones can also be studied in competition studies. If only one receptor or receptor state is involved in ligand binding and no allosteric interaction is present, the system is one of a simple bimolecular interaction and the following equations apply, where R_{free} = unbound receptor and L = ligand:



Where, $K_d = [R]_{free}[L]/[RL] = k_{-1}/k_1$ = dissociation constant. K_d can also be defined as the concentration of ligand at which $[R]_{free} = [RL]$; k_1 and k_{-1} are the bimolecular association and unimolecular dissociation rate constants, respectively.

2.1.1 Saturation Binding

Studies in which the total receptor concentration is held constant while the radioligand concentration is varied (over a 100-fold range encompassing the K_d if possible) are called saturation binding studies. As $[L]$ becomes very large (with respect to K_d), receptor ligand binding becomes saturated (i.e. $[RL] \gg [R]_{free}$). These experiments are carried out at equilibrium (i.e. the system has reached a point where $d[RL]/dt = 0$, there is no net change in the concentration of RL with respect to time). Proper analysis of such data demands

that the system is truly at equilibrium. Since this type of experiment produces data which is mathematically described by a rectangular hyperbola, it is not directly amenable to linear regression analysis. Before the advent of personal computers and sophisticated data analysis software, a linear transformation of the hyperbolic saturation isotherm was employed to determine K_d and receptor density (B_{max}): the so-called Scatchard (or more correctly Scatchard-Rosenthal) transformation (Scatchard, 1949; Rosenthal, 1967). The Scatchard-Rosenthal plot relates the fraction of occupied receptor sites to the concentration of free ligand. If \bar{Y} is the fraction of occupied sites:

$$\bar{Y} = RL/(R_{free} + RL)$$

Substitution using equation 1 yields:

$$\bar{Y}/L = (1 - \bar{Y})/K_d$$

A plot of fraction of receptor sites bound divided by free drug concentration (\bar{Y}/F) [ordinate] versus fraction bound (\bar{Y}) [abscissa] gives a straight line of slope equal to $-1/K_d$ and intercept equal to $1/K_d$. However, in a binding experiment, we know the absolute concentrations of binding sites, not the fraction bound. If we replace 'fraction bound' with absolute concentrations of bound receptors, $[RL]$, we obtain:

$$[RL]/[L] = ([R_t] - [RL])/K_d$$

where $[R_t]$ is the total receptor concentration, or receptor density (conventionally called B_{max}). A plot of concentration of receptor bound divided by free ligand concentration (B/F) versus concentration of bound (B) receptors gives a slope of $-1/K_d$ and an abscissa intercept of B_{max} (or $[R_t]$). The ordinate intercept in this plot equals $[R_t]/K_d$ and provides a check on K_d (using $[R_t]$ extrapolated by linear regression of the data).

A major disadvantage of this transformation is that the error associated with data points representing small B/F ratios becomes very large so that there is non-uniformity of variance along the plot. Since these points are required to obtain saturation and determine B_{\max} , this implies that a large error is associated with the determination of B_{\max} using this linearisation (Munson, 1984). In addition, it can be difficult to experimentally achieve saturation in some situations, necessitating a very large extrapolation of the data to derive B_{\max} [See: Klotz (1982); Munson (1984)]. By using computerised, non-linear regression analysis models can be created which take factors such as variance into account (Munson, 1984). One other advantage of non-linear regression analysis is the ability to analyse complicated binding isotherms consisting of multiple binding sites, affinity states or allosteric interactions (Unnerstall, 1990). When analysed by Scatchard-Rosenthal transformation, such data produce curvilinear plots which, of course, defeats the initial purpose of the transformation. It is, however, common practice to analyse saturation binding data by iterative, non-linear regression in order to determine K_d and B_{\max} but still graphically present the data using the Scatchard-Rosenthal coordinates. Thus a subtle distinction must be made between the Scatchard-Rosenthal plot and Scatchard-Rosenthal transformation. The former merely is a convenient means of graphically depicting the data while the latter implies a particular mathematical process of data analysis.

2.1.2 Competition Binding

In addition to saturation studies using a single radioligand, binding properties of a receptor for an unlabelled ligand can be studied by competition between varying concentrations of it and a fixed concentration of radioligand

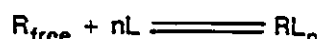
(Tomlinson, 1988). Like saturation binding, these are also equilibrium binding studies. Competition data are generally plotted as semi-logarithmic, sigmoidal curves which are best analysed by computerised non-linear regression, avoiding linearisation of the data via logit or probit transformations (Williams and Wood, 1986). (This is especially true if complicated curves are obtained indicating multiple binding sites). Usually, the labelled ligand concentration is fixed at or below the K_d value so that the free concentrations of receptor, radioligand and drug may be approximated by the total concentrations, then the displacement of specific binding is measured at increasing concentrations of unlabelled ligand and the concentration for 50% displacement of labelled ligand (IC_{50}) determined. If and only if, the two ligands interact in a simple competitive manner, then the K_i (binding affinity constant for unlabelled ligand) can be estimated from the IC_{50} value using the Cheng-Prusoff equation (Cheng and Prusoff, 1973):

$$K_i = \frac{IC_{50}}{1 + [L]/K_d}$$

Where $[L]$ is the radioligand concentration and K_d its dissociation constant. Note that this relationship is only an approximation which is valid if $[L]_{total} + [R]_{total} \ll K_d$. Thus with this approach, the amount of tissue should be kept small so that $[R]_{total}$ is less than 10% K_d .

2.1.3 Hill Plot

The Hill plot is an alternate way to represent binding data assuming that:



(i.e., not necessarily a bimolecular interaction)

We can define a phenomenological constant K_a :

$$[RL_n]/[R]_{\text{free}}[L]^n = K_a$$

such that K_a is the inverse of the half-saturating ligand concentration. This equation is linearised by taking logarithms:

$$\log \{[RL_n]/[R]_{\text{free}}\} = \log K_a + n \cdot \log [L]$$

Plotting $\log \{[RL_n]/[R]_{\text{free}}\}$ against $\log [L]$ yields a plot with slope n (Hill coefficient, or n_H). The intercepts on the abscissa and ordinate are $(1/n)\log K_d$ and $\log K_a$ ($= -\log K_d$), respectively. If $n = 1$, then the situation simplifies to that previously discussed (half-saturating ligand concentration is the apparent dissociation constant).

Note that the Hill procedure requires an independent knowledge of the concentration of free receptors. In practice this datum is obtained by subtracting the number of bound sites from the total (B_{max}). This last would have to be obtained from saturation binding experiments. Note also that this extrapolation is valid only when a single affinity constant defines binding; i.e., no cooperativity and no heterogeneity of binding sites.

Hill plots can be constructed for competition data and were in fact, together with logit and probit plots, the manner in which competition data were analysed before the days of the personal computer and non-linear analysis. Competition data is plotted as $\log[(B_T - B)/B]$ vs $\log [D]$; where $[D]$ is the molar

concentration of the competing drug, B is the % specific binding at a given $[D]$ and B_T is the total possible amount of specific binding (which is 100% by definition). The slope of the plot (which should be linear) is equal to n_H (the Hill coefficient, Hill value or Hill number). The y-intercept is equal to $-\log IC_{50}$ while the x-intercept is equal to $\log IC_{50}$. If $n = 1$, this indicates a homogeneous population (with respect to K_d) of non-cooperative binding sites. If $0 < n < 1$, this indicates either multiple types of binding sites, multiple affinity states or negative cooperativity between binding sites. If $n > 1$, this indicates positive cooperativity between binding sites. The Hill, logit and probit transformations are all sensitive to errors in the data at the extremes of the sigmoidal curves (i.e. the plateaus) and data values $< 10\%$ or $> 90\%$ of the control binding should not be used (Williams and Wood, 1986).

2.1.4 Kinetic Studies

The rate of association of receptor and radioligand as well as the dissociation rate of the receptor-ligand complex may also be studied by measuring changes in $[RL]$ with respect to time. By application of the appropriate exponential equations and logarithmic transformations (Weiland and Molinoff, 1981; Hrdina, 1986; Bylund and Yamamura, 1990) the association and dissociation rate constants can be determined. These provide an independent check on the value of K_d as derived from saturation experiments since $K_d = k_{-1}/k_1$. Once again, if complicated kinetics are observed then computerised, non-linear regression analysis of the data is the best approach.

2.1.5 Receptor Criteria

A set of criteria exist which must be fulfilled in order to define a receptor. Radioligand binding experiments can be employed to test many of these

criteria (Burt, 1985; Hrdina, 1986): saturability, reversibility, high affinity binding, tissue linearity of binding, specificity (stereospecificity if applicable), temperature sensitivity, pH sensitivity, protease sensitivity, tissue distribution, species distribution, subcellular distribution and pharmacological profile of drug binding. The only major criterion which cannot be addressed is that of functionality. The results of binding studies should, however, be complementary to those of functional studies.

2.2 Materials and Methods³

2.2.1 Materials

Na¹²⁵I (1800- 2175 Ci/mmol; 0.5- 0.8 mCi/μL in dilute NaOH, pH 7- 11, [IMS.300]) and [O-methyl-³H]melatonin (70- 85 Ci/mmol) were purchased from Amersham. [furanyl-5-³H]Prazosin (24.4 Ci/mmol) was bought from New England Nuclear while Scint-A scintillation cocktail was from Packard. 6-Chloro-melatonin and 6-methylmelatonin were kindly donated by Dr. M. Flaugh (Lilly Research Laboratories). N-acetyl-5-methoxykynurenamine was a gift of Dr. P. Marangos (NIMH, Bethesda). N-n-butyryl-5-methoxytryptamine, N-n-propionyl-5-methoxytryptamine and N-n-myristyl-5-methoxytryptamine were supplied by Dr. D.J. Kennaway (University of Adelaide, South Australia). Indoramin·HCl was provided by Wyeth Laboratories Inc., metergoline by Farmitalia, ipsapirone by Miles Inc., Ru 24969 by Roussel UCLAF and cyproheptadine·HCl by Merck Sharp & Dohme. Phentolamine was supplied by Ciba-Geigy and methysergide maleate was a gift of Sandoz Pharmaceuticals. Iodogen (1,3,4,6-tetrachloro-3α,6α-diphenyl-

³ Portions of the text and figures have been previously published (Niles et al., 1987; Pickering and Niles, 1989, 1990) and are reproduced here with the permission of the publishers.

glycouril), guanosine 5'-triphosphate, 6- γ -imidoguanosine 5'-triphosphate (sodium salt), prazosin, yohimbine·HCl, clonidine·HCl, chlorpromazine·HCl, pargyline·HCl, melatonin, N-acetylserotonin, tryptamine, haloperidol, diazepam, 6-methoxy-2-benzoxazolinone, trifluoroacetic anhydride, isobutyryl chloride, 4-fluoro-3-nitrophenylazide (FNPA), N-(5-azido-2-nitrobenzoyl)-oxysuccinimide (ANB-NOS), CAPS and CHES were purchased from Sigma Chemical Co. N-succinimidyl-6-(4'-azido-2'-nitrophenylamino)hexanoate (SANPAH) and 2-diazo-3,3,3-trifluoropropionyl chloride were obtained from Pierce. Spiperone, ketanserin, mianserin·HCl, (\pm)-verapamil·HCl, methoxyverapamil·HCl, diltiazem·HCl, amiloride·HCl, L-propranolol, 6-carboline 3-carboxylate ethyl ester (6-CCE), 1-(3-chlorophenyl)piperazine·HCl (mCPP), (\pm)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane·HCl (DOI), 3-tropanyl-3,5-dichlorobenzoate (TDCB), 1-(1-naphthyl)piperazine·HCl (NPP) and (\pm)-8-hydroxydipropylaminotetralin·HBr (8-OH-DPAT), benextramine·HCl, WB-4101·HCl and 2-iodo-melatonin were obtained from Research Biochemicals Inc. 5-Methoxytryptamine, 6-methoxytryptamine and N-t-butoxycarbonyloxyimino-2-phenylacetonitrile (BOC-ON) were purchased from Aldrich Chemical Co. Acetic anhydride (analytical grade) was from BDH. 1-[(p-carboxy)benzyl]melatonin was supplied by CIDtech (Hamilton, Ont). All solvents and acids used were reagent grade or HPLC grade and were obtained from standard suppliers. Other drugs, chemicals and reagents were obtained from Sigma Chemical Co. or similar standard commercial suppliers. Silica gel (60- 200 mesh) was purchased from Sargent-Welch. Silica gel polyester TLC plates, 0.25 mm thickness (with 254 nm fluorescent dye indicator) were from Kodak.

2.2.2 2-[¹²⁵I]Iodomelatonin Synthesis

[¹²⁵I]MEL was prepared using a modification of the method of Vakkuri et al. (1984b). A solution of melatonin (4 mg/mL) was prepared in 20% methanol:80% 0.2M KH₂PO₄ pH 6.0 (23°C) and 10 µL (40 µg melatonin) of this solution placed in a 0.5 mL eppendorf tube. A solution of Iodogen was made (1 mg/mL in CHCl₃) and 20 µL of this placed in another 0.5 mL eppendorf tube. After gently drying the chloroform under a stream of nitrogen gas, an even coating of 20 µg Iodogen was obtained on the tube bottom. Note that it is important to obtain an even coating of Iodogen on the bottom of the tube. Subsequent steps were carried out in a 'hot-lab' fumehood.

Na¹²⁵I (= 10 µL; = 5 mCi) was added to the melatonin solution using a Hamilton syringe. After mixing with the syringe, the melatonin/¹²⁵I mixture was transferred to the bottom of the Iodogen-coated eppendorf tube. Iodination was carried out for one minute at room temperature and then 100 µL chloroform added to stop the reaction. The tube was vortexed for 10- 20 sec, the CHCl₃ phase (lower layer) removed and transferred to a new 0.5 mL eppendorf tube. A second extraction of the aqueous phase with 100 µL of fresh chloroform was performed. The aqueous phase was discarded and the chloroform extracts combined and evaporated to dryness under a gentle nitrogen stream. The majority of unincorporated iodine was removed by these procedures. The iodinated melatonin was then purified by silica gel TLC or by HPLC.

2.2.3 Purification of [¹²⁵I]MEL

2.2.3.1 TLC

The dried extract was redissolved in a small (e.g. 20 μ L) volume of CHCl_3 and spotted on a TLC plate. The plate was eluted with either ethyl acetate or ethyl acetate : CHCl_3 (8:2). Strips (5 mm) of the plate were cut and counted in a Searle γ -counter (71% efficiency) to locate the [¹²⁵I]MEL product which was then eluted from the silica with methanol. After low-speed centrifugation of the methanol (to remove silica particles), the eluate was stored in brown glass vials at -70°C (long term) or -20°C (working stock).

2.2.3.2 HPLC

For HPLC purification, the dried extract was reconstituted in 100 μ L methanol with gentle vortexing, then 100 μ L distilled water added and the mixture vortexed. [¹²⁵I]MEL was purified by injection of this solution (50% methanol-water) with a Rheodyne injection valve onto an Alltech Econosphere 250 x 4.6 mm ODS (5 μ m particle size) column and elution with 50% methanol-water (isocratic) at a flow rate of 1 mL/min using a BioRad SS 1350 HPLC pump. The retention times of melatonin and (nonradioactive) iodomelatonin standards were confirmed using a Waters model 440 absorbance detector with a 280 nm UV filter connected to an HP 3394A integrator/recorder. [¹²⁵I]MEL was collected as a single peak using a Gilson model 203 programmable fraction collector. The purified label solution was diluted with an equal volume of methanol and 0.5 μ L δ -mercaptoethanol/mL added. Aliquots were stored in glass vials at -20°C (working stock) or -70°C (long term storage).

2.2.4 2-[¹²⁷I]Iodomelatonin Synthesis

Cold 2-iodomelatonin was prepared on a milligram scale essentially as described by Vakkuri et al. (1984a). The reaction was monitored by TLC. Increasing the reaction time from 1 to 45 min did not appreciably affect the overall yield as judged visually by TLC. Initial pilot experiments indicated that an optimal yield of iodinated product was obtained using a mass ratio of approximately 1:1:1 for melatonin:iodogen:KI. [³H]Melatonin (57 pmol) was added to the reaction as a tracer, the chloroform extract dried under nitrogen, the residue dissolved in ethyl acetate and iodinated product purified by silica gel column chromatography, eluting with ethyl acetate : chloroform (8:2). Fractions (15 mL) were collected and 20 µL aliquots removed, mixed with 5 mL Scint-A cocktail and counted for tritium. The peak of iodinated melatonin was pooled, and evaporated to dryness. Product structure was confirmed by ¹H- and ¹³C-NMR spectroscopy.

2.2.5 Other Syntheses

Pyridine and triethylamine (TEA) used in these syntheses were distilled and kept dry over 4 Å molecular sieves (Fisher Scientific). All reactions were performed at room temperature in darkness with constant stirring (on a magnetic stir plate) unless otherwise stated. Solutions were prepared and handled in a dry-bag under a nitrogen atmosphere until the reaction flasks were stoppered. TLC spots were visualised by UV (254 nm) illumination and/or I₂ vapour adsorption. HPLC analyses were conducted as described in Section 2.2.3.2.

2.2.5.1 N-t-butoxycarbonyl-5-methoxytryptamine

5-Methoxytryptamine (5-MT) (65.6 mg, 345 μ mol) was dissolved in 0.5 mL pyridine and 1 mL TEA added. N-t-butoxycarbonyloxyimino-2-phenylacetonitrile (BOC-ON) (85 mg, 345 μ mol) was dissolved in 1 mL TEA and added to the 5-MT solution. After overnight reaction the solution was evaporated under vacuum until a yellow-orange oil residue was obtained. This residue was dissolved in a small volume of CHCl_3 (+ 1% ethanol), applied to a 20 x 1 cm silica gel column and the product, N-t-BOC-5MT, eluted using the same solvent. Evaporation of the solvent yielded a white solid (m.pt. 122- 123°C) which migrated as a single spot on silica gel TLC (CHCl_3 + 1% ethanol) with R_f = 0.65. R_f values: 5-MT = 0.0; BOC-ON = 0.93; HO-N=C(Φ)CN (BOC-ON side product) = 0.28. HPLC analysis indicated a single peak for the product, indicating homogeneity. Mass spectrometry of this product indicated a molecular ion size consistent with that expected for the proposed structure.

2.2.5.2 6-Methoxy-N-acetyltryptamine

6-Methoxytryptamine (12 mg, 52 μ mol) was dissolved in 0.5 mL pyridine and 1 mL acetic anhydride added. After reacting overnight, the solution was evaporated under vacuum to yield a yellow oil which gave a yellow solid upon lyophilisation. This residue was dissolved in a small volume of solvent (1:9 = methanol: CHCl_3) and purified by silica gel column chromatography. The product was evaporated to give a clear oil from which a white solid was crystallised in cold methanol-water. TLC and HPLC analysis of the product indicated homogeneity. The product structure was confirmed to be 6-methoxy-N-acetyltryptamine by $^1\text{H-NMR}$ and mass spectrometry.

2.2.5.3 N-acetyltryptamine

Tryptamine (13 mg, 81 μmol) was dissolved in 0.5 mL pyridine, 50 μL (360 μmol) TEA added then 0.5 mL acetic anhydride added. After reacting overnight, the solution was evaporated under vacuum to yield a clear, pale yellow oil. This residue was dissolved in a small volume of methanol and a white solid crystallised by the gradual addition of cold water and cooling to -20°C . TLC and HPLC analysis of the product (m.pt. $74-75^{\circ}\text{C}$) indicated homogeneity. The product structure was confirmed as N-acetyltryptamine by $^1\text{H-NMR}$ and mass spectrometry.

2.2.5.4 O-acetyl-N-acetylserotonin

N-acetylserotonin (30 mg, 138 μmol) was dissolved in 0.5 mL pyridine and 0.5 mL acetic anhydride added. After 3 h reaction, the solution was evaporated under vacuum to give a clear, colourless oil. TLC (95% CHCl_3 + 5% ethanol) indicated one product spot or $R_f = 0.40$; (R_f (N-acetylserotonin) = 0.15, R_f (melatonin) = 0.48). HPLC analysis indicated this oil to be homogeneous. A solid product could not be obtained from the oil. $^1\text{H-NMR}$ and mass spectrometry indicated that the proposed structure of this product was correct.

2.2.5.5 O-isobutyryl-N-acetylserotonin

N-acetylserotonin (25 mg, 115 μmol) was dissolved in 0.5 mL pyridine, 50 μL (360 μmol) TEA added then 100 μL (955 μmol) isobutyryl chloride added. After overnight reaction the solution was evaporated under vacuum and the clear, colourless oil residue dissolved in 100 μL 60% methanol- 40% water. The product was purified using a preparative size (30 x 1 cm) ODS HPLC column (60% methanol solvent). Solvent evaporation gave a white solid. HPLC analysis of the solid indicated a single peak. Mass spectrometry of this product revealed a molecular ion of size consistent with O-isobutyryl-N-acetylserotonin (base peak @ 245; molecular ion @ 288, 48% base).

2.2.5.6 N-isobutyryl-5-methoxytryptamine

This compound was prepared (and purified) from 25 mg (115 μmol) N-acetylserotonin as described for O-isobutyryl-N-acetylserotonin. HPLC analysis of the white solid product (m.pt. 73- 74°C) indicated it to be homogeneous. Mass spectrometry of the solid yielded a molecular ion of size consistent with that calculated for N-isobutyryl-5-methoxytryptamine (base peak @ 189; molecular ion @ 260, 50% base).

2.2.5.7 N-trifluoroacetyl-5-methoxytryptamine

5-MT (109 mg, 0.57 mmol) was dissolved in 1 mL pyridine and cooled on ice to 0°C. Trifluoroacetic anhydride (160 μL , 1.15 mmol) was added and the reaction allowed to proceed for three hours at room temperature. Evaporation of the solution produced an orange oil which was dissolved in a small portion of methanol. A white solid (m.pt. 160- 161°C) was crystallised from this solution by the gradual addition of water and cooling on ice. TLC analysis (CHCl_3) of this solid revealed a single spot of $R_f = 0.24$ (5-MT $R_f = 0.0$). HPLC analysis also indicated one major peak (area = 95% of total). Mass spectrometry of the product gave a molecular ion of a size consistent with that for N-trifluoroacetyl-5-methoxytryptamine (base peak @ 160; molecular ion @ 286, 14% base).

2.2.5.8 N-(4-azido-2-nitrophenyl)-5-methoxytryptamine

5-MT (34.2 mg, 180 μmol) and 35.8 mg (197 μmol) fluoronitrophenylazide (FNPA) were dissolved together in 0.3 mL pyridine and 50 μL (360 μmol) TEA added. After reacting overnight, 100 μL concentrated NH_4OH was added and the solution left 2 h at room temperature with stirring. Evaporation under vacuum gave an orange-red residue which was washed with 2 mL 0.1M HCl then 2 mL distilled water. TLC (1:1 = ligroin: diethylether + 4% TEA) of the residue indicated an orange product spot of $R_f = 0.24$; [R_f (5-MT) = 0.0, R_f (FNPA) = 1.0].

The residue was dried under vacuum, dissolved in a minimal volume of pyridine and crystallised by the addition of 50% ethanol-water. Cooling the solution to -20°C completed crystallisation of a dark red solid. HPLC analysis of the solid (eluent = 75% methanol + 0.4% TEA) indicated the product to be 95 - 98% pure. A melting point of $134 - 136^{\circ}\text{C}$ was obtained for the solid (m.pt. FNPA = $52 - 53^{\circ}\text{C}$). Mass spectrometry indicated the expected molecular ion for N-(4-azido-2-nitrophenyl)-5-methoxytryptamine (base peak @ 174; molecular ion @ 353, 53% base).

2.2.5.9 N-(5-azido-2-nitrobenzoyl)-5-methoxytryptamine

5-MT (14.8 mg, 78 μmol) and N-(5-azido-2-nitrobenzoyl)oxysuccinimide (13.5 mg, 44 μmol) were separately dissolved in 0.2 mL pyridine and mixed. After overnight reaction, the solution was evaporated under vacuum and the residue washed with 1 mL 1M HCl then twice with 1 mL CHCl_3 to leave a brownish oil residue. This residue was purified using a preparative size (30 x 1 cm) ODS HPLC column (eluent = 75% methanol, 0.4% TEA) to give a yellow solid after solvent evaporation. HPLC analysis of the solid indicated a single peak. The solid decomposed upon heating past 50°C . Mass spectrometry of the product gave a molecular ion compatible with the proposed structure (base peak @ 355; molecular ion @ 381, 74% base).

2.2.5.10 N-(6-[4'-azido-2'-nitrophenylamino]hexanoyl)-5-MT

5-MT (13.3 mg, 70 μmol) was dissolved in 150 μL pyridine and 50 μL (360 μmol) TEA added. N-succinimidyl-6-(4'-azido-2'-nitrophenylamino)hexanoate (SANPAH) (28.2 mg, 72 μmol) was dissolved in 100 μL pyridine and added to the 5-MT solution. After reacting 4 h the solvent was evaporated under vacuum to yield an orange residue. TLC (CHCl_3) indicated one orange product spot of $R_f = 0.16$; [R_f (5-MT) = 0.0; R_f (SANPAH) = 0.61; R_f (N-hydroxysuccinimide) = 0.0]. The residue was washed with 0.5 mL 0.1M HCl and extracted twice with 1 mL ethyl ace-

tate. The combined ethyl acetate phase was washed with 1 mL water and evaporated to dryness under vacuum. The residue was then dissolved in 0.2 mL methanol and the product purified using a preparative size (30 x 1 cm) ODS HPLC column (60% methanol solvent). Evaporation of the solvent gave an orange solid (m.pt. 60-61°C). Mass spectrometry of this solid gave a molecular ion consistent with that of the expected product (base peak @ 427; molecular ion @ 464, 38% base).

2.2.5.11 N-(2'-diazo-3',3',3'-trifluoropropionyl)-5-methoxytryptamine

5-MT (24 mg, 126 μ mol) was dissolved in 1 mL pyridine and the solution evaporated to dryness under vacuum. The dried residue was redissolved in 0.2 mL pyridine and 50 μ L (360 μ mol) TEA added. 2-diazo-3,3,3-trifluoropropionyl chloride (= 10 μ L) was added under dim lighting and the reaction tube vortexed. After reacting 3 h in darkness, the solution was evaporated under vacuum and 1 mL 0.1M HCl added to the residue which was then extracted twice with 2 mL CHCl_3 . Evaporation of the combined CHCl_3 phase yielded a brown solid residue. This residue was dissolved in 100 μ L methanol and the product purified using a preparative size (30 x 1 cm) ODS HPLC column (50% methanol, 0.1% HCOOH solvent). The major product peak (R_t = 12.2 min) was collected and the solvent evaporated to give a pale yellow oil. Re-examination of this product by HPLC indicated it to be homogeneous. Mass spectrometry of the compound indicated a molecular ion size consistent with that calculated for the proposed product structure base peak @ 201; molecular ion @ 326, 21% base).

2.2.6 Tissue Preparation

Adult (2- 4 months old) male Syrian hamsters (High Oak Ranch, Ont. or Charles River, Que.) were group housed on a 16h L:8h D cycle with access to food (Purina Rat Chow) and water ad libitum. Animals were sacrificed by decapitation between 4 and 5 hours after lights-on. Brains were rapidly removed, cooled on

ice and dissected on a chilled glass plate according to the method of Glowinsky and Iversen (1966). Fresh brain regions (or frozen at -70°C and thawed) were homogenised by polytron in 20 volumes of 50 mM Tris-HCl pH 7.4 (4°C) [hereafter called assay buffer] and centrifuged at $30,000 \times g$ for 10 min at 4°C in a Beckman J-21C high-speed centrifuge. The pellet (whole homogenate membranes) was washed three times by resuspension in 20 volumes of this buffer and centrifugation. For inhibition and kinetic studies, whole homogenates were resuspended in 100- 200 volumes of assay buffer. In saturation binding experiments, the crude synaptosomal (P2) membrane fraction was prepared in assay buffer as described below and resuspended finally in approximately 5 volumes of assay buffer. In some experiments, frozen (-20°C) membrane preparations were thawed, washed 2- 3 times by resuspension in assay buffer and centrifugation 10 min at $30,000 \times g$ (at 4°C) and used in binding experiments.

2.2.7 Subcellular Fractionation

Differential centrifugation was employed to yield the P1, P2 and P3 fractions of brain tissues according to the method of Bennett and Snyder (1975). All steps were carried out on ice or at 4°C . Tissue was homogenised in 10 volumes of ice-cold 0.32 M sucrose using a teflon pestle and then centrifuged at $1,000 \times g$ for 10 min. The supernatant was removed and the pellet resuspended by polytron (5 sec, setting 5) in 10 volumes of sucrose and centrifuged at $1,000 \times g$ for 10 min. Sucrose supernatants were combined, leaving the washed P1 pellet, then centrifuged at $27,500 \times g$ for 10 min. The resulting supernatant was removed, leaving the crude P2 pellet, and centrifuged at $103,000 \times g$ for 1 h in a Beckman L5-50 ultracentrifuge to give the crude P3 pellet.

2.2.8 Binding Assays

2.2.8.1 [¹²⁵I]MEL Equilibrium Studies

Binding studies were performed in assay buffer in triplicate. Studies using cortical membranes were conducted in assay buffer containing 0.1% (5.7 mM) ascorbic acid + 10 μ M pargyline. In competition experiments 0.15 - 0.25 nM [¹²⁵I]MEL was used (at a specific activity of 1700 - 2175 Ci/mmol), in the presence of varying concentrations of drug. Tissue aliquots (50 - 200 μ L) were used in binding assays, containing 50 - 200 μ g protein as determined by the method of Lowry et al. (1951) using BSA as standard. For saturation binding studies, 0.070 - 20 nM [¹²⁵I]MEL was used, after isotopic dilution with [¹²⁷I]MEL to a specific activity in the range of 500 - 800 Ci/mmol.

Incubations were carried out for 30 - 60 minutes on ice and bound radioactivity separated by rapid vacuum filtration on Whatman GF/B filters (or Schleicher & Schuell #32 filters). Unbound radioactivity was removed by washing filters with 4 x 3.5 mL of ice-cold buffer with a total filtration time of less than 10 seconds. Bound radioactivity was determined with a Searle γ -counter (at 71% efficiency). Specific binding was defined as that radioactivity displaced by 1 μ M 6-chloromelatonin and ranged from 50 - 60% in synaptosomal membranes to 70 -85% in whole tissue homogenates.

2.2.8.1.1 Kinetic Studies

Association kinetics were examined using time points from 0.5 -60 min. Whole tissue homogenates (200 μ L), with or without 25 μ L 6-chloromelatonin (1 μ M final concentration), were incubated on ice and association was started by the addition of 25 μ L [¹²⁵I]MEL (0.52 nM final concentration). After further incubation on ice for the indicated times, samples were filtered as described

above. Dissociation kinetics were performed using time points from 0.25 - 30 min. Whole tissue homogenate (200 μ L) and 25 μ L [125 I]MEL (0.52 nM final concentration) were pre-incubated 30 min on ice since the association kinetics indicated that equilibrium is reached by this time. Dissociation was then initiated by the addition of 6-chloromelatonin (1 μ M final concentration) and samples were filtered at the times specified.

2.2.8.2 [3 H]Prazosin Equilibrium Studies

Hamster cerebral cortex membranes (whole homogenate) were prepared as described above and treated with or without 0.10 mM benextramine-HCl in assay buffer on ice (or at 25°C) for 3 h. [3 H]Prazosin binding was evaluated as described by Reader et al. (1986). Membranes were incubated at 25°C with [3 H]prazosin (0.8 or 4.8 nM) in 50 mM Tris-HCl, pH 7.4 (25°C) for 45 min. Non-specific binding was defined using 10 μ M phentolamine. Filters (Whatman GF/C) were pre-soaked in 50 mM Tris base + 0.1 % PEI. Samples were filtered and washed with 3 x 4 mL ice-cold assay buffer. Filters were placed in mini-vials and 5 mL Scint-A cocktail added. After overnight equilibration, samples were counted for tritium using a Beckman LS 7000 scintillation counter.

2.2.9 Data Analysis

Binding data were assessed by iterative, non-linear regression analyses using the programmes BDATA (EMF Software) for saturation studies and CDATA (EMF Software) for competition experiments. IC_{50} values of inhibitors were converted to 'apparent' K_i 's using the Cheng-Prusoff equation (Cheng and Prusoff, 1973). Kinetic experiments were analysed by logarithmic transformation of the binding data (Bylund and Yamamura, 1990) followed by linear regression using an HP 11C calculator. Where error measurements are given, S.D. is implied unless otherwise stated.

2.3 Results

2.3.1 Iodomelatonin Characterisation

In order to confirm the chemical structure of iodomelatonin, [^{127}I]iodomelatonin was prepared as described above and purified by silica gel column chromatography (Fig. 3). Proton NMR spectra were recorded at 500 MHz on a Bruker AM 500 spectrometer. Tetramethylsilane was used as the internal chemical shift reference. As shown in Fig. 4a, the melatonin proton spectrum exhibits peaks at about 7.2 ppm (proton 7), 7.1 ppm (proton 2), 7.0 ppm (proton 4) and 6.7 ppm (proton 6) in the aromatic region. The iodomelatonin proton spectrum (Fig. 4b) gives peaks for protons 7, 4 and 6 as found for melatonin; however, the NH peak at about 11 ppm no longer shows any proton coupling indicating that iodine has replaced proton 2. Consistent with the foregoing, the carbon-13 spectrum of iodomelatonin (recorded at 125 MHz; Fig. 4c,d) indicated that carbon 2 shifts from 123.2 ppm (as in melatonin) to 81.1 ppm, an effect typical of iodine substitution. This confirms the chemical structure of the iodinated melatonin as being 2-iodomelatonin. Purification of radioiodinated melatonin was achieved either by silica gel TLC (Fig. 5) or reversed phase HPLC (Fig. 6). In both cases, [^{127}I]iodomelatonin migrated/eluted in the same manner as [^{125}I]iodomelatonin. Therefore, prior to each batch HPLC purification of label, [^{127}I]MEL and melatonin standards were run to calibrate and confirm retention times.

Figure 3. Purification of 2-iodo[O-methyl-³H]melatonin by silica gel column chromatography. Elution was carried out with ethyl acetate: chloroform (8:2). Fractions (15 mL) were collected and 20 μ L aliquots counted by liquid scintillation spectrometry.

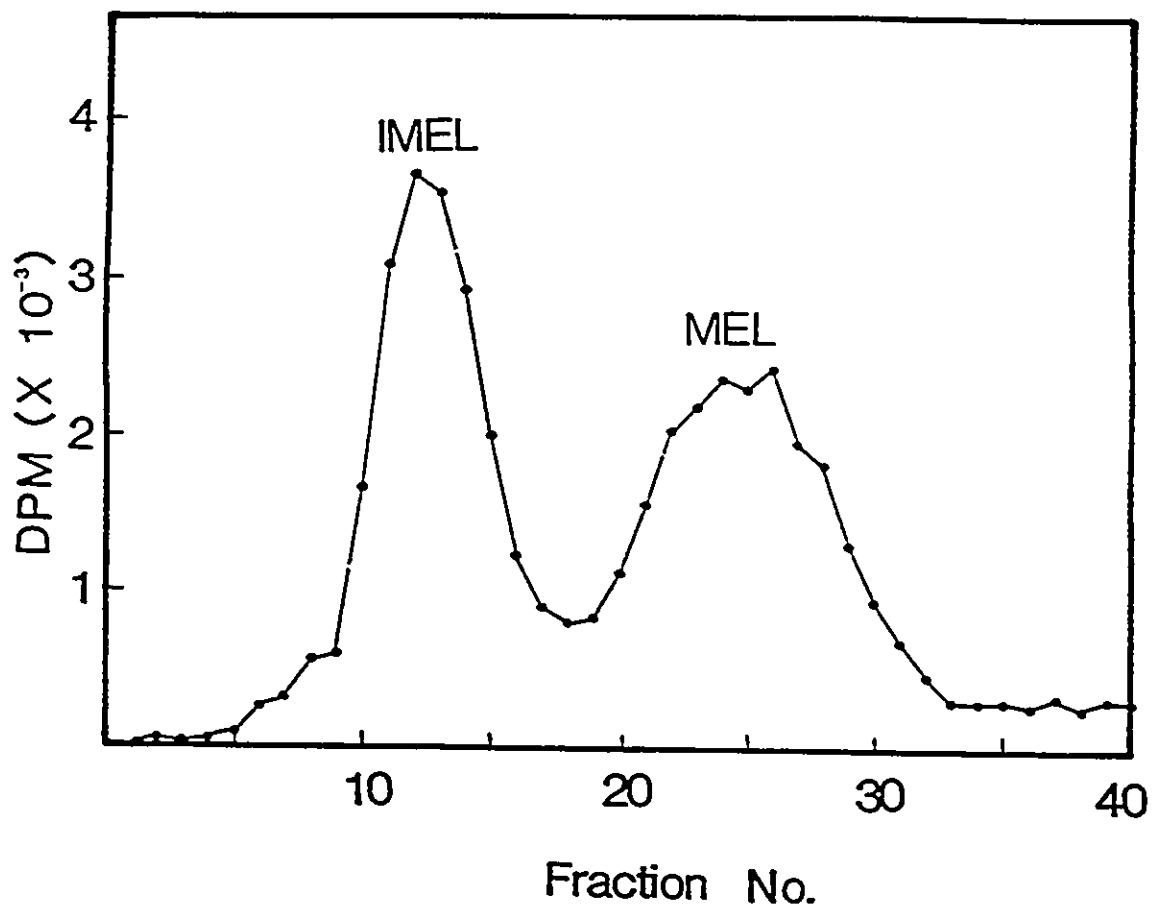
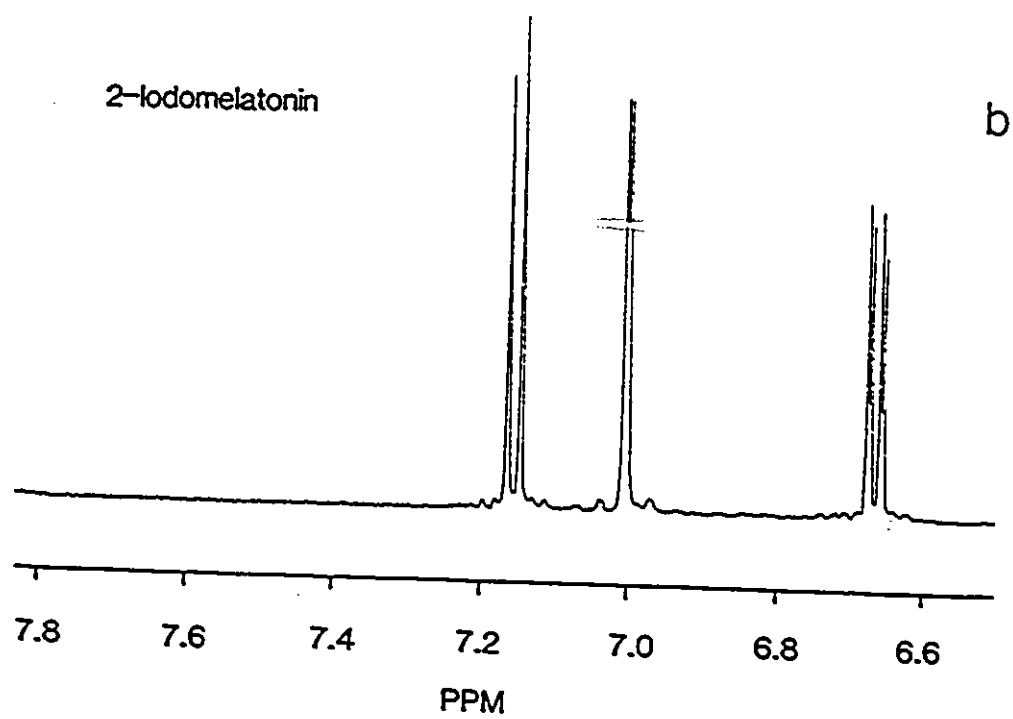
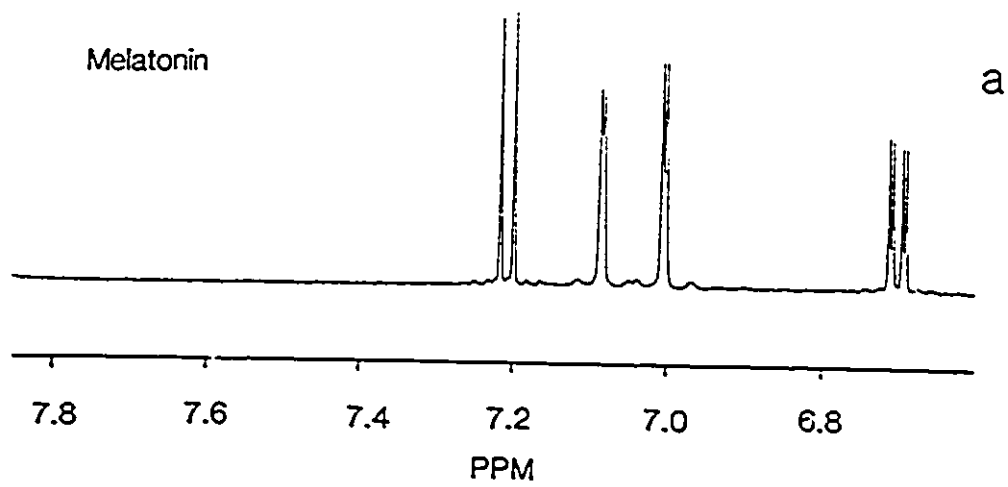


Figure 4. ^1H -NMR spectra for (a) melatonin and (b) 2-iodomelatonin in deuterated DMSO, recorded at 500 MHz with tetramethylsilane added as the internal chemical shift reference.

^{13}C -NMR spin sort spectra (natural abundance) for melatonin (c) and 2-iodomelatonin (d), recorded at 125 MHz in deuterated DMSO with -CH and -CH₃ down, -C- and -CH₂ up.



¹H-NMR (500 MHz)

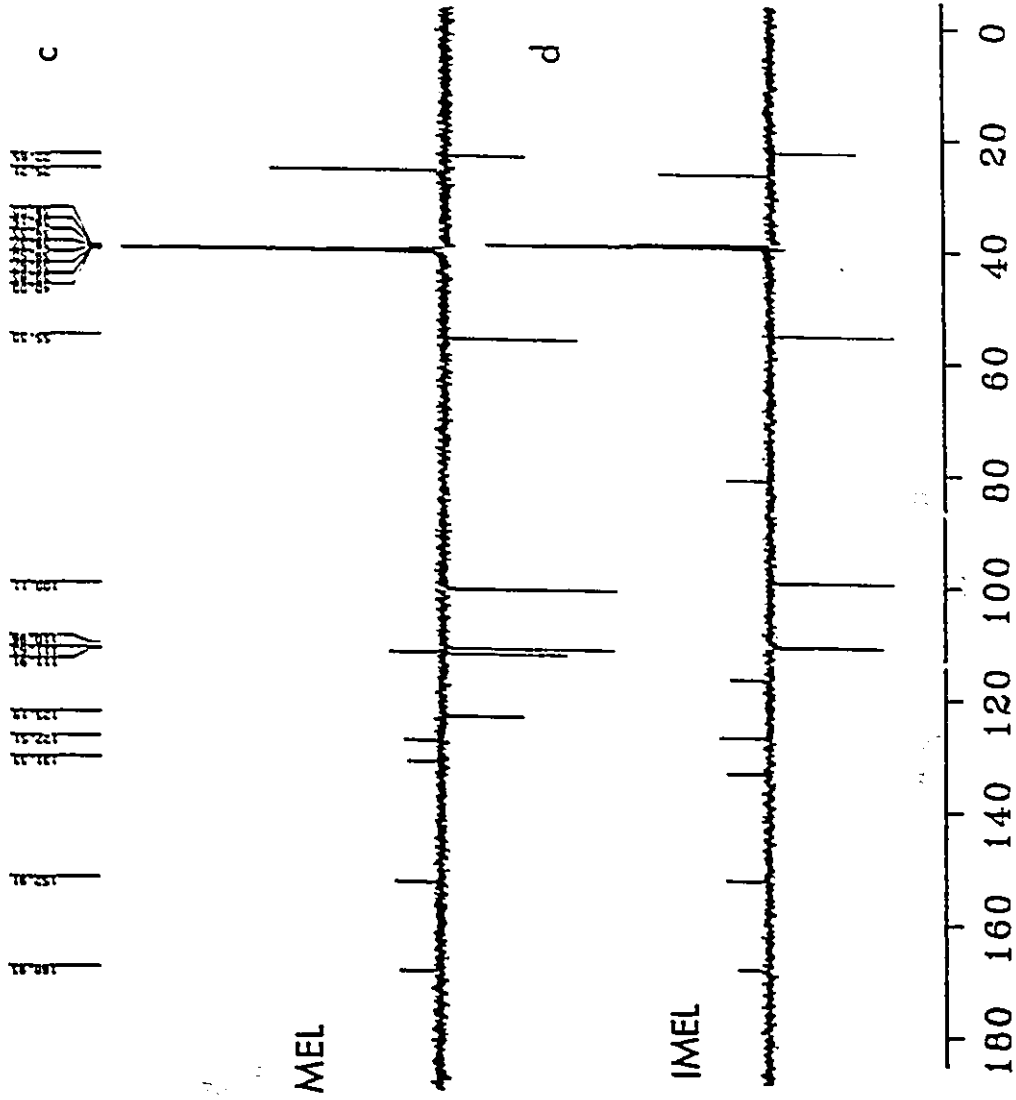


Figure 5. TLC chromatogram of 2-[¹²⁵I]iodomelatonin developed with ethyl acetate. Sections (5 mm) of the plastic-backed silica plate were cut and radioactivity measured with a γ -counter at 71% counting efficiency. R_f values of [¹²⁵I]MEL and melatonin (MEL) are indicated.

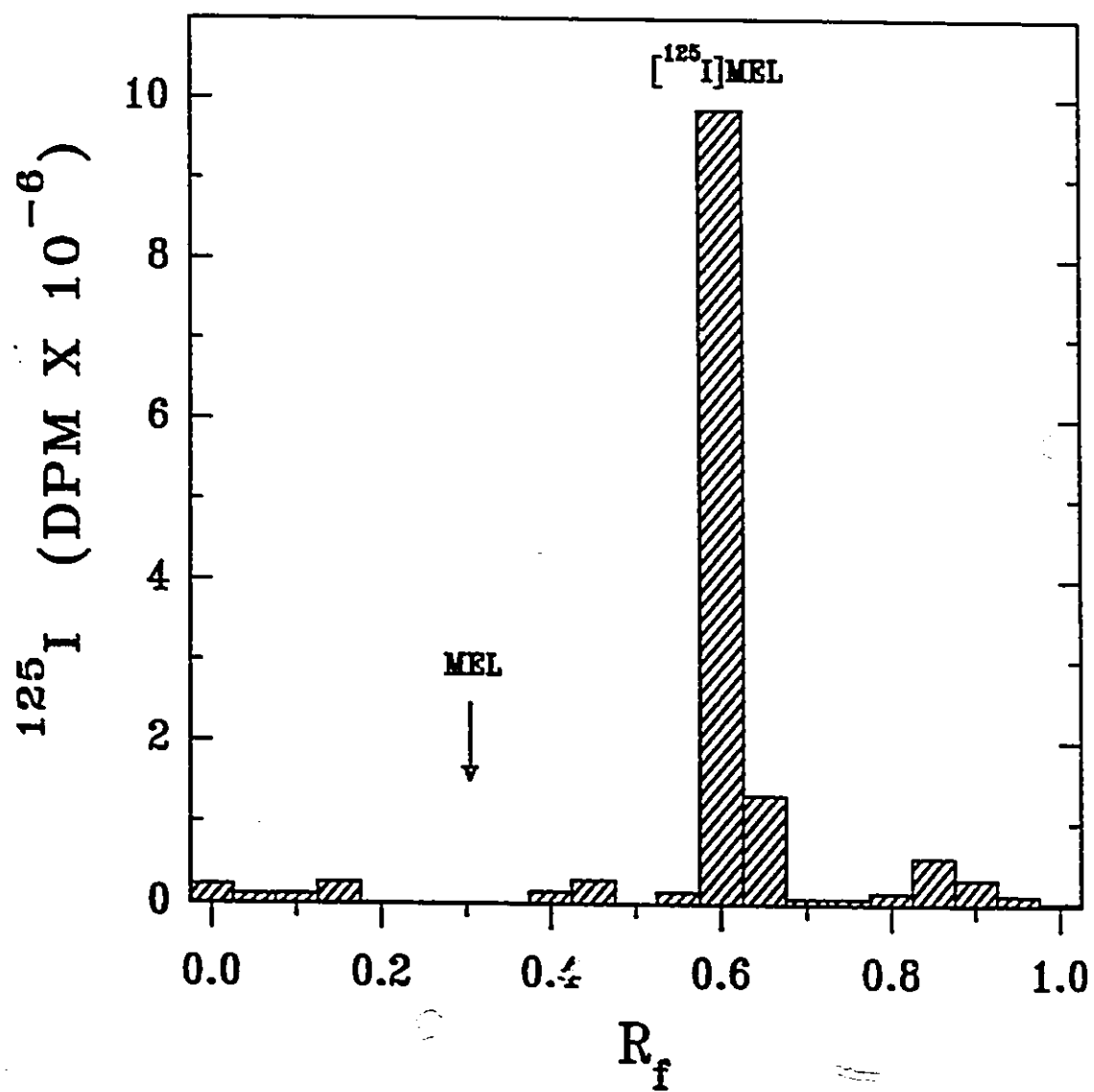


Figure 6. HPLC chromatogram of iodomelatonin purification. Melatonin (20 μ g) and iodomelatonin (20 μ g) were co-injected onto a 250 x 4.6 mm Alltech Econosphere ODS (5 μ m) column and eluted with 1 mL/min of 50% methanol-water. Absorbance at 280 nm was monitored (2 AUF). MEL = melatonin (R_t = 5.5 min), IMEL = iodomelatonin (R_t = 9.8 min). Scale bar = 1 min.

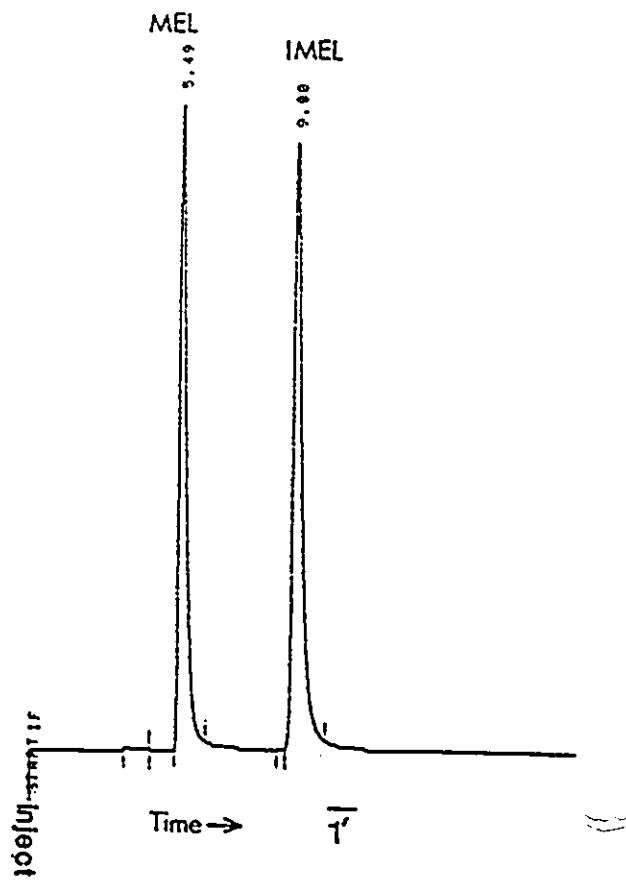
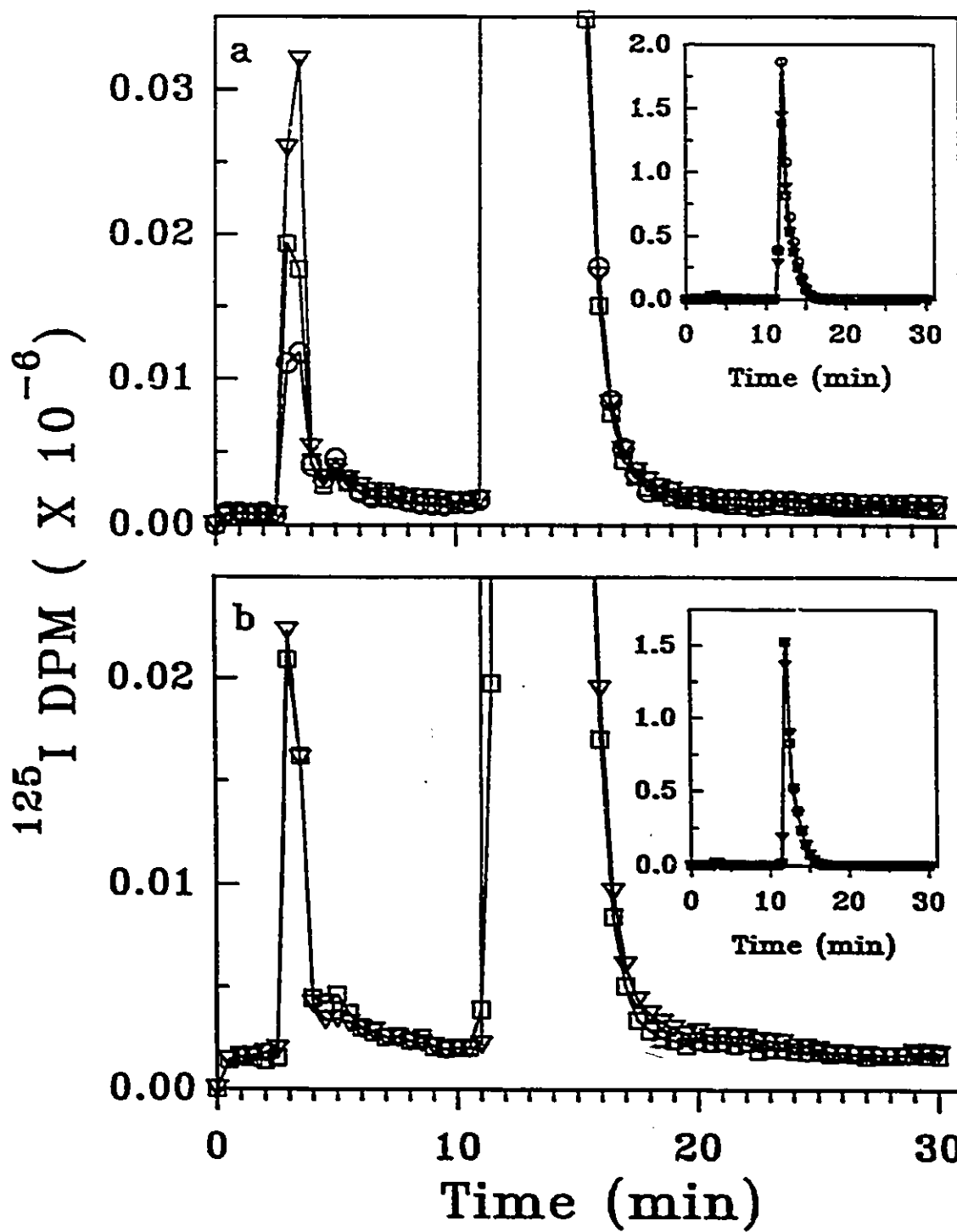


Figure 7. [¹²⁵I]MEL stability under assay conditions. Fresh hamster cortical membranes (whole homogenate) were prepared in assay buffer as described in Methods and incubated for 1 h at either 0°C or 37°C in the presence of 10.8 nM [¹²⁵I]MEL (1915 Ci/mmol). The tissue was then centrifuged and the pellet extracted with methanol. Aliquots of the supernatant (a) and extract (b) were then analysed by HPLC on a 250 x 4 mm LiChrosorb ODS column as described in Methods. Fractions (0.5 mL = 0.5 min) were collected and counted for radioactivity. ○, control (no tissue); □, 0°C incubation; ▼, 37°C incubation. *Insets: Full scale.*



2.3.2 Radioligand Stability

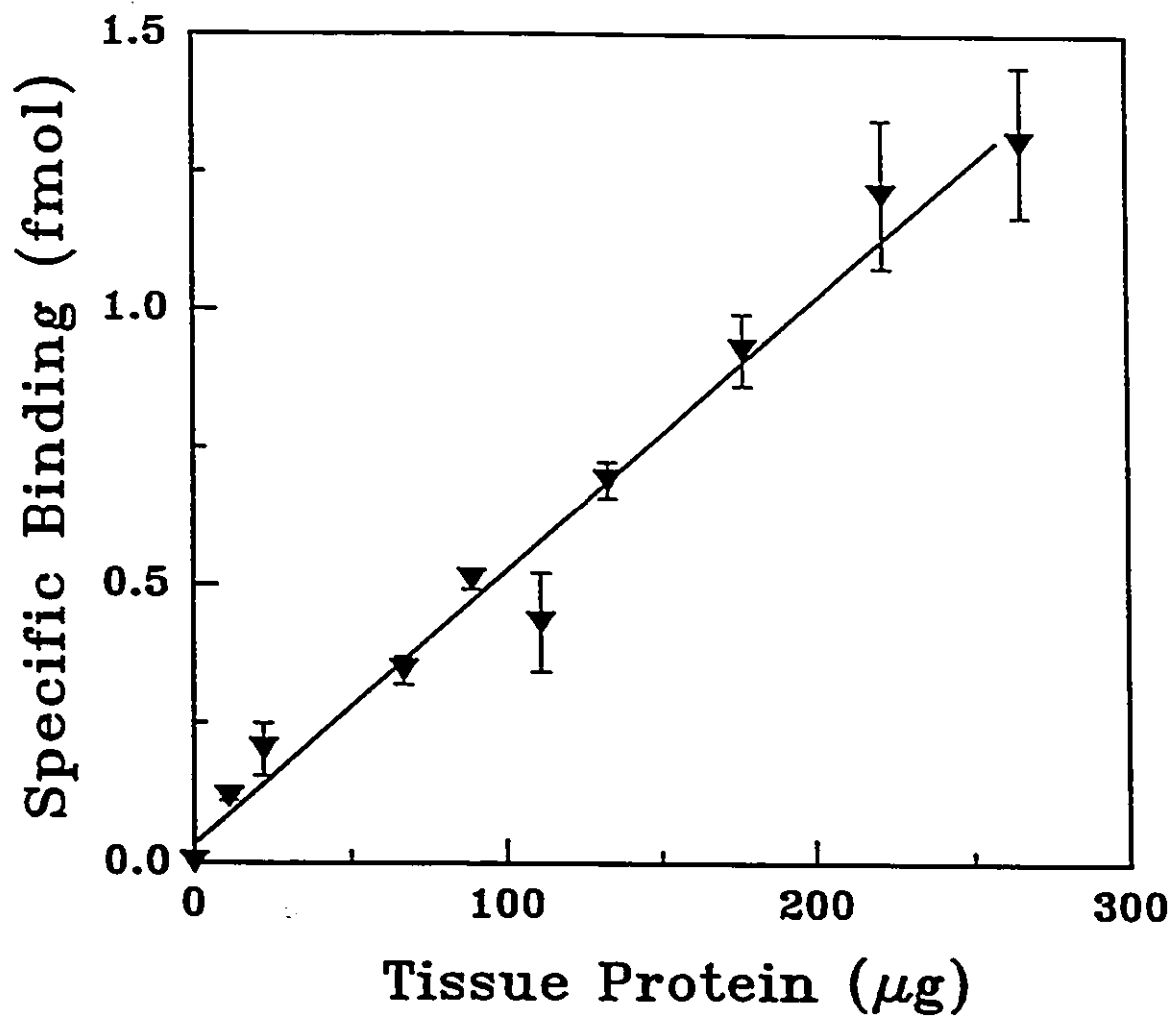
[¹²⁵I]MEL stored in methanol:water + 6-mercaptoethanol at -20°C (or -70°C) retained its binding properties for at least 3 months. Both the TLC purified and HPLC-purified labels exhibited similar binding characteristics. However, while the yield by TLC was about 15- 30%, purification by HPLC significantly enhanced the yield to about 30- 60%. An examination of the radioligand chemical stability under normal assay conditions was conducted. Incubation of the radiolabel (10.8 nM) with hamster cortical membranes (whole homogenate) for 1 h at 37°C indicated only a very minor degree of ligand degradation (= 1.2%) [Fig. 7].

Adsorption of [¹²⁵I]MEL to glass test tubes was evaluated at 0°C and 23°C (room temperature). [¹²⁵I]MEL was redissolved in 50 mM Tris-HCl pH 7.4, after evaporation under N₂, to give concentrations of 10.7, 1.07 and 0.107 nM. No change in the radiolabel concentration was noticed over a 3 h incubation period at either temperature at any of the three concentrations (data not shown).

2.3.3 Assay Optimisation

Inclusion of ascorbic acid (as anti-oxidant), pargyline (an MAO inhibitor) or EDTA (a multivalent-cation chelator and protease inhibitor) in the assay buffer did not affect [¹²⁵I]MEL binding. Therefore, these additives were not routinely used. Optimisation of the filtration assay included testing several different washing protocols. Presoaking the glass fibre filters in 50 mM Tris-HCl containing 0.1% BSA or 0.5% PEI or 10 µM idomelatonin or 0.1% BSA + 10 µM idomelatonin or 0.5% PEI + 10 µM idomelatonin did not alter the blank values. Washing volumes from 12- 20 mL of assay buffer were tried and 14 mL (4 x 3.5 mL) found to give maximal specific binding using hamster forebrain P2

Figure 8. Tissue linearity curve. Hamster cortical P2 membranes were prepared and incubated with 0.20 nM [125 I]MEL (2070 Ci/nmol) as detailed in Methods. Means \pm S.D. from one experiment are shown. Similar results were obtained using forebrain P2 membranes and hypothalamic whole homogenate membranes. $r = 0.99$



membranes. Specific binding of [125 I]MEL in brain tissue membrane homogenates was linear up to $\approx 250 \mu\text{g}$ protein per assay tube (Fig. 8). A centrifugation assay was tested in pilot experiments using hamster forebrain P2 membranes, however the trapping of label within the tissue pellet was extensive and little or no specific binding could be discerned so that filtration assays were exclusively employed for binding experiments. Binding assays at elevated temperatures (22-37°C) were unsuccessful due to high nonspecific and erratic binding.

2.3.4 Characterisation of [125 I]MEL Binding

Subcellular fractionation of brain tissue membranes indicated specific [125 I]MEL binding (using single point assays) in all fractions tested with the highest amount of binding in the P3 fraction (Table 1). A pH profile indicated specific binding to be stable in the pH 5 - 8 range in hamster brain tissues (Fig. 9). [125 I]MEL specific binding in hamster brain tissues was saturable (Fig. 10), reversible (Fig. 14), heat sensitive (98°C, 10 min) and trypsin sensitive (0.16% at 37°C, 1 h). Regional studies revealed the presence of highest binding in the hypothalamus (Table 2). A saturation isotherm of binding as a function of radioligand concentration in hypothalamic tissue is presented in Figure 10. Specific binding was saturable, with half-maximal binding occurring at about 1 nM [125 I]MEL. Nonspecific binding increased linearly with radioligand concentration as shown in the inset. Scatchard-Rosenthal transformation of saturation binding data indicated a single, high affinity site in hamster hypothalamus: $K_d = 1.8 \pm 0.3 \text{ nM}$, $B_{\text{max}} = 75 \pm 7 \text{ fmol/mg protein}$, ($n = 4$); and hippocampus: $K_d = 2.2 \pm 0.2 \text{ nM}$, $B_{\text{max}} = 49 \pm 5 \text{ fmol/mg protein}$, ($n = 4$) (Fig. 11). Multiple binding sites for [125 I]MEL were detected in the hamster cerebral

Table 1. Subcellular binding of 2-[¹²⁵I]iodomelatonin in hamster brain.

Specific Binding (fmol/mg protein/nM) ± S.D.				
Tissue	W.H. ^a	<u>Membrane Fraction</u>		
		P1	P2	P3
Whole Cortex ^b	23.1 ± 6.6	10.5 ± 0.30	16.7 ± 5.7	22.0 ± 0.50
Hypothalamus ^c	62.7 ± 8.0	21.8 ± 3.6	63.6 ± 1.3	83.0 ± 2.8

^a W.H. = whole homogenate membranes. ^b Means ± S.D. of three experiments conducted in triplicate. ^c Means ± S.D. of triplicate values from one experiment. Subcellular fractions were prepared and assayed as described in Methods. [¹²⁵I]MEL = 0.17- 0.27 nM.

Table 2. Regional binding of 2-[¹²⁵I]iodomelatonin in hamster brain.

<u>Tissue Homogenate</u>	<u>Specific Binding (fmol/mg protein)</u>
Hypothalamus	6.76 ± 0.24
Midbrain	5.11 ± 0.57
Cerebellum	4.34 ± 0.26
Occipital Cortex	3.90 ± 0.24
Hippocampus	3.89 ± 0.25
Pons/Medulla	3.87 ± 0.14
Frontal Cortex	3.32 ± 0.42
Striatum	3.31 ± 0.62
Spinal Cord	2.57 ± 0.42

Assays were carried out as detailed in the Materials and Methods.

Means ± S.E. from one experiment performed in quadruplicate using 0.18 nM [¹²⁵I]MEL are presented. This experiment was repeated twice with similar results.

Figure 9. pH profile of [125 I]MEL specific binding in freshly prepared hamster hypothalamic membranes (whole homogenate). Membranes were prepared and assayed in several different buffers on ice as described in Methods. Means \pm S.D. of triplicate values from one experiment are presented. Similar profiles were obtained with hippocampal and cortical membranes. [125 I]MEL] = 0.45 nM (1570 Ci/mmol). Buffers (each 50 mM): ∇ , sodium acetate; \circ , sodium phosphate; \square , Tris-HCl; \diamond , CHES; \triangle , CAPS.

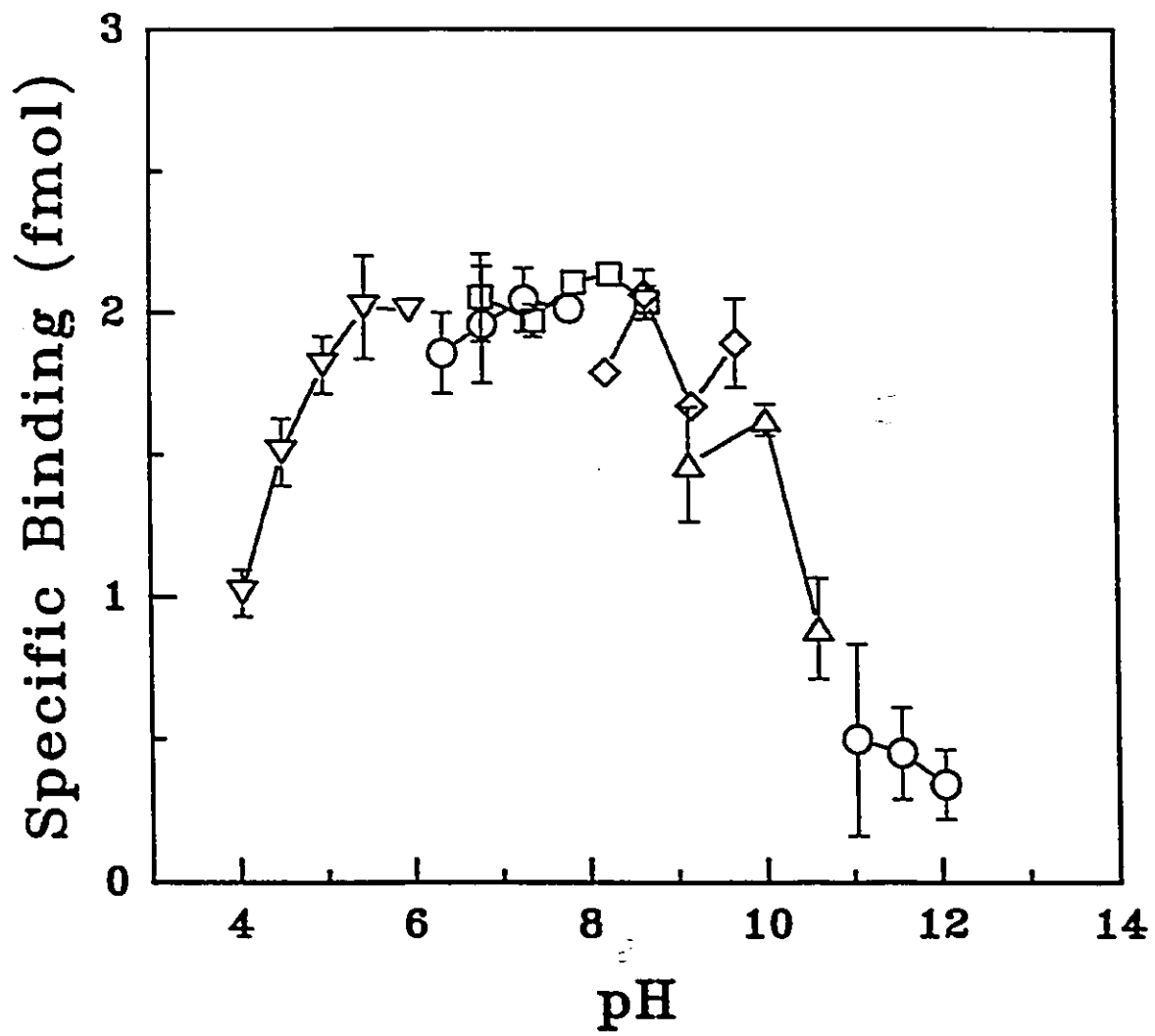


Figure 10. Saturation isotherm of specific [^{125}I]MEL binding in hamster hypothalamus, assayed as described in Methods. *Inset:* [^{125}I]MEL total (T), nonspecific (N) and specific binding (S) components in this tissue. Means of triplicate values from one experiment are shown. These data were used for Figure 11.

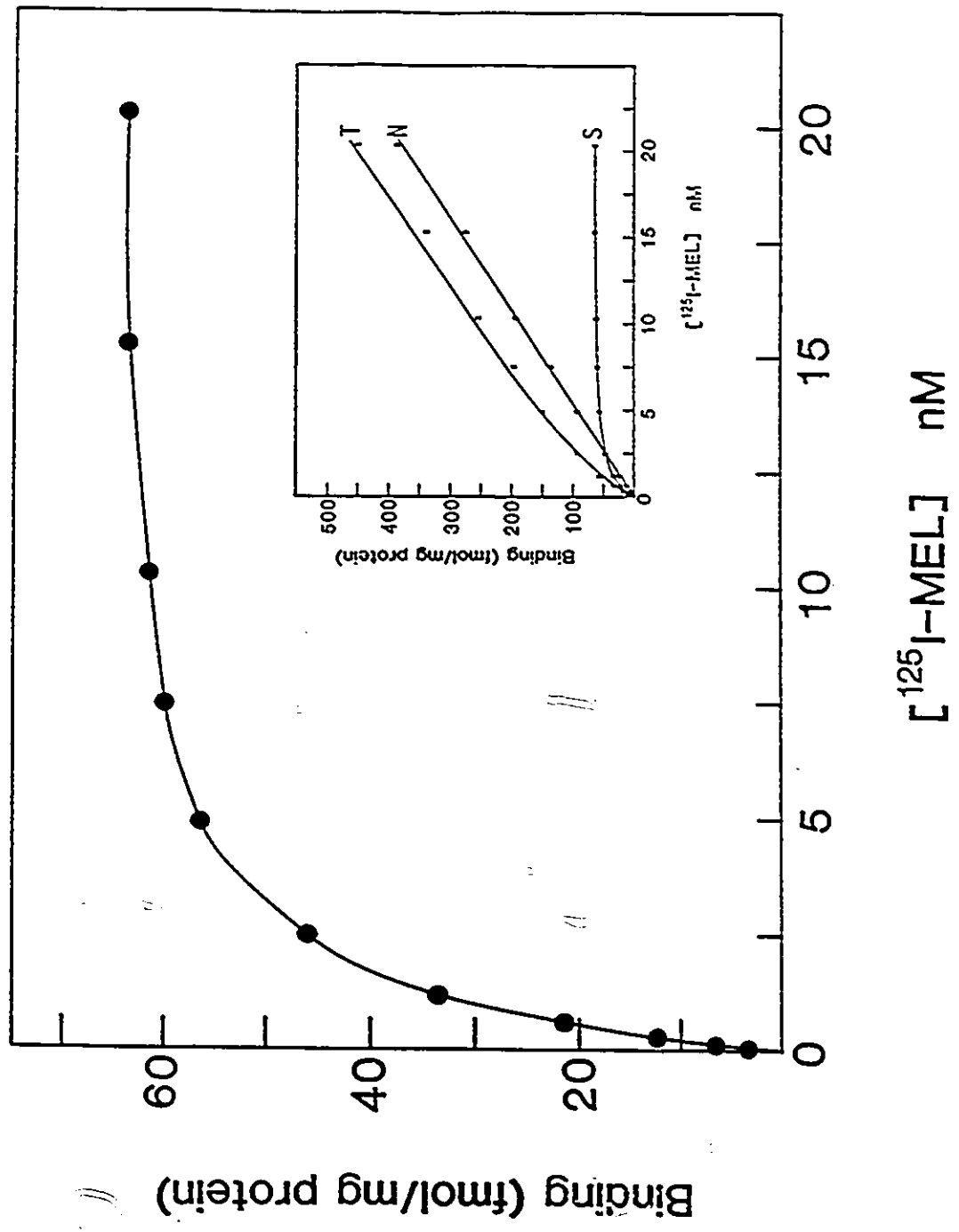


Figure 11. Scatchard-Rosenthal plots of saturation binding in hamster hypothalamus and hippocampus P2 membranes assayed as described in Methods. Means of triplicate values from one representative experiment, repeated thrice, are shown. [¹²⁵I]MEL was isotopically diluted to 600- 800 Ci/mmol and used in final concentrations of 0.070- 20 nM.

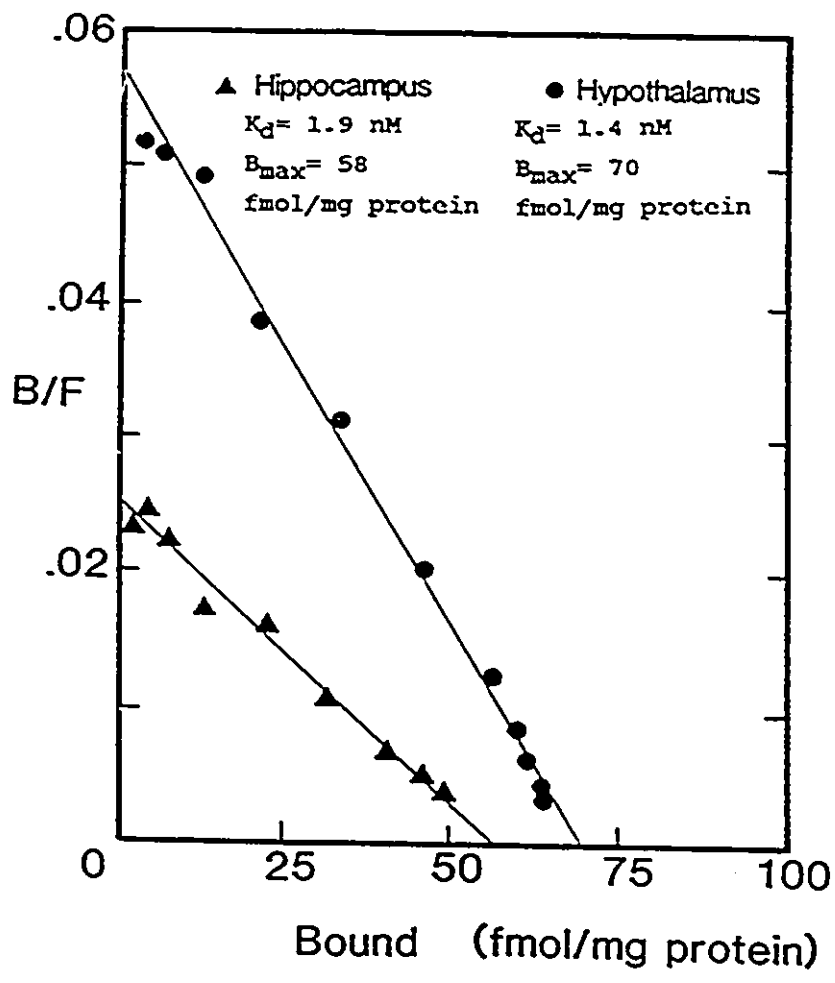


Figure 12. Scatchard-Rosenthal plot of specific [^{125}I]MEL binding in hamster cortical P2 membranes, prepared and assayed as described in Methods. Means of triplicate values from one experiment, repeated twice, are presented. [^{125}I]MEL was isotopically diluted to 530 Ci/mmol and used in a range from 0.147 - 30.2 nM. Protein = 45 $\mu\text{g}/\text{tube}$. *Dashed lines*: Individual high- and low-affinity binding components, resolved by non-linear regression of the binding data and plotted using the Scatchard-Rosenthal axes.

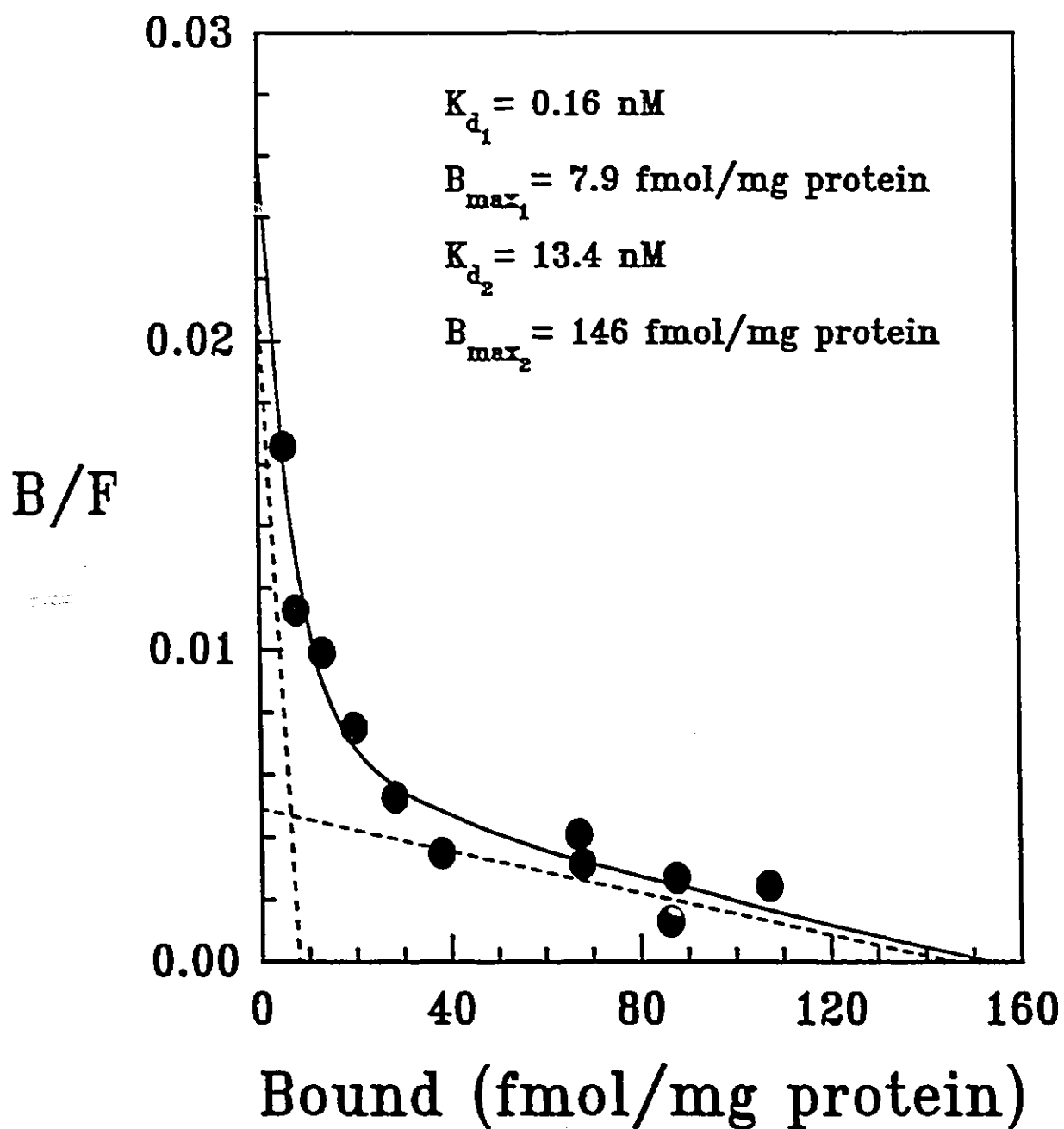
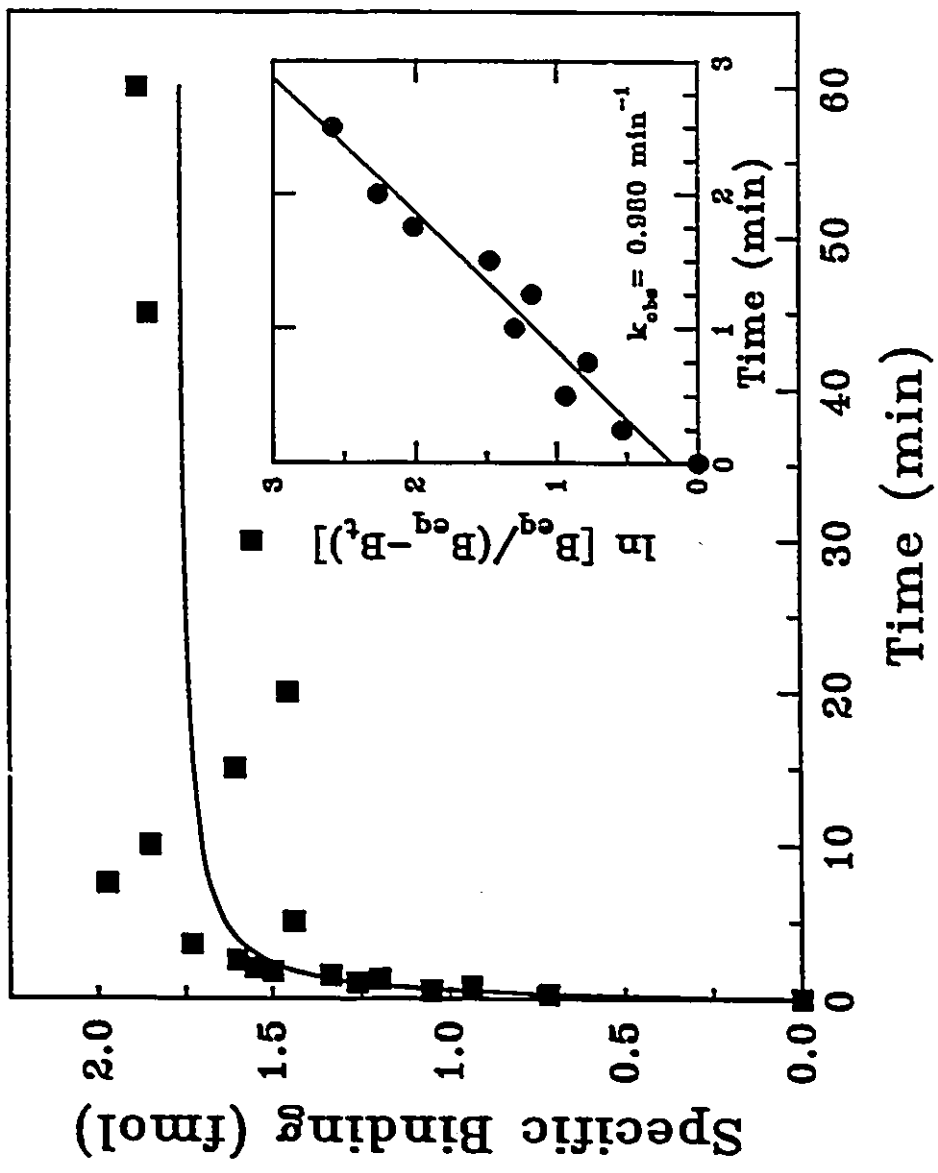
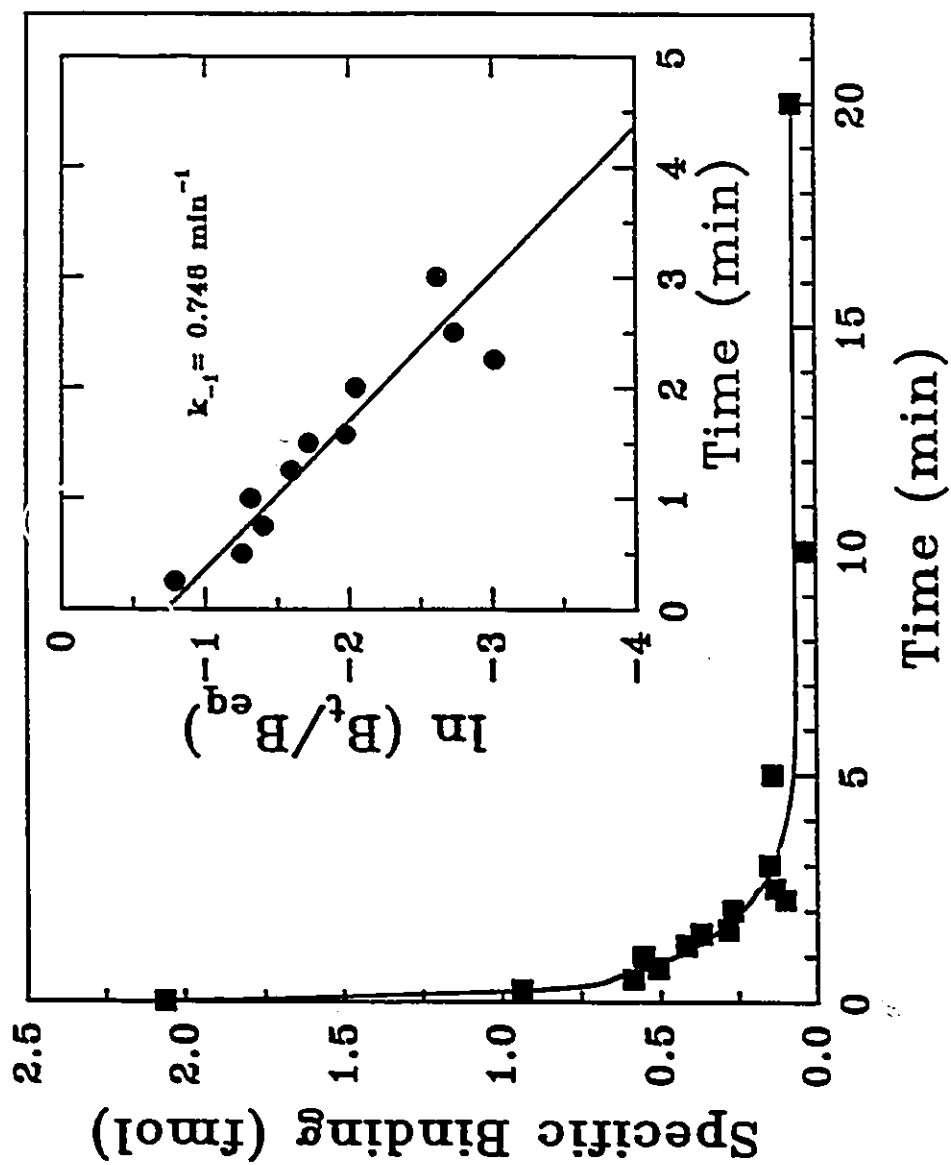


Figure 13. Association kinetics of [^{125}I]MEL specific binding in hamster hypothalamic membranes (whole homogenate). Membranes were prepared and assayed as described in Methods. Protein = 117 μg /assay tube. [^{125}I]MEL] = 0.52 nM, (1500 Ci/mmol). *Inset*: log transformation of association data. B_{eq} = specific binding at equilibrium, B_t = specific binding at time (t), $r = 0.98$, $k_{\text{obs}} = 0.980 \text{ min}^{-1}$.



0.8

Figure 14. Dissociation kinetics of [125 I]MEL specific binding in hamster hypothalamic membranes (whole homogenate). Membranes were prepared and assayed as described in Methods. Protein = 70 μ g/assay tube. [125 I]MEL] = 0.52 nM, (2000 Ci/mmol). *Inset*: log transformation of dissociation data. B_{eq} = specific binding at equilibrium, B_t = specific binding at time (t), $r = 0.93$, $k_{-1} = 0.748 \text{ min}^{-1}$.



cortex by Scatchard-Rosenthal transformation of saturation binding data (Fig. 12). Non-linear regression analysis of the binding data yielded: $K_d = 0.32 \pm 0.14$ nM, $B_{max} = 5.6 \pm 1.7$ fmol/mg protein; $K_d = 10.5 \pm 3.2$ nM, $B_{max} = 123 \pm 33$ fmol/mg protein, ($n = 3$). Rapid association (Fig. 13) and dissociation (Fig. 14) rates were observed in the hypothalamus ($k_1 = 4.46 \times 10^8 \text{ min}^{-1}\text{M}^{-1}$, $k_{-1} = 0.748 \text{ min}^{-1}$, $t_{1/2} = 56 \text{ s}$). Calculation of K_d from these gives a value of 1.7 nM, which is similar to that obtained in equilibrium studies.

The influence of several cations and guanine nucleotides upon specific binding of [^{125}I]MEL at 0°C in hypothalamus whole homogenate was investigated. NaCl (1- 200 mM) and KCl (1- 100 mM) were without effect. CaCl_2 (4 mM) enhanced binding by $\approx 36\%$ while MgCl_2 and MnCl_2 (0.02 - 16 mM) did not significantly affect binding. GTP and GppNHp also did not affect binding at concentrations up to 1 mM.

2.3.5 [^{125}I]MEL Binding Pharmacology

Several neurotransmitters and other drugs, including: serotonin, norepinephrine, epinephrine, dopamine, taurine, diazepam, verapamil, acetylcholine, histamine, γ -aminobutyric acid, glutamate, glycine, etc., all exhibited very poor binding affinity for the nanomolar-affinity site (Table 3). In contrast to the relatively high affinity exhibited by prazosin, several other α -adrenergic drugs were ineffective or weak inhibitors of binding. Competition curves of displacement of [^{125}I]MEL binding in hamster hypothalamic membranes are shown for six of the more potent indoles tested (Fig. 15). A detailed iodomelatonin displacement curve in hamster cortical membranes suggested the presence of three binding sites of IC_{50} values 43 pM, 4.3 nM and 3.4 μM in relative ratios of 2.5:5:1, respectively (Fig. 16).

Table 3. The regional pharmacology of 2-[¹²⁵I]iodomelatonin sites in hamster brain.

	K _i ³ (nM)			
	Hypothalamus ^b		Hippocampus ^c	Cerebral Cortex ^c
<u>Indoles</u> ^d		n _H		
2-iodomelatonin	1.5 ± 0.8	0.7	1.1 ± 0.7	0.30
6-chloromelatonin	6.9 ± 3.9	1.1	18 ± 1	6
N-acetylserotonin	25	1.4	67	63
Ru 24969	40 ± 9	0.9	30	100
N-(2'-diazo-3',3',3'-tri- fluoropropionyl)-5-MT	59 ± 19		74	-
N-trifluoroacetyl-5-MT	60 ± 31		-	-
melatonin	61 ± 16	1.3	64 ± 3	44
O-acetyl-N-acetylserotonin	88	1.0	91	51
N-acetyl-6-methoxytryptamine	106	1.1	130	86
6-methylmelatonin	194		201	60
N-(6-[4'-azido-2'-nitrophenyl- amino]hexanoyl)-5-MT	255 ± 84		-	-
N-t-BOC-5-MT	265		-	-
N-isobutyryl-5-MT	335 ± 54		380	-
N-(5-azido-2-nitrobenzoyl)-5-MT	341 ± 135		-	-
6-hydroxymelatonin	360	0.7	150	140
N-n-propionyl-5-MT	370		-	-
N-(4-azido-2-nitrophenyl)-5-MT	395 ± 54		-	-
5-methoxytryptophol	550	0.9	720	420
O-isobutyryl-N-acetylserotonin	616 ± 200		450	-
N-n-butyryl-5-MT	730		-	-
5-hydroxytryptophol	770	1.0	620	650
5-methoxyindole	850		-	-
4-methoxyindole	1300		-	-
N,N-dimethyl-5-methoxy- tryptamine	1500		3100	1600
5-methoxytryptamine	2000	1.0	3600	1400
N-acetyltryptamine	2600	1.5	1300	650
6-methoxyindole	3300		-	-
7-methoxyindole	3500		-	-

Table 3 - continued

	K_i^a (nM)			
	Hypothalamus ^b		Hippocampus ^c	Cerebral Cortex ^c
		n_H		
6-methoxytryptamine	3900	0.7	4800	9000
N,N-dimethylserotonin	4400		7900	11000
dihydroergotamine	5700		-	-
serotonin	9300 ± 4000	1.0	19000 ± 2300	19000
tryptophol	10000	0.8	10000	6000
indoramin	12000	0.5	11000	900
5-methoxyindoleacetic acid	25000		-	-
1-[(p-carboxy)benzyl]melatonin	32000 ± 990		-	-
N-methyltryptamine	42000	0.6	53000	5000
tryptamine	59000	0.6	76000	2000
5,6-dihydroxytryptamine	83000		-	-
<u>Non-idoles</u>				
prazosin	8.9 ± 1.3	0.7	9.3 ± 1.1	6.4 ± 0.9
N-acetyl-5-methoxykynurenamine	185	0.7	440	-
6-CCE	240	0.6	310	400
spiperone	670	0.7	580	2400
6-methoxy-2-benzoxazolinone	900	1.2	800	-
amiloride	-		1500	-
meteryoline	1900	0.5	2800	3100
WB-4101	2900	1.4	2400	2400
ketanserin	3200	1.3	6200	3400
methysergide	3600	0.9	13000	12000
L-propranolol	4400	0.7	6800	12000
chlorpromazine	5800	1.3	16000	16000
DOI	5900	0.6	7000	-
diazepam	13000	1.0	6400	3400
NPP	13000		>100000	-
theophylline	19000		18000	31000
alprenolol	37000		-	-
norepinephrine	38000		>100000	>100000

Table 3 - continued

	K_i^a (nM)		
	Hypothalamus ^b	Hippocampus ^c	Cerebral Cortex ^c
apomorphine	42000	-	-
yohimbine	68000	>100000	>100000
phentolamine	70000	>100000	>100000
mianserin	74000	>100000	>100000
quipazine	80000	-	-

Compounds with IC_{50} values > 100,000

mCPP, dopamine, epinephrine, clonidine, GABA, taurine, histamine, acetylcholine, glutamic acid, glycine, haloperidol, TDCB, (\pm)-8-OH-DPAT, ipsapirone, cyproheptadine, verapamil, diltiazem, methoxyverapamil, 5,7-dihydroxytryptamine, N-n-myristyl-5-MT, 5-hydroxyindoleacetic acid, N-acetyltryptophan, 5-methoxytryptophan, naloxone, bicuculline, picrotoxinin, morphine sulphate, pentobarbital, kynuramine, Ro 15-1788, 3-methoxytyramine, D,L-metanephrine, D,L-normetanephrine and D,L-isoproterenol.

Means \pm S.E. for 2- 4 experiments done in triplicate are presented.

^a K_i was calculated from the IC_{50} value (Cheng and Prusoff, 1973). Hill

coefficient (n_H) values for hypothalamus represent the slopes of Hill plots.

^b Whole tissue homogenate ^c Synaptosomal (P2) membranes.

^d See Appendix II for chemical structures.

Figure 15. Competition experiments of [125 I]MEL binding in hamster hypothalamic membranes (whole homogenate) assayed as described in Methods. Means of triplicate values from one experiment are shown. [125 I]MEL was used in final concentrations of 0.13- 0.37 nM. ○, icdomelatonin; □, melatonin; ▽, 6-chloromelatonin; ◇, Ru 24969; ▲, prazosin; ●, O-acetyl-N-acetylserotonin.

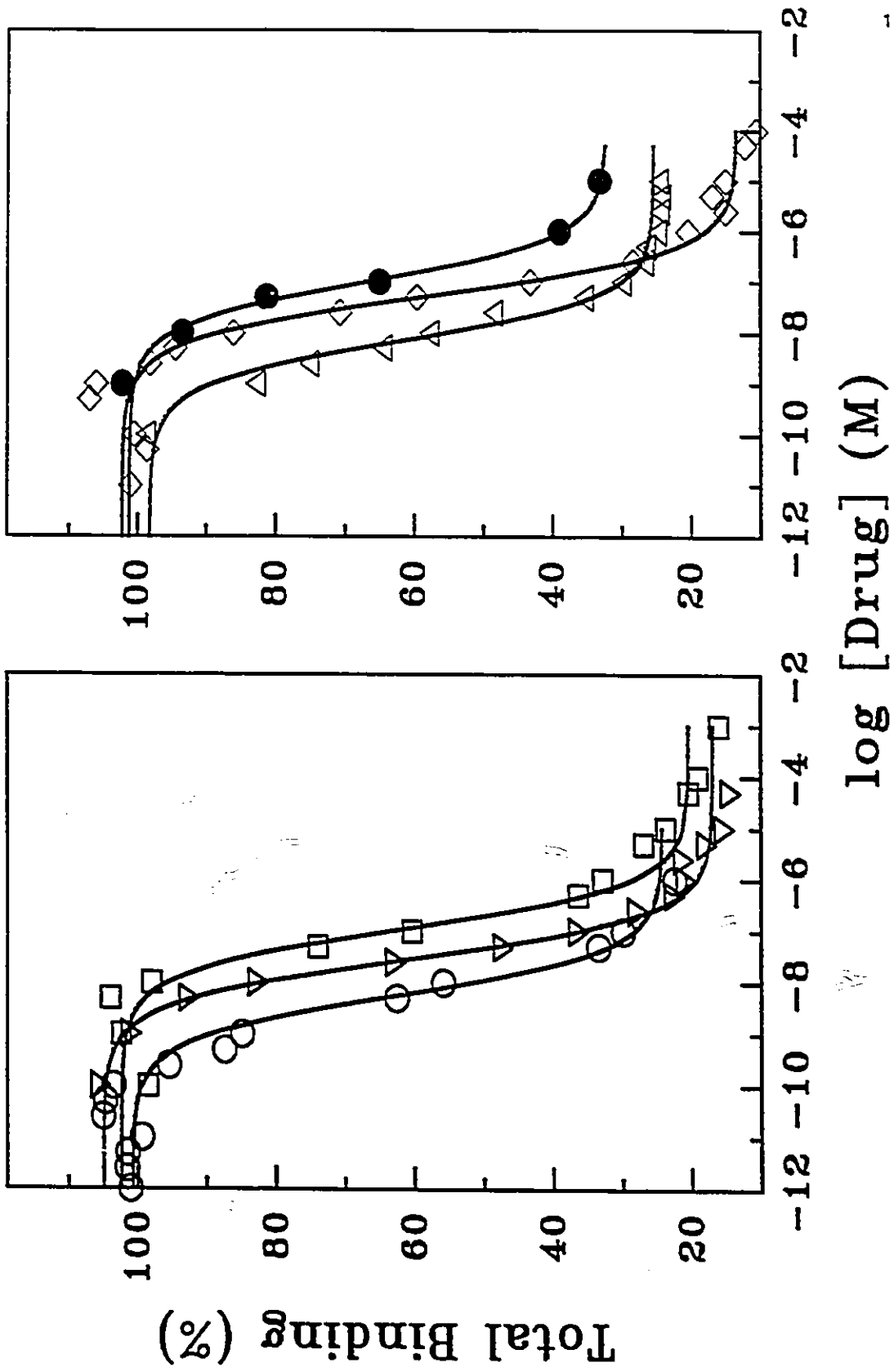


Figure 16. Iodomelatonin competition of [¹²⁵I]MEL binding in hamster cortical membranes (whole homogenate), prepared and assayed as described in Methods. Means of triplicate values from one experiment are presented. [¹²⁵I]MEL = 0.57 nM (914 Ci/mmol). Protein = 194 μg/tube. Three sites were found by nonlinear analysis: IC₅₀ = 43 pM, IC₅₀ = 4.3 nM, IC₅₀ = 3.4 μM, in a ratio of 2.5:5:1. Using the F test, a three site fit of the data was significantly better than a two (F = 9.36) or one (F = 10.66) site fit at p = 0.01.

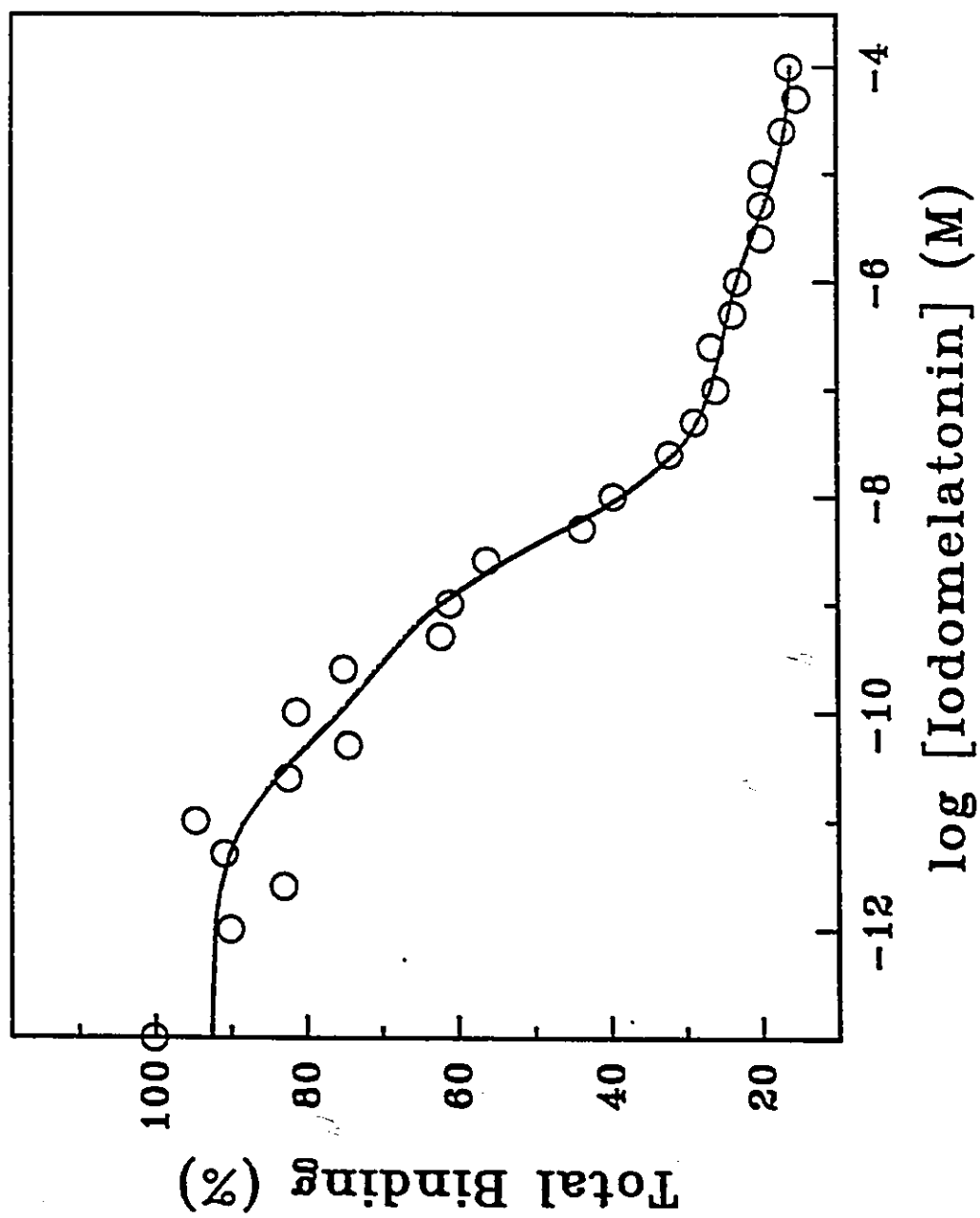


Figure 17. Correlation of the pharmacological profiles of 20 indoles
in cortex vs hypothalamus ($r = 0.96$, $m = 0.98$, $p < 0.01$),
hippocampus vs hypothalamus ($r = 0.98$, $m = 1.01$, $p < 0.01$),
cortex vs hippocampus ($r = 0.97$, $m = 0.98$, $p < 0.01$). Data from
Table 3.

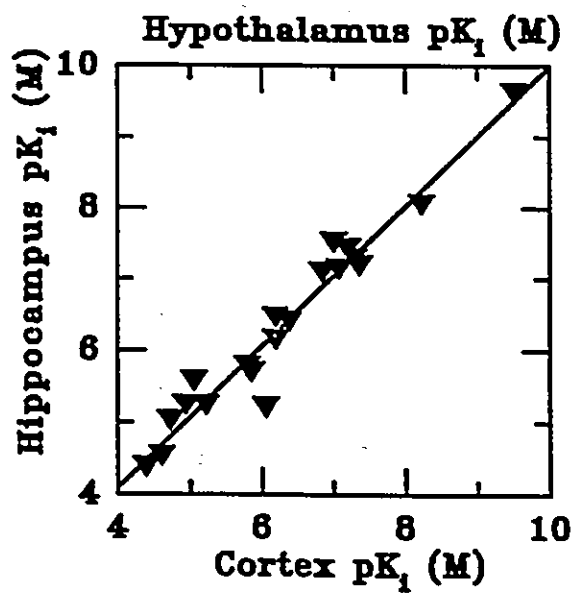
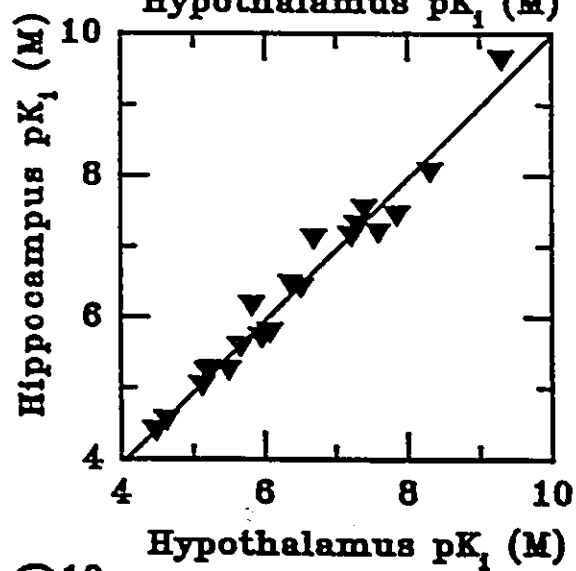
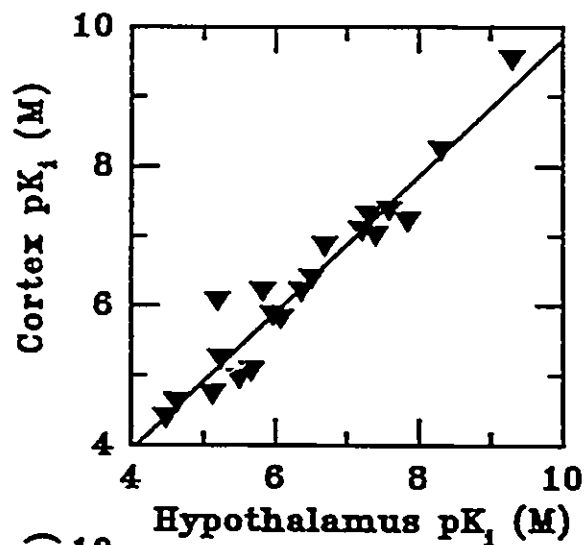
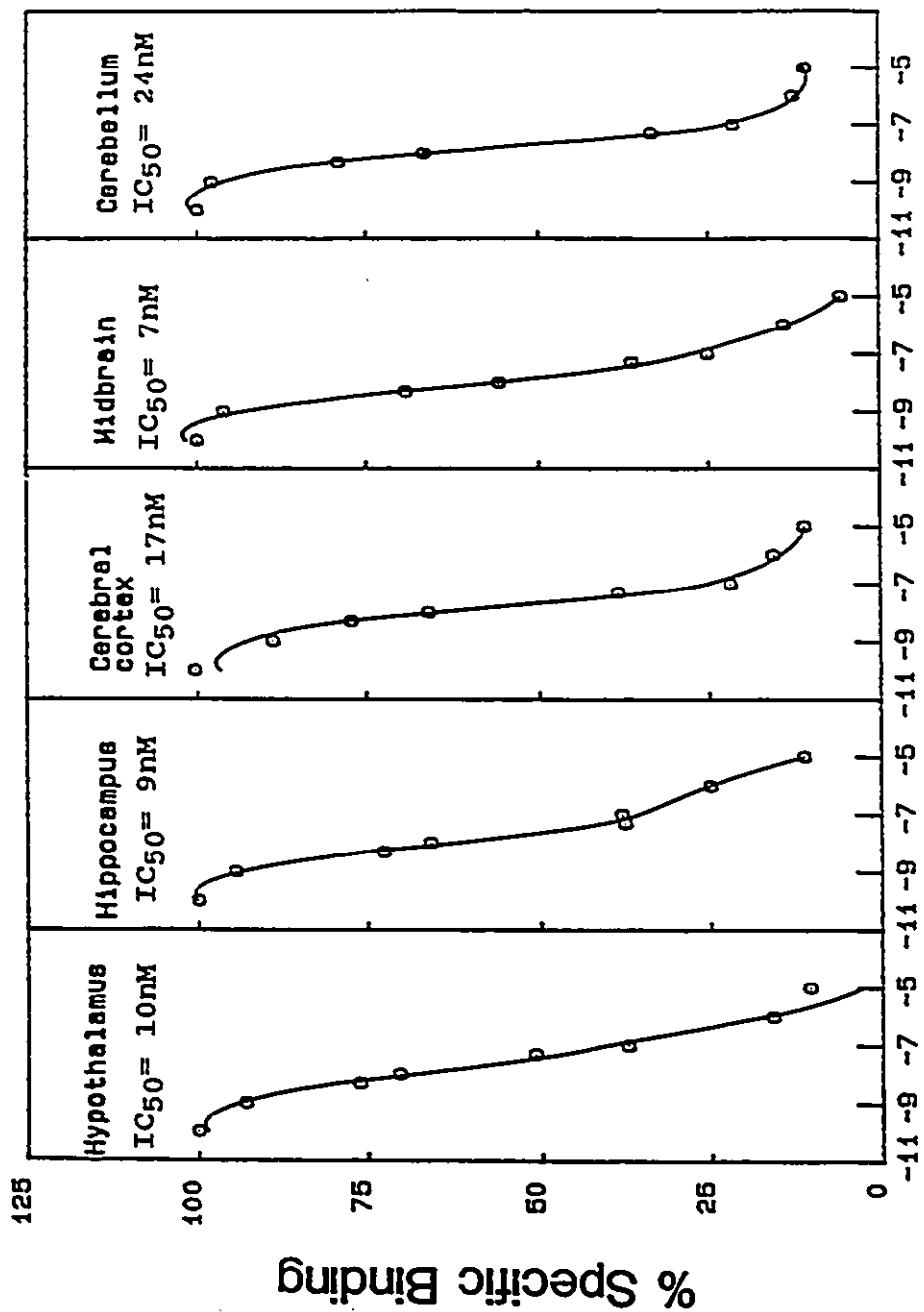


Figure 18. Competition curves of prazosin displacement of [^{125}I]MEL binding in several hamster brain regions. Whole tissue homogenates were prepared and assayed in 50 mM Tris-HCl, 0.1% ascorbic acid, 10 μM pargyline pH 7.4 (4°C) as described in Methods. [^{125}I]MEL = 0.16 nM. K_i values are presented in Table 4.



log [Prazosin]

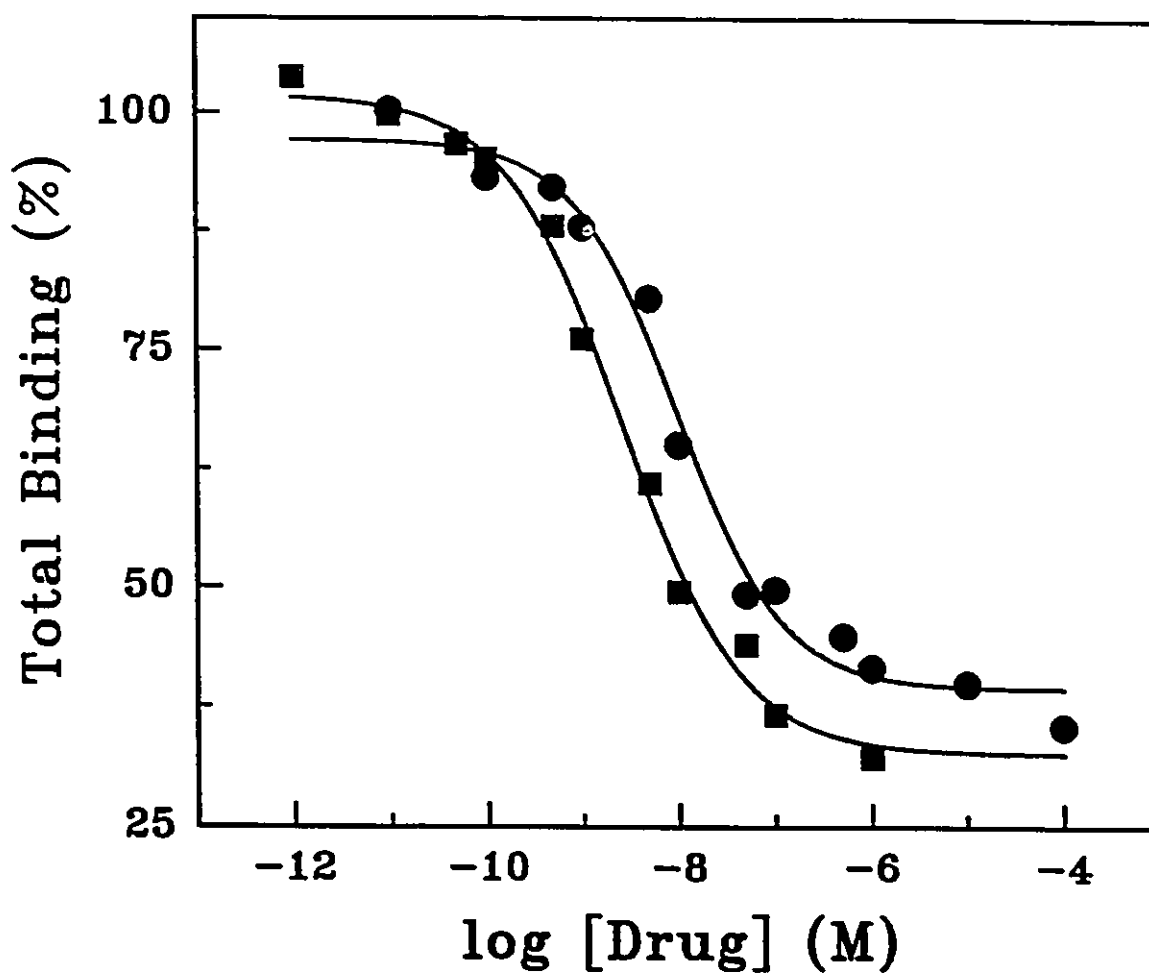
0

Table 4. Comparative Regional Prazosin Affinities

Tissue	K _i (nM)
Cortex	6
Hypothalamus	9
Hippocampus	8
Cerebellum	22
Midbrain	6
Testes	12

Whole tissue homogenates were prepared and assayed as described in Methods. Results from one experiment conducted with brain tissues and one separate experiment using testes are presented. [¹²⁵I]MEL = 0.16 nM for brain regions, 0.34 nM for testes.

Figure 19. Competition of [125 I]MEL binding in hamster hippocampal membranes (whole homogenate), prepared as detailed in Methods. Membranes were incubated with 1 mM benextramine 1h on ice and washed thrice with assay buffer before use. Means of triplicate values from one experiment are shown. [125 I]MEL = 0.25 nM (1434 Ci/mmol). ●, prazosin (K_i = 7.3 nM); ■, idomelatonin (K_i = 1.1 nM).



The pharmacological profile of binding in the hypothalamus was found to be similar to that of the hippocampus and the cerebral cortex (Table 3). Comparisons of the affinities of a series of indoles (Table 3) for binding sites in the various brain regions yielded correlation coefficients of 0.99 (hypothalamus vs. hippocampus), 0.96 (hypothalamus vs. cortex) and 0.97 (hippocampus vs. cortex) using linear regression (Fig. 17). The drug prazosin potently inhibited [125 I]MEL binding in the hypothalamus, hippocampus and cerebral cortex as shown in Table 3, and also in the midbrain and cerebellum (K_i 's = 6- 22 nM; Fig. 18, Table 4). In all the brain regions examined, prazosin produced shallow inhibition curves with a Hill slope ranging from 0.7 - 0.8.

Treatment of hamster cortical membranes with benextramine at either 0°C or 25°C caused a loss of 90- 95% of [3 H]prazosin specific binding while [125 I]MEL specific binding was not affected. Benextramine competition for [125 I]MEL binding sites indicated an $IC_{50} > 100 \mu\text{M}$. [125 I]MEL competition experiments carried out in brain tissue membranes which had been pre-treated with 1 mM benextramine and washed indicated affinities for prazosin and idomelatonin similar to the values obtained in untreated tissue (Fig. 19 vs Table 3).

2.4 Discussion

The low-affinity (nanomolar) binding site for [125 I]MEL examined here in the Syrian hamster CNS is assuredly distinct from the high-affinity (picomolar) melatonin receptor of the mammalian retina, SCN, area postrema and pars tuberalis (see Section 1.10) in terms of its radioligand binding properties. In fact, all aspects of the binding parameters were different: kinetics,

affinity, pharmacological profile, site distribution and lack of sodium ion and guanine nucleotide sensitivity.

After having initially validated the radioligand synthesis and having optimised the binding assay conditions for the low-affinity [^{125}I]MEL binding site, definition of specific binding was set as that amount of binding inhibited by the presence of 1 μM 6-chloromelatonin since competition experiments had indicated that the displacement of total binding begins to plateau at this concentration. A similar affinity for 6-chloromelatonin was reported by Duncan et al. (1988, 1989) in Syrian and Djungarian hamster brain membranes. Ideally, to define nonspecific binding, one should employ a compound which has a chemical structure as far removed from the radioligand as possible in order to minimise the possibility of displacement of nonspecific binding. Unfortunately, the only such compound available at the start of these studies was 6-chloromelatonin and its structure is very similar to that of melatonin and [^{125}I]MEL.

The affinity of 6-chloromelatonin is much higher at the picomolar-affinity melatonin receptor since complete displacement of specific binding is seen at a concentration of 10 nM in mammalian retina (Blazynski and Dubocovich, 1991) CNS (Stankov et al. 1991) or pars tuberalis (Carlson et al., 1989), this being another distinguishing point between the receptor and the low-affinity [^{125}I]MEL binding site.

Specific binding was observed to be heat and trypsin sensitive, suggesting binding to a protein site which is to be expected if binding is occurring to a receptor. Binding was stable over a rather broad pH range (= 5-8) while acidic and especially alkaline pH caused a sharp decrease in specific binding. Alkaline sensitivity is typically observed for many membrane recep-

tors and reflects a solubilisation and denaturation of the protein from its membrane phospholipid environment. Duncan et al. (1988) also reported that the level of specific binding of [¹²⁵I]MEL in hamster whole brain membranes remained relatively constant between pH 6.2- 8.2.

An examination of the regional distribution of specific binding indicated that the hypothalamus contained the highest amount of binding. However, significant amounts of binding were measured in all brain regions examined as well as in peripheral tissues (data not shown; Duncan et al. [1988, 1989]; Niles, [1989]). This is in sharp contrast to the CNS distribution of the high-affinity melatonin receptor which is found primarily in the pars tuberalis and less densely in a few other discrete areas such as the suprachiasmatic nuclei and paraventricular nuclei (Vaněček, 1988b; Williams, 1989; Williams et al., 1989; Weaver et al., 1989). Binding to these receptors in the hypothalamus could be an explanation for the observation that, in the studies conducted here, the hypothalamus was observed to have the highest amount of [¹²⁵I]MEL binding since, at the single concentration of radioligand used, binding would be a sum of the high and low-affinity binding components. However, saturation binding studies also revealed a greater site density (B_{max}) of the nanomolar-affinity site in the hypothalamus as compared to the hippocampus or cortex. Duncan et al. (1988) have also reported highest binding of [¹²⁵I]MEL to the nanomolar-affinity site to occur in hamster hypothalamic membranes.

Subcellular fractionation of hypothalamic and cortical membranes indicated that the greatest amount of binding occurred in the P3 (crude microsomal) fraction. Significant amounts of binding also were present in other subcellular fractions, possibly representing the relatively crude separation

of the subcellular fractions from one another with the protocol employed. Duncan et al. (1988) found highest binding in the P2 (crude synaptosomal) fraction but did not prepare and test the P3 fraction. Since melatonin is highly lipophilic and can readily permeate cell membranes, it is possible that it also binds to intracellular binding sites. This view is supported by the presence of low-affinity cytosolic binding sites for melatonin in the brain (Niles, 1987) and peripheral organs (Cohen et al., 1978).

An examination of the kinetics of binding revealed reversible binding and rapid association and dissociation at a temperature of 0°C, also reported by Duncan et al. (1988, 1989). Duncan et al. (1989) found an association rate constant of $7.3 \times 10^8 \text{ M}^{-1}\text{min}^{-1}$ and dissociation rate constant of 1.19 min^{-1} with a $t_{1/2}$ of 0.60 min in hamster whole brain membranes (at 0°C) which agrees moderately well with the values obtained in the present study ($4.46 \times 10^8 \text{ M}^{-1}\text{min}^{-1}$, 0.748 min^{-1} , 0.93 min, respectively). Because of these rapid kinetics, assays conducted at higher temperatures gave erratic or negligible specific binding.

The kinetics are another point of divergence between the nanomolar-affinity site and the picomolar receptor, which exhibits slow binding kinetics even at elevated temperatures (22- 37°C) (Vaněček, 1988b; Weaver et al., 1989; Williams et al., 1989; Morgan et al., 1989c; Sugden and Chong, 1991). The affinity of the radioligand for the nanomolar site calculated from the association and dissociation rate constants (1.7 nM) was in very good agreement with the equilibrium dissociation constant (K_d) derived from saturation analysis (1.8 nM), implying that the value is likely a true reflection of the affinity of the binding site for [125 I]MEL under the assay conditions used.

Saturation binding studies revealed a single [125 I]MEL binding site in

hamster hypothalamic and hippocampal membranes of similar affinity (1.8 and 2.2 nM, respectively) which is also similar to the affinity reported by Duncan et al. (1986, 1988, 1989) in Syrian and Djungarian hamster whole brain membranes (3.3 nM and 1.5 nM, respectively). Interestingly, in the present study two binding sites were resolved in cortical membranes using saturation binding experiments ($K_d = 0.32$ nM, $K_d = 10.5$ nM). The higher affinity component is of 6-fold higher affinity than the nanomolar [125 I]MEL binding site found in the hypothalamus and hippocampus. This discrepancy was resolved by a detailed competition analysis of [127 I]MEL displacement of [125 I]MEL binding in cortical membranes where non-linear analysis of this competition curve indicated a triphasic displacement. The cortex seems to contain a low density of high (picomolar)-affinity ($IC_{50} = 43$ pM) binding sites. These sites may be functional (or nonfunctional) picomolar receptors similar to those found in the pars tuberalis. The fact that other laboratories have not been able to detect these sites autoradiographically probably reflects their low density in cortex which would not permit them to be recognised by this technique. Autoradiography is likely not sufficiently sensitive to detect a diffuse distribution of high-affinity binding sites in tissue slices (Weaver et al., 1990, 1991), especially in the presence of a higher density of the nanomolar-affinity binding sites. The second site, of nanomolar affinity ($IC_{50} = 4.3$ nM), observed in competition analysis in the cortex is of similar affinity to the site found in the hypothalamus and hippocampus. In saturation studies, the presence of a low density of the picomolar-affinity sites could skew the observed affinity of the nanomolar sites to a higher affinity if the two sites were not properly resolved. The K_d of 0.32 nM obtained in the cortex by saturation analysis may in fact be a

composite value of the picomolar and nanomolar binding affinities. It would indeed be difficult to resolve a triphasic saturation isotherm into its three components, especially if the relative site density of one component was small in comparison to the others.

The third, lowest affinity site found in the cortex by saturation binding analysis ($K_d = 11$ nM) or competition analysis ($IC_{50} = 3.4$ μ M) may correspond to the low-affinity binding site ($K_d = 40-280$ nM) that has been reported in the rat and hamster brain (Zisapel and Anis, 1988; Laitinen et al., 1989; Anis, Nir and Zisapel, 1989; Anis and Zisapel, 1991). Zisapel et al. suggest that this site is involved in the mediation of reproductive functions because this site seems to be regulated by circulating testosterone. These studies have not been corroborated by other laboratories and as such remain tenuous. The extremely low affinity of this site is suggestive of a non-functional receptor site.

Guanine nucleotides and sodium ion have been demonstrated to have an inhibitory effect upon binding of [125 I]MEL to the picomolar-affinity melatonin receptor (Dubocovich and Takahashi, 1987; Morgan et al., 1989b; Niles, 1990; Laitinen and Saavedra, 1990a,b; Ying and Niles, 1991) whereas binding to the nanomolar-affinity site in the hypothalamus observed here and elsewhere (Niles, 1990) was insensitive to these agents. Duncan et al. (1988) also reported that GTP had no effect upon [125 I]MEL specific binding or upon the IC_{50} of 6-chloromelatonin in competition experiments. They did however, find a slight inhibition of binding at high (0.1- 0.2 M) NaCl and KCl concentrations. Also, they found no effects of $CaCl_2$ upon binding while in the studies reported here an enhancement of binding by calcium ion was found. These differences may

be due to the fact that Duncan et al. had used whole brain membranes but in the present studies hypothalamic membranes were used.

The pharmacological (binding) profile of the nanomolar-affinity [^{125}I]MEL binding site was examined using a wide variety of neurotransmitters and drugs in competition experiments in hypothalamic, hippocampal and cortical membranes. An overall specificity for melatonin and closely related indoles was noted and the profiles in these three brain regions were significantly correlated with one another indicating that the site seems identical in these regions.

The Hill coefficients (n_H) for selected indoles and non-indoles in the hypothalamus are shown in Table 3. Most of the indoles (with the notable exception of 2-iodomelatonin) have Hill slopes near unity, indicating interaction at a single class of binding sites in the hypothalamus. In the hippocampus and cerebral cortex, both melatonin and 6-chloromelatonin have n_H values near unity indicating interaction of these compounds with a single population of binding sites in these regions. In contrast, [^{127}I]MEL inhibition of specific [^{125}I]MEL binding is shallow in hypothalamus ($n_H = 0.7$) and cerebral cortex ($n_H = 0.3$) indicating the presence of multiple binding sites or negative cooperativity in these areas. Interestingly, in the hippocampus [^{127}I]MEL exhibits a Hill slope of ≈ 1 suggesting a single population of nanomolar-affinity sites in this region.

The pharmacological profiles reported by Duncan et al. (1988, 1989) in Syrian and Djungarian hamster whole brain membranes were significantly correlated with each other, indicating that the nanomolar-affinity site is similar or identical in the two strains of hamster. The rank ordering of affini-

ties reported here for various indoles and drugs was also similar to that reported by Duncan et al. Only melatonin and closely related indoles with 5-methoxy and/or N-acetyl substituents showed significant affinity in inhibition experiments. Moreover, as observed in the present studies, they found that serotonin is a weak inhibitor while catecholamines are virtually ineffective. Thus, it appears that the nanomolar-affinity melatonin binding site observed in the hypothalamus and other brain regions in this study is similar to that described by Duncan et al. (1988, 1989) in hamster whole brain membranes.

Dopaminergic and adrenergic compounds did not exhibit potency in inhibition of [125 I]MEL binding, the sole exception being the drug prazosin (discussed below). Displacement of [125 I]MEL by 6-CCE suggested binding of the radioligand to a benzodiazepine receptor site. However, the poor affinity of diazepam in displacement of [125 I]MEL revealed that a benzodiazepine site is not involved. The moderate affinities of the non-selective serotonergic antagonists, metergoline and methysergide, suggested labelling of a 5-HT receptor subtype by [125 I]MEL. The poor affinities displayed by serotonin and 5-methoxytryptamine argue against labelling of 5-HT₁ sites, for which these compounds have high affinity. Drugs selective for the various 5-HT₁ binding site subtypes: 5-HT_{1A}, 5-HT_{1B} and 5-HT_{1C} (Peroutka, 1986) were tested for inhibition of [125 I]MEL binding. Ipsapirone and 8-OH-DPAT, which both have high affinity for the 5-HT_{1A} site, did not inhibit [125 I]MEL binding at a concentration of 10 μ M. The 5-HT_{1B} selective compound Ru 24969 was a relatively potent inhibitor of [125 I]MEL binding (K_i = 40 nM). However, this affinity is some two orders of magnitude lower than its affinity for 5-HT_{1B} sites and is likely due to the indole nature of the drug since mCPP, a 5-HT_{1B} selective drug, was a very poor

inhibitor of binding. Mianserin has selectivity for 5-HT_{1C} sites but was a poor inhibitor of [¹²⁵I]MEL binding. The apparent lack of involvement of any of the 5-HT₁ binding site subtypes leads one to consider the involvement, if any, of 5-HT₂ sites. The moderate inhibition produced by spiperone and ketanserin suggested that [¹²⁵I]MEL binding to 5-HT₂ sites may be occurring. However, the affinities of these drugs were somewhat low if they are to be attributed to 5-HT₂ sites. In addition, NPP, DOI and cyproheptadine, which bind to 5-HT₂ sites, were all poor inhibitors of [¹²⁵I]MEL binding. The drug TDCB, which is selective for and potent at 5-HT₃ sites (Richardson et al., 1985), did not inhibit binding at 10 μM. The evidence presented here makes it very unlikely that any of these serotonin receptor sites are labelled by low nanomolar concentrations of [¹²⁵I]MEL.

Only one non-indolic compound, prazosin, exhibited appreciable affinity for the nanomolar-affinity binding site in all brain regions tested. The high affinity exhibited by this α₁-adrenergic antagonist (Greengrass and Bremner, 1979) for the nanomolar-affinity [¹²⁵I]MEL binding site strongly suggested initially that this radioligand could be labelling α₁-adrenergic sites in the hamster CNS. However, the failure, of the α-adrenergic antagonist phentolamine, the α₁-antagonist WB-4101, and the α-adrenergic agonists epinephrine and norepinephrine to inhibit binding did not support the involvement of α₁-sites. Similarly, the inability of the α₂-selective agents yohimbine and clonidine and poor ability of the β-adrenergic agents propranolol and alprenolol to compete for binding indicated that α₂- and β-adrenergic sites are not labelled by [¹²⁵I]MEL. The possibility that [¹²⁵I]MEL was labelling an α₁-adrenoceptor was most convincingly eliminated by the use of the irreversible

α -adrenoceptor alkylating agent, benextramine (Melichorre, 1981). In competition experiments, benextramine had negligible affinity for the nanomolar-affinity site labelled by [125 I]MEL. However, pretreatment of brain membranes with benextramine abolished all specific binding of [3 H]prazosin while not affecting the displacement of [125 I]MEL binding by unlabelled prazosin or 2-iodomelatonin. This implied that the potency exhibited by prazosin in competition studies is likely due to its affinity for unique, non- α -adrenergic binding sites labelled by [125 I]MEL in the hamster CNS. Prazosin is reported to have negligible affinity for the picomolar-affinity receptor (Pickering and Niles, 1989; Rivkees et al., 1989b; Laitinen et al., 1990a; Viswanathan, Laitinen and Saavedra, 1990; Stankov et al., 1991) and is therefore a useful drug for discriminating between the nanomolar-affinity binding site and the picomolar-affinity receptor. A somewhat higher affinity of prazosin for the solubilised chick brain receptor has been reported (Ying and Niles, 1991) and may reflect the solubilised nature of the receptors (DeLisi and Wiegel, 1981).

Prazosin's affinity for the nanomolar-affinity site may be related to the presence of two non-polar methoxy substituents on its ring structure as suggested by the increased affinity of 6-chloromelatonin (compared to melatonin and 6-hydroxymelatonin) which has a non-polar halogen substituent on carbon 6. One substituent in the 6 position of prazosin corresponds to the 5-methoxy group on the indole ring which, as discussed below, is important for binding affinity. Prazosin also has a 7-methoxy group which corresponds to a substitution on carbon 6 of the indole ring. This second methoxy group would presumably enhance the affinity of prazosin for methoxyindole binding sites. Duncan et al. (1989) found that 6-methoxymelatonin, an indole resembling

prazosin in having dimethoxy substituents, has high affinity for the nanomolar-affinity [¹²⁵I]MEL binding site, being 35-fold more potent than melatonin in competition experiments in Djungarian hamster brain membranes. This supports the idea that it is the dimethoxy structure of prazosin which is largely responsible for its binding affinity. Although it is generally accepted that prazosin is a specific α_1 -adrenergic antagonist, its binding to the nanomolar-affinity [¹²⁵I]MEL sites throughout the brain suggests that it could have pharmacological actions at these sites as well.

Structure-activity relationships can be derived by comparison of the affinities of a series of indoles for the nanomolar-affinity [¹²⁵I]MEL binding site. Among the tryptamines examined in inhibition experiments, the most potent were those possessing both 5-methoxy and ethyl N-acetyl substituents suggesting the importance of these groups for binding affinity. However, N-acetylserotonin which lacks a 5-methoxy group was about 20- 80 times more potent than 5-methoxytryptophol or 5-methoxytryptamine suggesting that the N-acetyl group may be the more important in conferring binding affinity. In keeping with this view, serotonin, which does not have an N-acetyl substituent but is otherwise identical to N-acetylserotonin, displayed negligible potency. The affinity of N-acetylserotonin for the picomolar melatonin receptor is very poor (Vaněček et al., 1987; Rivkees et al., 1989b; Laitinen and Saavedra, 1990a) and therefore makes this compound of use in the discrimination of the nanomolar and picomolar sites.

A methoxy group at position 5 of the indole ring also is important for potent inhibition. This requirement seemed less stringent than the need for N-acetylation since N-acetyl-6-methoxytryptamine, a structural isomer of

melatonin, was similar in potency to melatonin. Also, N-acetylserotonin and O-acetyl-N-acetylserotonin had affinities similar to that of melatonin. A degree of variability is tolerated in this part of the molecular structure by the binding site.

A nonpolar group is favoured at position 6 (e.g.: 6-chloromelatonin) while a polar group in this position (e.g.: 6-hydroxymelatonin) decreased affinity. In contrast, at position 5 on the ring a substituent more polar than the methoxy group of melatonin is tolerated (e.g.: N-acetylserotonin, O-acetyl-N-acetylserotonin). Increasing the hydrophobicity of melatonin by halogenation (e.g.: 6-chloromelatonin, 2-iodomelatonin) or by inclusion of other hydrophobic substituents on the indole ring was seen to increase the affinity of the compound indicating a hydrophobic, protein binding domain for melatonin. Removal of the positively charged amino group of the tryptamines was required for potency, as for example, by N-acetylation of serotonin which increased the affinity > 370- fold. Neutralisation of the positive charge of indolealkylamines may be a biological mechanism for directing the affinity of these compounds towards melatonin binding sites, while the presence of this charge allows interaction at serotonergic receptors. Neutralisation of the positive charge by itself was insufficient for producing tight binding compounds since 5-methoxytryptophol bears no positive charge but had an affinity intermediate between that of melatonin and 5-methoxytryptamine. The presence of an amide linkage seemed to be needed for maximal affinity. Perhaps a positively charged amino acid residue is present in this binding domain which normally hydrogen bonds to the amide of melatonin. Compounds with positively charged amino groups, such as serotonin, would experience electrostatic repul-

sions and have poor affinity for this site. Compounds containing hydroxyl groups, such as the tryptophols, would hydrogen bond less well than melatonin at this domain and have lower affinity.

Several melatonin analogues were synthesised in order to further explore structure-affinity relationships of binding to the nanomolar-affinity site. The motivation behind this was to explore possible structural modifications which could be used to create radiolabelled photoaffinity ligands and/or ligands which could be used to create affinity column chromatography media. This would facilitate attempts at purification of the site and studies towards its biochemical and physical characterisation. Structure-activity relationships have previously been derived for the picomolar-affinity melatonin receptors using both radioligand binding data and functional assays (see Section 1.10 and Lewis et al. [1990]).

Modification of the indole ring structure by alkylation of the indolic nitrogen to give 1-[(p-carboxy)benzyl]melatonin lowered the binding affinity over 500- fold. Thus, either steric hindrance of the bulky carboxybenzyl group interferes with binding or a disturbance of the pseudo-aromaticity of the indolic ring is responsible for the loss of affinity. Hence, this position in the molecular structure did not seem to be of promise for chemical modification if one wished to retain appreciable binding affinity and other positions were examined.

Increasing the alkyl chain size of the N-acetyl moiety brought about a gradual loss of affinity as can be seen from the series melatonin > N-t-BOC-5-MT > N-n-propionyl-5-MT ≈ N-isobutyryl-5-MT > N-n-butyryl-5-MT >>> N-n-myristyl-5-MT. Increasing the N-acetyl group in size up to that of N-isobutyryl

only resulted in a decrease in affinity of some 5-fold, suggesting that moderately small changes in the structure at this position are tolerated. Modifications in the 5-methoxy group of melatonin were also explored. Changing this moiety from an ether to an ester (O-acetyl-N-acetylserotonin) decreased the binding affinity only very slightly while increasing the size of the substituent to that of an O-isobutyryl group caused an approximately 10- fold drop in affinity. Consequently, because of the somewhat greater acceptance of the binding site for structural modifications at the amide site (as well as because of the easier chemistry of modification of a primary amine vs an aromatic hydroxyl group), further efforts were aimed at exploration of modifications at this position.

With the goal in mind of producing useful photoaffinity ligands, structural modifications including an azido or diazo group were designed. Each of these photoactivatable groups has its own advantages and disadvantages. Both are reasonably stable in aqueous solutions in the physiological pH range. The azido group is relatively easy to incorporate into aromatic ring structures with the additional bonus that radioiodination of the ring structure is also possible in many cases. The wavelengths for photolysis of the azido group are > 300 nm and as such one can avoid exposure of the biological sample to strong ultraviolet wavelengths (< 280 nm) which may damage the protein of interest. Also, several arylazido precursor compounds can be (and were) commercially obtained, making the synthetic strategy more straightforward. The major drawback of these groups are that they are relatively big, bulky, groups and may reduce the binding affinity of the parent molecule substantially. The diazo group, while requiring shorter (higher energy) wavelength irradiation, is

small and may be better tolerated by a binding site. In particular a commercially available precursor of an especially small size is presently available, 2-diazo-3',3',3'-trifluoropropionyl chloride (Chowdhry et al. 1976). For a more detailed discussion of the photochemical properties of these and other photoreactive groups the interested reader is referred to Bayley, (1983); Ruoho, Rashidbaigi and Roeder (1984).

Of the azidonitrophenyl compounds synthesised, all were less potent than melatonin in competition experiments and none were judged to be of sufficiently high affinity to be used as photoaffinity ligands. Photolysis of a 0.1 mM solution of the compound N-(5-azido-2-nitrobenzoyl)-5-MT in 50 mM Tris·HCl pH 7.4 ($\lambda_{\text{max}} = 301 \text{ nm}$; $\epsilon_{301} = 8775$) for 1 min using a 1,000 W Xe/Hg lamp, with wavelengths under 360 nm and infrared filtered out, reduced A_{301} by 52%. Photolysis (75 sec) of hamster hippocampal membranes using 10 nM - 50 μM of this compound with subsequent washing of the membranes was seen to irreversibly abolish specific [^{125}I]MEL binding with an observed EC_{50} of 4.7 μM (data not shown). Pilot experiments at radioiodination and photoaffinity labelling with these compounds yielded high nonspecific labelling (as seen using SDS-PAGE) which could not be circumvented (data not shown).

The compound N-trifluoroacetyl-5-MT was synthesised and found to be equipotent with melatonin in competition experiments. Radioiodination of this compound (using the same methodology as for melatonin) produced the putative radioligand 2-[^{125}I]iodo-N-trifluoroacetyl-5-MT (not chemically characterised) which had an affinity of $\approx 0.12 \text{ nM}$ in hamster brain membranes as evaluated from a pilot saturation binding study. The increased hydrophobicity due to trifluoro substitution of the acetyl group and iodination may account for the high

binding affinity (as discussed above) which is higher than even [^{125}I]MEL. This information indicated that the compound 2-[^{125}I]iodo-N-(2'-diazo-3',3',3'-trifluoropropionyl)-5-MT might be a potential high affinity radiolabelled photoaffinity probe for the nanomolar-affinity binding site since the structural homology to 2-[^{125}I]iodo-N-trifluoroacetyl-5-MT is large. In addition, as discussed above, N-isobutyryl sized substituents seem to be tolerated in the amide position of the structure of melatonin. The unlabelled photoaffinity compound N-(2'-diazo-3',3',3'-trifluoropropionyl)-5-MT was synthesised and found to be equipotent with melatonin in competition experiments, as anticipated. It was predicted that radioiodination of this compound would increase its affinity substantially (10- 100 fold), as is the case for iodination of melatonin. Unfortunately, attempts at synthesis of the radioiodinated compound were unsuccessful. Direct iodination of the compound was not possible due to the instability of the diazo group under the iodination reaction conditions. A multi-step synthetic scheme was attempted with prior incorporation of radioiodine before attaching the diazo moiety, however this was also unsuccessful. The potential of this radioligand as a photoaffinity probe remains to be explored.

In summary, using radioligand binding assays, it has been demonstrated that a unique, nanomolar-affinity site for [^{125}I]MEL is found in the hamster CNS that is distinct from the picomolar-affinity melatonin receptor and that fulfils many of the criteria for the classification of receptor. Binding is: saturable, reversible, of high affinity, pharmacologically specific, pH sensitive, trypsin sensitive and temperature sensitive. The ubiquitous presence of the nanomolar site in the CNS makes it difficult to surmise the possible

physiological role of these sites. However, the physiological effects of melatonin seem to be more wide-ranging than simply the neuroendocrine role most often ascribed to it and which the picomolar-affinity receptors of the pars tuberalis are thought to mediate. Binding sites found in the spleen (Niles, 1989) may suggest an interaction between melatonin and the immune system (a neuroendocrine-immune axis) (Maestroni et al., 1986). Melatonin antagonists have been observed to have an antidepressant-like activity in the C3H/HeN mouse behavioural despair test (Siuciak et al., 1990; Dubocovich et al., 1990a). Melatonin has been shown to modify the density of benzodiazepine receptors and GABA receptors in the rat cortex (Lowenstein et al., 1985; Acuña-Castroviejo et al., 1986a,b). Melatonin has also been reported to have anti-oncogenic activity in a human breast cancer cell line (Blask and Hill, 1986). It is possible that this nanomolar-affinity melatonin binding site is involved in mediating one or more of the many responses which melatonin has been observed to affect. For a detailed discussion of the many effects attributed to melatonin see the review: Cardinali (1981).

CHAPTER THREE

PHYSICAL CHARACTERISATION OF
MELATONIN BINDING SITES

3.1 Introduction

3.1.1 Target-Size Analysis

Target-size analysis (also known as radiation inactivation) is a method of determining the in situ functional molecular mass of a biological 'target' which could be an enzyme, DNA, RNA, multi-molecular complex (e.g. ribosome), etc. A measurable function (e.g. transcription) of the target must exist which can be quantified after exposure to a dose of ionising radiation. This technique is particularly useful for measuring the holomeric size of membrane proteins such as receptors since the target can be measured in its native membrane state, with minimal experimental perturbation. For detailed accountings of the theory and practice of target-size analysis refer to: Lea (1955), Pollard et al. (1955), Kempner and Schlegel (1979), Pollard (1987).

3.1.1.1 Theory

As high energy radiation (ionising radiation) passes through matter, energy is transferred to the matter. This energy is absorbed by electrons which then dissociate from atoms to produce ion pairs (primary ionisation). These electrons may have sufficient energy to produce secondary ionisations. The formation of unstable ions during irradiation leads to cleavage of chemical bonds in the ionised molecule, changing its chemical structure. The energy deposition by the incident radiation (high energy electrons, X-rays, γ -rays etc.) occurs randomly along the path of its movement through matter due to the large separation (e.g. ≈ 30 nm for 1 MeV electrons) between primary ionisations. The random nature of the energy deposition means that the probability of a target being 'hit' (ionised) is proportional to the size (volume) of the target.

The larger the target (i.e. the larger its molecular weight), the more volume it occupies and therefore the more likely it is to be 'hit' using a given dose of radiation.

Lea (1955) proposed the single-target, single-hit hypothesis to explain the relationship between radiation dose and loss of biological activity of a target molecule. Since the energy deposition is so large (one primary ionisation averages about 66 electron volts [Kepner and Macey, 1968]), he assumed that one primary ionisation event occurring *anywhere* within the volume of the target is sufficient to cause complete functional inactivation. This assumption is corroborated by experimental observations of a wide range of targets. A second assumption is that only one functional target exists within the molecular target (i.e. multiple *independent* functional sites or domains are not present in the molecule). The latter assumption, of course, is dependent upon the given target molecule being studied whereas the former assumption is universally applicable.

3.1.1.2 Mathematical Derivations

According to the single-target, single-hit hypothesis, the biological activity of the target is destroyed by one high energy 'hit' occurring within its volume, V (the molecular volume of the target carrying the activity), following the Poisson probability equation such that⁴:

$$A = A_0 e^{-VD} \quad (1)$$

since $M = N_0 V / \bar{v} \quad (2)$

⁴ A_0 = initial activity, A = activity remaining after dose D , D = radiation dose (in inactivating events/cm³), D_{37} = radiation dose (rads) reducing the initial activity by 63%, M = molecular weight of the target (g/mole), N_0 = Avogadro's number (6.02×10^{23} particles/ mole), V = volume of the target molecule (cm³), \bar{v} = partial specific volume of the target (cm³/g).

substituting, $A = A_0 e^{-(\bar{v}D/N_0)}$

$$\ln (A/A_0) = -(\bar{v}/N_0) \cdot M \cdot D$$

letting \bar{v}/N_0 be a constant, k: $\ln (A/A_0) = -k \cdot M \cdot D$ (3)

Plotting $\ln (A/A_0)$ vs D will yield a straight line of slope $-kM$. When $A = 37\% A_0$,

$\ln (A/A_0) = -1$. Therefore, equation 3 reduces to: $M = k/D_{37}$ (4)

where D_{37} represents the radiation dose required to reduce the initial activity to 37% of its value.

Using equation 4, Kepner and Macey (1968) empirically determined the value of the constant k to be 6.4×10^{11} (when D_{37} is in rads). This value agrees quite well with a theoretically calculated value of 5.8×10^{11} (Pollard, 1987). Therefore, knowing the D_{37} value of a target, the molecular weight can be calculated by the empirical equation:

$$M = 6.4 \times 10^{11} / D_{37} \quad (5)$$

A basic assumption of this equation is, of course, that the partial specific volume, \bar{v} , of the biological targets (usually proteins) does not vary greatly and can therefore be approximated by a constant. Since most proteins have partial specific volumes between 0.69 and 0.75 cm^3/g (Cantor and Schimmel, 1980), this seems to be a reasonable approximation.

3.1.1.3 Limitations

In addition to the direct action of radiation upon the target, indirect effects can occur. Since most biological samples contain water, formation of reactive species (e.g. H^+ , OH^- , $\cdot\text{OH}$, H_2O_2 , etc.) can occur by radiolysis of water which can chemically inactivate the target molecule causing loss of function (Kempner and Schlegel, 1979). In order to avoid this problem, freeze-dried (if possible) or frozen samples can be used and antioxidants (e.g. dithiothreitol,

ascorbic acid) can be included in the sample to act as free radical scavengers. Molecular oxygen dissolved in the sample can also drastically increase the radiation sensitivity of the target (Butler and Robins, 1962; Kempner and Schlegel, 1979). This problem can be minimised by degassing buffers prior to sample preparation and by using antioxidants. The choice of sample buffer can also affect the radiation sensitivity (D_{37} value) of a target (Parkinson, 1987).

The energy absorbed by the sample during irradiation is ultimately converted to heat which can thermally damage sensitive biological molecules. To overcome this, the irradiation process can take place under conditions of low temperature (e.g. in liquid nitrogen). This introduces further problems to the use of equation 5 because the D_{37} value for a target is a temperature-dependent parameter (Fluke, 1987).

A major technical obstacle associated with the implementation of equation 5 is the determination of the *absolute* dose of radiation received by samples because of beam attenuation, instrument variability, etc. Dosimetry can be problematic depending upon the method used (Kempner and Haigler, 1982; Jung, 1984). The *relative* doses within an experiment can, however, be accurately determined.

A more serious limitation of the technique comes from a consideration of what shall be called the target volume. The target may consist of several subunits and if the measured function resides in one (or a subgroup) of these, a target size less than that of the complete oligomer may be observed (Lai et al., 1987). Similarly, since proteins are generally composed of discrete domains, it is not inconceivable that the target size of a functional domain

could be obtained in lieu of the size of the entire protein. Other factors also come into play such as: Should carbohydrate moieties of membrane glycoproteins be considered as part of the target volume? Does sufficient energy transfer occur between non-covalently associated proteins (e.g. subunits of the nicotinic acetylcholine receptor) such that the true target size can be obtained by measuring the loss of ligand binding to one subunit? Unfortunately, the only answer available is an empirical one, depending upon the given target being studied (Vaidhyanathan and Jung, 1987). However, the majority of cases reported to date support the single-target, single-hit theory and the target molecular weight determined by this technique agrees rather well with that measured by other means (Fraser and Venter, 1987). It is only in our understanding of the 'target' that apparent incongruities arise. Obviously, no technique can stand alone and other methods must be used to test any predictions derived from target-size analysis results.

3.1.1.4 Molecular Mass Standardisation

To overcome the problems mentioned above, one can use a set of proteins (enzymes) of known molecular weight, subunit composition, etc. as internal or (less desirably) external standards to create an inactivation standard curve for the particular experimental conditions being used (Lo et al., 1982; Lai et al., 1987).

3.1.1.4.1 Theory

A set of enzyme standards is irradiated with a series of apparent doses of radiation and their activity then measured. From this data, a plot of target sensitivity is constructed as per equation 6:

$$\ln (A/A_0) = -\kappa \cdot M \cdot \bar{D} \quad (6)$$

where \bar{D} is the apparent dose (Mrad) and κ is a complex constant combining k (i.e. σ/N_0) with other empirical constants which adjust for temperature, quenching, sample composition, etc. Although the absolute value of κ is unknown, it will be constant for all samples irradiated within a given experiment. This means that the inactivation slopes for all targets (unknowns and standards) will be affected by the same amount and since we are only concerned with the relative values of the slopes, κ can be disregarded. Linear regression analysis of the data ($\ln [A/A_0]$ vs \bar{D}) allows calculation of the \bar{D}_{37} for each enzyme standard. A plot of M_r vs $1/\bar{D}_{37}$ produces a linear molecular weight calibration curve. Knowing the \bar{D}_{37} value for an unknown target, its molecular weight can be interpolated from this standard curve.

3.1.1.5 Summary

According to classical target theory, the following assumptions apply (Kepner and Macey, 1968): (1) The primary energy releases generated by ionising radiation in matter are discrete, highly localised clusters of ionisation. (2) One primary ionisation is assumed to completely inactivate the function of the target molecule if it occurs within the 'effective volume' for that function. (3) The primary ionisations are randomly distributed with respect to volume, i.e. sparsely ionizing radiations (having low linear energy transfer {LET} values) are used (such as ^{60}Co γ -rays or electrons from a linear accelerator). (4) There are no indirect effects due to free radical diffusion (i.e. solvent effects).

3.2 Materials and Methods⁵

3.2.1 Animals

Adult male Syrian hamsters (2- 3 months old) were obtained from Charles River, Quebec and High Oak Ranch, Ontario. Animals were housed in groups of six, on a 14hr L:10hr D cycle (lights-on at 7:00 a.m.), at 23- 27°C with free access to food (Purina Rodent Chow) and water. Male Leghorn chicks (2- 3 days old) were purchased from Martindale Hatchery, Ontario. Chicks were housed in a brooder at 35°C under 12L:12D illumination for 2- 3 days until sacrifice. Water and feed were available ad libitum.

3.2.2 Tissue Preparation

Hamsters were sacrificed by decapitation between 6- 7 hours after lights-on. Heads were put into ice-cold 0.85% (w/v) saline and kept at 0°C. Brains were removed and dissected on a chilled glass plate according to Glowinsky and Iversen (1966) within an hour after sacrifice. Hypothalami were pooled, weighed and homogenised by polytron (5 sec, setting 5) in 20 volumes of ice-cold homogenisation buffer (50 mM Tris-HCl, 5 mM ascorbic acid, 5 mM EDTA, 1 mM L-leucine hydroxamic acid, 10 µg/mL bacitracin, 1 mM iodoacetamide, 0.1 mM D,L-benzylsuccinic acid, and 10 µg/mL trypsin inhibitor, pH 7.4 at 4°C). The tissue homogenate was then centrifuged for 5 min at 30,000 x g (4°C). The pellet was resuspended in 20 volumes of homogenisation buffer and washed twice by centrifugation. A final wash was performed using 20 volumes of sample buffer (50 mM Tris-HCl, 5 mM ascorbic acid and 5 mg/mL BSA, Ph 7.4 at 4°C) and the pellet was

⁵ These studies have been previously published (Pickering et al., 1990) and are reprinted by permission of the publisher, John Wiley & Sons, Ltd.

resuspended in 10 volumes of this buffer. Since one (or more) of the inhibitors used in the homogenisation buffer were found to depress [125 I]MEL binding slightly (= 7% decrease in specific binding), inhibitors were omitted from the irradiation sample buffer. Aliquots (0.8 mL) of suspension were transferred into aluminum sample trays and immediately frozen in liquid nitrogen. Samples were stored at -70°C for 1- 2 days prior to (and up to 9 days after) irradiation. On the day of irradiation the samples were transported on dry ice.

Chicks were sacrificed by decapitation and heads kept on ice until removal of the eyes. The retinal tissue homogenate was prepared as detailed above (Section 2.2.6) with the exception that homogenisation buffer was used for washing of the pellet. The pellet was washed one final time in sample buffer and then resuspended in approximately 3 volumes of this buffer. Aliquots (0.8 mL) were frozen as described above.

3.2.3.1 Target-Size Enzyme Standards

All enzymes were purchased from Boehringer Mannheim. They were subjected to discontinuous SDS-polyacrylamide electrophoresis with a 10% or 12.5% separating gel (Laemmli, 1970) using the BioRad mini-gel system. SDS-PAGE molecular weight standards (Sigma Chemical Co.) were included: bovine erythrocyte carbonic anhydrase [E.C. 4.2.1.1] ($M_r = 29,000$), chicken ovalbumin ($M_r = 45,000$), BSA ($M_r = 66,000$), rabbit muscle phosphorylase b [E.C. 2.4.1.1] ($M_r = 97,400$), E. coli β -galactosidase [E.C. 3.2.1.23] ($M_r = 116,000$), rabbit muscle myosin ($M_r = 205,000$).

Enzyme standards were prepared on the day of irradiation in sample buffer at the following concentrations: 7 μg alkaline phosphatase/mL, 0.3 mg

alcohol dehydrogenase/mL, 32 μ g α -amylase/mL, 8 mg ribonuclease/mL, 20 μ g lactate dehydrogenase/mL and 60 μ g carboxypeptidase A/mL. Aliquots were frozen in aluminium trays as described above and stored on dry ice until irradiation.

3.2.3.2 Gel Filtration Chromatography

The functional molecular size of LDH in this buffer system was examined by gel filtration chromatography on a 50 cm x 1 cm (I.D.) Sephadex G-100 (Pharmacia) column (at 4°). Protein standards were used to create a molecular weight calibration curve: bovine pancreatic ribonuclease [E.C. 3.1.4.22] (M_r = 14,000); horse skeletal muscle myoglobin (M_r = 18,800); bovine pancreatic α -chymotrypsin [E.C. 3.4.21.1] (M_r = 25,100); porcine gastric mucosal pepsin [E.C. 3.4.23.1] (M_r = 35,000); chicken ovalbumin (M_r = 45,000); BSA (M_r = 66,000) and bovine serum transferrin (M_r = 77,000). These proteins were purchased from Boehringer Mannheim except for transferrin and myoglobin (Calbiochem) and BSA (Sigma Chemical Co.). Standards were eluted in 50 mM Tris·HCl pH 7.4 (4°C) while LDH was eluted in irradiation sample buffer (50 mM Tris·HCl, 5 mM ascorbic acid, 5 mg BSA/mL, pH 7.4 at 4°C). Fractions (\approx 1 mL) were collected and protein concentration monitored by measuring the absorbance at 280 nm. LDH elution was monitored by measurement of enzymatic activity [vide infra].

3.2.4 Reagents

Pyruvate, NAD and NADH were purchased from Boehringer Mannheim. L-leucine hydroxamic acid, N-(3-[2-furyl]acryloyl)-L-phenylalanine-L-phenylalanine, turkey egg white trypsin inhibitor, iodoacetamide, bacitracin, D,L-benzylsuccinic acid, p-nitrophenyl phosphate, cyclic cytidine 2':3'-monophosphate, soluble potato starch, semicarbazide hydrochloride, and 3,5-di-

nitrosalicylic acid were obtained from Sigma Chemical Co. L-propranolol was a gift of Imperial Chemical Industries, U.K. Polyacrylamide electrophoresis reagents were obtained from BioRad. All other reagents were obtained from standard commercial sources.

3.2.5 Sample Irradiation

Enzymes standards and duplicate samples of frozen hypothalamic tissue homogenates were exposed to 0- 30 Mrad of radiation as described previously (Saccomani et al., 1981; Jung, 1984). Briefly, aluminum trays are irradiated with a Van de Graff generator producing a 0.5 mA beam of 1.5 MeV electrons at a dose-rate of 0.5 Mrad/min. The irradiation chamber was cooled with a stream of liquid nitrogen flowing under the samples in a metal cooling manifold and the chamber temperature maintained between -60 and -45°C. Apparent radiation doses were measured using the Blue Cellophane (du Pont) film-bleaching technique calibrated against a chemical dosimeter. Irradiated samples were stored at -70°C for 2- 9 days post-irradiation before analysis. This experiment was repeated once, with the inclusion of triplicate samples of retinal tissue homogenate.

3.2.6 Receptor Assays

Irradiated (or control) membrane samples were thawed at room temperature (23°C), diluted into 8- 10 mL of assay buffer (50 mM Tris-HCl, pH 7.4) and homogenised by polytron (5 sec, setting 3). [¹²⁵I]MEL was prepared and used in the concentration range 0.2- 0.8 nM for melatonin receptor assays as described above (Section 2.2.8.1) using 0.4 mL aliquots of tissue (0.1- 0.2 mg protein) per assay tube.

[¹²⁵I]iodocyanopindolol ([¹²⁵I]CYP) (= 2,000 Ci/mmol) was obtained from New England Nuclear and used to label β-adrenoceptors (Engel et al., 1981) in hamster hypothalamic membranes. Tissue aliquots (0.4 mL) in 50 mM Tris-HCl, pH 7.4 (37°C) were incubated with [¹²⁵I]CYP (in the range 25- 50 pM) in the absence or presence of 10 μM L-propranolol for 1 hr at 37°C. Samples were then filtered over Schleicher & Schuell #32 glass fibre filters and washed with 4 x 4 mL ice-cold 50 mM Tris-HCl, pH 7.4 (4°C). All samples were assayed in quintuplicate for both [¹²⁵I]MEL and [¹²⁵I]CYP. Radioactivity was determined using a Searle γ-counter at 71% counting efficiency.

3.2.7 Enzyme Assays

Irradiated (or control) enzyme samples were thawed at room temperature and then transferred to ice until used. All enzyme assays were carried out at room temperature (23°C). Aliquots (25 μL) of enzyme solution were added to 2.97 mL of assay solution and kinetics monitored using a Perkin-Elmer Lambda 4B UV/VIS spectrophotometer. All samples were assayed in quintuplicate.

Alcohol dehydrogenase and lactate dehydrogenase activities were determined by recording changes in A_{340} due to changes in the NAD^+/NADH concentration ratio (Das, 1980). RNase activity was determined by monitoring increases in A_{286} upon hydrolysis of cyclic cytidine 2':3'-monophosphate (Crook et al., 1960). Carboxypeptidase A activity was monitored by recording the decrease in A_{330} upon hydrolysis of 0.2 mM N-(3-[2-furyl]acryloyl)-L-phenylalanine-L-phenylalanine (Peterson et al., 1982). Alkaline phosphatase activity was followed by recording the increase in A_{405} upon hydrolysis of p-nitrophenyl phosphate (2 mM) in 0.1M Na_2CO_3 , pH 10.1 (at 23°C) containing 5 mM

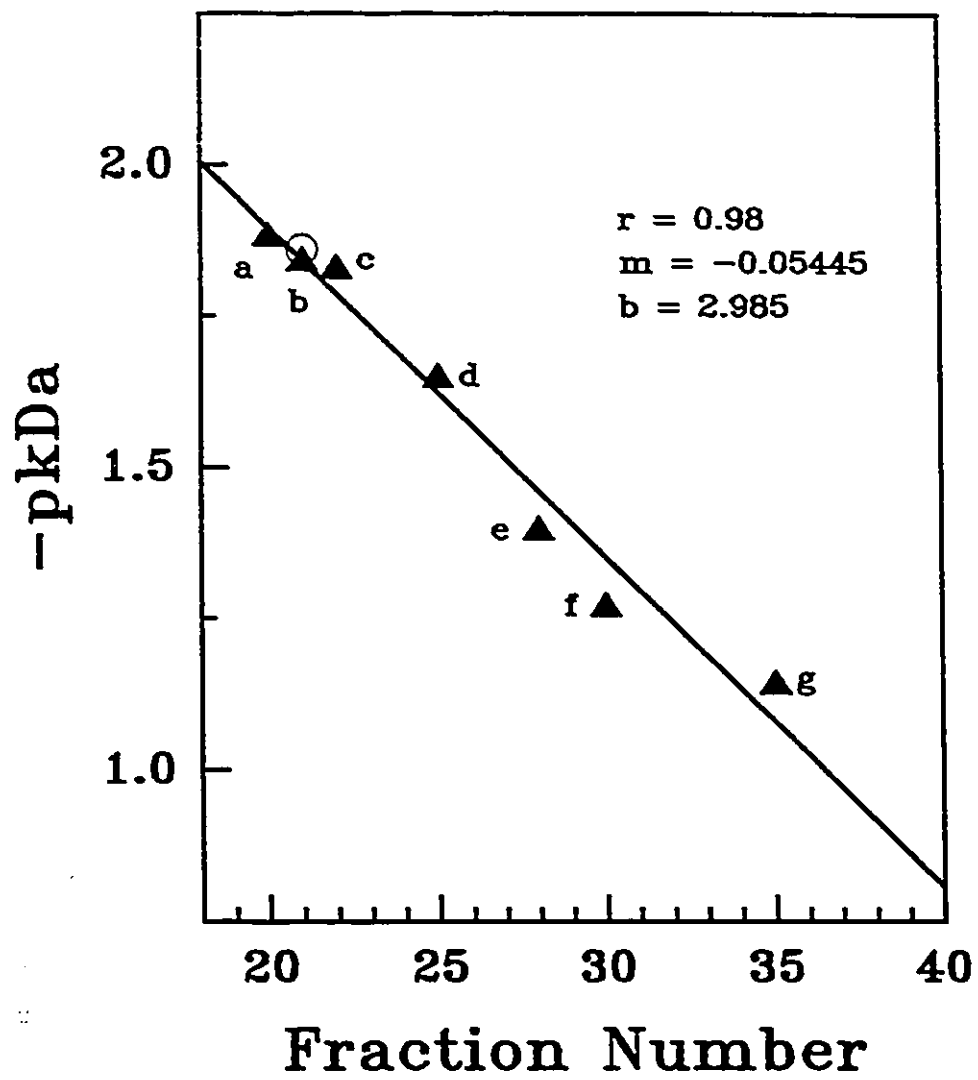
MgCl₂ (Engström, 1964). α -Amylase activity was determined by the hydrolysis of soluble starch using a modification of the assay described by Fischer and Stein (1961). Enzyme (25 μ L) was added to 225 μ L of 5% (w/v) soluble starch in 0.1 M NaHPO₄, 50 mM NaCl, 0.1 mM CaCl₂, pH 5.9 (at 23°C). After 8 min, 250 μ L of 3,5-dinitrosalicylate stop solution is added. The tube is then boiled 5 min, diluted with 4.5 mL water, centrifuged 5 min at 2,800 x g and a 1 mL aliquot of the supernatant diluted into 2 mL of water. The A₄₇₃ of the solution is used as a measure of enzymatic activity.

3.3 Results

3.3.1 Electrophoresis

The target-size enzyme standards were found to be homogeneous by one dimensional SDS-PAGE as indicated by Coomassie Blue staining. The apparent molecular weight of each enzyme corresponded to the accepted subunit value in all cases: calf intestine alkaline phosphatase [E.C. 3.1.3.1], 62 kDa (Hua et al., 1986); bovine pancreatic ribonuclease [E.C. 3.1.4.22], 14 kDa (Blackburn and Moore, 1982); rabbit muscle lactate dehydrogenase [E.C. 1.1.1.27], 35 kDa (Sass et al., 1989); horse liver alcohol dehydrogenase [E.C. 1.1.1.1], 40 kDa (Pietruszko, 1982); bacillus subtilis α -amylase [E.C. 3.2.1.1], 48 kDa (Stein and Fischer, 1961); bovine pancreatic carboxypeptidase A [E.C. 3.4.17.1], 34 kDa (Bradshaw et al., 1969).

Figure 20. Sephadex G-100 gel filtration molecular weight standard curve. Standards: a. bovine transferrin ($M_r = 77,000$); b. porcine pepsin ($M_r = 70,000$); c. bovine serum albumin ($M_r = 66,000$); d. chicken ovalbumin ($M_r = 45,000$); e. bovine α -chymotrypsin ($M_r = 25,000$); f. horse myoglobin ($M_r = 18,800$); g. bovine ribonuclease ($M_r = 14,000$). \circ , rabbit muscle lactate dehydrogenase.



3.3.2 Gel Filtration

Gel filtration molecular weight standard proteins were eluted from the Sephadex G-100 column in 50 mM Tris-HCl pH 7.4 (4°C) and the peak fraction of the elution profile taken as the elution volume. A linear ($r = 0.98$) calibration curve was constructed of elution fraction vs molecular size, -pKDa (Fig. 20). From this calibration curve, the molecular size of lactate dehydrogenase was found to be 69 kDa (dimeric size) under the buffer conditions used for target-size analysis.

3.3.3 Irradiation of Enzyme Standards

Plots of $\ln(A/A_0)$ vs apparent radiation dose were linear (Fig. 21), having correlation coefficients ≥ 0.96 in all cases. A \bar{D}_{37} value for each enzyme was calculated by linear regression of the data and was then used to create a linear ($r = 0.94$) molecular mass calibration curve of M_r vs $1/\bar{D}_{37}$ (Fig. 22).

3.3.4 Receptor Target-Size Determination

Binding of [125 I]MEL was used in hamster hypothalamus and chick retina as a measure of inactivation of the low-affinity melatonin binding site and high-affinity receptor, respectively. Linear ($r = 0.98$) inactivation plots were obtained (Fig. 23) and \bar{D}_{37} values determined as described above. From the calibration curve, the molecular size of the low-affinity hypothalamic binding site was found to be 30 ± 4 kDa ($n = 4$) while the high-affinity retinal receptor was 44 ± 9 kDa ($n = 3$) (Table 5). As an internal control, [125 I]CYP was used to measure inactivation of δ -adrenoceptors in hamster hypothalamus (Fig. 23) and a target size of 48 ± 8 kDa ($n = 4$) was found (Table 5).

Figure 21. Radiation inactivation of enzyme standards. Frozen samples were irradiated at -45°C and assayed as described in Methods. \circ , ribonuclease; \bullet , carboxypeptidase A; \square , α -amylase; \blacksquare , alkaline phosphatase; ∇ , lactate dehydrogenase; \blacktriangle , alcohol dehydrogenase. $r > 0.96$ for all lines.

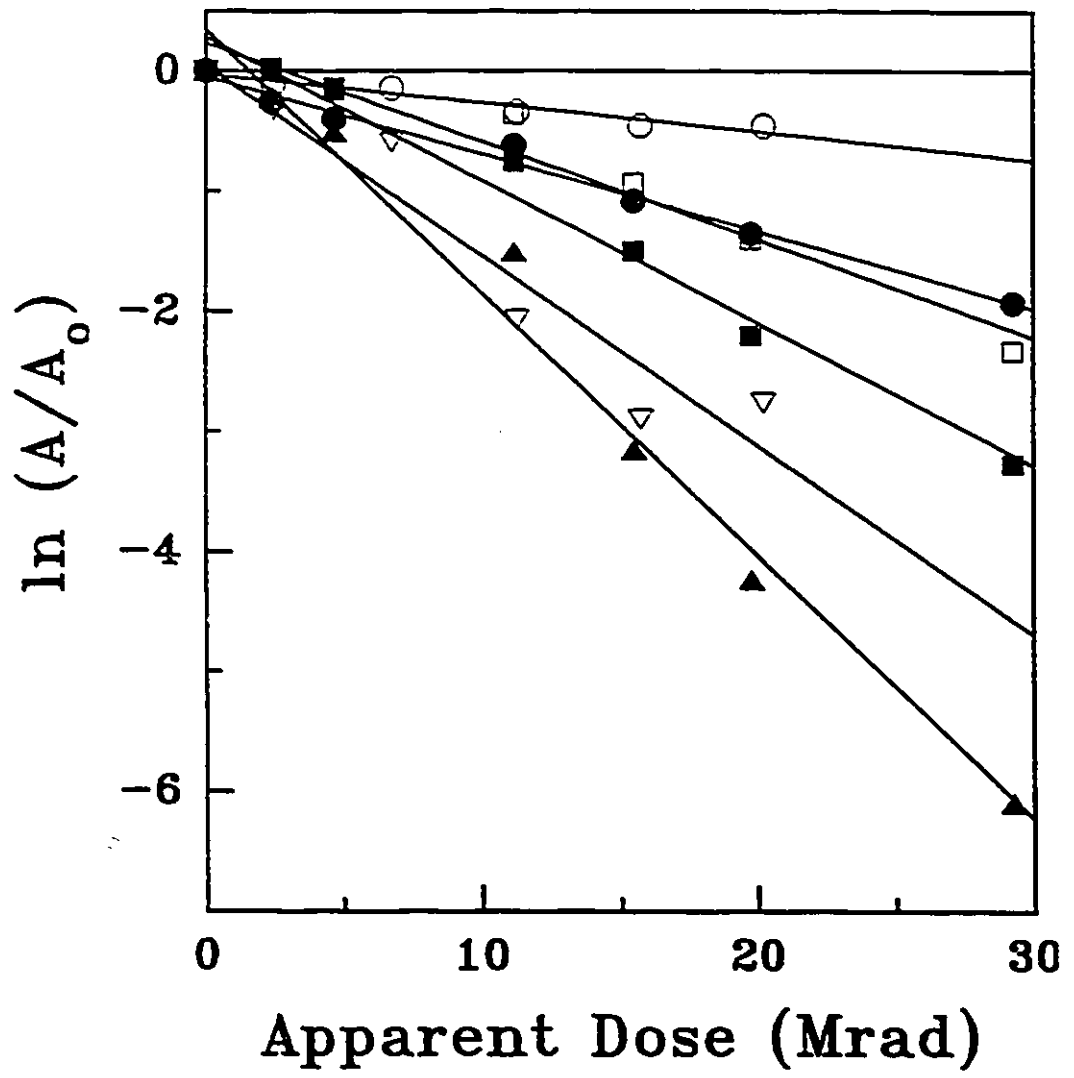


Figure 22. Molecular mass calibration curve. \bar{D}_{37} values were calculated for each enzyme as described in Methods. \circ , enzyme set for first experiment; ∇ , enzyme set for second experiment. All samples were analyzed using this composite calibration curve. ($r = 0.94$, $m = 0.00204$, $b = -0.01307$)

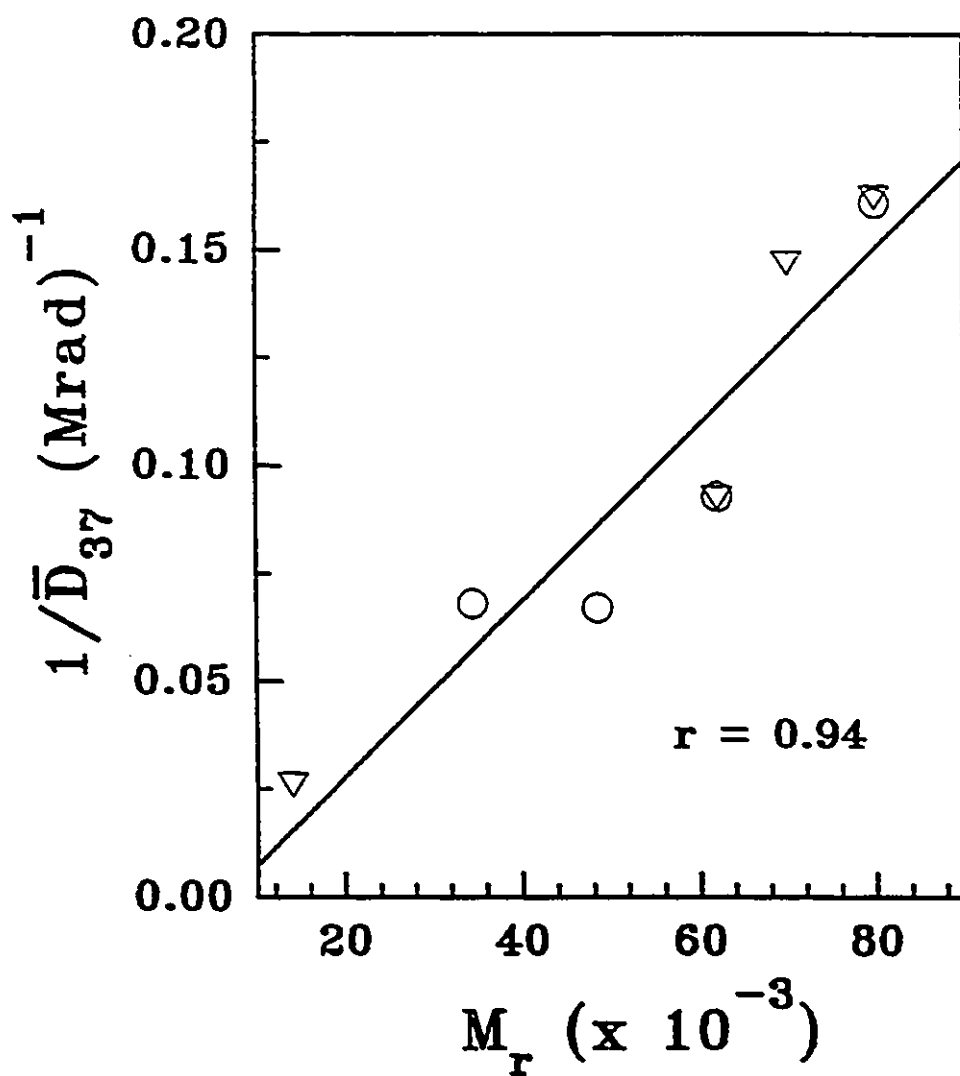


Figure 23. Radiation inactivation of melatonin receptors and β -adrenoceptors. Frozen tissue homogenates were inactivated at -45°C and assayed as described in Methods. Presented are data from one set of samples assayed in quintuplicate; $r = 0.98$ for each line. Hamster hypothalamus: \circ , low-affinity melatonin binding site; \bullet , β -adrenoceptors. Chick retina: \square , high-affinity melatonin receptor.

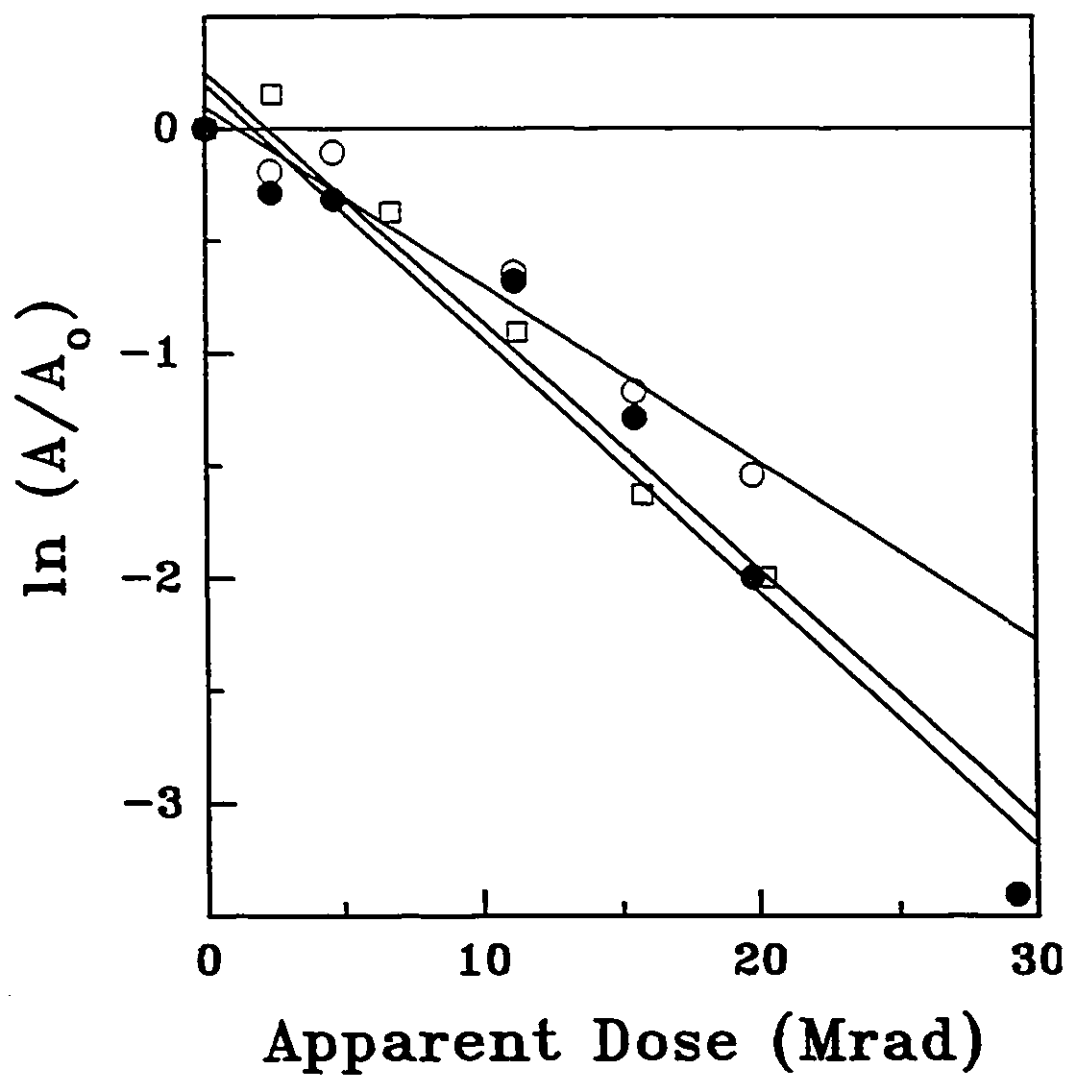


Table 5. Molecular mass of melatonin receptors and δ -adrenoceptors.

Mass (kDa)					
<u>Melatonin Receptor</u>				<u>δ-Adrenoceptor</u>	
Hamster Hypothalamus		Chick Retina		Hamster Hypothalamus	
Standard curve	K-M [†]	Standard curve	K-M	Standard curve	K-M
30 [*] ± 4	31 [*] ± 6	44 ± 9	50 ± 12	48 ± 8	55 ± 10
(4)	(4)	(3)	(3)	(4)	(4)

Means ± S.D. are presented for (n) experiments conducted in quintuplicate. [†] Calculated using the Kepner-Macey equation (Kepner and Macey, 1968).

* p < 0.04 versus retinal melatonin receptor, using an unpaired t-test.

3.3.5 Alternative Molecular Mass Calculation

Many investigators use the empirical equation developed by Kepner and Macey (1968):

$$M_r = 6.4 \times 10^{11} / D_{37} \quad (4)$$

to calculate the target size. Using this equation, the following molecular mass values were obtained (Table 5): hamster hypothalamic melatonin binding site, 31 ± 6 kDa ($n = 4$); hamster hypothalamic δ -adrenoceptor, 55 ± 10 kDa ($n = 4$) and chick retinal melatonin receptor, 50 ± 12 kDa ($n = 3$).

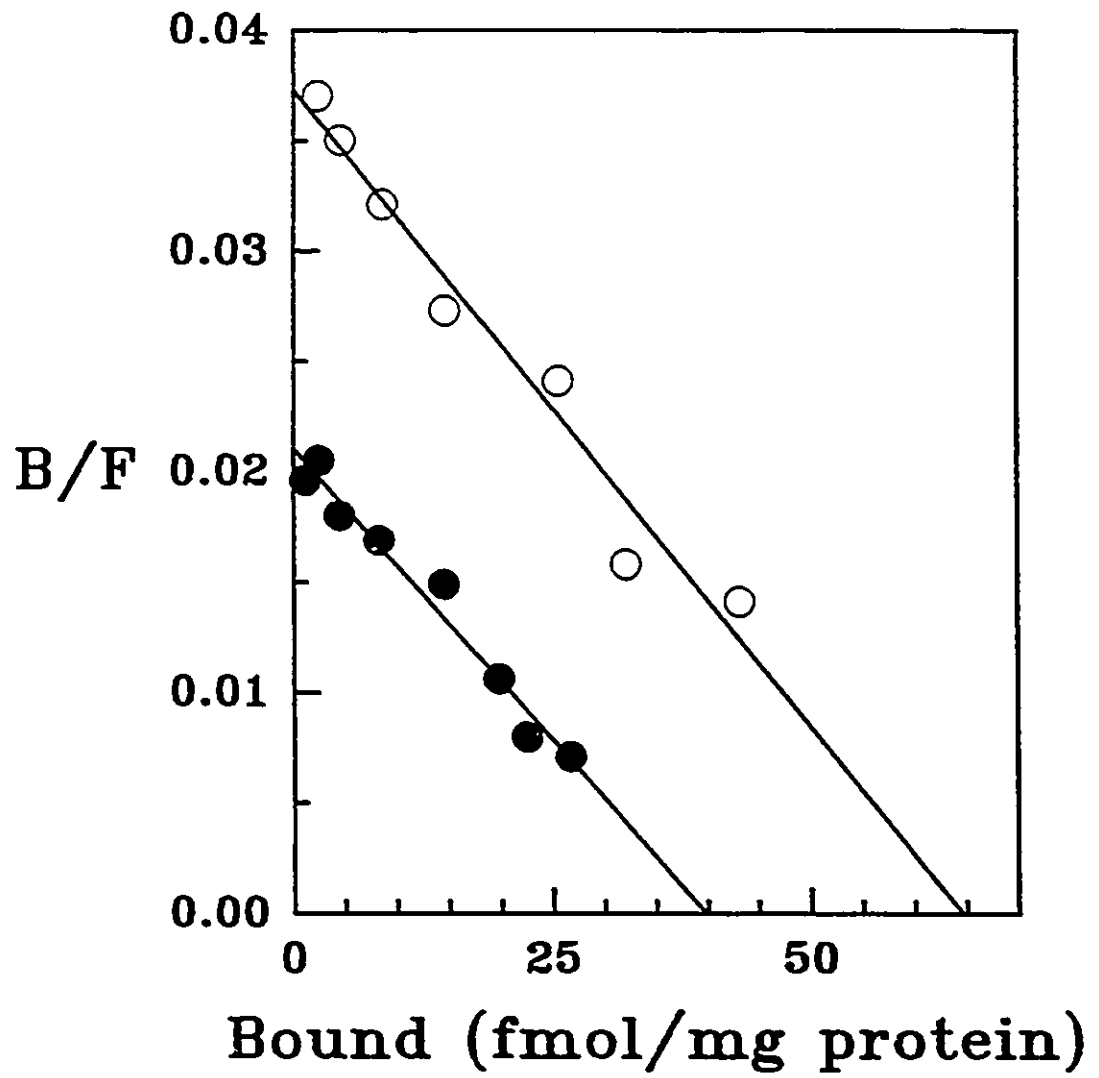
3.3.6 Scatchard-Rosenthal Analysis of Irradiated Tissue

A frozen sample of hamster hypothalamus homogenate was exposed to an apparent dose of 11.3 Mrad of radiation. Melatonin binding was quantitated as described above using 0.12- 5.5 nM [125 I]MEL and the data evaluated by Scatchard-Rosenthal analysis (Scatchard, 1949; Rosenthal, 1967) (Fig. 24). The control (unirradiated) tissue and irradiated tissue both had similar dissociation constants for [125 I]MEL: $K_d = 3.1$ nM and $K_d = 3.7$ nM, respectively. The number of binding sites (B_{max}) in the irradiated tissue (40 fmol/mg protein) was 61% of the control value (65 fmol/mg protein).

3.4 Discussion

In the preparation of tissue homogenates care was taken to include a variety of protease inhibitors to minimise possible proteolysis of the melatonin receptor and δ -adrenoceptors and all steps were performed on ice. Ascorbic acid (an antioxidant) and BSA were included in the sample buffer as a measure to protect samples from chemical inactivation by reactive species (e.g.

Figure 24. Effects of radiation inactivation on saturation binding of 2-[¹²⁵I]iodomelatonin (0.12- 5.5 nM) to hamster hypothalamus whole homogenate. Samples were prepared, irradiated and assayed as described in Methods using 0.44- 0.49 mg protein per tube. ○, unirradiated control tissue; ●, irradiated (11.3 Mrad apparent dose) tissue. Control: $K_d = 3.1$ nM, $B_{max} = 65$ fmol/mg protein, $r = 0.98$. Irradiated: $K_d = 3.7$ nM, $B_{max} = 40$ fmol/mg protein, $r = 0.99$. The data were analyzed as described by Rosenthal (1967) and the means of quintuplicate values from one experiment are presented.



H₂O₂, OH⁻) which are produced by the action of ionising radiation on the solvent molecules and dissolved molecular oxygen. Storage of the irradiated samples for several days post-irradiation at -70°C also allows time for neutralisation of any reactive species which have been formed. Inactivation plots of the enzyme standards used, as well as of the receptors, were linear in all instances ($r \geq 0.96$) as predicted by the single-target, single-hit theory. This suggests that the protective measures employed were effective.

While the linear nature of the inactivation plots signifies that these targets are behaving according to target theory, a necessary control experiment involves verification of the inactivation of the melatonin receptor by Scatchard-Rosenthal analysis. Again, according to the single-target, single-hit theory, one 'hit' is supposed to completely inactivate the target. Supporting this, the receptor number (B_{max}) of irradiated tissue homogenate was found to be 61% that of the control value, while the affinity of the receptors for [¹²⁵I]MEL was not appreciably changed. These results demonstrate that what is actually being measured is a loss of target molecules (i.e. complete functional inactivation) as assumed by target theory.

In order to obtain an estimate of the target size of the melatonin receptor, the approach of Lai et al. (1987) was chosen whereby a molecular mass calibration curve is used to determine the size of an unknown target molecule. The enzyme standard LDH was found to give a target size of ≈ 70 kDa in these studies, indicating a dimeric structure. Radiation inactivation of lyophilised LDH has previously yielded a monomeric target size (Fluke and Hochachka, 1965) whereas the in vivo form of the enzyme is tetrameric (Markert, 1963). However, gel filtration chromatography verified that under the current assay conditions, LDH has a dimeric molecular size.

A criticism of the external standard method of target-size analysis, as applied to receptors, is that soluble enzymes may not present the same type of target as membrane-bound proteins. The protein environments are vastly different in the two cases and possible effects of a lipid environment upon target size cannot be discounted offhand. Therefore, in order to validate the use of this procedure, the δ -adrenoceptor of hamster hypothalamus was used as an internal control since this target should have a similar environment as the melatonin low-affinity binding site.

The molecular mass of the hamster δ_2 -adrenoceptor is 46 kDa as deduced from a cDNA clone (Dixon et al., 1986) or 49 kDa as obtained with purified, deglycosylated receptor (Stiles et al., 1984). The hamster δ_1 -adrenoceptor has not yet been cloned or purified, however, a cDNA clone of the rat δ_1 -adrenoceptor gave a deduced molecular weight of 50.5 kDa (Machida et al., 1990). The radioligand used to detect δ -adrenoceptors in the present experiments ($[^{125}\text{I}]\text{CYP}$) does not distinguish between subtypes (Engel et al., 1981) so that both δ_1 and δ_2 subtypes would be labelled by this ligand. L-propranolol, which is used to define nonspecific binding, also does not discriminate between subtypes. $[^{125}\text{I}]\text{CYP}$ has been demonstrated as well to label 5-HT_{1B} receptors in rat brain (Hoyer et al., 1985). The affinity of the radioligand for this site ($K_d = 230$ pM) is about one order of magnitude less than its affinity for δ -adrenoceptors (Engel et al., 1981). In the present experiments, at the concentrations of $[^{125}\text{I}]\text{CYP}$ used (25- 50 pM), one would not anticipate significant labelling of 5-HT_{1B} receptors.

The ratio of δ_1 : δ_2 adrenoceptors in rat forebrain and hippocampus is 4:1 (Minneman et al., 1979). Assuming a similar situation in the hamster hypo-

thalamus, a weighted average δ -adrenoceptor molecular mass of 49.6 kDa would be anticipated by using [125 I]CYP to label these sites. The target size determined here for the δ -adrenoceptor (48 kDa) agrees very well with this value. Although it is not presently known if the hamster contains the newly discovered δ_3 subtype, the molecular weight of this receptor in the human (as deduced from a cDNA clone) is 42.9 kDa (Emorine et al., 1989) which is similar in size to the other subtypes. Therefore, the target-size results obtained with the δ -adrenoceptor further strengthen the data obtained on the low-affinity melatonin binding site and melatonin receptor, suggesting that the molecular sizes reported are indeed valid.

The target size of the melatonin receptor in the chick retina was found to be 44 ± 9 kDa. This is significantly larger (47% , $p < 0.04$) than the hamster hypothalamic low-affinity binding site. Solubilised high-affinity melatonin receptors (Ying and Niles, 1991) have been estimated to have a molecular size of 46 ± 5 kDa ($n = 4$) for the chick brain receptor and 52 ± 5 kDa ($n = 3$) for the chick retinal receptor by Sephadex G-100 (and S-300) gel filtration chromatography (private communication, Dr. S.-W. Ying). These values are in close agreement with the value found in the present work in the retina. Interestingly, N-bromoacetyl-2- [125 I]iodo-5-methoxytryptamine has recently been used to covalently and specifically label proteins of molecular sizes 92, 55 and 45 kDa in rat brain as estimated by SDS-PAGE analysis (Laudon and Zisapel, 1991). The molecular mass values calculated from the target-size calibration curve were also in close agreement with the values calculated using the Kepner-Macey equation. The size calculated for the melatonin low-affinity binding site in hamster hypothalamus is 30 ± 4 kDa. From this value, using equations 5 and 3, it

can be calculated that a radiation dose of 11.3 Mrad received by the tissue used in Scatchard-Rosenthal analysis should result in a loss of 41% of B_{max} . The experimentally observed decrease in B_{max} is 39% which agrees well with this calculated value.

The finding of different target sizes for the low-affinity melatonin binding site of hamster brain and the high-affinity site of chicken retina agrees with the pharmacological data (Chapter 2) where different pharmacological profiles were seen in the two tissues, suggesting that the two sites are distinct proteins. Interestingly, the molecular sizes found for these sites are in the same range as the target sizes reported for several of the serotonin receptor subtypes (Ferry et al., 1988). Considering the structural similarity of melatonin and serotonin, one might expect some structural similarity between the receptors. In particular, one or more of the 5-HT₁ receptor subtypes may share considerable structural homology to the melatonin receptor since all can be coupled to inhibition of adenylate cyclase (Yocca and Maayani, 1990). Evidence for this hypothesis awaits the purification and/or cloning of the melatonin receptor as has been accomplished for some of the serotonin receptor subtypes (Hartig, 1989).

CHAPTER FOUR

FUNCTIONAL CHARACTERISATION OF THE LOW-AFFINITY
MELATONIN BINDING SITE

4.1 Introduction

The high-affinity melatonin receptor of mammalian pars tuberalis, chicken retina and brain is coupled to inhibition of adenylate cyclase [vide supra]. In addition, a physiological response may be correlated with this second messenger activity in at least two model systems: (1) antagonism of LHRH-induced gonadotropin release in the neonatal rat anterior pituitary [vide supra] and (2) inhibition of calcium-dependent dopamine release in retina. In contrast, the physiological and biochemical functions of the low-affinity melatonin binding site in hamster brain are unknown. In order to 'promote' this site to the status of a receptor, functionality must be demonstrated.

When assaying a receptor-mediated biochemical response, such as changes in adenylate cyclase activity, it is advantageous to use a cell population that is as homogeneous as possible to maximise detection of responses. If using tissue (e.g. brain) explants, a very heterogeneous cell population is obtained, all of which contain adenylate cyclase but only a small percentage of which may contain the receptor of interest (e.g. melatonin receptor). The consequence of this could then be a very large 'background' activity which could mask any specific changes in the activity being sought (i.e. a situation of a very low signal-to-noise ratio). This problem was particularly evident in the case of the high-affinity melatonin receptor which is very discretely localised in the mammalian brain and is coupled to *inhibition* of adenylate cyclase, necessitating stimulation of activity (with forskolin usually) to be able to observe its inhibition. In an attempt to circumvent this potential obstacle in the functional studies of the low-affinity melatonin

binding site. it was decided to try to obtain an in vitro continuous cell line expressing this site which could then be used in these biochemical studies. The Syrian hamster-derived melanoma cell line RPMI 1846 was discovered to contain this low-affinity site and was investigated for possible coupling to various known signal transduction pathways.

4.2 Materials and Methods⁶

4.2.1 Materials

Adult male Syrian hamsters (2- 3 months old) were obtained from Charles River, Quebec. Animals were housed in groups of six, on a 14hr L:10hr D cycle (lights-on at 7:00 a.m.), at 23- 27°C with free access to food (Purina Rat Chow) and water. [¹²⁵I]MEL was synthesised as described above (Section 2.2.2). Frozen RPMI 1846 cells (and other cell lines used in screening [vide infra]) were obtained from the American Type Culture Collection, MD. Tissue culture reagents and powdered medium were purchased from GIBCO BRL. [8-³H]adenine (23.5 Ci/mmol), [adenine-U-¹⁴C]adenosine 3',5'-cyclic phosphate ammonium salt (276 mCi/mmol), [2,8-³H]adenosine 3',5'-cyclic phosphate ammonium salt (30-50 Ci/mmol) and adenosine 5'-[α -³²P]triphosphate triethylammonium salt (= 3000 Ci/mmol, 1 mCi/mL) were obtained from Amersham. ⁴⁵CaCl₂ (11.3- 14.5 mCi/mg Ca; 19.8- 25.4 mCi/mL) and Ecolume and Universol scintillation cocktails were purchased from ICN Biomedicals, Inc., while myo-[2-³H]inositol (50 μ M; 20 Ci/mmol) and D-myo-[2-³H]inositol-1,4,5-trisphosphate (25 μ M; 1.0 Ci/mmol) were purchased from American Radiolabeled Chemicals, Inc. Rolipram was a gift of Schering AG. DOI-HCl was obtained from Research Biochemicals Inc. Dowex AG

⁶ Portions of this are in press (Pickering and Niles, 1991).

50W-X4 (200-400 mesh, hydrogen form) was from BioRad. Bovine pancreatic DNase I, creatine phosphokinase, creatine phosphate and Tris base were supplied by Boehringer Mannheim. BSA, DTT, alumina (activity grade I, type WN-3, neutral), adenine·HCl, turkey egg white trypsin inhibitor, EGTA, forskolin, bacitracin, cAMP (sodium salt), GTP (sodium salt) type III, ATP (sodium salt) grade I, sodium formate, $\text{LaCl}_3 \cdot 7 \text{H}_2\text{O}$, ammonium formate, Dowex-1 AG 1-X8 (100- 200 mesh, formate form) and percoll were obtained from Sigma Chemical Co. Bromophenol blue, LiCl, $\text{EDTA} \cdot \text{Na}_2$, formic acid and trichloroacetic acid were obtained from BDH Chemicals. $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10 \text{H}_2\text{O}$ was from Baker Chemical Co. Other chemicals and reagents were from Sigma or similar commercial suppliers.

4.2.2 Cell Screening

Crude membranes were prepared from cell suspensions by homogenisation (polytron, setting #5, 2 x 10 sec, 4°C) in 50 mM Tris·HCl, pH 7.4 (4°C) followed by centrifugation 10 min at 39,000 x g (at 4°C). Pellets (crude membranes) were washed 2- 4 times by resuspension in fresh buffer and centrifugation. Membranes were used in [^{125}I]MEL binding assays as described above (Section 2.2.8) using a range of radioligand concentrations from ≈ 0.02 - 3 nM.

4.2.3 Cell Culture

RPMI 1846 cells were grown on 10 cm Falcon disposable plastic dishes in filter (0.20 μm)-sterilised complete medium [DMEM/F-12 (1:1) medium containing: 5% calf supreme serum, 1% antibiotic/antimycotic (100 units penicillin G/mL, 100 μg streptomycin/mL, 250 ng amphotericin B/mL), 30 mM HEPES, 4.5 mg/mL D-glucose, 14.3 mM NaHCO_3 , 2.5 mM L-glutamine, 0.5 mM sodium

pyruvate, phenol red, pH 7.4 (by NaOH)] in a humidified, 2% CO₂/air, 37°C incubator. A doubling time of approximately 1.5- 2 days was routinely observed. Confluent cells were subcultured using 0.05% trypsin - 0.53 mM EDTA in PBS.

4.2.4 Cell Harvesting

RPMI 1846 cells were harvested by aspiration of the culture medium, addition of 3 mL Dulbecco's PBS + 10 mM EDTA, pH 7.4 (PBS/EDTA) and incubation for = 30 min at 37°C. Detached cells were collected into 50 mL centrifuge tubes, rinsing the plates with a small volume of PBS if necessary. After centrifugation for 5 min at = 300 x g (4°C), the supernatant was discarded. Cell pellets were washed twice by resuspension in PBS (without EDTA) by gentle vortexing and centrifugation. The final, washed pellet of cells was then used for the preparation of membranes or for functional studies.

4.2.5 Membrane Preparation

Crude membranes of RPMI 1846 cells were prepared following the method of Gerst et al. (1986). All steps were carried out at 4°C. Cell pellets were lysed by polytron (setting #5, 2 x 10 sec) in 2 mL/dish of lysis buffer (1 mM NaHCO₃, 1 mM DTT, 0.2 mM MgCl₂, 0.1 mg/mL bacitracin, 0.1 mg/mL turkey egg white trypsin inhibitor, 0.05 mg/mL DNase I, pH 7.0). After incubation for 30 min on ice, the lysis solution was layered onto a cushion of 40% sucrose + 1 mM NaHCO₃ + 1 mM DTT, pH 7.0 and centrifuged at 103,000 x g for 45 min. Crude membranes were collected as a cream-coloured band, free of melanin contamination, at the 0- 40% sucrose interface then diluted with 50 mM Tris-HCl, pH 7.4 and centrifuged at 48,000 x g for 10 min. The membrane pellet was washed once more in this buffer

and then resuspended and used in radioligand binding experiments or adenylate cyclase assays.

4.2.6 [¹²⁵I]MEL Binding Assay

Saturation and inhibition experiments were carried out at 0°C as detailed above (Section 2.2.8) using 0.1- 0.5 mg RPMI 1846 membrane protein per assay tube. Protein concentrations were measured by the method of Lowry et al. (1951) using bovine serum albumin as the standard.

4.2.7 Cyclic AMP Experiments

4.2.7.1 Adenylate Cyclase Assay

Membrane adenylate cyclase activity was measured as previously described (Niles and Hashemi, 1990). Membrane aliquots containing 30- 200 µg RPMI 1846 membrane protein were incubated with drugs at 30°C in a reaction mixture containing: 75 mM Tris-HCl, pH 7.4 (at 30°C), 4 mM MgCl₂, 0.1 mM GTP, 1 mM EGTA, 1 mM DTT, 1 mg BSA/mL, 0.5 mM cAMP, 0.5 mM ATP, 0.5- 1 µCi [α -³²P]ATP (= 3,000 Ci/mmol), 5 mM creatine phosphate, 20 units creatine phosphokinase, 10 µM rolipram (in place of IBMX or theophylline) in the presence or absence of 25 µM forskolin in a final volume of 150 µL. Incubation times from 15- 30 min were utilised. Melatonin solutions were prepared in assay buffer and added to the reaction solution prior to membrane addition which was done last to initiate the reaction. Drugs were initially dissolved in DMSO such that the final solvent concentration was always less than 0.3% which had no effect on adenylate cyclase activity. Reactions were terminated by the addition of 150 µL 4 mM cAMP and boiling for 5 min. [³H]cAMP (= 3- 5 x 10⁴ DPM) was added to each sample as a

recovery marker. Samples were centrifuged at 12,000 x g in a refrigerated (4°C) Mikro Rapid centrifuge. [³²P]cAMP formed was isolated by sequential chromatography on Dowex AG-50 and neutral alumina columns (Salomon et al., 1974). Samples were counted in 12 mL Ecolume cocktail using a Beckman LS 7500 scintillation counter. Protein concentrations were measured by the method of Lowry et al. (1951) using BSA as standard. CPM data were corrected for column recovery and converted to pmol cAMP/min/mg protein using a programme written for the HP-11C calculator (see Appendix I).

4.2.7.2 [³H]Adenine Prelabelling

RPMI 1846 cells were harvested as described above (Section 4.2.4) in 10 mL siliconised glass tubes, resuspended finally in HBSS containing 30 mM HEPES, pH 7.4 (37°C) (H/H buffer) by gentle vortexing to give approximately 10⁷ cells/mL and kept on ice until needed. Prelabelling was carried out using a modification of the protocol of Gerst et al. (1986).

[³H]Adenine was isotopically diluted to a specific activity of 1- 2 Ci/mmol in some experiments but was typically used at 11.75 Ci/mmol. It was added to the ice-cold cell suspension to give a concentration of 0.50 μM. Three 50 μL aliquots of the suspension were immediately removed and added to 50 μL of 20% TCA (w/v)/0.2% TX-100 to provide 'zerotime uptake' samples ([³H]cAMP background). The suspension was incubated at 37°C for 15 min (except in kinetic studies) and then transferred to ice. Two 50 μL aliquots of cells were removed and one aliquot was added directly to scintillation cocktail to determine the total tritium present. The second aliquot was centrifuged 1 min at 1,000 x g (4°C) in a refrigerated microfuge. Radioactivity in 25 μL of supernatant was determined and the percentage uptake of [³H]adenine calculated. Uptake was

usually 40- 80% of the total added. The cell suspension was then centrifuged 5 min at 300 x g (4°C), the supernatant decanted and the pellet resuspended in an equal volume of ice-cold H/H buffer (containing 100 µM rolipram in most experiments). The cells were aliquoted (50 µL) into 12 x 75 mm glass test tubes and kept on ice until needed. Cell viability was generally greater than 80% as judged by the trypan blue dye-exclusion test (Fry, 1981).

4.2.7.2.1 [³H]cAMP Measurements

Prior to the addition of 50 µL of drugs (in H/H at 37°C) to the cells, they were transferred to a 37°C water bath for about 30 sec. After incubation at 37°C for 15- 30 min, 0.1 mL 20% TCA/0.2% TX-100 was rapidly added and the tube vortexed. To each tube was added 750 µL of 10% TCA containing ≈ 2,500 DPM [¹⁴C]cAMP as recovery marker. [³H]- and [¹⁴C]cAMP were isolated by the method of Maurice and Haslam (1990) (using 10% TCA) and counted in 12 mL Ecolume cocktail using a Beckman LSC 3501 scintillation counter programmed for ³H/¹⁴C dual label counting.

4.2.8 Calcium Flux Experiments

4.2.8.1.1 Synaptosome Preparation

Synaptosomes were prepared from male (1- 3 months old) Syrian hamster forebrains by the method of Dunkley et al. (1986). Briefly, a normal P2 membrane preparation was made (Section 2.2.6) and gently resuspended in centrifugation buffer (0.32 M sucrose, 1 mM EDTA, 0.25 mM DTT, pH 7.4 [by NaOH]) to give 3.5- 4

mg protein/mL⁷. One volume of this suspension was carefully layered on top of a step gradient of 3%/10%/15%/23% percoll in centrifugation buffer (1 volume each). Following centrifugation at 32,500 x g for 5 min (4°C), synaptosomes were obtained as a white, fluffy band at the 15%/23% percoll interface. Synaptosomes were diluted = 20- fold with ice-cold centrifugation buffer and centrifuged at 48,000 x g for 10 min (4°C). Decanting this supernatant removed most of the percoll. The synaptosome pellet was slowly diluted (10- 15 min) with = 20 volumes of ice-cold low K⁺/low Ca²⁺ (LK/LCa) buffer (145 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 10 mM D-glucose, 0.02 mM CaCl₂, 10 mM HEPES, pH 7.5 (23°C) with Tris base) followed by centrifugation at 39,000 x g for 10 min (4°C). The pellet was then resuspended by gentle vortexing in LK/LCa buffer and kept on ice until used in calcium flux studies.

4.2.8.1.2 Calcium Flux in Synaptosomes

Calcium uptake was assayed according to the method of Harris and Bruno (1985). Synaptosomes in LK/LCa buffer were aliquoted (100 µL containing 100-200 µg protein) into 12 x 75 mm glass test tubes and kept on ice. Tubes were preincubated 10 min at 30°C with or without drugs prior to initiation of uptake which was commenced by the addition of 100 µL ⁴⁵Ca²⁺ (= 0.5 µCi) in LK/LCa buffer (control) or in high potassium buffer (HK/HCa) [5 mM NaCl, 145 mM KCl, 1 mM MgCl₂, 10 mM D-glucose, 10 mM HEPES, pH 7.5 (23°C) with Tris base], prewarmed to 30°C. This gave a final, depolarising concentration of 75 mM KCl. The final calcium concentration was = 130 µM, depending upon the specific activity of the radionuclide. After incubation for two min (except in kinetic studies) uptake

⁷ Approximately 1% of the initial wet tissue mass is recovered as protein in the normal P2 membrane preparation.

was terminated by filtration of the synaptosomes onto 0.45 μm nitrocellulose (Millipore HAWP) filters and washing 4 x 5 mL with ice-cold PBS + 10 mM EDTA, pH 7.4 (4°C). The filter was transferred to a 5 mL scintillation vial and 4 mL Ecolume cocktail added. Samples were equilibrated overnight at room temperature before counting in a Beckman LS 7500 scintillation counter.

4.2.8.2 Calcium Flux in RPMI 1846 Cells

RPMI 1846 cells were grown (Section 4.2.3) in 24-well culture dishes and assayed as monolayers or were harvested from 10 cm dishes (Section 4.2.4) and used as a cell suspension. Cell viability was > 80% for cell suspensions as judged by the trypan blue dye exclusion test (Fry, 1981).

4.2.8.2.1 Monolayer Assay

The medium was gently aspirated from each well which was then washed with 2 mL 37°C PBS and aspirated. One mL of calcium-free⁸ H/H buffer was added and the 24-well plate incubated at 37°C = 30 min. Uptake was initiated by aspiration of the buffer and the addition of 200 μL 37°C H/H buffer containing 2 mM CaCl_2 (1- 2 μCi $^{45}\text{Ca}^{2+}$) with or without drugs. After incubation for 30 min (except in kinetic studies) at 37°C, uptake was terminated by the addition of 2 mL ice-cold stop solution (145 mM NaCl, 5 mM KCl, 5 mM LaCl_3 , pH 7.4 with NaOH) and aspiration. Each well was washed 2 x 2 mL with ice-cold stop solution and aspiration. To each well 0.4 mL 10% TX-100 was then added. After 0.5- 1 h lysis at room temperature, the lysis solution was transferred to a 5 mL scintillation vial and 4 mL Ecolume cocktail added. Samples were counted as described above.

⁸ No added calcium.

4.2.8.2.2 Cell Suspension Assay

RPMI 1846 cells were harvested in siliconised tubes as described above and washed once in calcium-free⁸ H/H buffer by resuspension and centrifugation (5 min at 300 x g [4°C]). Cells were then resuspended in 1- 2 mL fresh buffer (per 10 cm culture dish), aliquoted (50 µL) into 12 x 75 mm plastic test tubes and kept on ice until needed. Tubes were transferred to a 37°C water bath prior to uptake which was initiated by the addition of 50 µL H/H buffer containing 2 mM CaCl₂ (= 0.5- 1 µCi ⁴⁵Ca²⁺) ± 20 µM melatonin, prewarmed to 37°C. At various times (0- 60 min) samples were filtered onto 0.45 µm HAWP filters, washed and counted as described above.

4.2.9 Inositol Phosphate Experiments

4.2.9.1 myo-[³H]inositol Prelabelling

Prelabelling was carried out using a modified version of the protocol of Slominski et al. (1989). RPMI 1846 cells were grown on 10 cm Falcon plastic culture dishes as described above (Section 4.2.3). At confluence, the medium was aseptically aspirated and the dishes rinsed with 10 mL sterile PBS followed by aspiration. To each dish was added 3 mL of H/H buffer containing = 10 µCi (= 0.17 µM) myo-[2-³H]inositol (20 Ci/mmol) which had been filter (0.20 µm)-sterilised. An aliquot of this solution was counted to determine the precise myo-inositol concentration. The dishes were incubated overnight (18- 24 h) at 37°C in a humidified culture incubator under an atmosphere of 2% CO₂/air. The medium was collected from each dish, combined and centrifuged 5 min at 300 x g. An aliquot of the supernatant was counted to determine tritium content. The percentage uptake of radiolabel was then calculated. Cells were harvested from

the culture dishes into siliconised tubes as detailed above (Section 4.2.4). The cell pellet was washed once by resuspension (gentle vortexing) in 10 mL PBS and centrifugation 5 min at 300 x g (4°C). The pellet was then resuspended in ice-cold modified⁹ H/H buffer (1- 1.5 mL/10 cm culture dish) to give $\approx 10^7$ cells/mL. The cells were kept on ice until needed.

4.2.9.2 Label Incorporation

To determine the extent of membrane labelling, 100 μ L of the cell suspension was added to 900 μ L 1% TCA (w/v) in an eppendorf tube. After 30 min lysis on ice, the tube was centrifuged 5 min at 12,000 x g (4°C). An aliquot (500 μ L) of the supernatant was counted to determine soluble radioactivity. The pellet was washed 2 x 1 mL with PBS by resuspension and centrifugation and then 1.2 mL Ecolume cocktail was added to the eppendorf tube. After overnight equilibration, the tube was counted to determine membrane radioactivity and the percentage label incorporation calculated.

4.2.9.3 Phosphoinositide Hydrolysis Assay

Cells were aliquoted (100 μ L containing 1- 1.5 x 10⁶ cells) into 12 x 75 mm plastic test tubes and kept on ice. Cells were preincubated 30 min at 37°C to allow intracellular equilibration with LiCl. Stimulation was initiated by the addition of 100 μ L drug solution dissolved in modified H/H buffer, prewarmed to 37°C. After incubation for various times (0- 120 min) at 37°C, the reaction was terminated by the addition of 750 μ L ice-cold 12.7% TCA (w/v) and vortexing. Samples were allowed to stand 30 min on ice (or overnight at 4°C) to lyse and equilibrate and were then neutralised by the addition of 50 μ L 0.64 M NaHPO₄ (pH

⁹ Modified H/H = Hanks' balanced salt solution containing 30 mM HEPES, pH 7.4 (by NaOH) with [NaCl] = 127 mM; [LiCl] = 10 mM.

7.0) + 560 μ L 1 M NaOH. (One set of buffer blanks was used to confirm complete neutralisation to pH 6- 7 using bromophenol blue pH indicator). Samples were then diluted with 3 mL distilled water, centrifuged 10 min at 3,000 x g (4°C) and inositol phosphates isolated by column chromatography.

4.2.9.4 Inositol Phosphate Column Chromatography

Inositol phosphates were eluted stepwise from a Dowex-1 column using the method developed by Berridge et al. (1983). Each sample was applied by pasteur pipette to a column of 1.25 mL Dowex-1 anion exchange resin (AG 1-X8, 100- 200 mesh, formate form) packed in a plastic disposable 10 mL serological pipette. Columns were calibrated using [3 H]I(1,4,5)P₃ standard and approximately 70% recovery obtained. Inositol phosphates were eluted as follows¹⁰:

3 x 5 mL distilled H₂O (removes [3 H]inositol)

2 x 5 mL Buffer #1 (elutes [3 H]glycerophosphoinositol)

4 x 5 mL Buffer #2 (elutes [3 H]IP₁ + [3 H]IP₂)

2 x 5 mL Buffer #3 (elutes [3 H]IP₃ + [3 H]IP₄)

The washings of buffer #2 were pooled, mixed and a 2 mL aliquot added to 4 mL Universol scintillation cocktail in a scintillation minivial. The two washings of buffer #3 were collected separately in 20 mL scintillation vials and 12 mL Universol added. After overnight equilibration samples were counted (15- 20 min each) in a Beckman LSC 3501 scintillation counter.

¹⁰ Buffer #1 = 60 mM sodium formate + 5 mM disodium tetraborate; Buffer #2 = 0.4 M ammonium formate + 0.1 M HCOOH; Buffer #3 = 1 M ammonium formate + 0.1 M HCOOH.

4.3 Results

4.3.1 Cell Types Screened

Several different types of cells were screened for specific [125 I]MEL binding (Table 6). Only the melanoma cell lines tested had specific binding. The Syrian hamster cell line RPMI 1846 was chosen for subsequent studies.

4.3.2 [125 I]MEL Binding

The melanin present in RPMI 1846 cells caused high non-specific binding of [125 I]MEL. To overcome this, crude RPMI 1846 membranes free of melanin were prepared as described above. Removal of the pigment increased the percentage of specific binding from $\approx 20\%$ (using whole cell homogenates) to $\approx 50\%$ (crude membranes). The temperature- and time-dependency of binding to RPMI 1846 membranes was examined in a single-point study (Table 7). Since the highest amount of specific binding was observed using incubation on ice (0°C), the assay conditions used for [125 I]MEL binding in hamster brain membranes (Section 2.2.8) were also employed for RPMI 1846 membranes. Scatchard-Rosenthal analysis of saturation binding data indicated a single class of binding sites for [125 I]MEL: $K_d = 0.89 \pm 0.08$ nM, $B_{\text{max}} = 6.2 \pm 2.9$ fmol/mg protein, ($n = 3$) (Fig. 25). Inhibition experiments with a series of indoles and other compounds revealed a similar rank ordering of affinities (Table 8) as seen in the Syrian hamster hypothalamus [vide supra]. A high correlation was noted between the K_i in hypothalamus and RPMI 1846 membranes: $r = 0.94$, $m = 0.93$, $p < 0.01$, $n = 14$, (Fig. 26). GTP (10^{-5} - 10^{-2} M) and NaCl (1- 300 mM) had no significant effects on [125 I]MEL binding (data not shown). An examination of the radioligand binding kinetics to RPMI 1846 membranes was made at 37°C in the same buffer system

(modified H/H) used for the phosphoinositide studies (Fig. 27). Negligible specific binding was detected under these assay conditions.

4.3.3 Cyclic AMP Studies

4.3.3.1 Membrane Adenylate Cyclase Activity

The concentration-dependent stimulation of adenylate cyclase activity by forskolin ($EC_{50} = 4.3 \mu\text{M}$) indicated the presence of a functional enzyme in RPMI 1846 membranes (Fig. 28). Inclusion of phosphodiesterase (PDE) inhibitors in the assay did not appreciably enhance basal or stimulated cAMP levels, however 10 μM rolipram was used routinely in the assays as a PDE inhibitor. Melatonin (10^{-10} - 10^{-5} M) did not alter either basal or 25 μM forskolin-stimulated adenylate cyclase activity (Fig. 29).

4.3.3.2 In Situ Cyclic AMP Measurements

The uptake kinetics of [^3H]adenine into RPMI 1846 cells was studied at 37°C (Fig. 30). Uptake was maximal by 20- 25 min and represented approximately 80% of the total radioactivity added. The time course for basal [^3H]cAMP formation closely paralleled that of adenine uptake reaching maximal values by 30 min [i.e. 5- 10 min after maximal uptake had occurred] (Fig. 31). A comparison of the basal and forskolin-stimulated [^3H]cAMP levels in the presence of rolipram vs IBMX is shown in Fig. 32. Rolipram was observed to be more potent and efficacious than IBMX in inhibiting PDE activity. In order to further optimise assay conditions, the time course for forskolin stimulation of [^3H]cAMP accumulation in the presence of rolipram was obtained (Fig. 33). Maximal cAMP levels were attained by 15 min at 37°C.

Table 6. Cell types investigated for specific binding of 2-[¹²⁵I]iodomelatonin.

Cell Line	Origin	Specific Binding ^a
CHO-K1	Chinese hamster ovary	-
Y-79	Human retinoblastoma	-
MCF-7	Human breast tumour	-
Blood cells	Chick blood	-
RPMI 1846	Syrian hamster melanoma	+
FF	Hamster melanoma ^b	+
M-6	Human melanoma ^c	+

^a +, detected; -, not detected.

^b A virally transformed RPMI 1846 cell line.

^c Personal communication, Dr. S.-W. Ying.

Table 7. Specific 2-[¹²⁵I]iodomelatonin binding in RPMI 1846 membranes at various temperatures and incubation times.

Temperature (°C)	Incubation Time (min)	
	10	30
0	64%	51%
23	25%	25%
30	20%	23%
37	6.5%	18%

Binding assays were carried out as described in Methods in modified H/H buffer. [¹²⁵I]MEL = 0.71 nM. Protein = 403 µg/assay tube.

Table 8. Inhibition of 2-[¹²⁵I]iodomelatonin binding to RPMI 1846 membranes.

Drug	K _i (nM)	n _H
iodomelatonin	8	0.7
prazosin	13	0.5
6-chloromelatonin	14	1.0
O-acetyl-N-acetylserotonin	24	0.6
N-acetyl-6-methoxytryptamine	65	1.3
melatonin	104	1.0
N-acetylserotonin	141	1.0
Ru 24969	161	0.4
6-hydroxymelatonin	330	1.6
5-methoxytryptophol	1,130	0.9
5-methoxytryptamine	1,500	1.1
5-hydroxytryptophol	1,800	0.9
N-acetyltryptamine	11,700	0.9
serotonin	25,300	-

Means of triplicate values from one experiment. K_i was calculated from the IC₅₀ value using the equation of Cheng and Prusoff (1973).

Figure 25. Binding of 2-[¹²⁵I]iodomelatonin to RPMI 1846 membranes.

Incubation was carried out for 2 h at 0° C with 190 µg membrane protein per assay tube. Means ± S.D. of triplicate values from one experiment, repeated twice, are shown. *Inset*: Scatchard-Rosenthal transformation of the binding data. $K_d = 0.80$ nM, $B_{max} = 5.9$ fmol/mg protein, ($r = 0.83$).

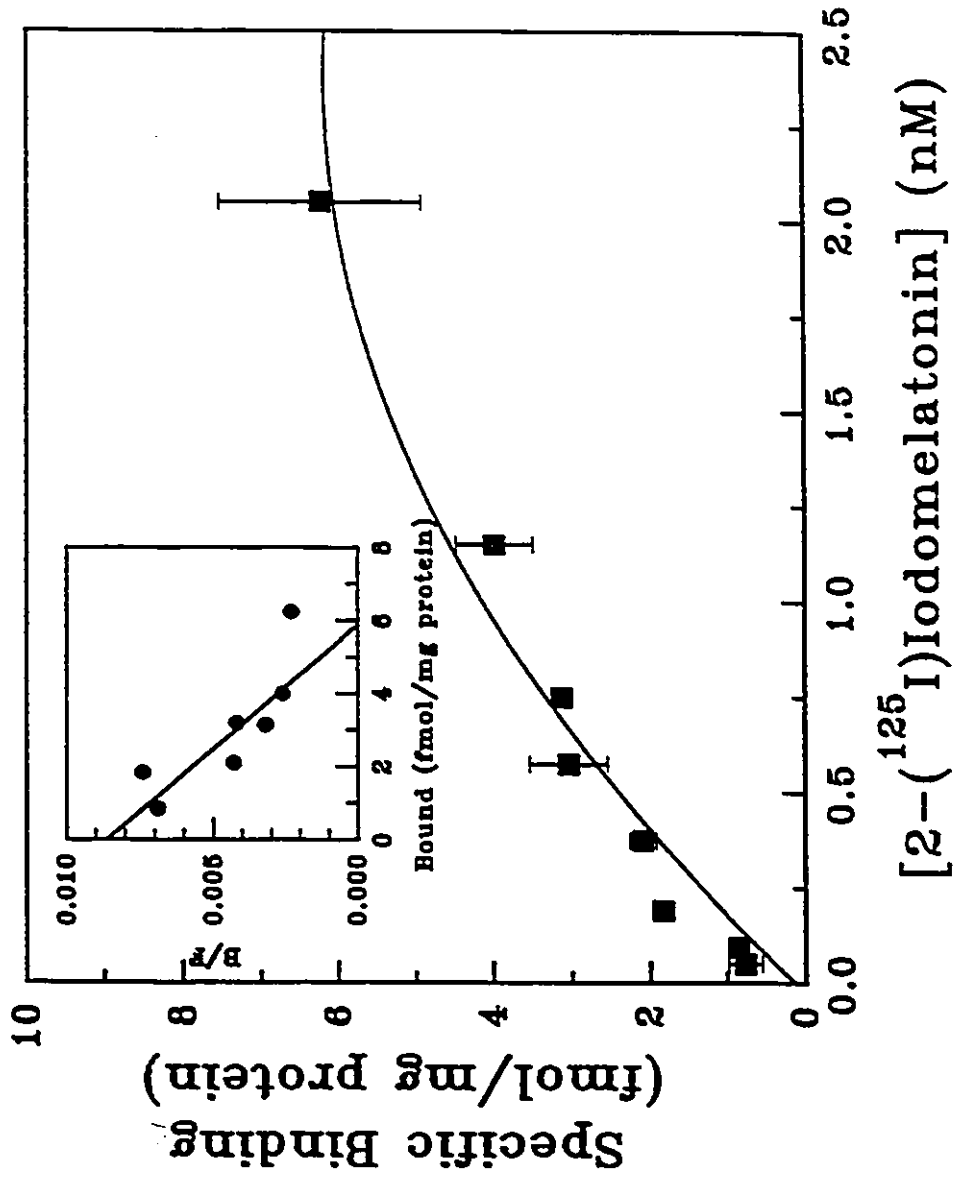


Figure 26. Correlation of the pharmacological profiles of the low-affinity melatonin binding site in hamster hypothalamic membranes to that of RPMI 1846 membranes (data from Tables 3 and 8). Legend: a, iodomelatonin; b, prazosin; c, 6-chloromelatonin; d, O-acetyl-N-acetylserotonin; e, N-acetyl-6-methoxytryptamine; f, melatonin; g, N-acetylserotonin; h, Ru 24969; i, 6-hydroxymelatonin; j, 5-methoxytryptophol; k, 5-methoxytryptamine; l, 5-hydroxytryptophol; m, N-acetyltryptamine; n, serotonin.

($r = 0.94$, $m = 0.93$, $p < 0.01$)

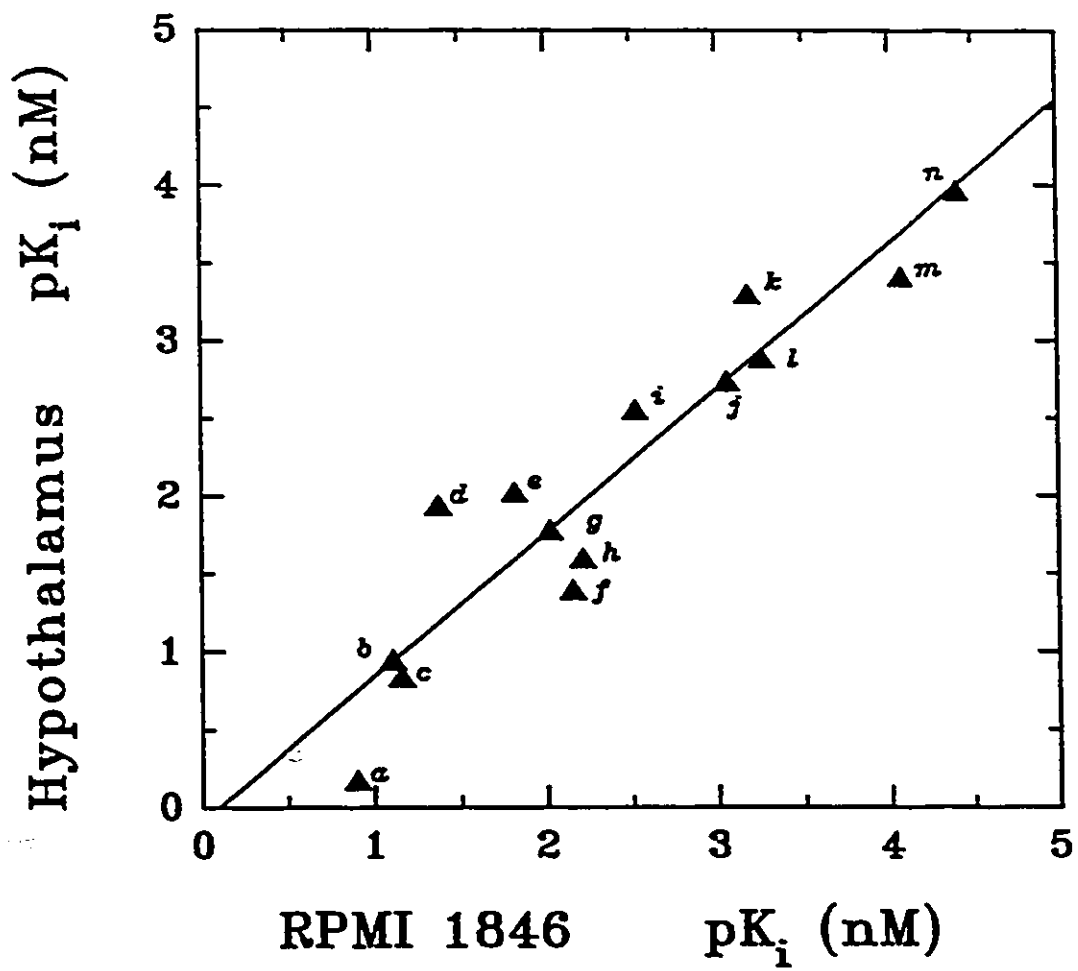


Figure 27. Kinetics of 2-[¹²⁵I]iodomelatonin binding to RPMI 1846

cell membranes at 37°C. Membranes were prepared and assayed as described in Methods. Membranes were assayed in modified H/H buffer (HBSS + 30 mM HEPES pH 7.4, [NaCl] = 127 mM, [LiCl] = 10 mM). Protein = 473 µg/assay tube. [¹²⁵I]MEL = 0.65 nM. Means ± S.D. of triplicate values from one experiment are presented. ○, total binding; ▽, nonspecific binding (in the presence of 1 µM 6-chloromelatonin); ▼, specific binding.

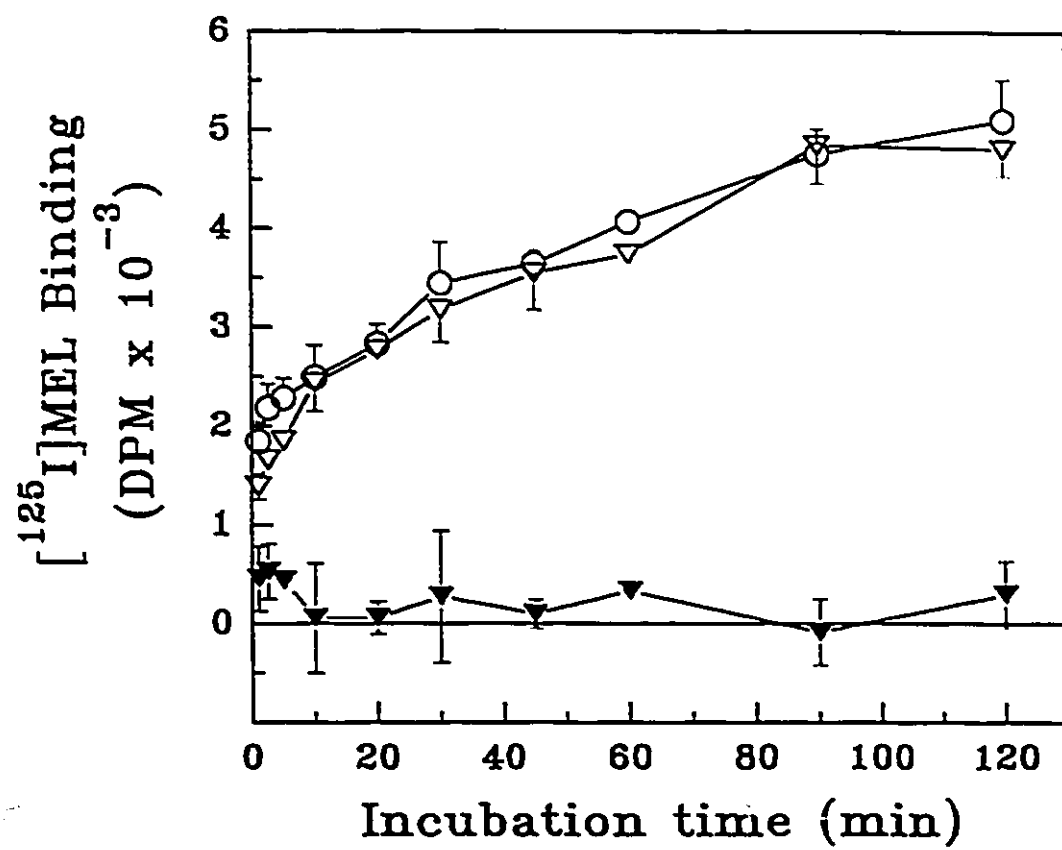


Figure 28. Dose-response of forskolin stimulation of adenylyate cyclase activity in RPMI 1846 membranes (165 μg protein/assay tube) in the presence of 10 μM rolipram. $\text{EC}_{50} = 4.3 \mu\text{M}$. [DMSO] = 6.7% at 1 mM forskolin. Means \pm S.D. of triplicate values from one experiment, repeated once, are shown.

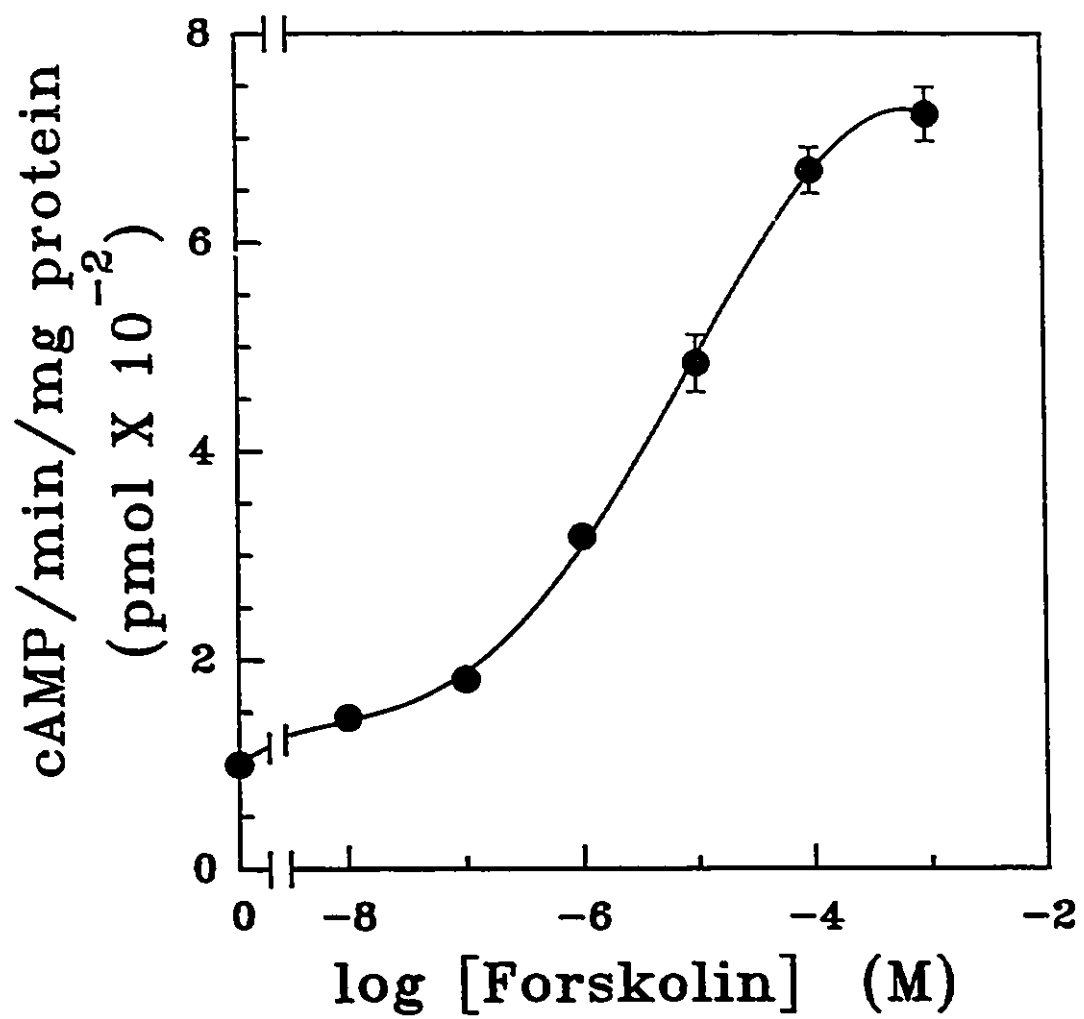
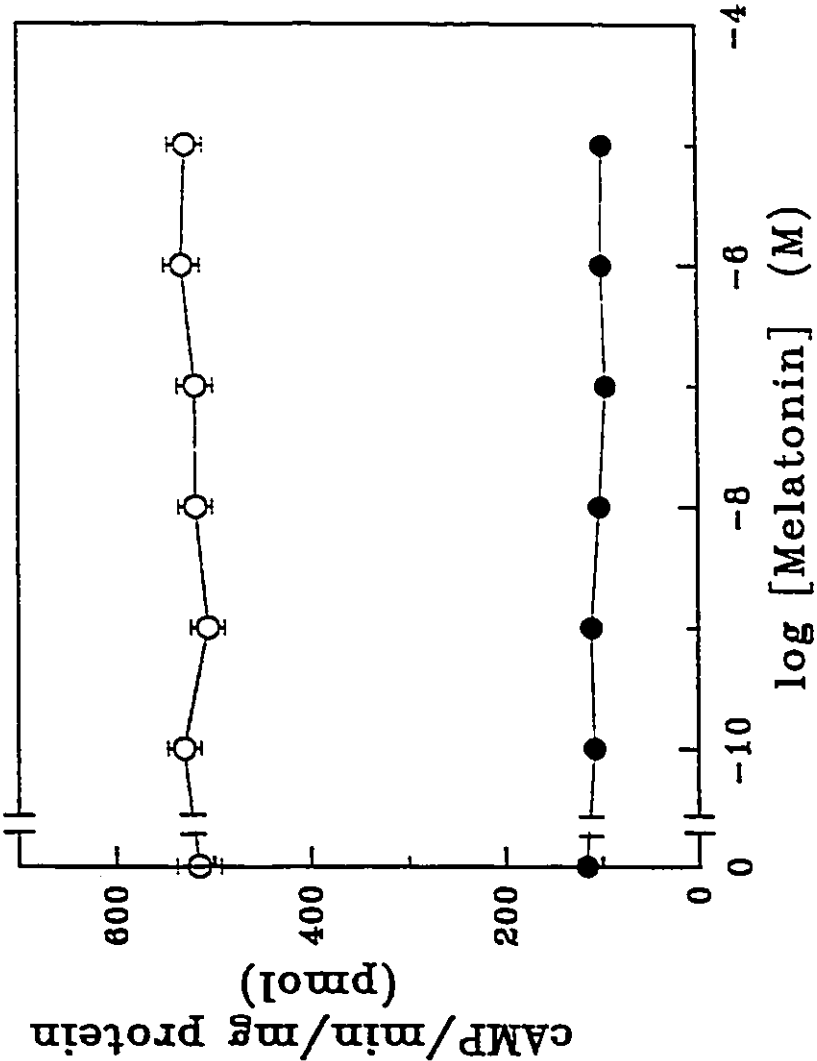


Figure 29. Effects of melatonin on RPMI 1846 membrane adenylate cyclase activity. [Rolipram] = 10 μ M; 163 μ g membrane protein/assay tube. Means \pm S.D. from one experiment conducted in triplicate, repeated twice, are shown. ●, basal activity; ○, 25 μ M forskolin.



Basal or forskolin-stimulated cAMP accumulation, in the absence of PDE inhibitors, was not affected by melatonin (10^{-12} - 10^{-5} M) (Fig. 34). The effects of melatonin (10^{-12} - 10^{-5} M) and N-acetylserotonin (10^{-11} - 10^{-6} M) on intracellular cAMP levels were then investigated in the presence of rolipram to increase the [3 H]cAMP signal (Fig. 35). Neither melatonin nor N-acetylserotonin affected cAMP accumulation in situ. Similar experiments conducted on RPMI 1846 cells grown and assayed as monolayers in 24-well culture dishes also revealed no effects of melatonin or iodomelatonin upon basal or forskolin-stimulated in situ cAMP levels (data not shown).

4.3.4 Calcium Flux Studies

Filter blanks and boiled tissue blanks were typically 100- 300 CPM. A time-course of basal and potassium-stimulated uptake in synaptosomes indicated maximal uptake occurring within 1- 2 min at 30°C (Fig. 36). A dose-response of melatonin (10^{-12} - 10^{-4} M) revealed no effects upon basal or K⁺-depolarised induced calcium uptake (Fig. 37). The kinetics of basal calcium uptake into RPMI 1846 cell monolayers indicated a slow increase in intracellular $^{45}\text{Ca}^{2+}$ over 1 h at 37°C, equilibrium being attained by \approx 2 h (Fig. 38). Dose-responses of melatonin (10^{-11} - 10^{-5} M) indicated no significant effect upon calcium uptake in RPMI 1846 cell monolayers (Fig. 39). Iodomelatonin and NAS were similarly without effect (data not shown). The kinetics of calcium uptake in cell suspensions were not significantly altered by the presence of 10 μ M melatonin (Fig. 40).

Figure 30. Uptake kinetics of [³H]adenine by RPMI 1846 cells (3.8 x 10⁵/assay tube) at 37°C. [³H]Adenine = 0.50 μM; 1.17 Ci/mmol. Means ± S.D. of triplicate values from one experiment, repeated twice, are shown.

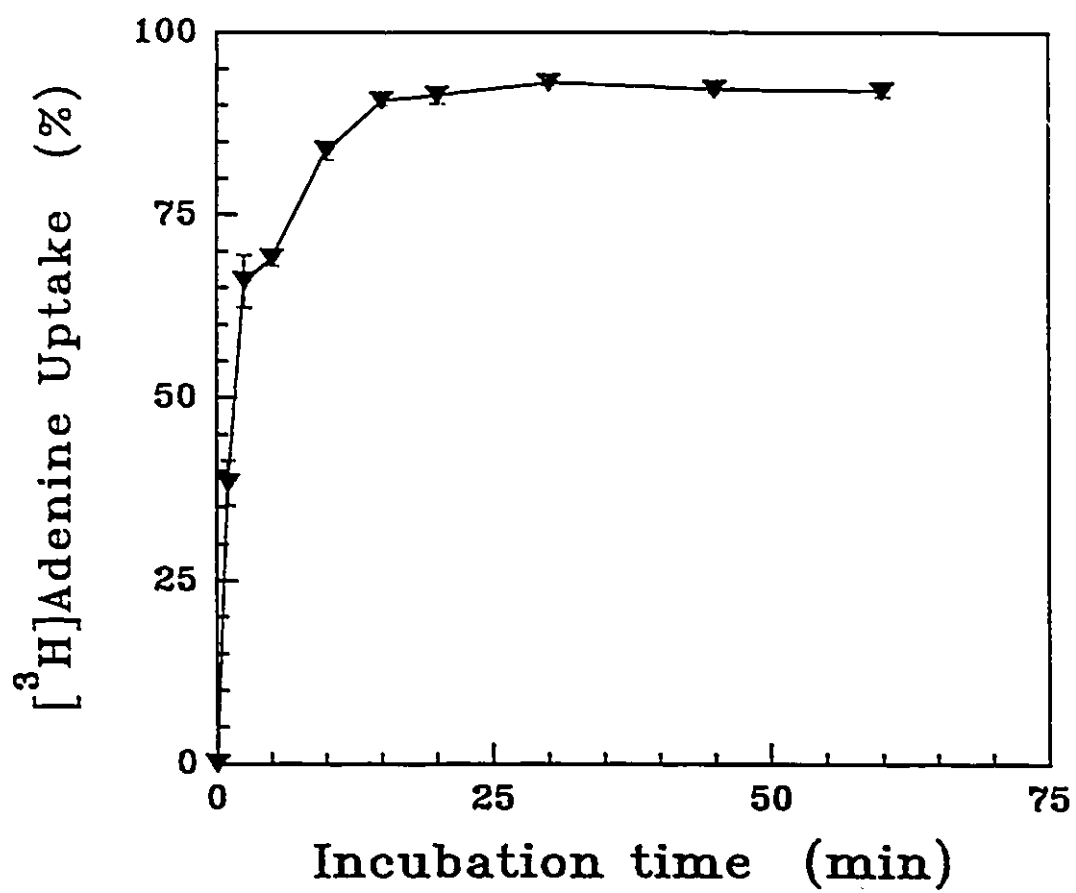


Figure 31. Kinetics of basal [³H]cAMP formation from [³H]adenine.

RPMI 1846 cells (5.2×10^5 /assay tube) were incubated with 0.50 μ M [³H]adenine (17.1 Ci/mmol) at 37°C for various times and [³H]cAMP formation measured as described in Methods. Means \pm S.D. of triplicate values from one experiment are presented.

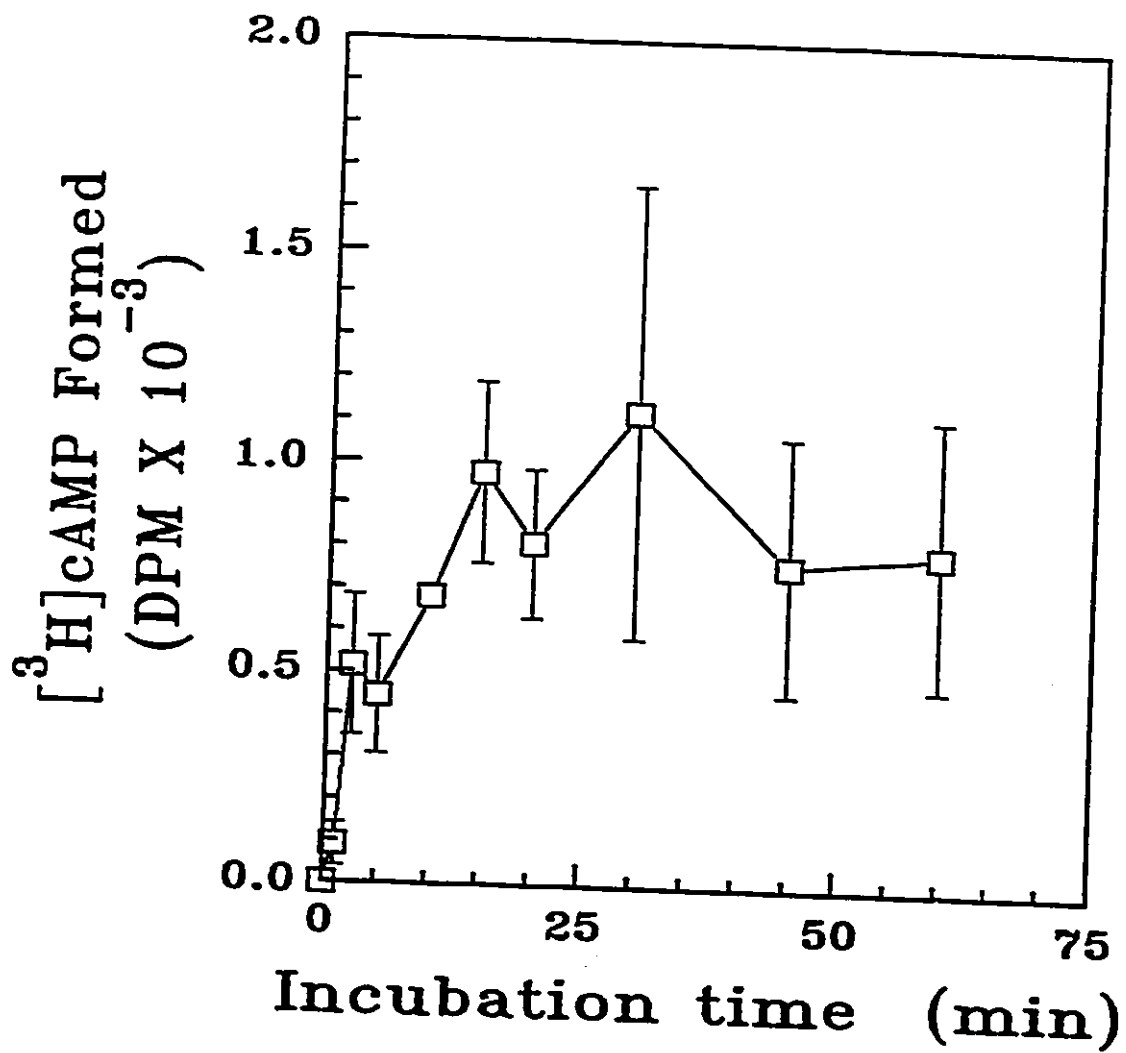


Figure 32. Potentiation of [^3H]cAMP accumulation in RPMI 1846 cells (6.4×10^5 /assay tube) by rolipram and IBMX. Cells ($= 10^7$ /mL) were prelabelled with $0.50 \mu\text{M}$ [^3H]adenine (11.75 Ci/mmol) in H/H for 15 min at 37°C , centrifuged and resuspended in fresh buffer prior to drug addition. [DMSO] < 0.5%. ●, basal activity; ▽, 25 μM forskolin activity. Means \pm S.D. from one experiment conducted in triplicate, repeated twice, are shown.

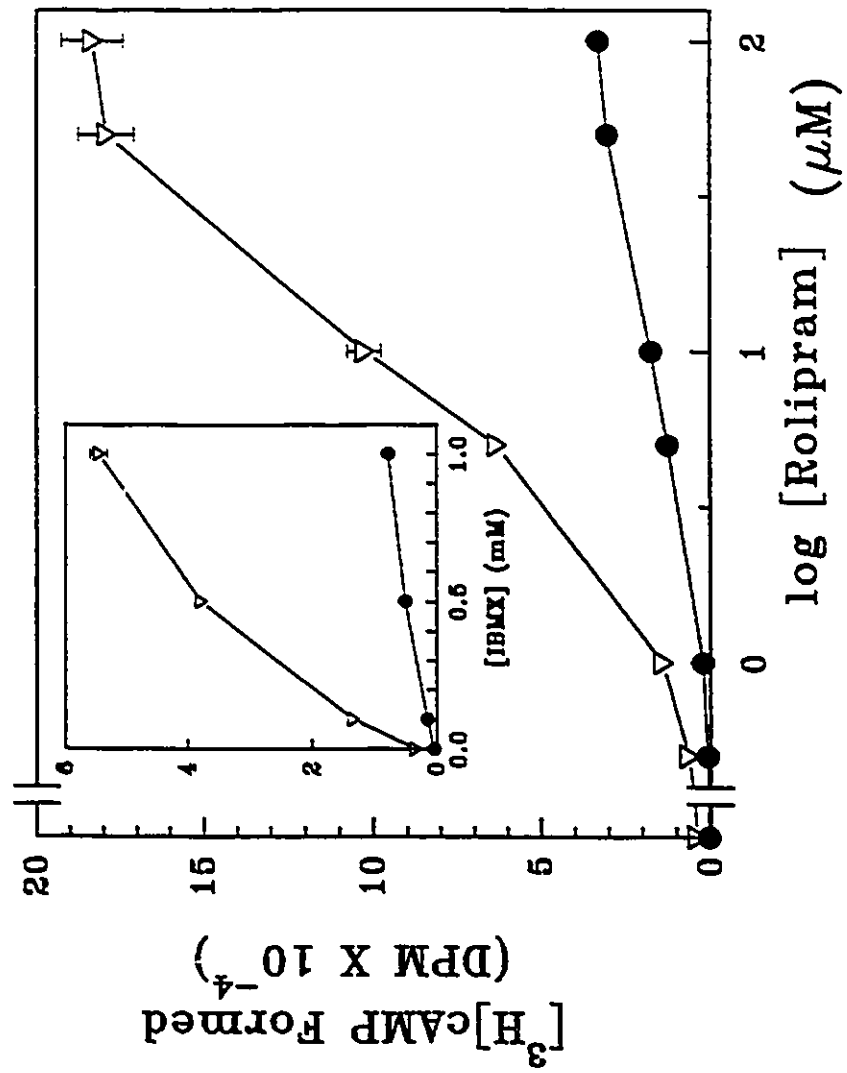


Figure 33. Kinetics of forskolin-stimulated [^3H]cAMP formation in the presence of rolipram. RPMI 1846 cells ($= 10^7/\text{mL}$) were incubated with $0.50 \mu\text{M}$ [^3H]adenine (4.7 Ci/mmol) in H/H buffer at 37°C for 15 min, centrifuged and resuspended in fresh buffer prior to forskolin stimulation. [Rolipram] = $50 \mu\text{M}$; [Forskolin] = $25 \mu\text{M}$; cells = $4.1 \times 10^5/\text{assay tube}$. [DMSO] = 0.3% . Means \pm S.D. from one experiment are presented.

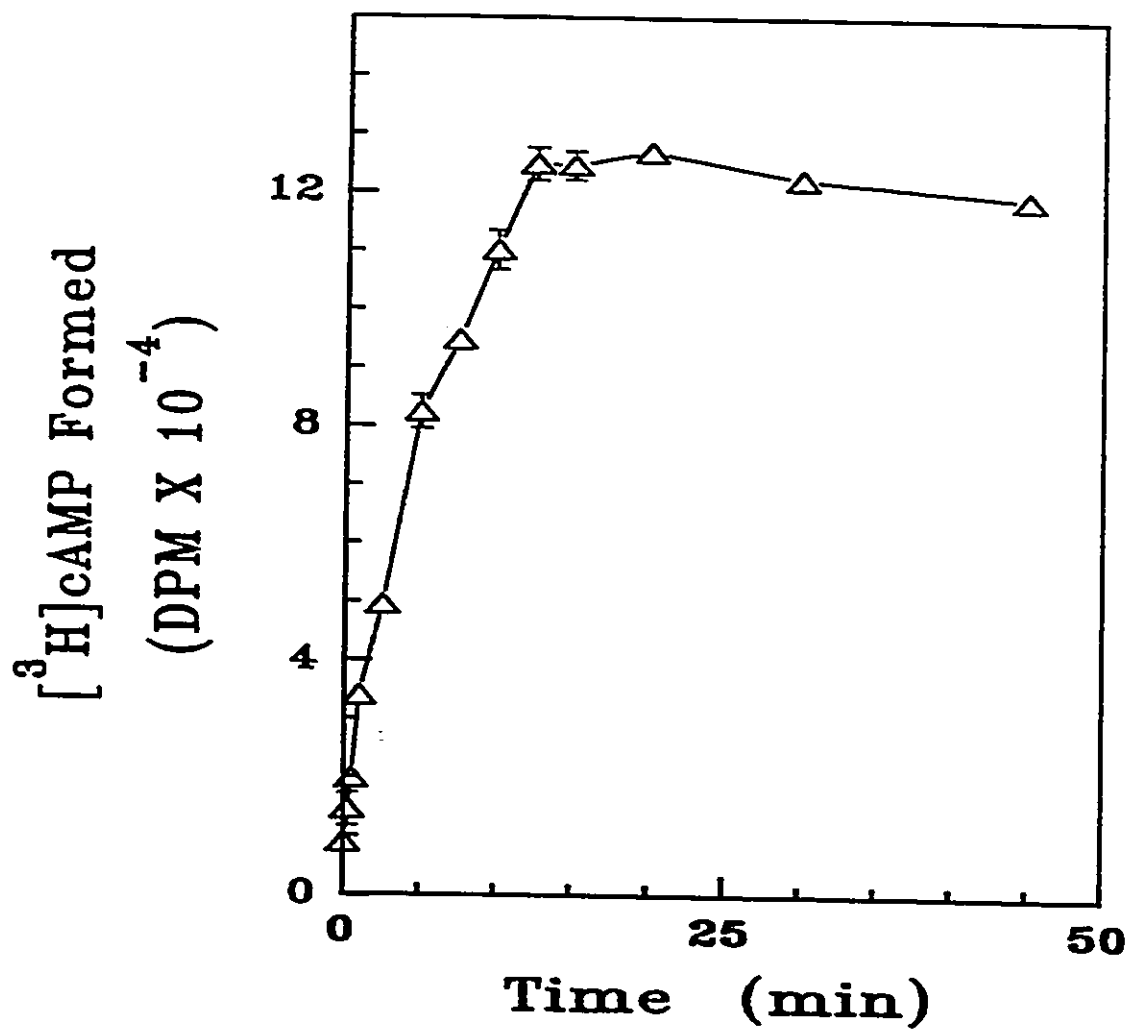


Figure 34. Effects of melatonin upon intracellular [^3H]cAMP accumulation in the absence of PDE inhibitors. RPMI 1846 cells ($= 10^7/\text{mL}$) were incubated with $0.50 \mu\text{M}$ [^3H]adenine (11.75 Ci/mmol) in H/H buffer for 15 min at 37°C , centrifuged and resuspended in fresh buffer prior to drug treatment. Means \pm S.D. of triplicate values from one experiment are shown. cells $= 5.8 \times 10^5/\text{assay tube}$. \circ , basal activity; \bullet , $25 \mu\text{M}$ forskolin.

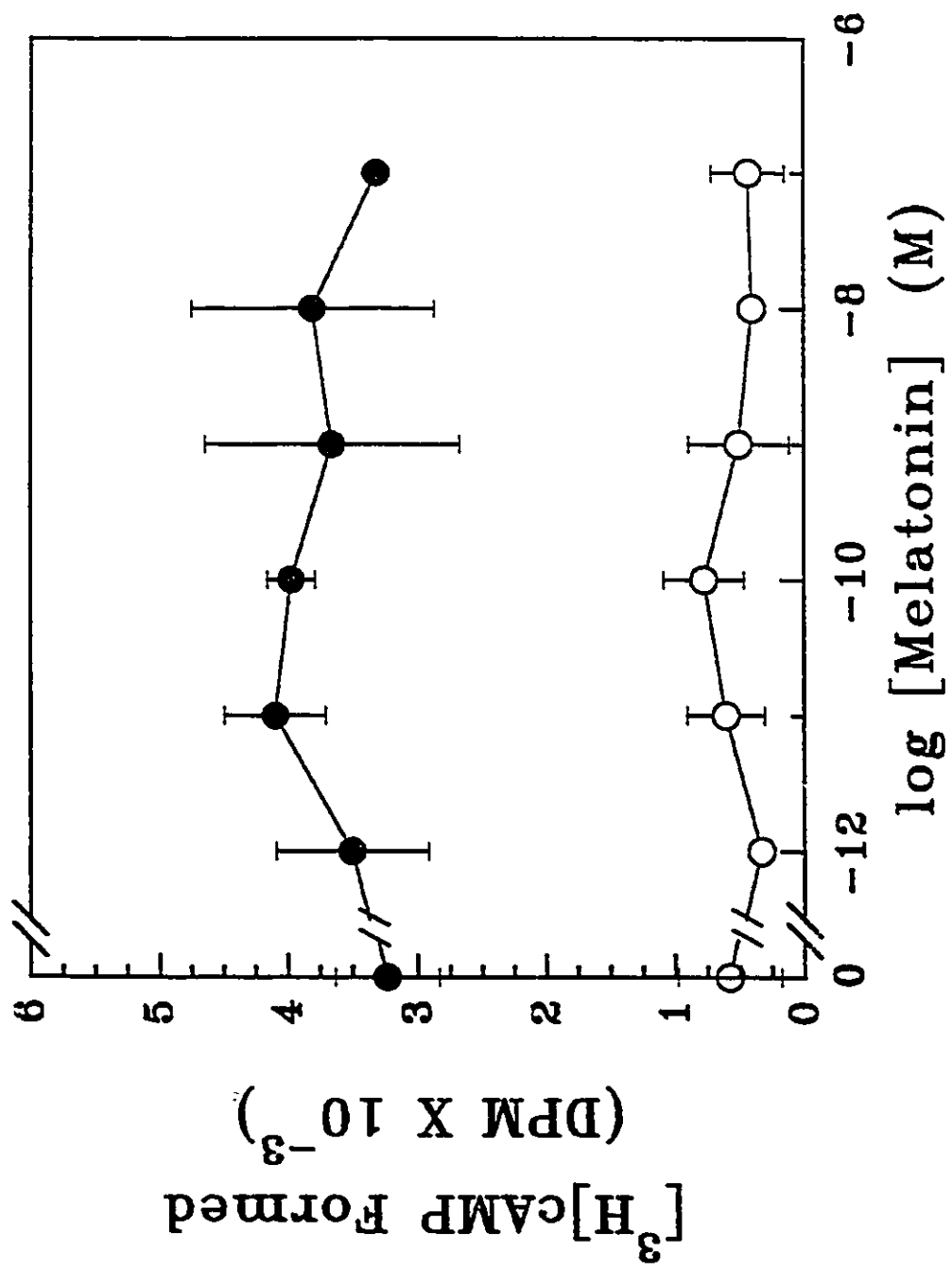


Figure 35. Effects of melatonin and N-acetylserotonin (NAS) on (a) 25 μM forskolin-stimulated and (b) basal [^3H]cAMP levels in RPMI 1846 cells (7.0×10^5 /assay tube) in the presence of 50 μM rolipram. Cells were prelabelled with 0.50 μM [^3H]adenine (11.75 Ci/mmol) in H/H buffer for 15 min at 37°C, centrifuged and resuspended in fresh buffer prior to drug addition. Means \pm S.D. of triplicate values from one experiment, repeated twice, are shown. ●, NAS; ▼, melatonin

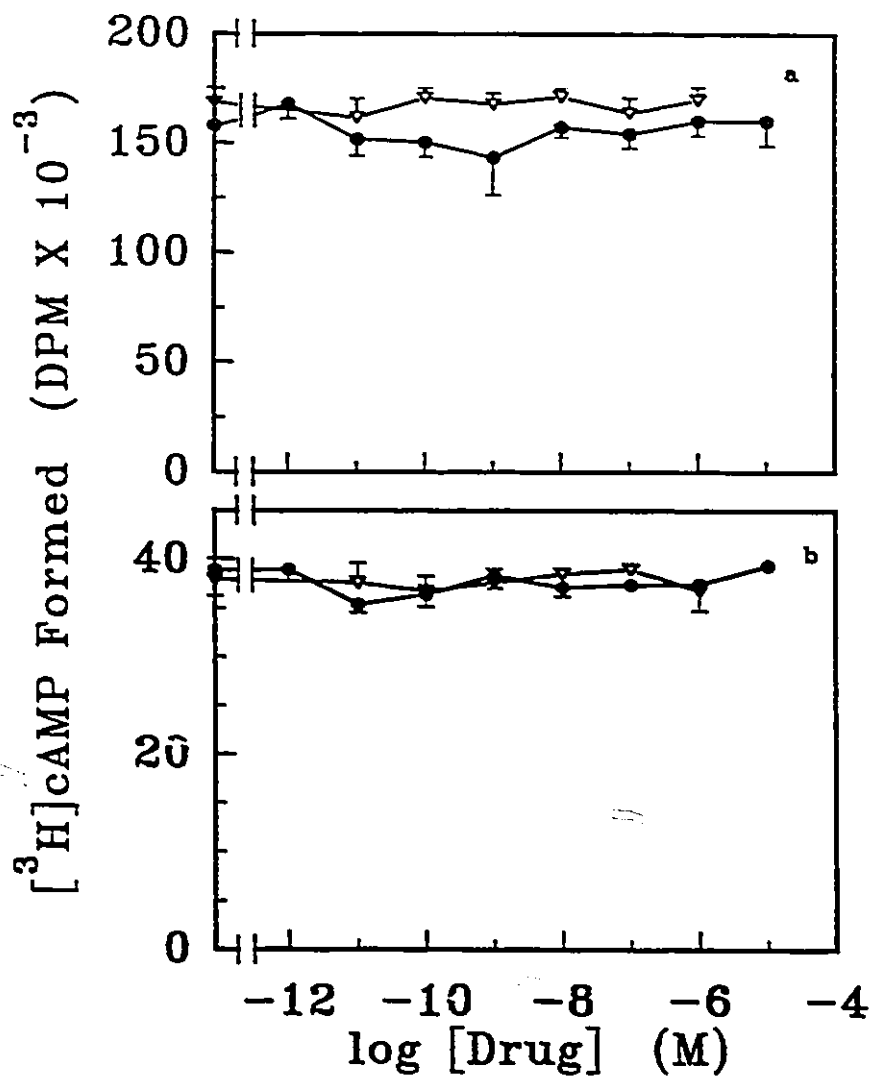


Figure 36. Kinetics of calcium uptake in hamster brain synaptosomes at 30°C as described in Methods. $[Ca^{2+}]_o = 0.13 \text{ mM}$; $0.85 \mu\text{Ci } ^{45}\text{Ca}^{2+}$ /assay tube. Protein = $113 \mu\text{g}$ /assay tube. Means \pm S.D. of triplicate values from one experiment, repeated once, are shown. ●, basal uptake; ○, uptake in 75 mM K^+ , ■, potassium-dependent uptake.

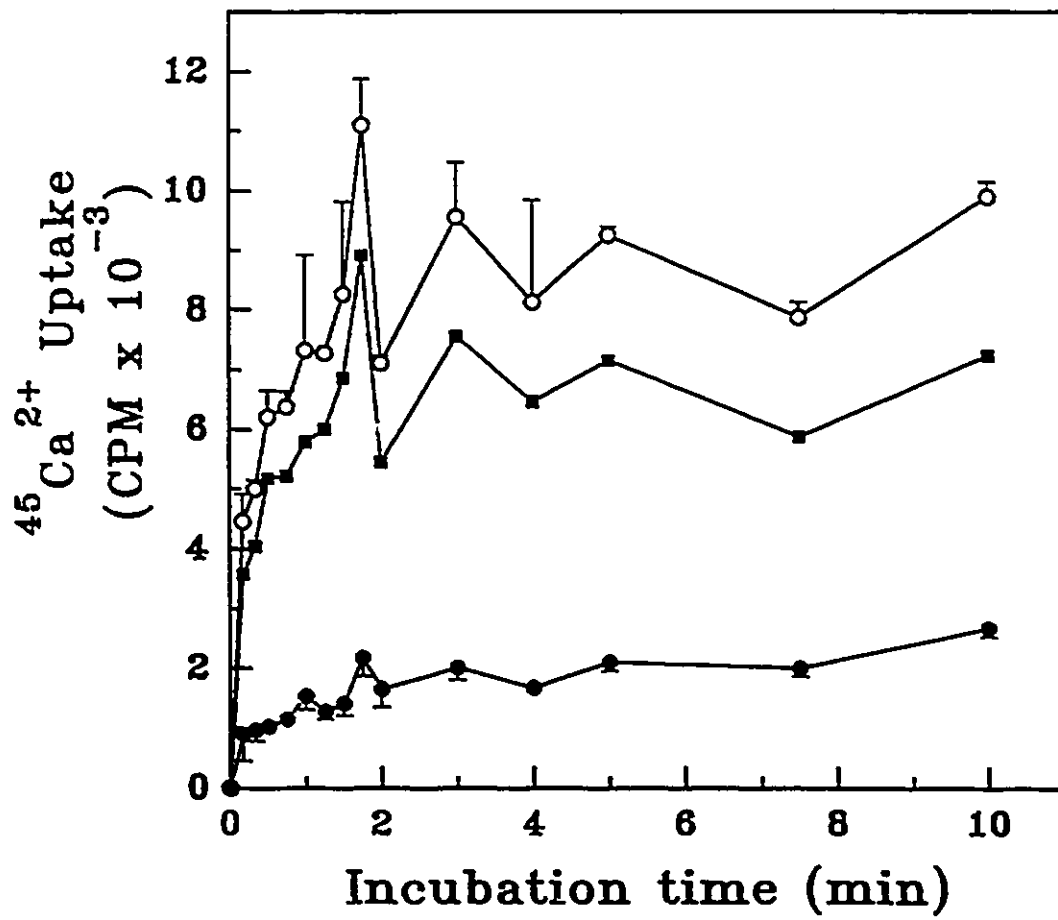


Figure 37. Dose-response of melatonin upon basal and potassium-stimulated calcium uptake in hamster brain synaptosomes at 30°C as described in Methods. $[Ca^{2+}]_o = 0.13$ mM; $0.70 \mu Ci$ $^{45}Ca^{2+}$ /assay tube. Protein = $156 \mu g$ /assay tube. Means \pm S.D. of triplicate values from one experiment, repeated twice, are shown. \circ , basal uptake; \bullet , uptake in 75 mM K^+ .

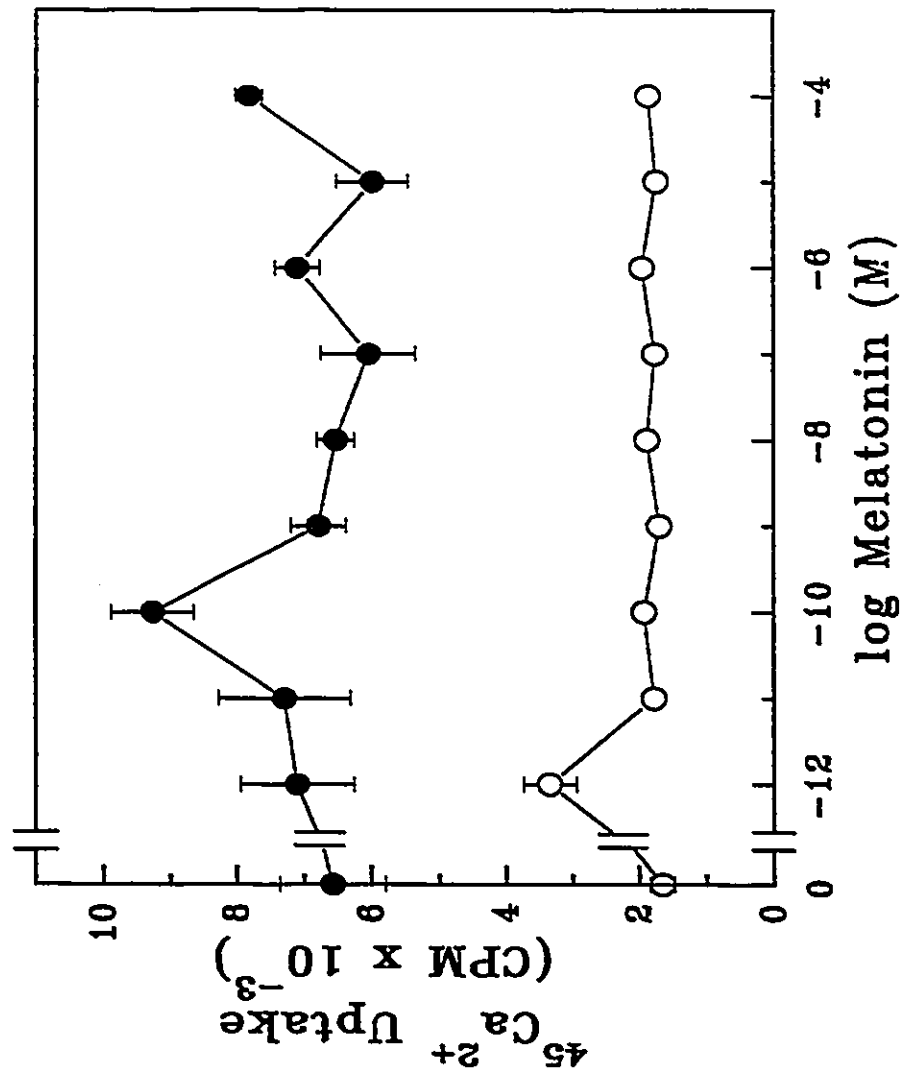


Figure 38. Time course of calcium uptake in RPMI 1846 cell monolayers at 37°C as described in Methods. $[\text{Ca}^{2+}]_o = 2 \text{ mM}$; 1.65 $\mu\text{Ci/well}$. Cell count = $5.0 \times 10^5/\text{well}$. Means \pm S.D. of triplicate values from one experiment, repeated once, are shown.

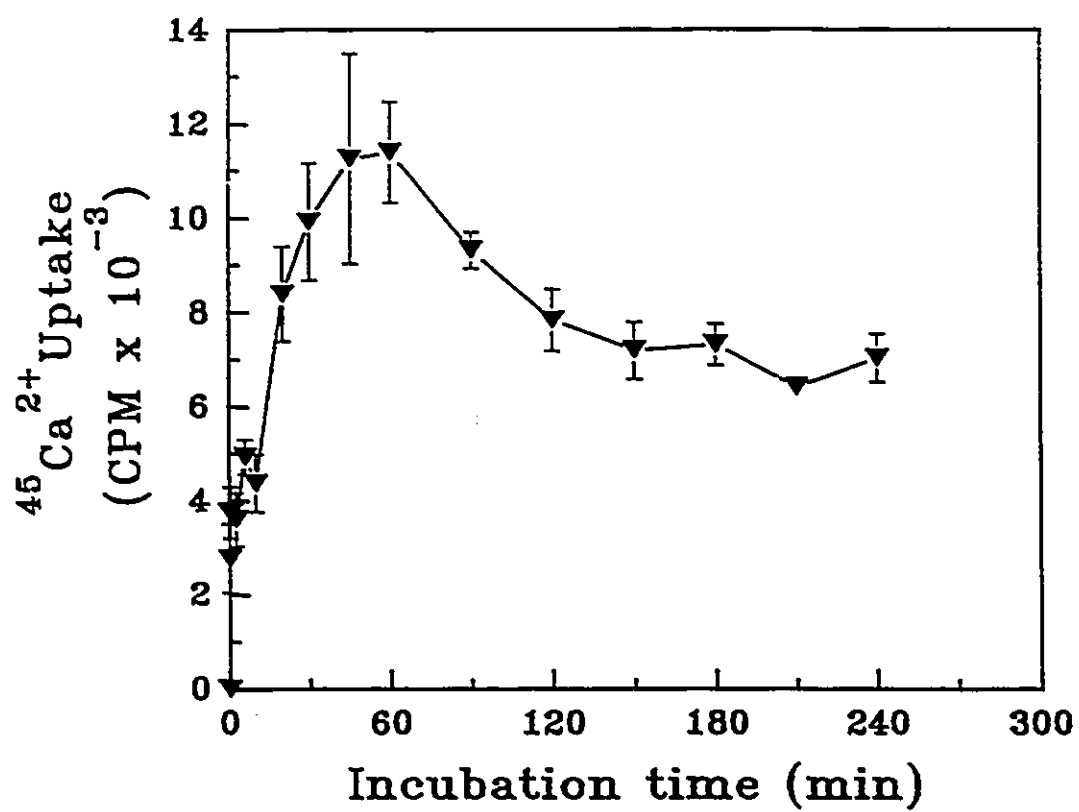


Figure 39. Effect of melatonin on calcium uptake in RPMI 1846 cell monolayers. Cells were incubated 30 min at 37°C as described in Methods. $[Ca^{2+}]_o = 2 \text{ mM}$; $1.56 \mu\text{Ci } ^{45}\text{Ca}^{2+}/\text{well}$. $[\text{Ethanol}] < 0.002\%$. Means \pm S.D. of triplicate values from one experiment, repeated twice, are shown.

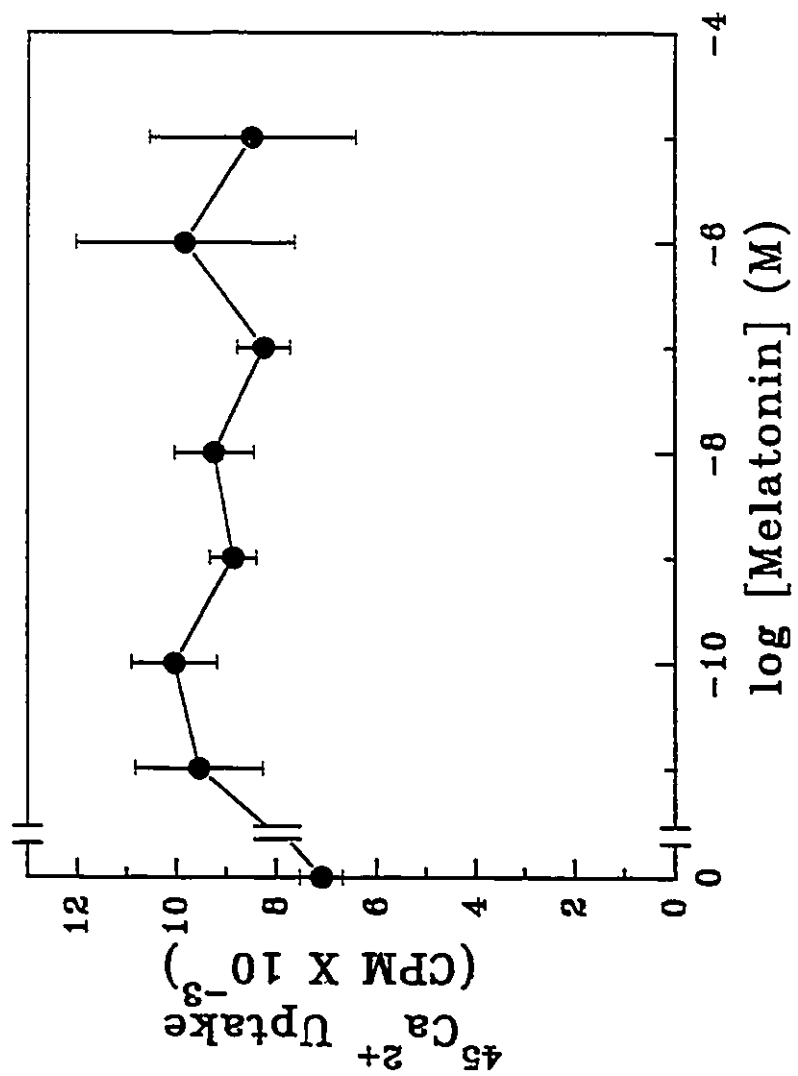


Figure 40. Effects of melatonin on the kinetics of calcium uptake in RPMI 1846 cells in suspension at 37°C. $[Ca^{2+}]_o = 1$ mM; $0.63 \mu Ci$ $^{45}Ca^{2+}$ /assay tube. Cell count = 1.1×10^6 /assay tube. Means \pm S.D. of triplicate values from one experiment, repeated twice, are shown. \square , basal uptake; \blacksquare , $10 \mu M$ melatonin.

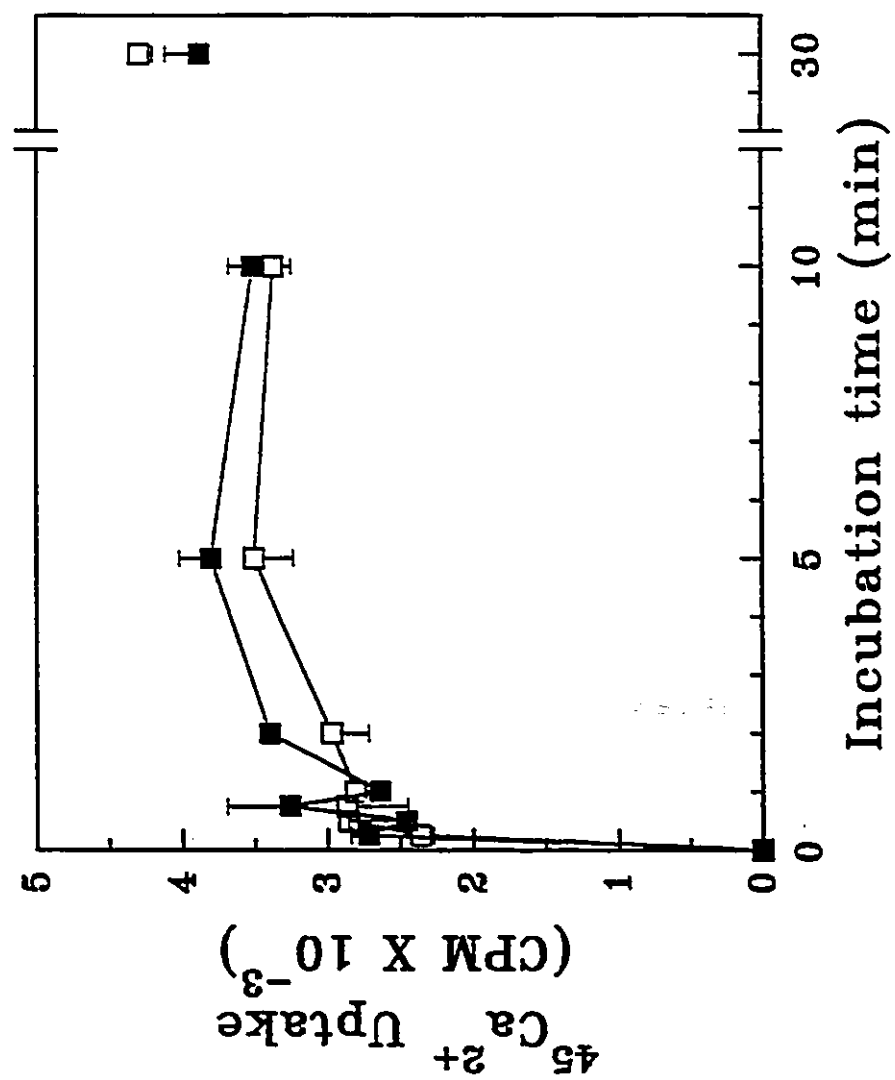


Figure 41. Uptake of myo-[2-³H]inositol into RPMI 1846 cell confluent monolayers. Incubation was carried out using 35 mm culture dishes in a humidified incubator at 37°C under an atmosphere of 2% CO₂/air as detailed in Methods. [myo-inositol] = 14 nM (0.57 μCi/dish). Means ± S.D. of triplicate values from one experiment, repeated once, are shown. ●, cytosol; ○, medium; ▽, membranes.

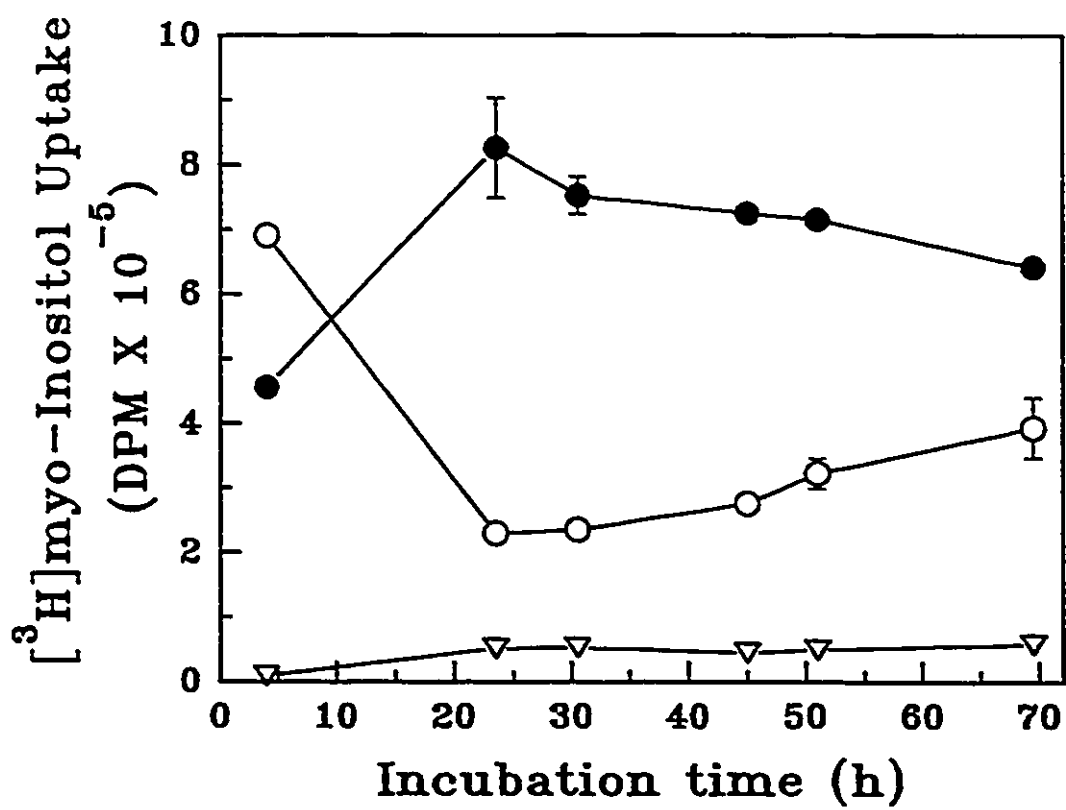


Figure 42. Time course of resting [^3H]IP $_3$ levels in RPMI 1846 cells during incubation at 37°C in modified H/H buffer. Cells were prelabelled for 24.5 h with 99 nM myo-[2- ^3H]inositol as described in Methods with 91% uptake. [LiCl] = 10 mM. Means \pm S.D. of triplicate values from one experiment, repeated once, are presented. Cell count = 1.5×10^6 /assay tube.

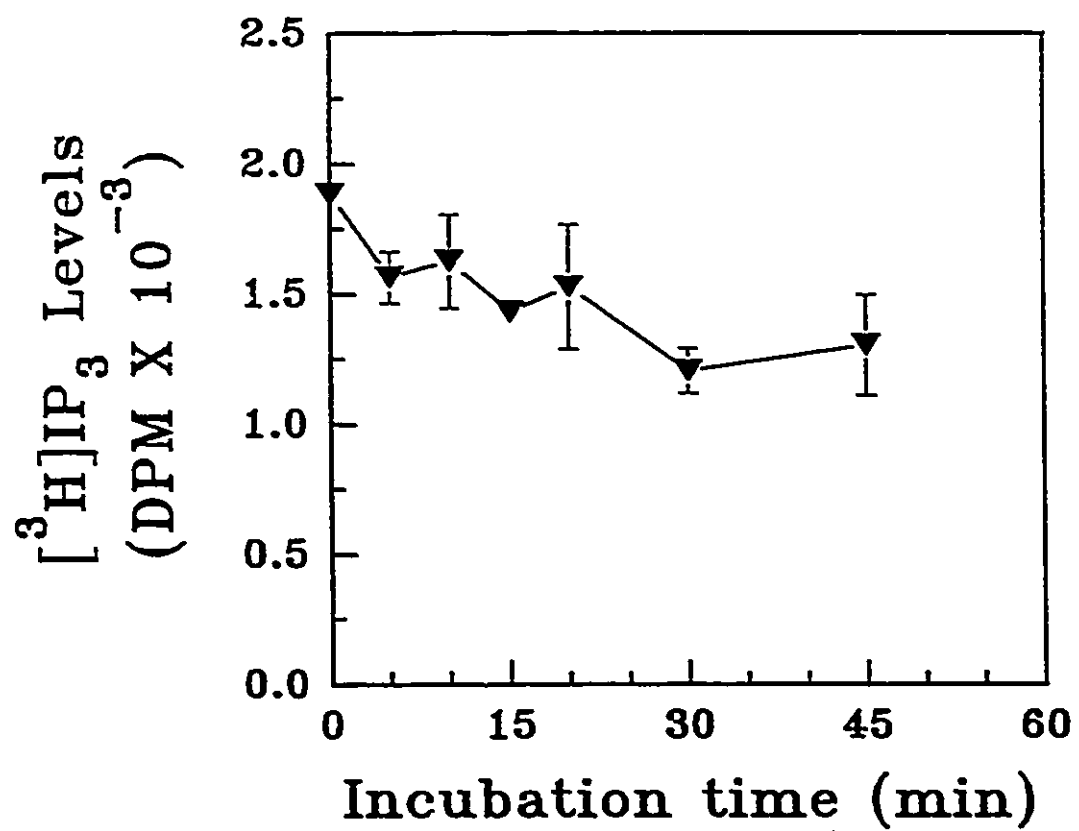
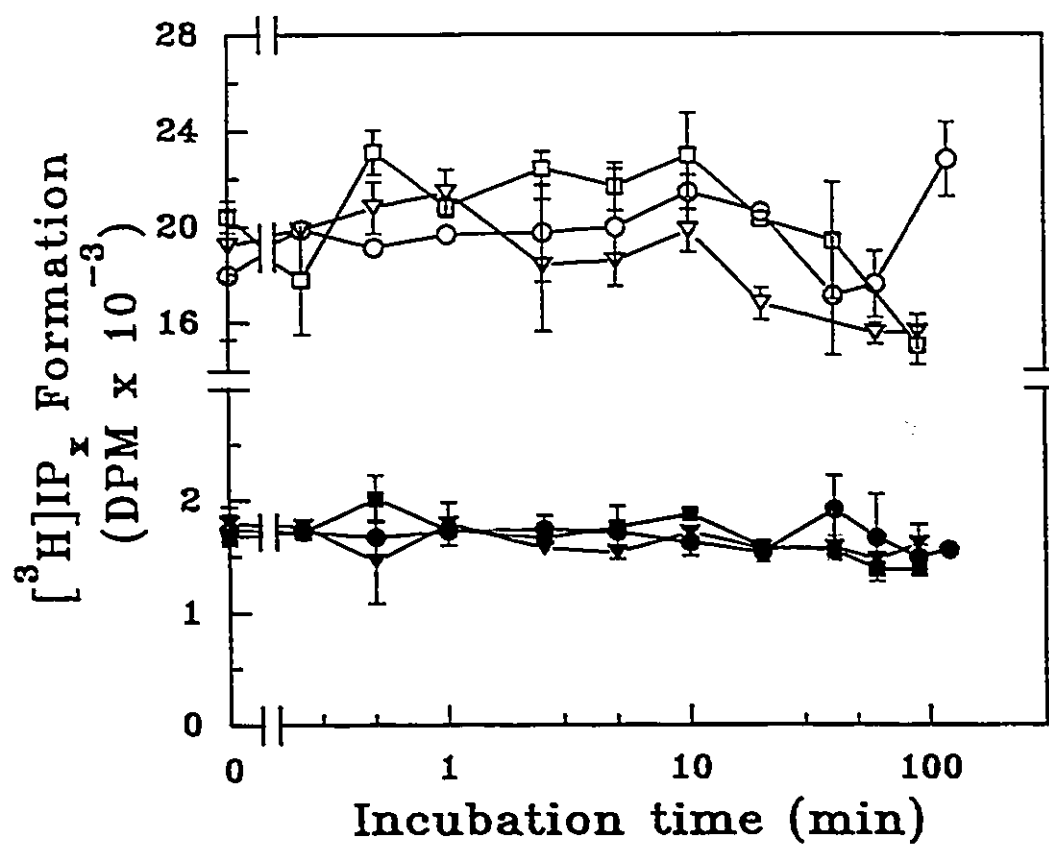


Figure 43. Effects of melatonin and NAS on the kinetics of accumulation of inositol phosphates at 37°C. Cells were prelabelled for 19 hours with 188 nM myo-[2-³H]inositol as described in Methods with 97% uptake. [LiCl] = 10 mM. Means ± S.D. of triplicate values from one experiment, repeated twice, are presented. Cell count = 1.2×10^6 /assay tube. [Ethanol] = 0.008%. IP₃, filled symbols; IP₁₊₂, open symbols. ○, control; ▽, 10 μM melatonin; □, 10 μM NAS.



4.3.5 Inositol Phosphate Studies

An examination of the time course of prelabelling indicated that maximal labelling of RPMI 1846 cell membranes had occurred by = 24 h of incubation at 37°C (Fig. 41). After cell prelabelling and harvesting, the stability of the basal IP₃ levels during the course of the 37°C incubation were studied (Fig. 42). A slow decrease (= 36%) in the resting levels of IP₃ was observed over a 45 min incubation period (ANOVA: df = (6,14), F = 7.14, p < 0.01). The effects of 10 µM melatonin and NAS on the kinetics of the accumulation of inositol phosphates were investigated next (Fig. 43). No statistically significant effects were seen. Similarly, no effect was seen using a lower concentration of 0.10 µM melatonin or NAS.

4.4 Discussion

4.4.1 Binding Pharmacology

The Syrian hamster-derived melanoma cell line RPMI 1846 was discovered to contain a nanomolar-affinity [¹²⁵I]MEL binding site. The low receptor density made detection of the RPMI 1846 binding site difficult. It was necessary to remove as much melanin as possible from RPMI 1846 cell homogenates since it was found to interfere with the radioligand binding assay. Laitinen and Saavedra (1990b) suggest that melanin may be acting as a low-affinity, high-capacity binding site for [¹²⁵I]MEL. An attempt was made to conduct binding experiments at 37°C in the physiological buffer used for the inositol phosphate studies. Unfortunately, at this temperature, specific binding was not retained using the filtration assay. The same problem had been noted in radioligand

binding studies using hamster brain membranes and can be attributed to the rapid dissociation rate constant of the low-affinity site at higher temperatures (Section 2.3.3).

Inhibition experiments indicated that the RPMI 1846 binding site had a pharmacological profile comparable to the low-affinity site in hamster brain (Duncan et al., 1988, 1989; Chapter 2). Prazosin exhibited virtually identical affinity for the RPMI 1846 and brain low-affinity sites while NAS displayed an affinity comparable to that of melatonin, which is also the situation for the brain site. These data strongly suggest that the two sites are the same. The picomolar-affinity site has relatively poor affinity for NAS and prazosin (Ying and Niles, 1991) and can thus be pharmacologically distinguished from the low-affinity site using these compounds. The affinity of the RPMI 1846 site for [¹²⁵I]MEL obtained from Scatchard-Rosenthal analysis was comparable to that for the low-affinity brain site (0.94 vs 1.8 nM, respectively) while competition experiments using unlabelled 2-iodomelatonin indicated a somewhat lower affinity (8 nM). The key indoles (most closely resembling melatonin) generally had Hill values close to unity, signifying displacement from a single site. Neither GTP nor sodium ion affected [¹²⁵I]MEL binding, consistent with the lack of sensitivity of the low-affinity brain site to these agents. Since these pharmacological data indicated that the RPMI 1846 cell line expresses a low-affinity melatonin binding site which is similar to that in brain, it was decided to use these cells as an in vitro model for the brain site.

In order to truly call the low-affinity site a receptor, one needs to be able to ascribe some function to it. Cultured RPMI 1846 cells represent a homogeneous cell population, unlike primary cultures derived from tissue

explants. Therefore, they are particularly suitable for studies directed at investigating signal transduction mechanisms.

4.4.2 Cyclic AMP Studies

The concentration-dependent effect of forskolin on adenylate cyclase activity in RPMI 1846 membranes is similar to that observed in Syrian hamster brain membranes (Niles and Hashemi, 1990). However, the absence of a melatonin effect on basal or forskolin-stimulated adenylate cyclase activity seemed to preclude either stimulatory or inhibitory coupling of the low-affinity site to adenylate cyclase. In order to rule out possible indirect (i.e. non-adenylate cyclase mediated) effects on cAMP levels, [³H]adenine prelabelling was employed to measure the in situ intracellular cAMP levels since RPMI 1846 cells take up extracellular [³H]adenine quite quickly and completely.

Many researchers use IBMX (typically at 0.1- 5 mM) to inhibit PDE activity in experiments designed to measure changes in cAMP in response to various stimuli. This may not be a good practice because IBMX has been demonstrated to interact directly at G_i (Parsons et al., 1988), making it a poor choice as a PDE inhibitor in this type of assay. In addition, IBMX is an antagonist at adenosine receptors and is therefore capable of affecting cAMP levels via these receptors (Daly, 1985). Hence, it was decided to compare IBMX to another class of PDE inhibitor, rolipram, which is a much more potent and selective inhibitor of cAMP-dependent PDE activity than IBMX (nanomolar vs micromolar potencies, respectively) (Schneider et al., 1986). In agreement with this, the EC₅₀ for potentiation of forskolin-stimulated cAMP levels in RPMI 1846 cells by rolipram was found to be ≈ 9 μM while that for IBMX was ≈ 0.5 mM. Hence, rolipram is about

55- fold more potent than IBMX in inhibiting PDE activity in RPMI 1846 cells. In addition, the maximal amount of potentiation is much greater for rolipram which has about a 3.3- fold higher efficacy than IBMX. Since = 10- fold lower concentrations of rolipram than IBMX may be used, there is less likelihood of nonspecific drug effects occurring and the concentration of organic solvent (DMSO in this case) in the assay can be minimised.

Both melatonin and its precursor, NAS, are endogenous compounds showing similar affinities for the low-affinity binding site. Neither melatonin nor NAS affected basal or forskolin-stimulated intracellular cAMP levels in the presence of 50 μ M rolipram. In the absence of PDE inhibitors, melatonin still did not alter cAMP levels. This rules out possible activation or inhibition of PDE activity by melatonin as a mechanism for modulating cAMP levels (which could have been obscured by the presence of PDE inhibitors). Thus, while the high (picomolar)-affinity melatonin receptor inhibits adenylate cyclase activity leading to suppression of cAMP levels, the low (nanomolar)-affinity binding site does not alter cAMP levels and does not appear to be coupled to adenylate cyclase.

4.4.3 Calcium Flux Studies

Melatonin has been suggested to regulate calcium influx in various rat brain tissues. Zisapel and Laudon (1982) and Zisapel et al. (1982) found that nanomolar to micromolar (maximal effects at 10^{-5} M) concentrations of melatonin inhibited K^+ -depolarised or electrically-stimulated release of [3 H]dopamine from preloaded rat hypothalamic tissue slices in vitro. The extent of neurotransmitter release was dependent upon the external calcium concentration. It

was then found that micromolar concentrations of melatonin also inhibited K^+ -depolarised or electrically-stimulated calcium influx in these tissue preparations (Zisapel and Laudon, 1983). Using crude synaptosomes prepared from whole rat brain, Vacas et al. (1984) were able to demonstrate an inhibitory effect of micromolar concentrations of melatonin upon $^{45}Ca^{2+}$ influx induced by high potassium. This suggestion of a relationship between melatonin and calcium flux was further reinforced by the well-documented calcium-dependent inhibition of dopamine release in the retina by melatonin (Dubocovich, 1983) (Section 1.8.1). An inhibition by melatonin of calcium-stimulated (using the calcium ionophore A23187) LH release in prepubertal female rat pituitary cells *in vitro* also implied an involvement of melatonin in the regulation of calcium-mediated hormone secretion (Symons et al., 1985). Since Ca^{2+} influx is required for neurotransmitter release and hormone secretion (Schweizer et al., 1991), it is conceivable that melatonin could be acting, in these instances, to somehow antagonise the effects of increased cytosolic calcium levels.

The ubiquitous presence of the low-affinity melatonin binding site in the hamster CNS (Chapter 2) suggested that synaptosomal preparations from Syrian hamster brain tissue could be used to test the possible effects of melatonin upon synaptosomal calcium uptake. Since the hamster is generally more responsive to the effects of melatonin than the rat (Section 1.6.1.1), a repetition of the work of Vacas et al. (1984) seemed warranted. Unfortunately, no effect of melatonin was seen on the basal or potassium-depolarised calcium influx in hamster brain synaptosomes. However, the rapid kinetics of the control basal and K^+ -stimulated uptake were in close agreement with the literature values obtained with rat brain synaptosomes (Harris and Hood, 1980;

Nachshen and Blaustein, 1980; Vacas et al., 1984), maximal effects being achieved within 1- 2 min. Also, the extent of K^+ -stimulated uptake (= 4- 5-fold basal uptake) was comparable with reported values in the literature. The lack of agreement with the work of Vacas and Zisapel is puzzling. Since rat brain has not been convincingly demonstrated to contain the low (nanomolar)-affinity melatonin binding site using [^{125}I]MEL receptor binding or autoradiographic techniques, one would expect the hamster to provide a better model for the functional studies of these sites. One difference in the approach taken here was to use purified synaptosomes whereas Vacas et al. used an unpurified, crude synaptosomal preparation containing other subcellular fractions. It is imaginable that the effects on calcium uptake being observed by Vacas et al. were on some non-synaptosomal component such as mitochondria. Recently, Rosenstein et al. (1991) have repeated the studies of Vacas et al. in rat hypothalamic crude synaptosomes. In this study it was found that the pre-incubation time with melatonin was crucial to the effect seen. A 10 min pre-incubation gave an enhancement of K^+ -stimulated calcium uptake while a 30 min pre-incubation gave inhibition of uptake, no effects were seen upon basal calcium uptake. No explanation was given for this bimodal effect of melatonin but it may relate to the use of unpurified synaptosomes. In the present studies, a 10 min pre-incubation with melatonin was employed, however K^+ -stimulated uptake was not affected either positively or negatively. Rosenstein et al. also found that both the enhancement and inhibition of uptake were maximal in animals sacrificed at lights-off (L:D = 14:10). At a sacrifice time similar to that used in the present study (4 h after lights-on), Rosenstein et al. report that only an inhibition of K^+ -stimulated calcium uptake was seen by melatonin pre-incubation, and only at micromolar concentrations. The discrepancy between these results cannot be presently explained.

Melatonin was found to be without effect upon calcium uptake in RPMI 1846 cells, both as a monolayer and cell suspension. The inference drawn from this is that melatonin itself does not stimulate calcium influx in these cells. It remains possible however that melatonin could influence some as yet unknown stimulator or inhibitor of calcium influx in these cells or modulate some intracellular calcium-mediated event. It may be of interest to investigate the effects of melatonin on intracellular calcium concentrations under a variety of experimental conditions known to affect calcium levels in other systems (e.g. receptors coupled to phosphoinositide turnover such as the muscarinic acetylcholine receptor). The use of calcium-binding fluorescent dyes such as FURA-2 could be employed for such studies (Roe et al., 1990). Unfortunately, the lack of available biochemical information on RPMI 1846 cells (in particular on the presence of known receptors) would necessitate a 'shotgun' type of approach for such studies.

Recently, Benítez-King et al. (1990) have observed effects of low nanomolar concentrations of melatonin upon the cytoskeleton of MDCK and N1E-115 (neuroblastoma) cells in culture. Subsequently, it was reported that low concentrations (10 pM- 100 nM) of melatonin have an effect upon calmodulin levels in these cells (Benítez-King et al., 1991). A slight enhancement of levels was observed at three days of exposure to melatonin while an inhibition was seen after six days. More interestingly, melatonin was also noted to be a very potent inhibitor of calmodulin-dependent cAMP-PDE (bovine heart) activity in vitro ($IC_{50} = 1 \text{ nM}$) while basal PDE activity was unaffected. In addition, [^3H]melatonin was found to interact directly with calmodulin in a calcium-dependent fashion, as assessed by non-denaturing PAGE. Calmodulin, like the

nanomolar-affinity [^{125}I]MEL binding site, is a low molecular weight protein, yielding a size of 25-30 kDa by gel filtration (Dedman and Kaetzel, 1983). This is very similar to the size of the nanomolar-affinity site found by target-size analysis (Chapter 3) suggesting that the two proteins could be one and the same. However, several points argue against this. (1) The major portion of calmodulin in most tissues is soluble while the nanomolar-affinity [^{125}I]MEL binding site was not detected here in cytosolic fractions in preliminary experiments. (2) A unique feature of calmodulin is its thermal stability (i.e. it can withstand 100°C for several minutes) while the nanomolar-affinity site was shown to be heat labile (Chapter 2) with respect to radioligand binding. (3) Calmodulin is a ubiquitous protein found in many (if not all) animals and even plants while the nanomolar-affinity [^{125}I]MEL binding site seems to be only found in the hamster. It has not been detected in bovine or rat brain membrane preparations (data not shown) which are rich sources of calmodulin. Notwithstanding these caveats, the relationship between the low-affinity melatonin binding site in hamster brain and calmodulin should be investigated. Isolation methods for calmodulin are well-documented (Dedman and Kaetzel, 1983) and its purification from hamster brain and subsequent assay in [^{125}I]MEL binding experiments should conclusively answer the question of its possible identity to the nanomolar-affinity [^{125}I]MEL binding site.

4.4.4 Inositol Phosphate Studies

RPMI 1846 cells have not been previously used for phosphoinositide (PI) signal transduction investigations. However, uptake of myo- ^3H]inositol by these cells is similar to that seen in the Bomirski Ab melanotic hamster melanoma cell line in terms of the time requirement for maximal uptake

(Slominski et al., 1989). The basal levels of [^3H]IP $_3$ (per $\approx 10^6$ cells), after using similar prelabelling conditions, are also quite comparable between the two cell lines. A major disadvantage in using the RPMI 1846 cell line (as discussed above) is that virtually no information is known regarding the signal transduction system(s) and receptors contained by these cells. Consequently, no positive control was available to stimulate phosphoinositide turnover in these cells. In pilot experiments, a variety of potential stimulating agents (e.g. carbachol, glutamate, phenylephrine, DOI-HCl) did not increase intracellular IP $_3$ levels (data not shown).

Rather than perform dose-responses with a wide range of concentrations of melatonin, it was decided to focus upon the kinetics of PI turnover using selected concentrations. Because the low-affinity melatonin binding site exhibits an affinity of 10^{-8} - 10^{-7} for melatonin and N-acetylserotonin (in radioligand binding experiments), a concentration of 10^{-5} M should be virtually saturating for this site. If this site then were indeed coupled to stimulation of phospholipase C and PI turnover, then effects should be noticeable at this saturating concentration of melatonin. Both rapid (< 30 sec) responses and slower (several minutes) responses have been observed in studies examining receptor coupling to this second messenger system (e.g. Francel and Dawson [1986] vs Kohn et al. [1990]). However, in the present investigations, melatonin and N-acetylserotonin did not stimulate PI turnover at the two concentrations used (100 nM and 10 μM) at any assay time used. It seems unlikely therefore that the low-affinity melatonin binding site is coupled to stimulation of PI turnover in RPMI 1846 cells. Notwithstanding, the possibility has not been ruled out that melatonin acting at this site could inhibit pre-stimulated PI

hydrolysis or have some effect on the phosphoinositide signal transduction system distal to phospholipase C activity.

4.4.5 Speculations

The physiological function of this low-affinity melatonin binding site remains unknown. The wide tissue distribution of the site makes it difficult to surmise a possible function. It is, of course, conceivable that functions may vary from one tissue to another. The similar (and somewhat low) affinity of melatonin and NAS for the site is also perplexing. Peak serum melatonin levels in the male Syrian hamster are generally reported to be in the range of 0.1- 0.2 nM (Vaughan et al., 1986), which is significantly lower than the in vitro affinity of melatonin for the site. Extrapineal synthesis of melatonin occurs in the retina, Harderian gland and gut (Gern and Karn, 1983) so that it is possible that local concentrations of melatonin could reach much higher values than in serum and that melatonin acts as a paracrine hormone in these tissues, as is the case in the retina. Alternatively, the actual endogenous ligand for the site may not be melatonin but instead its precursor, NAS. The peak circulating serum levels of NAS in male rats is reported to be \approx 13 nM (Pang et al., 1980b) while those in male hamsters are \approx 5 nM (Pang et al., 1981). Thus, NAS is a possible candidate as the endogenous ligand for this binding site.

The origin of blood NAS is not completely known. Pinealectomy of rats reduces, but does not eliminate, serum NAS levels (Yu et al., 1981a). This implies that a substantial component of serum NAS originates extrapineally. Both the retina and rodent Harderian gland synthesise NAS (Brown et al., 1987); however, it is unclear whether secretion of NAS into the blood occurs from these tissues. The liver contains N-acetyltransferase activity and it is likely that

acetylation of plasma serotonin accounts for a portion of blood NAS. NAS has also been localised in the rat CNS using immunohistochemistry (Brown et al., 1984) and [^3H]NAS binding sites have been demonstrated in rat brain (Niles et al., 1983). When injected into rat brain, NAS has been reported to have analgesic effects (Psarakis et al., 1988). Thus, evidence is mounting that NAS has neurotransmitter or neuromodulator properties of its own which are distinct from the serotonergic or melatonergic receptor systems. Much further work is needed for clarification of both the biochemical and physiological characteristics of the nanomolar-affinity site labelled by [^{125}I]MEL in the hamster and the role of melatonin and NAS at this site. The Syrian hamster melanoma cell line (RPMI 1846) provides a model for future studies of this site.

CHAPTER FIVE

GENERAL DISCUSSION

The low-affinity melatonin binding site characterised in this thesis could be responsible for mediating one or more of the non-reproductive physiological effects attributed to melatonin. Melatonin can induce sedation, torpor and sleep in many species (Datta and King, 1980; Cardinali, 1981), has also been postulated to have effects on the neuro-immune axis (Maestroni et al., 1986) and can counteract the proconvulsive effects of pinealectomy in some species (Albertson et al., 1981). In fact, the gerbil will undergo spontaneous seizures upon pinealectomy which are prevented by melatonin administration (Philo and Reiter, 1978; Rudeen et al., 1980; Philo, 1982). Recently, pinealectomy has been demonstrated to increase ouabain high-affinity sites (Na^+ , K^+ -ATPase pump) in rat cerebral cortex, while melatonin administration counteracted this (Acuña-Castroviejo et al., 1991). The wide distribution of the low-affinity binding site in the hamster brain is in accordance with such a proposed role of modulation of neuroexcitation and maintaining brain homeostasis.

Further evidence for a neurosuppressive role of melatonin comes from the finding that melatonin antagonists seem to have anti-depressant-like actions in some animal models (Siuciak et al., 1990; Dubocovich et al., 1990a). Melatonin itself has been shown to modulate benzodiazepine and GABA receptors in rat cortex (Lowenstein et al., 1985; Acuña-Castroviejo et al., 1986a,b). The presence of the nanomolar-affinity melatonin binding site throughout the hamster CNS, especially in the cortex and hippocampus, supports the notion that melatonin could act by way of these sites to mediate its neurosuppressive effects, perhaps via GABA or benzodiazepine receptors.

Nanomolar to micromolar concentrations of melatonin have been reported to inhibit K^+ -depolarised or electrically-stimulated release of dopamine from rat hypothalamic tissue slices *in vitro* (Zisapel and Laudon, 1982; Zisapel et al., 1982). The low-affinity binding site might well be responsible for producing such effects, again fitting in with the idea of a modulation of neuroexcitability. The presence of the low-affinity melatonin binding site in the hamster hypothalamus raises the interesting possibility that this site somehow regulates SCN neural activity, perhaps providing a feedback regulation of melatonin biosynthesis which is controlled by the SCN.

The circulating plasma levels of melatonin are far below the concentrations that would be required for 50% occupation of this low-affinity binding site. However, it is conceivable that under physiological temperature and conditions, the affinity of melatonin (and N-acetylserotonin) for this site could be higher such that the endogenous levels of the two compounds could cause substantial site occupancy. Alternatively, it is possible that, due to post-receptor events such as the efficiency of signal transduction coupling, or the presence of a large receptor reserve, maximal responses could be attained by occupation of only a small percentage of the total receptors present in a tissue. Indeed, such a system is very fine-tuned to the circulating levels of hormone since small changes in this level translate into relatively large changes in the basal level of receptor occupation and activation. There are precedents in the literature for the existence of both high- and low-affinity receptor subtypes for hormones and neurotransmitters, such as the 5-HT₁ vs the 5-HT₂ subtypes which have nanomolar and micromolar affinities for serotonin, respectively (Hamon et al., 1990).

In the hamster and gerbil, there is a evidence that melatonin can be secreted directly into the CSF due to the anatomy of the deep pineal in these species (Das Gupta, 1968; Hewing and Bergmann, 1985; Welsh, 1983, 1987). Thus, the CSF in these species may periodically contain locally high concentrations of melatonin which could reach the low-affinity binding sites in the brain via the third ventricle.

Melatonin's actions as a paracrine hormone in the retina [see: Chapter 1] suggest that perhaps melatonin also acts in a paracrine fashion at the low-affinity binding site. The presence of melatonin in extrapineal and extra-retinal tissues such as the gut [see: Chapter 1], in which it has been shown to be implicated in relaxation processes, supports the notion that melatonin could have paracrine effects mediated by the low-affinity binding site.

The finding that calmodulin-dependent phosphodiesterase activity is inhibited by an interaction of melatonin with calmodulin (Benítez-King et al., 1991) raises the interesting possibility that the low-affinity binding site may be calmodulin and that melatonin acts to enhance cAMP levels by inhibition of its rate of degradation. This is an effect opposite to that seen for the picomolar-affinity melatonin receptor which suppresses cAMP levels by inhibiting adenylate cyclase activity. Many precedents exist for subtypes of hormone or neurotransmitter receptors which differentially affect cAMP levels (e.g. dopamine D₁ and D₂ receptors, δ_2 - vs α_2 -adrenoceptors, etc.) In support of this idea, a biphasic effect of melatonin upon cAMP was seen in the hamster hypothalamus, where low (picomolar) concentrations suppressed and higher concentrations (nanomolar to micromolar) de-suppressed cAMP levels (Niles and Hashemi, 1990).

Recently, micromolar concentrations of melatonin and N-acetylserotonin have been implicated in stimulation of phosphoinositide turnover in chick brain tissue slice preparations. The picomolar-affinity melatonin receptor does not seem to be coupled to phosphoinositide turnover or calcium flux in the ovine pars tuberalis (Thompson et al., 1991). Hence, these effects seen in the chick brain may be mediated by the nanomolar-affinity melatonin binding site. While this tissue has not been rigorously examined for the presence of the nanomolar-affinity site, preliminary observations suggest that it may be present (unpublished observations, Dr. L.P. Niles). Therefore, the lack of effect of melatonin upon phosphoinositide turnover in the RPMI 1846 cell line cannot definitively exclude the possibility that a low-affinity melatonin binding site may be involved with phosphoinositide turnover in the brain.

An alternative possibility for the role of the nanomolar-affinity binding site is that perhaps N-acetylserotonin, which exhibits similar affinity as melatonin, is the true endogenous agonist for the site. NAS has been observed in the rat CNS immunohistochemically (Brown et al., 1984) and [³H]NAS binding sites found in rat brain (Niles et al., 1983). The wide tissue distribution seen for these [³H]NAS sites in rat brain is similar to, but not identical with, the wide distribution of the nanomolar-affinity binding site in the hamster CNS. NAS has been reported to have anti-nociceptive effects when injected intraventricularly in the rat (Psarakis et al., 1988). Thus, the nanomolar-affinity binding site in the hamster may be involved in such phenomena. Using radioiodinated N-acetylserotonin it would be possible to examine whether the NAS binding sites observed are the same as the low-affinity melatonin binding site characterised in this thesis.

APPENDIX I

HP 11C programme¹¹:

<u>Line #</u>	<u>Code</u>	<u>Explanation</u>
001	* LBL A	(Programme 'A')
002	STO .0	(Store H#)
003	R↓	
004	STO 1	(Store Channel 2 CPM)
005	R↓	
006	STO 9	(Store Channel 1 CPM)
007	RCI .1	(Recall Channel 1 background)
008	-	
009	STO 9	(Store Channel 1 - background)
010	RCI 1	(Recall Channel 2 CPM)
011	RCI .4	(Recall Channel 2 background)
012	-	
013	STO 1	(Store Channel 2 - background)
014	RCI 6	(Recall % Channel 1 β ² P overlap)
015	X	
016	STO .5	(Store CPM β ² P overlap)
017	CHS	
018	RCI 9	(Recall Channel 1 - background)
019	+	
020	STO 9	(Store CH1 - bkgd - β ² P overlap)
021	RCI 1	(Recall Channel 2 - background)
022	RCI .5	(Recall CPM β ² P overlap)
023	+	
024	STO 1	(Store total β ² P CPM)
025	RCI .0	(Recall H#)
026	f(y,r)	
027	1/x	
028	RCL 9	(Recall β H CPM)
029	X	
030	RCI 8	(Recall [β H]cAMP standard DPM)
031	+	
032	STO 9	(Store % [β H]cAMP recovery)
033	-	
034	1	
035	x \leq y	(If \geq 100% β H recovery then abort)
036	GTO 1	
037	RCI 7	(Recall β ² P counting efficiency)
038	x = 0	
039	GTO 0	
040	1	
041	STO 7	(Store β ² P counting efficiency)
042	* LBL 0	

¹¹ See a Hewlett-Packard calculator manual for explanation of the programming notations.

```

043          RCI 1          (Recall total corrected 32P CPM)
044          RCI 7          (Recall 32P counting efficiency)
045          -
046          RCI 9          (Recall % [3H]cAMP recovery)
047          -
048          STO 9
049          RCI .2         (Recall specific activity)
050          -
051          RCI .3         (Recall assay time)
052          -
053          RCI 9          (Recall 32P DPM)
054          x=y           (Exchange x and y registers)
055          GTO 2
056          * LBL 1
057          0
058          1/x
059          * LBL 2
060          RTN
end of file

```

Instructions

Before using cAMP programme:

1. Clear all statistics registers and the stack. (Clear Σ).
2. Place ³H standard curve in calculator as a linear regression of quenched standards. Enter % efficiency (y) then H# (x) then $\Sigma +$ and f(L.R.) at finish. Check r which should be > 0.97.
3. Store ³²P CPM (Channel 1/Channel2) ratio in register address 6.
4. Store ³²P counting efficiency in register address 7 (as a fraction). The default value is unity.
5. Store DPM of [³H]cAMP internal standard in register address 8.
6. Store Channel 1 background CPM in register address .1. Store Channel 2 background CPM in register address .4.
7. Store [α -³²P]ATP specific activity (DPM/pmol) in register address .2.
8. Store reaction time (min) in register address .3.

Running programme A:

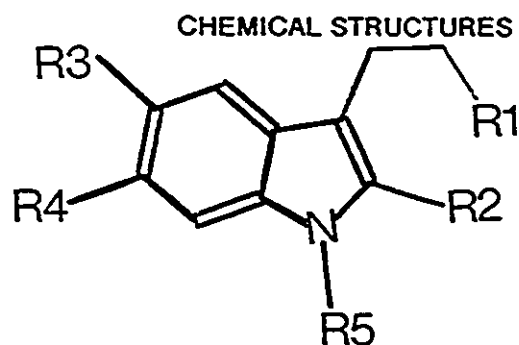
Use following keystrokes: CH1, ←, CH2, ←, H#, f(A).

The number displayed will be pmol cAMP/min formed (less the background). The raw total ³²P DPM (corrected) are in the y register.

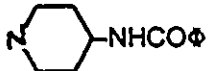
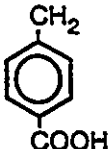
Assumptions for these calculations:

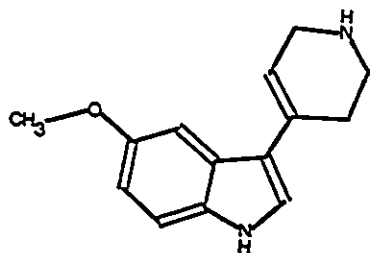
1. All samples (including ³²P and ³H total tubes) contain the same volume of 0.1 M Imidazole-HCl buffer.
2. 0.1 M imidazole doesn't quench severely.
3. The quench curve for standards is linear.
4. The quench (H#'s) for all samples is similar.
5. ³²P CPM in CH2 are relatively quench resistant.
6. The ³²P CPM ratio (CH1/CH2) is constant for all samples.
7. [³H]cAMP CPM in CH2 are minimal (i.e. CPM in CH2 << CPM in CH1).
8. The ³²P counting efficiency is assumed to be unity unless otherwise changed.

APPENDIX II

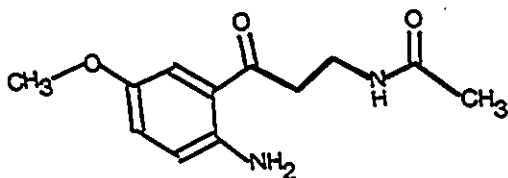


Compound	R1	R2	R3	R4	R5
2-iodomelatonin	NHCOCH ₃	I	OCH ₃	H	H
6-chloromelatonin	NHCOCH ₃	H	OCH ₃	Cl	H
N-acetylserotonin	NHCOCH ₃	H	OH	H	H
N-(2'-diazo-3',3',3'-tri-fluoropropionyl)-5-MT	NHCOCN ₂ CF ₃	H	OCH ₃	H	H
N-trifluoroacetyl-5-MT	NHCOCF ₃	H	OCH ₃	H	H
melatonin	NHCOCH ₃	H	OCH ₃	H	H
O-acetyl-N-acetyl-5-HT	NHCOCH ₃	H	OCOCH ₃	H	H
N-acetyl-6-MT	NHCOCH ₃	H	H	OCH ₃	H
6-methylmelatonin	NHCOCH ₃	H	OCH ₃	CH ₃	H
N-(6-[4'-azido-2'-nitrophenyl-amino]hexanoyl)-5-MT	NHCO(CH ₂) ₅ NHΦ(NO ₂) ² (N ₃) ⁴ H		OCH ₃	H	H
N-t-SOC-5-MT	NHCOOC(CH ₃) ₃	H	OCH ₃	H	H
N-isobutyryl-5-MT	NHCOCH(CH ₃) ₂	H	OCH ₃	H	H
N-(5-azido-2-nitrobenzoyl)-5-MT	NHCO-Φ(NO ₂) ² (N ₃) ⁵	H	OCH ₃	H	H
6-hydroxymelatonin	NHCOCH ₃	H	OCH ₃	OH	H
N-n-propionyl-5-MT	NHCOCH ₂ CH ₃	H	OCH ₃	H	H
5-methoxytryptophol	OH	H	OCH ₃	H	H
O-isobutyryl-NAS	NHCOCH ₃	H	OCOCH(CH ₃) ₂	H	H
N-n-butyryl-5-MT	NHCO(CH ₂) ₂ CH ₃	H	OCH ₃	H	H
5-hydroxytryptophol	OH	H	OH	H	H

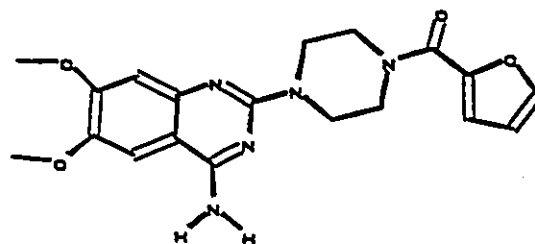
Compound	R1	R2	R3	R4	R5
N-(4-azido-2-nitrophenyl)-5-MT	$\text{NH}-\Phi(\text{NO}_2)^2(\text{N}_3)^4$	H	OCH_3	H	H
N,N-dimethyl-5-methoxytryptamine	$\text{NH}(\text{CH}_3)_2$	H	OCH_3	H	H
5-methoxytryptamine	NH_2	H	OCH_3	H	H
N-acetyltryptamine	NHCOCH_3	H	H	H	H
6-methoxytryptamine	NH_2	H	H	OCH_3	H
N,N-dimethylserotonin	$\text{NH}(\text{CH}_3)_2$	H	OH	H	H
serotonin	NH_2	H	OH	H	H
tryptophol	OH	H	H	H	H
indoramin		H	H	H	H
1-[(p-carboxy)benzyl]melatonin	NHCOCH_3	H	OCH_3	H	
N-methyltryptamine	NH_2CH_3	H	H	H	H
tryptamine	NH_2	H	H	H	H
5,6-dihydroxytryptamine	NH_2	H	OH	OH	H



Ru 24969



N-acetyl-5-methoxykynurenamine



prazosin

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