Melatonin and its Receptors in the Eye

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ABSTRACT

Melatonin is present within various eye tissues, in particular the retina, of many species and evidence exists for a local synthetic capacity. Melatonin is also reported to have several actions within the eye. The human ocular melatonin system, however, has previously been little studied. In these investigations, melatonin was quantified in human neural retina (NR) and choroid-retinal pigment epithelium (C-RPE) tissues of 46 post-mortem human eyes, using an existing radioimmunoassay which was fully validated for use in these tissues. Melatonin was present in 26 specimens in at least one tissue (range 10 to 486.8 pg/g wet weight tissue), but levels were not related to any donor parameter (sex; age; time, month and cause of death; specimen age; and post-mortem interval).

Additional studies investigated 2-[^125]I]iodomelatonin binding sites within the eye. The quail displays a robust retinal melatonin rhythm under the control of an intra-ocular clock. Membrane radioreceptor assays indicated a single class of high affinity bindings sites in both NR and C-RPE tissues. Pharmacologically the sites in the two tissues were very similar. Characterisation studies indicated that binding was saturable, reversible and specific. Photoperiod duration had little effect on either binding site density or affinity in both tissues. Autoradiographic studies confirmed the existence of a binding site in both tissues.

Using the methodology developed in the quail, 2-[^125]I]iodomelatonin binding sites were subsequently investigated in post-mortem human NR and C-RPE membranes. Saturable, specific binding which could be Scatchard transformed was demonstrated in NR and/or C-RPE membranes of only 3/18 specimens. Autoradiography confirmed this low occurrence of binding (1/11). Preliminary evidence was also obtained for a melatonin binding site in human iris sphincter muscle.

This study has detected melatonin and its binding sites in a proportion of human eyes. It is likely that the data were influenced by the post-mortem nature of the specimens.
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ABBREVIATIONS

5-HT  5-hydroxytryptamine (serotonin)
5-HTP  5-hydroxytryptophan
5-ML  5-methoxytryptophol
5-MT  5-methoxytryptamine
5MIAA  5-methoxy-3-indoleacetic acid
6MeOTHBC  6-methoxy-1,2,3,4-tetrahydro-9H-pyrido(3,4-f)indole
AADC  5-hydroxytryptophan-decarboxylase (aromatic-L-amino acid decarboxylase)
ATP  adenosine 5'-triphosphate
C-RPE  choroid-retinal pigment epithelium
cDNA  complementary DNA
COD  cause of death
DOD  date of death
GC-MS  gas chromatography-mass spectrometry
GTP  guanosine 5′-triphosphate
HIAA  5-hydroxyindole-3-acetic acid
HIOMT  hydroxyindole-O-methyltransferase
ICB  iris-ciliary body
M  ciliary muscle
MAO  monoamine oxidase
MIAA  5-methoxyindole-3-acetic acid
MOD  month of death
NAS  N-acetyl-5-hydroxytryptamine (N-acetylserotonin)
NAT  N-acetyltransferase
NR  neural retina
PMI  post-mortem interval
RHT  retinohypothalamic tract
RIA  radioimmunoassay
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>ROD</td>
<td>relative optical density measurement</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcriptase - polymerase chain reaction</td>
</tr>
<tr>
<td>SCN</td>
<td>suprachiasmatic nuclei</td>
</tr>
<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
</tr>
<tr>
<td>TOD</td>
<td>time of death</td>
</tr>
<tr>
<td>TPH</td>
<td>tryptophan-5-hydroxylase</td>
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CHAPTER 1

REVIEW OF THE LITERATURE
1.1 Introduction

1.1.1 Biological rhythms

Eukaryotic organisms are highly ordered within the temporal dimension. Not only are organisms directed through strictly organised stages of development as part of their life cycle but also their biology is structured around a framework of rhythms. Most, if not all, biochemical and physiological events occurring within living things display controlled fluctuations with time in order to optimise the internal milieu for function in the prevailing environmental conditions. Biological rhythmicity also confers anticipatory advantage to the organism allowing the required changes to occur in preparation for the expected alteration in environmental condition.

The most significant environmental variant is the daily light-dark cycle. Accordingly, most rhythms occur in synchrony with this 24 hour cycle (diurnal, or daily cycles). Such cycles may exist entirely as the product of the direct effects of light and darkness or as is more frequently the case, may result from a combination of these photoperiod effects and the output of an endogenous rhythm generator. Some diurnal rhythms are in fact circadian rhythms (Latin; *circa* about, *dies* day). True circadian rhythms are characterised by certain features. Firstly, they are of endogenous origin, that is, in the absence of any external time cues they are self-sustaining and free run with a repeating period approximating 24 hours. Secondly, they should be capable of being synchronised in phase and period (entrained) by periodic stimuli of the environment (Pittendrigh, 1981a). Principal among these entraining stimuli or *zeitgebers* (German; time-givers) is the daily light-dark cycle. Other non-photic cues include social interaction, temperature, and feeding time. The magnitude and direction of the phase shift induced by light varies as a function of subjective time. This rhythm of sensitivity is a universal property of circadian systems: light exposure during the subjective day has little or no effect on the timing of the oscillator, however applied during the early or late subjective night it delays and advances, respectively the phase of the circadian oscillator. Through the action of the *zeitgeber*(s) the period of the rhythm is set daily to 24 hours precisely. Many species also display circannual (seasonal) rhythms which have a period of approximately a year. The most obvious example of such a rhythm is the reproductive cycle in seasonal breeders.

All circadian rhythms are the outward manifestation of an internal oscillator or clock which acts as the pacemaker. Within multicellular organisms the circadian system is generally in fact multi-oscillator in nature, that is, more than one circadian clock exists. Normally all the circadian rhythms displayed by an organism are in fixed phase relationships with each other implying that when multiple clocks exist in individual organisms they are physiologically coupled to one another in some way (Pittendrigh, 1981b). One oscillator may act as the pacemaker controlling the phase and period of the secondary oscillators, or they may be mutually coupled with each other such that they all contribute to the pacemaking function. The innate rhythmicity of biological oscillators is subsequently translated into neural and hormonal outputs for the genesis of circadian functions at distant sites.

In vertebrates, circadian oscillators have been located to several anatomical areas; the suprachiasmatic nuclei (SCN) of the anterior hypothalamus (review, Klein
et al., 1991), the pineal gland (review, Underwood, 1989) and the lateral eyes (review, Cahill and Besharse, 1995). The relative contributions that these make to circadian control appears to be class dependent. In mammalian species, for example, there is much evidence to suggest that the principal circadian oscillator, or master pacemaker, which drives the circadian rhythms is situated within the SCN (Moore, 1983). Not only does the SCN function to generate circadian rhythms, however, but also, it subsequently acts to couple them with zeitgeber information in the process of entrainment. In order for light to act as a zeitgeber in mammals, it must be able to input at some level to the SCN. Light information is received by the retina and conveyed to the SCN via the retinohypothalamic tract (RHT) which leaves the eyes within the optic nerves (Moore 1983). Anatomical evidence has been provided for the existence of such a tract in a large number of species (Moore, 1973) including man (Sadun et al., 1984).

1.1.2 Pineal melatonin

The 5-methoxyindole melatonin (Figure 1.1) has been demonstrated in the pineal glands of all animals studied (Wurtman et al., 1968).

![Figure 1.1](image)

The production of melatonin by the pineal is one of the many physiological events under circadian control (Ralph et al., 1971). Synthetic rate is higher during the dark phase. Circadian production of melatonin may be under the control of a variety of oscillators depending on the species. In avian species and several lower vertebrates the pineal gland is itself a circadian oscillator and displays autonomous rhythmicity (Underwood, 1989). In mammals, pineal melatonin production is entirely dictated by sympathetic input from the superior cervical ganglion. In addition to the circadian component of production, in all animals, synthesis is a light-sensitive process (Illnerova et al., 1978; Lewy et al., 1980), light being inhibitory.

The pineal gland has been termed a neurochemical transducer (Axelrod, 1974). It translates light:dark information into a chemical messenger, melatonin. Melatonin then conveys the photoperiodic message to the organ systems of the body. The highly lipophilic nature of the molecule facilitates its passage into every fluid compartment and therefore essentially all cells. In this way, melatonin can be considered as representing the daily “chemical expression of darkness” (Reiter, 1991). Furthermore, the duration of the nocturnal melatonin surge is directly related to dark phase length and
hence it is able to provide information to the organism about absolute day and night length. Long term, therefore, melatonin is able to indicate increasing or decreasing day length and in doing so acts as a seasonal cue, for example in the control of reproduction. Consequently, the melatonin rhythm has been described as both a "clock and calendar" (Reiter, 1993). It has also been proposed that melatonin acts as an internal zeitgeber, its periodic nocturnal release serving to synchronise the internal rhythms and enabling adjustment of their phase (Armstrong, 1989). The demonstration of melatonin receptors in the mammalian SCN (Reppert et al., 1988) lends support to this hypothesis.

1.1.3 Extra-pineal melatonin

Although once believed to be a product unique to the pineal gland, evidence has now accumulated for the synthesis of melatonin in several extra-pineal tissues. Currently these include the gastrointestinal tract (Bubenik et al., 1977; Holloway et al., 1980; and Raikhlin et al., 1975); leukocytes (Finocchiaro et al., 1988; 1991); the testis (Tijmes et al., 1996), Harderian gland (Vivien-Roels et al., 1981) and the eye.

The first indication that the eye may be a site of melatonin synthesis was provided six years after the discovery of pineal melatonin. Quay (1965) demonstrated the capacity of retinal tissue, from several classes of lower vertebrate, to synthesise melatonin from N-acetylserotonin. Thus the presence of local hydroxyindole-O-methyltransferase (HIOMT) activity was confirmed. This enzyme, responsible for catalysis of melatonin formation from its precursor, had originally been localised in pineal tissue (Axelrod and Weissbach, 1960). The concept of the eye as an additional, independent source of melatonin is not an unlikely one given the many parallels, developmental, morphological, and functional, that exist between retinal and pineal tissue (reviewed in Flight, 1979; Wiechmann, 1986). Interest in ocular melatonin and its physiological relevance has since grown considerably. Discussion of various aspects of the ocular melatonin system forms the basis of this review.

1.2 Structure of the eye

The structure of the eye is shown in Figure 1.2. The globe of the eye consists of three concentric coats, the fibrous, uveal and retinal tunics, enclosing various transparent refractive media (Davson, 1980).
Figure 1.2  Structure of the eye

The three tunics
1. fibrous: sclera and cornea
2. uveal: choroid, ciliary body and iris
3. retinal

Substructure of the retina is shown in Figure 1.3

The outermost fibrous tunic is comprised of the tough, collagenous sclera and cornea. The choroid, ciliary body and iris together comprise the intermediate layer, the uvea. The choroid is highly vascularised and therefore serves a nutritive function. It contains large amounts of the pigment melanin which is synthesised in cellular organelles termed melanosomes. Melanin serves to absorb light thus preventing stimulation of the retinal cells by light reflected within the eye. The ciliary body is responsible for accommodation and secretes aqueous humor. The iris, the third, and most anterior, component of the uvea extends as a diaphragm across the front of the lens. It is suspended from the anterior surface of the ciliary body by means of a root (or ciliary border). It possesses a circular opening at its centre (the pupil) and thus forms an aperture through which light enters the eye. The iris contains two muscles; the sphincter pupillae muscle (circular, or constrictor muscle) and the dilator pupillae (radial, or dilator muscle). The posterior face of the iris is lined with a layer of heavily pigmented epithelial cells.

The innermost tunic of the eye is the photosensitive retina which lines the posterior compartment of the eye. The main organisation of the retina is in the vertical plane. It is a lamellar structure consisting of 10 layers, the specific layering pattern being common to all vertebrates (Fig 1.3). Three basic cell types comprise the retina. These are neuron support cells (Müller cells), pigmented epithelial cells and neurons.

Müller cells are glial-like elements extending in their height the full thickness of the retina having their cell bodies located in the inner nuclear layer. Their function is to provide structural support for the retinal neurons and they may also mediate essential metabolite transfer to the neurons.

The retinal pigment epithelium (RPE), a single layer of pigmented epithelial cells represents the outermost layer of the retina. This is an essential component of the vertebrate retina. The RPE cells possess long microvilli at their apical surface which project amongst the photoreceptor outer segments. Within the RPE cells and within their processes are found numerous melanosomes. In certain species, under specific environmental conditions, these granules move within the processes (section 1.7.2). The RPE is multifunctional. Its melanin content serves to reduce light scatter and it acts as an intermediary between the choroidal blood supply and the photoreceptors thus fulfils a nutritive and metabolic function. It also participates in the process of photoreceptor renewal via intermittent phagocytosis of detached distal portions of these structures (Young and Bok, 1969) (section 1.7.6) and is involved with the uptake, processing and transport of the visual retinoids (Dowling, 1960).

The neuronal component of the retina (neural retina, NR) is subdivided functionally into three components placed one on another, and forming synapses in between. (Figure 1.3) The photoreceptors (rods and cones) are the visual cells (Figure 1.4). Interior to these are the integrating neurons (bipolar cells, horizontal cells and amacrine cells) which serve to integrate sensory input from the photoreceptors before its transmission to the various brain centres. At the innermost face of the retina, are the ganglion cells the axons of which leave the retina in the optic nerve.

The eye is well supplied with blood via two separate systems: the retinal vessels which supply the inner part of the retina, and the uveal, or ciliary, blood vessels, which supply the rest of the eye.
Figure 1.3 Schema of retinal components

1. RPE.
2. Photoreceptor layer - 2 layers comprising the outer (a) and inner segments (b) of rods and cones. The outer segments project into the long microvilli of the RPE cells.
3. Outer limiting membrane - the line of intercellular junctions between Müller cells and the photoreceptors.
4. Outer nuclear layer - the cell bodies of the photoreceptors.
5. Outer plexiform layer - a zone of synaptic interaction between the photoreceptors and integrating neurons (bipolar and horizontal cells). The photoreceptors also display lateral connections with each other.
6. Inner nuclear layer - the cell bodies of the integrating neurons and the Müller cells.
7. Inner plexiform layer - a zone of synaptic interaction between the integrating neurons and the retinal ganglion cells.
8. Ganglion cell layer - the cell bodies of these cells.
9. Optic fibre (afferent fibre) layer - unmyelinated ganglion cell axons passing towards the optic disc to form the optic nerve.

Inner limiting membrane - a true membrane representing the basement membrane of the Müller cells resting on the vitreous body. Müller cells are not shown.

Figure 1.4

Photoreceptor structure; (a) rod, (b) cone

Rods and cones share several features. The cells are bipolar in form. Their cell bodies lie in the outer nuclear layer and their synaptic terminals in the outer plexiform layer. An elongated portion of the cell protrudes from the cell body. This is structurally divided into two portions, the outer and inner segments. The light sensitive outer segment is composed of a stack of membranous discs which contain photopigment. The discs are continuously shed from the end of each photoreceptor and subsequently phagocytosed by the RPE cells. The discs are continuously replaced by the inner segment. Control of the disc shedding process is discussed in section 1.7.2.6.

The interior of the eye consists of two fluid-filled cavities, the anterior and posterior compartments created by the combined partitioning effect of the lens, suspensory ligament and ciliary body. The posterior compartment is filled with a clear transparent hydrogel mass (the vitreous body). The anterior compartment contains a fluid, the aqueous humor. This is plasma-derived and secreted continuously by the cells of the ciliary epithelium into the posterior chamber from where it subsequently passes into the anterior chamber via the pupil. The pressure exerted by the aqueous humor represents the intraocular pressure (IOP). IOP is a function of the relative rates of aqueous humor formation and drainage via the canal of Schlemm (Figure 1.2).

1.3 Ocular melatonin

1.3.1 Quantification

Melatonin has been quantified in ocular tissue from members of all the animal classes (Table 1.1). A variety of analytical techniques have been employed. The earliest study demonstrating melatonin in ocular tissue (from *Xenopus laevis*) (Van de Veerdonk, 1967) used the bioassay developed by Bagnara (1960) in which melatonin is estimated on the basis of its skin lightening ability. The bioassay has now been superseded by a variety of more powerful analytical techniques. Many workers have utilised immunochemical techniques such as radioimmunoassay (RIA) and immunohistology. This latter poses special technical problems for the demonstration of melatonin. In particular the melatonin molecule is difficult to preserve as the commonly used fixatives (for example, formaldehyde) and dehydrating agents (for example, alcohol) solubilise it (Mennenga et al., 1991). Conversely, inadequate fixing results in poor tissue preservation thus allowing melatonin to diffuse (Falcón and Collin, 1991). Immunohistological studies provide semi-quantitative data only. However, their main advantage is that they enable localisation of the ligand. Data generated by both RIA and immunohistology relate to melatonin-like immunoreactivity and the possibility of interference by a cross-reactant cannot be excluded. Chromatographic techniques such as high pressure liquid chromatography (HPLC) (utilising electrochemical or fluorescence detection) and thin layer chromatography (TLC) allow more definite identification of endogenous melatonin by co-elution with authentic melatonin. The golden standard method for melatonin measurement, and that by which others are frequently validated, utilises the resolving capacity of gas chromatography coupled with mass spectrometric detection (GC-MS). This technique offers a means of unequivocal ligand identification.
<table>
<thead>
<tr>
<th>Class</th>
<th>Species</th>
<th>Technique</th>
<th>Tissue</th>
<th>Time of day</th>
<th>Amount</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fish (freshwater)</td>
<td>trout (?) (Salmo gairdneri)</td>
<td>RIA</td>
<td>neural retina</td>
<td>mid-dark; mid-light</td>
<td>55; 70</td>
<td>Gern et al., 1978</td>
</tr>
<tr>
<td></td>
<td>trout (?) Salmo fario</td>
<td>Immunohistology</td>
<td>retina: outer nuclear layer</td>
<td>light phase</td>
<td>present</td>
<td>Vivien-Roels et al., 1981</td>
</tr>
<tr>
<td></td>
<td>Trout (?) Salvelinus fontinalis</td>
<td>RIA</td>
<td>retina</td>
<td>mid-dark; mid-light</td>
<td>15; 30</td>
<td>Zachmann et al., 1992</td>
</tr>
<tr>
<td></td>
<td>Pike (M/F) Esox lucius</td>
<td>Immunohistology</td>
<td>neural retina: outer nuclear layer; outer plexiform layer; cell bodies of Müller cells of inner nuclear layer</td>
<td>dark phase; light phase</td>
<td>high levels present in all areas; low levels in INL only high; low</td>
<td>Falcón and Collin, 1991</td>
</tr>
<tr>
<td></td>
<td>Five beard rockling (Ciliata mustella)</td>
<td>Immunohistology</td>
<td>retina- outer nuclear layer</td>
<td>light phase</td>
<td>present</td>
<td>Vivien-Roels et al., 1981</td>
</tr>
<tr>
<td></td>
<td>Seascorpion (?) Taurulus bubalis</td>
<td>Immunohistology</td>
<td>retina- outer nuclear layer</td>
<td>light phase</td>
<td>present</td>
<td>Vivien-Roels et al., 1981</td>
</tr>
<tr>
<td>Amphibia</td>
<td>Frog (?) Rana pipiens</td>
<td>RIA</td>
<td>neural retina</td>
<td>mid-dark; mid-light</td>
<td>192; 96 pg/retina</td>
<td>Wiechmann et al., 1986</td>
</tr>
<tr>
<td></td>
<td>Frog (M/F) Rana perezi</td>
<td>RIA</td>
<td>whole eye minus cornea and lens</td>
<td>mid-dark; mid-light</td>
<td>200; 50 pg/eye</td>
<td>Delgado and Vivien-Roels, 1989</td>
</tr>
<tr>
<td></td>
<td>Frog (M) Rana esculenta</td>
<td>RIA</td>
<td>retina-RPE</td>
<td>mid-dark; mid-light</td>
<td>10,000; 6,000 pg/g</td>
<td>Skene et al., 1991</td>
</tr>
</tbody>
</table>

Table 1.1 Melatonin and melatonin-like immunoreactivity in the eye

Notes and abbreviations
All animals are adult; M/F = male/female; (?) = sex not stated; RIA = radioimmunoassay GC-MS = gas chromatography-mass spectrometry; HPLC-EC = high pressure liquid chromatography with electrochemical detection.
Classes Agnatha, Chondrichthyes and Osteichthyes are grouped under the heading "Fish.
Values represent means; ranges have been omitted for clarity.
<table>
<thead>
<tr>
<th>Reptilia</th>
<th>Immunohistology</th>
<th>retina- outer nuclear layer</th>
<th>light phase</th>
<th></th>
<th></th>
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<tr>
<td>Tortoise (?)</td>
<td>Immunohistology</td>
<td>retina- outer nuclear layer</td>
<td>light phase</td>
<td>present</td>
<td>Vivien-Roels et al., 1981</td>
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<tr>
<td>Testudo hermanni</td>
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<td></td>
<td></td>
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<tr>
<td>Lizard (?)</td>
<td>Immunohistology</td>
<td>retina- outer nuclear layer</td>
<td>light phase</td>
<td>present</td>
<td>Vivien-Roels et al., 1981</td>
</tr>
<tr>
<td>Lacerta agilis</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Snake (?)</td>
<td>Immunohistology</td>
<td>retina- outer nuclear layer</td>
<td>light phase</td>
<td>present</td>
<td>Vivien-Roels et al., 1981</td>
</tr>
<tr>
<td>Natrix tessellata</td>
<td></td>
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<tr>
<td>Aves</td>
<td></td>
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<tr>
<td>Chicken (?) (9 - 12d)</td>
<td>RIA</td>
<td>retina-RPE</td>
<td>dark phase</td>
<td>1050 pg/retina</td>
<td>Pang et al., 1977</td>
</tr>
<tr>
<td>White Leghorn,</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gallus domesticus</td>
<td>RIA</td>
<td>retina-RPE</td>
<td>mid-dark; mid-light</td>
<td>600; 160 pg/mg protein 920; 500 200; 200</td>
<td>Hamm and Menaker, 1980</td>
</tr>
<tr>
<td>Chicken (?) (19 d)</td>
<td>RIA and HPLC-EC</td>
<td>retina-RPE</td>
<td>mid-dark; mid-light</td>
<td>3,400; 600 pg/retina-RPE</td>
<td>Reppert and Sagar, 1983</td>
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<tr>
<td>White Leghorn,</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gallus domesticus</td>
<td>RIA</td>
<td>retina-choroid iris ciliary body</td>
<td>dark phase; light phase</td>
<td>63; 11 pg/mg protein 54; 27 61; 21</td>
<td>Aimoto et al., 1985</td>
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<tr>
<td>Chicken (?)</td>
<td>RIA</td>
<td>retina-choroid iris ciliary body</td>
<td>light phase</td>
<td>120 pg/mg protein 30</td>
<td>Rohde and Chiou, 1987</td>
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<td>White Leghorn,</td>
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<tr>
<td>Gallus domesticus</td>
<td>RIA</td>
<td>retina-choroid iris ciliary body</td>
<td>light phase</td>
<td>700; 30 pg/retina-RPE</td>
<td>Zawilska and Iwunoe, 1989</td>
</tr>
<tr>
<td>Chicken (M)</td>
<td>RIA</td>
<td>retina-RPE</td>
<td>dark; light</td>
<td>50,000; 16,000 pg/g tissue</td>
<td>Skene et al., 1991</td>
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<tr>
<td>White Leghorn,</td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>Gallus domesticus</td>
<td>RIA</td>
<td>retina-RPE</td>
<td>mid-dark; mid-light</td>
<td>15,000 - 2,000 pg/g</td>
<td>Zawilska and Wawrocka, 1993</td>
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<td>Chicken (?)</td>
<td>RIA</td>
<td>retina-RPE</td>
<td>mid-dark; mid-light</td>
<td>46,000; 7,000 pg/g retina-RPE</td>
<td>Skene et al., 1991</td>
</tr>
<tr>
<td>Mammalia</td>
<td>Immunohistology</td>
<td>retina- outer nuclear layer</td>
<td>light phase</td>
<td>present</td>
<td>References</td>
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<td>---------------------------------</td>
</tr>
<tr>
<td>Hamster, Golden (?) Mesocricetus auratus</td>
<td>GC-MS</td>
<td>retina</td>
<td>all times L:D 14:10</td>
<td>present</td>
<td>Vivien-Roels et al., 1981</td>
</tr>
<tr>
<td>Hamster, Golden (M) Mesocricetus auratus</td>
<td>RIA</td>
<td>retina-RPE</td>
<td>mid-dark; mid-light</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hamster, European (M) Cricetus cricetus</td>
<td>RIA</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Rat, Wistar (M/F)</td>
<td>Immunohistology</td>
<td>retina - outer nuclear layer</td>
<td>light phase</td>
<td>present</td>
<td></td>
</tr>
<tr>
<td>Rat, Wistar (M)</td>
<td>RIA</td>
<td>retina</td>
<td>dark phase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat, Sprague-Dawley (M)</td>
<td>RIA</td>
<td>retina</td>
<td>dark acrophase; light nadir</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat, Sprague-Dawley (M)</td>
<td>RIA</td>
<td>retina</td>
<td>dark; light phase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat, Wistar (?)</td>
<td>Immunohistology</td>
<td>retina- outer nuclear layer</td>
<td>light phase</td>
<td>present</td>
<td></td>
</tr>
<tr>
<td>Rat, Sprague-Dawley albino (M)</td>
<td>immunohistology</td>
<td>inner nuclear layer and outer nuclear layer</td>
<td>dark; light phase</td>
<td>high; low</td>
<td></td>
</tr>
<tr>
<td>Rat, Sprague-Dawley (M)</td>
<td>RIA</td>
<td>retina</td>
<td>2 h after lights on; all other times</td>
<td>58; 25 pg/retina</td>
<td>Reiter et al., 1983</td>
</tr>
<tr>
<td>Rat, Wistar albino (M)</td>
<td>RIA</td>
<td>retina</td>
<td>mid-dark; mid-light</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat, Wistar (M/F)</td>
<td>Immunohistology</td>
<td>retina - outer nuclear layer</td>
<td>mid-dark; mid-light</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse (?) Mus musculus</td>
<td>RIA</td>
<td>retina</td>
<td>light phase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Guinea pig. pigmented (M) Cavia porcellus</td>
<td>RIA</td>
<td>retina</td>
<td>close to end of dark; light phases</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Richardson’s ground squirrel (?) Spermophilus richardsoni</td>
<td>RIA</td>
<td>retina</td>
<td>all times</td>
<td>50 pg/retina - no rhythm</td>
<td>Reiter et al., 1981</td>
</tr>
<tr>
<td>Table 1.1 continued</td>
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<td></td>
</tr>
<tr>
<td><strong>Rabbit (M/F)</strong></td>
<td><strong>RIA</strong></td>
<td><strong>retina</strong></td>
<td><strong>mid-dark; mid-light</strong></td>
<td><strong>600: 300 pg/g tissue</strong></td>
<td><strong>Nowak et al., 1989</strong></td>
</tr>
<tr>
<td>Rabbit (F), albino</td>
<td>RIA</td>
<td>retina-choroid iris ciliary body</td>
<td>dark phase; light phase</td>
<td>28; 27 pg/mg protein 34; 20 23; 16</td>
<td>Aimoto et al., 1985</td>
</tr>
<tr>
<td>Rabbit, pigmented (M)</td>
<td>RIA</td>
<td>aqueous humor</td>
<td>mid-dark; mid-light</td>
<td>24; 5pg/ml</td>
<td>Yu et al., 1990</td>
</tr>
<tr>
<td>Rabbit, pigmented and albino (M/F)</td>
<td>RIA</td>
<td>aqueous humor</td>
<td>2 hrs before/after lights off</td>
<td>20; 45 pg/ml</td>
<td>Liu and Dacus, 1991</td>
</tr>
<tr>
<td>Rabbit (F)</td>
<td>RIA</td>
<td>retina choroid iris iris root-ciliary body</td>
<td>light phase</td>
<td>28.6 pg/mg protein 22.7 22.6 25.8</td>
<td>Rohde et al., 1993</td>
</tr>
<tr>
<td>Rabbit (F) New Zealand White</td>
<td>RIA</td>
<td>retina iris ciliary body</td>
<td>light phase</td>
<td>278 pg/mg protein 419 258</td>
<td>Rohde et al., 1993</td>
</tr>
<tr>
<td>Cow (?)</td>
<td>HPLC-EC</td>
<td>neural retina</td>
<td>early light phase</td>
<td>6,155 pg/g wet weight</td>
<td>Hall et al., 1985</td>
</tr>
<tr>
<td>Human (M/F)</td>
<td>RIA</td>
<td>neural retina-RPE</td>
<td></td>
<td>347-1971 pg/g wet weight</td>
<td>Osol and Schwartz, 1984</td>
</tr>
<tr>
<td>Human (?)</td>
<td>GC-MS</td>
<td>neural retina</td>
<td></td>
<td>750-10,100 pg/g wet weight</td>
<td>Leino, 1984</td>
</tr>
<tr>
<td>Human (M/F)</td>
<td>HPLC-EC</td>
<td>aqueous humor</td>
<td></td>
<td>nd - 2,300 pg/ml</td>
<td>Martin et al., 1992</td>
</tr>
</tbody>
</table>
The disparate data units employed by research groups make inter-species comparison of absolute ocular melatonin level difficult. Variations in experimental procedure, and animal age and breed differences, etc., contribute further problems. Moreover, it is likely that these factors are responsible for creating the apparent differences in data generated from the same species but by different groups (Table 1.1). One such factor is that melatonin levels of individual animals are highly dependent on the lighting schedule they are maintained under, their photoperiodic history and on the precise time of sacrifice. This is because melatonin levels within the eye do not remain constant across a 24 hour period: in a large number of species a diurnal rhythm of melatonin level has been demonstrated. In the majority of those species for which this is the case highest levels were demonstrated at night with lowest occurring during the light phase (Table 1.1). Hence ocular melatonin concentrations are in phase with melatonin synthesis in the pineal. The rhythms in ocular melatonin level and their genesis are described below.

1.3.2 Ocular distribution

1.3.2.1 NR and RPE

Studies of ocular melatonin have focused predominantly upon the content of NR, sometimes in association with the retinal pigment epithelium (RPE). Frequently it is not stated whether or not the RPE is included in the tissue termed “retina”. Melatonin appears to be present in both compartments, however. Isolated trout (Gern et al., 1978), bovine (Hall et al., 1985) and chicken (Hamm and Menaker, 1980) NR tissues contain melatonin. Immunohistochemical studies in several species indicate that within the substructure of the NR melatonin-like immunoreactivity is almost exclusively confined to the outer nuclear layer, that is, the cell bodies of the photoreceptors (Bubenik et al., 1978; Vivien-Roels et al., 1981 and Mennenga et al., 1991). The specific cell type was not determined. In some instances, melatonin has been located in the inner nuclear layer (Vivien-Roels et al., 1981) although these authors have attributed their own results to diffusion of the molecule. The studies of Falcon and Collin (1991) showed that, in addition to the outer nuclear layer, pike retina contained melatonin-like immunoreactivity in both the outer plexiform layer and the cell bodies of Müller cells. These authors acknowledged, however, that the methodology employed may have visualised the indole 5-methoxytryptamine as well as melatonin.

The small number of studies which have investigated RPE only, found it to contain melatonin (for example, chicken Hamm and Menaker, 1980). Also, the melatonin content of combined NR and RPE is higher than that of NR (Zawilska and Iuvone, 1989) further implying that the RPE contains melatonin.

1.3.2.2 Uveal tissue

One group have reported the presence of melatonin within rabbit choroidal tissue (Rohde et al., 1993). Also in the rabbit, melatonin has been demonstrated in uveal tissue from the anterior eye, namely the iris and ciliary body (Aimoto et al., 1985; Rohde et al., 1993a,b). Similarly, chicken iris and ciliary body contain...
melatonin (Aimoto et al., 1985; Rohde and Chiou, 1987). The precise location of melatonin within these areas has not been investigated.

1.3.2.3 Aqueous humor

Melatonin has been quantified in both human (Martin et al., 1992) and rabbit (Yu et al., 1990; Liu and Dacus, 1991) aqueous humor.

1.3.3 Origin

That melatonin which is quantified within the eye may derive from three possible sources; local, de novo synthesis; active uptake of melatonin originating in other, distant sites of melatonin synthesis, in practical terms the pineal gland; or it may simply represent that present within the ocular blood circulation. Available evidence favours the former. However, the possibility of the entrance of melatonin into the eye from the systemic circulation, that is, accumulation of circulating pineal melatonin has, been neither proved nor disproved conclusively. Kopin and co-workers (1961) demonstrated that following intravenous administration of radiolabelled melatonin to rats there was an extensive distribution of melatonin throughout the body. Although the eye itself was not examined in this study it was shown that other organs, for example, the adrenal gland, were capable of concentrating melatonin relative to plasma levels. Subsequently it was shown in similar experiments that this in fact was also the case for the eye: in the cat, the iris-choroid layer of the eye was observed to concentrate the circulating hormone tenfold (Wurtman et al., 1964). Bubenik and co-workers (1978) have shown that following both melatonin injection and implant, the amount of melatonin in the outer nuclear layer of the rat retina is greatly increased and suggest that this may indicate some form of local uptake mechanism, for example, a receptor. However, because of the supra-physiological levels of melatonin employed the physiological significance of such findings is questionable. Similar studies were undertaken by Cassone et al. (1986) who demonstrated that circulating exogenous melatonin was able to enter the chicken eye (NR and RPE). Yu and colleagues (1990) suggest that the melatonin content of rabbit aqueous humor is derived from the circulating plasma pool but provide only circumstantial evidence.

No specific mechanism for accumulation of melatonin by the eye, for example facilitated or active transport, has been reported. Furthermore bi-directional movement of melatonin across the RPE has been demonstrated to occur as a result of simple passive diffusion facilitated by the high permeability coefficient of the melatonin molecule (Pautler and Hall, 1987).

Melatonin content of rabbit aqueous humor is not decreased following unilateral transection of the cervical sympathetic trunk which would decrease night-time pineal melatonin synthesis (Liu and Dacus, 1991a). It is possible, therefore that in conflict with the proposals of Yu and co-workers (1990) the melatonin content of aqueous humor does in fact derive from some intraocular source.

The most plausible explanation for the existence of melatonin within the eye remains local synthesis. This is supported by many facts. Firstly, the appropriate precursor and intermediary molecules of synthesis and anabolic enzymatic machinery have been shown to be present in ocular tissue. These aspects of synthesis are discussed in detail in section 1.4. Additional evidence is provided indirectly by studies
on pinealectomised animals. Under these circumstances, ocular melatonin in various tissues remains demonstrable despite removal of the primary source of circulating melatonin (rats: Yu et al., 1981a, b; Reiter et al., 1983; quail: Underwood et al., 1984; chicken: Reppert and Sagar, 1983; Rohde et al., 1985; Hamm and Menaker, 1980).

1.4 Synthesis of ocular melatonin

The concept that the eye may be capable of de novo synthesis of melatonin was originally not widely accepted. Later this view changed when evidence for retinal HIOMT in a number of species was offered (Quay, 1965).

Numerous studies have since investigated the potential components of the retinal melatonin generating system. The elucidation of the metabolic pathway for ocular melatonin synthesis has been based on observations made regarding pineal melatonin synthesis. It is now apparent that the ocular pathway is extensively similar with that described for the pineal (review, Axelrod, 1974). Thus the synthetic precursor of melatonin is the essential amino acid L-tryptophan which is supplied via dietary sources. The pathway of intermediates and enzymes involved is shown in Figure 1.5.
Fig. 1.5
Pathway of retinal synthesis of melatonin

tryptophan-5-hydroxylase (TPH)

5-hydroxytryptophan-decarboxylase (AADC)

5-hydroxytryptophan (5-HTP)

serotonin-N-acetyltransferase (NAT)

5-hydroxytryptamine (5-HT)

hydroxyindole-O-methyltransferase (HIOMT)

N-acetyl-5-hydroxytryptamine

melatonin
1.4.1 5-Hydroxytryptamine

In the first stage of melatonin synthesis L-tryptophan is sequentially hydroxylated and decarboxylated by the enzymes tryptophan-5-hydroxylase (TPH) and 5-hydroxytryptophan-decarboxylase (aromatic-L-amino acid decarboxylase, AADC), respectively, through the intermediate 5-hydroxytryptophan (5-HTP) to yield 5-hydroxytryptamine (5-HT, serotonin).

The presence of 5-HT in NR has now been confirmed in many species, both non-mammalian (Tornqvist et al., 1983; Schütte and Witkovsky, 1990; Siuciak et al., 1992) and mammalian (Ehinger et al., 1981; Mitchell and Redburn, 1985; Wilhelm et al., 1993; Steinlechner et al., 1995) albeit at lower concentrations in the latter. Within the retina, endogenous 5-HT is reported to be found primarily in amacrine cell bodies and terminals situated in the inner plexiform layer, and bipolar cells (Osborne et al., 1981; Schütte and Witkovsky, 1990; Wilhelm et al., 1993). 5-HT has also been demonstrated in RPE and iris-ciliary body tissues (Bondy et al., 1983; Klyce et al., 1982; Uusitalo et al., 1982; Osborne, 1983), and in aqueous humor (Martin et al., 1988, 1992). All of these sites of 5-HT correspond with the reported ocular distribution of melatonin.

Much evidence exists for the local synthesis of 5-HT from tryptophan. Both TPH (Osborne, 1980; Osborne et al., 1984; Thomas and Iuvone, 1991; Wilhelm et al., 1993) and AADC (Nguyen-Legros et al., 1994) have been demonstrated within the retina and isolated retina is able to synthesise radiolabelled 5-HT from tritiated tryptophan (for example, Osborne, 1980; 1981; 1984; Redburn and Mitchell, 1989). A complementary DNA (cDNA) probe for TPH has been produced (Green and Besharse, 1994) and used to demonstrate that within the retina, mRNA encoding TPH is present in photoreceptors (Green et al., 1995). Similarly, AADC immunoreactivity has been localised to the photoreceptor cell inner segments (Nguyen-Legros et al., 1994).

1.4.2 N-acetyltransferase (NAT)

Melatonin is subsequently synthesised from 5-HT in a two-step sequence. Firstly, it is acetylated in the presence of acetyl CoA by N-acetyltransferase (NAT; arylalkylamine N-acetyltransferase) to form N-acetyl-5-hydroxytryptamine (N-acetylserotonin; NAS).

Within the eye, the majority of studies demonstrating NAT activity have investigated homogenates of either NR alone or combined with the RPE (Table 1.2). As with several melatonin quantification studies, some authors have not stipulated whether the term “retina” they employ refers to NR only or NR combined with RPE. NR and RPE tissue compartments have been investigated independently in the chicken however and activity has been demonstrated in both (Binkley et al., 1979 and Hamm and Menaker, 1980). Data concerning the precise localisation of NAT has not been available due to the (initial) lack of specific probes: early studies investigated NAT activity by radioenzymatic assay in whole retina homogenate (Table 1.2). There is indirect evidence, however, that NAT activity is associated primarily with the photoreceptors. Firstly, NAT activity is maintained in the retina of chicken which have been treated with intravitreal kainic acid to destroy ganglion and integrating neurons (Zawilska and Iuvone, 1992). Secondly, NAT activity in chick retinal cell
cultures is highly, positively correlated with their relative photoreceptor contents (Iuvone et al., 1990). Additionally in these latter experiments, agents which stimulated NAT activity were more effective in those cultures containing the highest photoreceptor density.

Another possible location of NAT activity includes rabbit iris-ciliary body (Rohde and Chiou, 1987) and in the chicken, both the iris and ciliary body have been demonstrated to possess NAT activity (Aimoto et al., 1985). Martin and co-workers (1992) have also reported NAT activity in human ciliary body however, this finding is unconfirmed.

In 1995 the cDNA encoding ovine pineal serotonin-NAT was cloned (Coon et al., 1995) and subsequently NAT mRNA was found to be present in the ovine retina. Shortly afterwards, information concerning the human NAT gene was provided (Coon et al., 1996) and NAT was found to be expressed in human retina also. It is envisaged that availability of the NAT cDNA clone will soon enable cellular distribution of NAT within the retina and other ocular tissues to be determined.

The product of NAT action is the intermediate NAS. Evidence of its occurrence provides some support for the presence of NAT. Accordingly, NAS-like immunoreactivity has been identified in retinal tissue from several avian species including quail, pigeon and chicken (Pang et al., 1983) and rat (Pang et al., 1977 and 1981), and NAS is synthesised by primary cultures of guinea pig retina (Yu et al., 1982). Also, NAS is produced following incubation of rat NR with radiolabelled 5-HT (Cardinali and Rosner, 1971b; Redburn and Mitchell, 1989).

1.4.3 Hydroxyindole-O-methyltransferase (HIOMT)

In the final stage of melatonin synthesis, NAS is O-methylated on the indole ring by hydroxyindole-O-methyltransferase (HIOMT). Discovery of this enzyme in ocular tissue by Quay (1965) was responsible for initiating investigations in the field of retinal melatonin. As shown in Table 1.3, much evidence exists for the presence of HIOMT activity, within the eye. As with NAT, measurements of HIOMT activity have concentrated on NR or NR combined with RPE. Quay and co-workers (1969) reported that in trout, HIOMT was present in both NR and RPE but with greater amounts in the latter. In contrast, Eichler and Moore (1975) demonstrated that within the frog eye HIOMT was highly localised within the NR only. Other ocular tissues have been much less thoroughly investigated. Cardinali and Rosner (1971a) demonstrated the iris, sclerotic-choroid, lens and corneal tissue of the rat to be devoid of HIOMT activity, in contrast to their findings in retina. In conjunction with their studies on ciliary body in NAT, Martin and co-workers (1992) also reported HIOMT activity in human ciliary body.
<table>
<thead>
<tr>
<th>Class</th>
<th>Species</th>
<th>Tissue/Location</th>
<th>Authors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fish</td>
<td>pike</td>
<td>NR-RPE</td>
<td>Falcón and Collin, 1991</td>
</tr>
<tr>
<td>Amphibia</td>
<td>frog</td>
<td>retina</td>
<td>Besharse and Iuvone, 1983; Serino et al., 1993</td>
</tr>
<tr>
<td></td>
<td>toad</td>
<td>retina</td>
<td>Serino et al., 1993</td>
</tr>
<tr>
<td>Aves</td>
<td>chicken</td>
<td>NR-RPE</td>
<td>Binkley et al., 1979</td>
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<tr>
<td></td>
<td>chicken</td>
<td>NR; RPE</td>
<td>Hamm and Menaker, 1980</td>
</tr>
<tr>
<td></td>
<td>chicken</td>
<td>retina</td>
<td>Hamm et al., 1983</td>
</tr>
<tr>
<td></td>
<td>chicken</td>
<td>retina; iris; ciliary body</td>
<td>Nowak et al., 1989; Nowak and Zawilska and Iuvone, 1992; Nowak and Wawroska 1993</td>
</tr>
<tr>
<td></td>
<td>chicken</td>
<td>NR</td>
<td>Iuvone, 1990; Zawilska et al., 1991</td>
</tr>
<tr>
<td></td>
<td>sparrow</td>
<td>retina-RPE</td>
<td>Aimoto et al., 1985</td>
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<td></td>
<td>Binkley et al., 1979</td>
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<tr>
<td>Mammalia</td>
<td>rabbit</td>
<td>retina</td>
<td>Nowak et al., 1988; Nowak et al., 1989</td>
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<td></td>
<td>rabbit</td>
<td>iris-ciliary body</td>
<td>Rohde and Chiou, 1987</td>
</tr>
<tr>
<td></td>
<td>rat</td>
<td>NR-RPE</td>
<td>Binkley et al., 1979</td>
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<td>rat</td>
<td>retina</td>
<td>Miller et al., 1980; Nowak et al., 1989</td>
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<td>gerbil</td>
<td>retina</td>
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<td>Binkley et al., 1979</td>
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<tr>
<td></td>
<td>human</td>
<td>ciliary body</td>
<td>Martin et al., 1992</td>
</tr>
<tr>
<td></td>
<td>sheep</td>
<td>retina</td>
<td>Coon et al., 1995</td>
</tr>
<tr>
<td></td>
<td>human</td>
<td>retina</td>
<td>Coon et al., 1996</td>
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</tbody>
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Table 1.2
Ocular NAT activity and NAT expression in vertebrates. NAT activity was determined by radioenzymatic assay (measurement of the transfer of a radiolabelled acetyl group from acetyl CoA to tryptamine), or by measurement of unlabelled product. Expression of NAT was assessed by probing for mRNA.

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<table>
<thead>
<tr>
<th>Class</th>
<th>Species</th>
<th>Tissue/Localisation</th>
<th>Authors</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Fish</em></td>
<td>4 species, including trout and goldfish&lt;br&gt;trout&lt;br&gt;pike</td>
<td>retina&lt;br&gt;NR; RPE&lt;br&gt;NR-RPE</td>
<td>Quay, 1965&lt;br&gt;Quay <em>et al.</em>, 1969&lt;br&gt;Falcón and Collin, 1991</td>
</tr>
<tr>
<td><em>Amphibia</em></td>
<td>frog, toad; mud puppy&lt;br&gt;frog</td>
<td>retina&lt;br&gt;NR</td>
<td>Quay, 1965&lt;br&gt;Eichler and Moore, 1975</td>
</tr>
<tr>
<td><em>Reptilia</em></td>
<td>turtle, lizard, iguana</td>
<td>retina</td>
<td>Quay, 1965</td>
</tr>
<tr>
<td><em>Aves</em></td>
<td>chicken; dove&lt;br&gt;chicken</td>
<td>retina&lt;br&gt;retina&lt;br&gt;photoreceptors§§</td>
<td>Zawilska and Irvone, 1992; Voisin <em>et al.</em>, 1988&lt;br&gt;Wiechmann and Craft, 1993</td>
</tr>
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**Table 1.3**
Ocular HIOMT activity and HIOMT expression in vertebrates. HIOMT activity was determined by radioenzymatic assay (measurement of production of labelled melatonin from [methyl-3H/14C]-S-adenosyl-L-methionine), or by measurement of unlabelled product§. Expression of NAT was assessed by probing for mRNA: in situ hybridisation of mRNA§§, Southern blot analysis of reverse transcriptase-polymerase chain reaction products†, or ribonuclease protection assay‡.
Unlike NAT, probes for HIOMT, both immunoreactive and molecular, have been available for some time. Much controversy has been associated with the former, however. Wiechmann and colleagues reported production of a HIOMT-specific antiserum in 1985 which they subsequently used to study the occurrence and distribution of HIOMT in several species (Wiechmann et al., 1985; Wiechmann and Hollyfield, 1987 and 1989; Wiechmann and O'Steen, 1990). Later these claims were retracted on the basis that the antiserum was not in fact HIOMT-specific as previously indicated (Wiechmann, 1993). Using a different antiserum however, Voisin and co-workers (1988) have reported immunoprecipitation of HIOMT activity in chicken retina homogenate.

More recently molecular techniques have been applied to the study of HIOMT. In the chicken retina, \textit{in situ} hybridisation studies have localised HIOMT mRNA to the photoreceptor layer (Wiechmann and Craft, 1993). Using a highly sensitive technique (Southern blot analysis of reverse transcriptase-polymerase chain reaction products), HIOMT mRNA has also been demonstrated at low level in human retina (Rodriguez et al., 1994; Bernard et al., 1995) although Bernard and co-workers (1995) were unable to detect HIOMT enzyme activity or immunoreactive protein in human retina. HIOMT mRNA has also been identified in rat retina (Gauer et al., 1996). An earlier study was unable to detect HIOMT mRNA in bovine retina (Ishida et al., 1987).

1.4.4 Studies of melatonin synthesis \textit{in vitro}

In addition to data concerning measurement of individual enzymes and precursor molecules involved in melatonin synthesis there is a large body of data which provides evidence for an intact, functional synthetic capacity of various ocular tissues \textit{in vitro}. The first evidence came from the isolated rat retina which when incubated with radiolabelled 5-HT produced both NAS and melatonin (Cardinali and Rosner, 1971b). This was later confirmed in trout NR by Gern and Ralph (1979) and subsequently in rat retina by Redburn and Mitchell (1989). Production of melatonin by isolated guinea pig retina has been demonstrated in the presence of exogenous unlabelled 5-HT (Yu et al., 1982). In rat retina also, synthesis of melatonin from radiolabelled tryptophan is possible indicating the presence of the full complement of metabolic enzymes (Redburn and Mitchell, 1989). Melatonin production has also been observed in the absence of exogenous substrate in eyecup preparations of the goldfish (Grace et al., 1991); frog (Cahill and Besharse, 1990); hamster (Tosini and Menaker, 1996); and in isolated frog NR (Flight et al., 1983). Supplementation of the culture medium with 5-HTP or indirectly with 5-HT, via the addition of the monoamine oxidase inhibitor (MAO) pargyline, does however enhance melatonin synthesis in the frog eyecup, although addition of the primary precursor tryptophan does not have the same effect (Cahill and Besharse, 1989 and 1990).

Inclusion of TPH and HIOMT inhibitors in the rat NR incubation system results in the effective blockade of 5-HT and melatonin synthesis, respectively, thus confirming the participation of these enzymes in melatonin synthesis (Redburn and Mitchell, 1989).

Data obtained with whole eyecup preparations do not provide information regarding the precise area of melatonin synthesis. However, culture of isolated NR indicates that this tissue specifically is capable of melatonin synthesis (Redburn and
Mitchell, 1989; Flight et al., 1983). Within the NR it is unlikely that melatonin synthesis occurs in the cells of the inner retina. *Xenopus* eyecups in which the inner retina has been destroyed by detergent retain their capacity for melatonin synthesis from 5-HTP (Cahill and Besharse, 1992). Similarly, if treated with kainic acid, a neurotoxic excitatory amino acid analogue, another agent which destroys inner retinal neurons, chicken NR-RPE is still able to synthesise melatonin from 5-HTP (Cahill and Besharse, 1992). These findings combined with data regarding the cellular distribution of the synthetic enzymes (sections 1.4.1 to 1.4.3) indicate that within the retina the photoreceptors are likely to be the principal site of melatonin synthesis. Additional evidence supporting the hypothesis that melatonin is synthesised within the photoreceptors comes from studies performed on retinally degenerate chicken. In such birds there is a negative correlation between extent of loss rods and cones and retinal melatonin content (Pang et al., 1989).

Few other ocular tissues have been investigated individually for their ability to synthesise melatonin. Frog RPE was however shown not to synthesise melatonin (Flight et al., 1983) although no substrates were provided and in addition, the possibility of rapid breakdown of any melatonin which may have been produced cannot be excluded.

1.4.5 Release of melatonin

Unlike most of the classical neurotransmitters of the retina, release of melatonin appears not to involve stimulus-coupled secretion requiring depolarisation: isolated rat retina does not release endogenously synthesised melatonin in response to potassium-induced depolarisation (Redburn and Mitchell, 1989). In this same experimental model, melatonin synthesis was associated with an immediate increase in melatonin release - there was no storage. This confirms the general assumption that melatonin simply diffuses from its site of synthesis as a result of its lipophilic nature. Not all the available evidence is consistent with the theory of immediate release however. In the quail, for example, despite the fact that the eyes contain more melatonin than the pineal, their contribution of melatonin to the circulating level is less than 40% (Underwood et al., 1984) suggesting either that melatonin is released immediately and degraded, or that some melatonin may be stored in the eye and not immediately secreted.
1.5 Regulation of ocular melatonin levels

1.5.1 Photic regulation

Melatonin levels within the retina of several species display day/night differences, that is, they exhibit diurnal rhythmicity (Table 1.1). The ocular melatonin rhythms observed are independent of those in the pineal as they persist following pinealectomy (for example, Yu et al., 1981; Reppert and Sagar, 1983). In most instances, the maxima and minima occur in darkness and light, respectively: at the end of the light phase melatonin content increases reaching a peak around the middle of the dark phase (for example, Pang et al., 1983; quail, pigeon, chicken: Underwood et al., 1984). Similar patterns are apparent regardless of whether the animal is diurnally or nocturnally active or exhibits crepuscular activity (Zawilska, 1992). Amongst the animal classes the magnitude of nocturnal increase is generally lowest in mammals (Nowak et al., 1989). Overall the cyclic changes of retinal melatonin are not as robust as those of the pineal, that is, the amplitude of the rhythm, is much less (for example, Zawilska and Iuvone, 1989), but there are exceptions. The lizard, Dipsosaurus dorsalis, has a very distinct night day variation in retinal melatonin, whereas the pineal of this species is reported to show no rhythm in its production of melatonin (Menaker, 1985).

There are a number of inconsistencies concerning retinal melatonin rhythms. For example, there has been a failure to demonstrate diurnally rhythmic ocular melatonin levels in the following species; Richardson's ground squirrel (Reiter et al., 1981), hamster Mesocricetus auratus (Beck and Pévet, 1984) and lizard Anolis carolinensis (Underwood, 1985). Also, in the trout, melatonin levels are out of phase with the light-dark cycle, that is, the melatonin peak occurs in the middle of the light phase (Zachmann et al., 1992; Gern et al., 1978).

These anomalous findings may be of physiological relevance. The possibility exists however that they are in fact attributable to various aspects of methodology. In the rat, for example, Reiter and co-workers (1983) demonstrated an unusual melatonin rhythm consisting of a short term rise two hours after lights on. These findings were not in agreement with other studies on the rat (for example, Pang et al., 1980) which demonstrated a more prolonged rise in melatonin occurring towards the end of the dark phase. Consequently the authors postulated that the differences were related to the different photoperiods that the research groups had employed. As a further example, Pang and Allen (1986) have suggested that the irregular data reported by Gern et al. (1978) and Reiter et al. (1981) may in fact be artefactual and related to the antiserum used in both instances. M. auratus hamster retina has also recently been shown to synthesise melatonin rhythmically (Steinlechner, et al., 1995; Tosini and Menaker, 1996) thus calling into question the significance of the findings of Beck and Pévet (1984).

The diurnal rhythm of retinal melatonin appears to be the product of a complex interaction of at least two factors. The first of these involves the direct effects of light and darkness. When the guinea pig is subjected to an extended period of darkness, the corresponding level of retinal melatonin is increased (Pang et al., 1982). In contrast, light suppresses the dark associated increase in retinal melatonin content (Yu et al, 1982). Furthermore, as in the pineal (section 1.1.2), light acutely inhibits
1.5.1.1 Molecular basis of melatonin rhythmicity

Melatonin is released immediately upon synthesis (Redburn and Mitchell, 1989), hence the nocturnal increases in melatonin levels observed must be accounted for by either an increase in its synthesis or decrease in its metabolic degradation. Regarding the latter, there is evidence of retinal metabolism of melatonin in some species (section 1.6) however there is no information concerning possible regulation of the enzymes involved. Available experimental evidence suggests that fluctuations in synthetic rate are of particular importance in the control of retinal melatonin. The synthetic enzymes and precursor molecules are obvious candidates for components which can be manipulated to effect changes in melatonin production. Fluctuating NAT activity plays an essential role in the regulation of ocular melatonin synthesis. Other factors are also involved.

NAT activity levels parallel the diurnal fluctuations of melatonin level occurring within the retina. The activity nadir occurs around the middle of the light phase. Activity subsequently increases just prior to the dark phase and reaches its maximum towards the middle of the dark phase. Similar activity profiles have been demonstrated in many species, including mammals (for example, chicken: Hamm and Menaker, 1980; rabbit: Nowak et al., 1988). The product of NAT activity, NAS, can be measured and its levels follow the diurnal variation of NAT activity, that is, high at night and low in the day (Pang et al., 1983). It is likely therefore that NAT makes an important contribution to the regulation of rhythmic melatonin biosynthesis: by increasing production of NAS, melatonin production is subsequently increased through a mass action effect.

The effect of acute exposure to light is also mediated through NAT: upon exposure to light during the dark phase NAT activity decreases (Binkley et al., 1979). This sensitivity of NAT to light is partially responsible for the low level of NAT activity during the day time. The period of illumination required for effective inhibition of NAT activity to occur is short. In the chicken, for example, five minutes is sufficient (Hamm et al., 1983). The decrease is rapid. In the chicken, the NAT halving time is about 10 minutes (Hamm and Menaker, 1980 and Hamm et al., 1983). The effect of light on NAT activity is specific to that in the retina (and pineal) - activity in various areas of the brain, for example the hypothalamus, is unaffected (Hamm et al., 1983). Monochromatic lights (blue, green or red) are equally effective as white light in suppressing chick retina NAT activity (Zawilska et al., 1995).
1.5.1.2 Control of NAT activity: Roles of cAMP, Ca^{2+} and protein synthesis

The surge in NAT activity which occurs in the dark phase is mediated by a cyclic AMP- and Ca^{2+}-dependent mechanism. Protein synthesis may also play an important role. These same factors are also implicated in the suppressive effects of light on NAT activity.

Agents which elevate intracellular cAMP levels in a variety of ways, for example, adenylate cyclase activators such as forskolin; phosphodiesterase inhibitors; and non-hydrolysable analogues (for example, dibutyryl cAMP) cause an increase in retinal NAT of the chicken and hen in vivo when applied during the light phase (Nowak and Zurawska, 1989; Nowak and Wawrocka, 1993 and Zawilska et al., 1991). Similar effects have been demonstrated in chick retina cell culture (Iuvone, 1990; Iuvone et al., 1990). In contrast, in rat and Xenopus in vitro and rat and rabbit in vivo application of cAMP enhancing agents will only induce increases in retinal NAT activity if applied during the dark phase (Iuvone and Besharse, 1983; Nowak, 1990). Hence there may be some mechanism by which the endogenous rhythm generator can overcome the effect of cAMP on NAT (Cahill et al., 1991). In accordance with the effects of cAMP on retinal NAT in the rabbit, application of topical forskolin in light also had no effect on iris-ciliary body NAT activity or melatonin level (Rohde and Chiou, 1987). Its effect in darkness was not investigated.

The effect of cAMP is specific to this nucleotide: others such as adenosine and dibutyryl cyclic-GMP do not have similar effects on NAT activity (Iuvone and Besharse, 1983).

Both the natural night-time increase in NAT activity and the stimulation of NAT activity induced by exogenous cAMP are inhibited by RNA and protein synthesis inhibitors (Iuvone and Besharse, 1983 and 1986; Iuvone et al., 1990). It is likely therefore that the regulation of NAT by cAMP occurs at the molecular level through activation of cAMP-dependent protein kinase and subsequent transcription of mRNA. Whether the increase in NAT occurs as a result of de novo synthesis of NAT or is stimulated via synthesis of some NAT-activating protein is unknown. In the absence of a probe for NAT, as was until recently the case, these problems could not be addressed. It is anticipated that the newly cloned cDNA for NAT will enable many questions concerning the regulation of NAT gene expression to be answered (Klein et al., 1996).

Calcium is another important factor involved in the control of NAT activity: transmembrane transport of calcium into the cell appears to play an important role in the induction of NAT activity. In Xenopus (Iuvone and Besharse, 1986), and rat (Nowak, 1990) retina, omission of calcium medium or introduction of Ca^{2+}-channel blockers can impede both the natural night-time rise in NAT activity and that induced pharmacologically. In cultured chick retinal cells the induction of NAT activity caused by K^+-evoked depolarisation is blocked by calcium chelators and voltage-sensitive calcium channels (VSCC) antagonists (Avendano et al., 1990). Influx of calcium through the L-type VSCC is of particular importance - the dark induced increase in NAT activity is inhibited and potentiated by L-type VSCC antagonists and agonists, respectively (Iuvone and Besharse, 1986; Avendano et al., 1990; Zawilska and Nowak, 1990).
Whether the role calcium plays in the regulation of NAT activity within the retina is in fact independent of or related to any action involved with the cAMP mediated stimulation of NAT activity is not clear at present. Accumulating evidence suggests that calcium may regulate NAT activity indirectly through its interactions with intracellular cAMP content. For example, the stimulatory effect of cAMP on NAT activity in rat and hen retina is highly dependent on Ca\(^{2+}\) concentration (Nowak, 1990).

The photoreceptor has been established as the primary site of melatonin synthesis within the retina (section 1.4.4). Zawilska and Nowak (1992) propose the following hypothesis in explanation of the calcium-dependent night-time induction of NAT which occurs in this cell type. During the hours of darkness, photoreceptor intracellular calcium levels increase as a result of calcium entrance through the VSCC which open in darkness. Once a critical level of calcium is reached, this activates the cAMP generating system which, in turn induces NAT activity. Conversely, during the light phase, VSCC are closed and calcium levels are consequently too low to have any effect on cAMP. This system may also be responsible for part of the acute suppressive effects of light on melatonin synthesis: light hyperpolarises photoreceptors, causing closure of the VSCC (for example, Corey et al., 1984) thus inhibiting NAT activity.

1.5.2 Circadian regulation of melatonin

Like the photic regulation of melatonin, its circadian control also results, at least in part, from fluctuations in NAT activity. The first evidence that NAT was under circadian control came from \textit{in vitro} studies with \textit{Xenopus} eyecups. The NAT activity rhythm expressed by this preparation conformed to both the essential criteria of a circadian rhythm, that is the rhythm displayed a persistent period of 24 hours in constant darkness (over a 3 day period), and could be phase reversed in response to a phase reversal of the light dark cycle (re-entrained) (Besharse and Iuvone, 1983). Subsequently in the same model, corresponding circadian production of melatonin was also demonstrated: when \textit{Xenopus} eyecups were maintained individually in superfusion culture systems, circadian overflow of melatonin was observed for up to 5 cycles in constant darkness (Cahill and Besharse, 1990; 1991).

Studies performed \textit{in vivo} in the Japanese quail also provide very convincing evidence for a true circadian rhythm of ocular melatonin production under the control of a local circadian oscillator. The existence of a melatonin rhythm in the quail retina is well documented (for example, Underwood et al., 1984; 1990b). This is retained following pinealectomy (Underwood et al., 1984) and optic nerve section (Underwood and Siopes, 1985). Exposure of individual eyes of the same bird to oppositely phased light-dark cycles resulted in oppositely phased ocular melatonin rhythms (Underwood et al., 1988). Furthermore, a similar effect is obtained despite optic nerve section and ablation of ocular sympathetic innervation (Underwood et al., 1990). Together these data provide evidence that an ocular circadian oscillator controlling melatonin synthesis exists in this species.

Studies performed on \textit{Xenopus} have located the specific site of the ocular circadian oscillator regulating ocular melatonin synthesis to the photoreceptors. Cultured whole NR continue to produce melatonin, albeit at reduced levels, in a
circadian manner, that is, independent of influence from other regions of the eye (Cahill and Besharse, 1993). Furthermore, in eyecups which had been lesioned by removal of the inner retina, circadian melatonin production was maintained (Cahill and Besharse, 1992). Ultimate proof that the clock controlling melatonin production is actually within the photoreceptors of the Xenopus eye was gained from studies on cultured isolated rod and cone photoreceptors (Cahill and Besharse, 1993). These preparations were found to rhythmically synthesise melatonin in constant darkness.

For several years the *Xenopus* eyecup represented the only functional *in vitro* preparation for study of the ocular circadian clock. Very recently however, circadian rhythmicity of melatonin synthesis has been demonstrated in cultured NR of a mammalian species, the golden hamster (Tosini and Menaker, 1996). Rhythmic melatonin production was maintained over a 5 day period of constant darkness. Furthermore, the rhythm was entrainable to particular light cycles. In the past, several researchers had experienced difficulty in demonstrating an ocular clock in mammalian species. The key factor in this hamster study appeared to be the much reduced culture temperature employed (27 compared with 37 °C previously). Although use of this temperature was justified on the basis that the hamster is a hibernating species, the physiological relevance of these findings should perhaps be questioned. However this aside the hamster model provides irrefutable proof for the first time that the mammalian retina does at least possess the intrinsic capacity for circadian production of melatonin.

Circadian rhythms are characterised by their susceptibility to entrainment by external stimuli and, in accordance with classical circadian theory, in *Xenopus* eyecups, for example, the specific effect of a light pulse (an entraining agent) on the subsequent phase of the melatonin rhythm has been shown to be dependent on the circadian phase during which the pulse was applied (Cahill and Besharse, 1991). That entrainment of the retinal melatonin/NAT rhythms can occur in isolated eyecups indicates that the entrainment mechanism is itself located within the eye. In *Xenopus* this is actually within the photoreceptors themselves (Cahill and Besharse, 1993).

1.5.3 Involvement of HIOMT

Early studies with HIOMT indicated that like NAT, its activity followed a low amplitude diurnal rhythm with maximum activity occurring in the light phase (Nagle *et al.*, 1972; Cardinali *et al.*, 1972). Subsequent studies have failed to confirm these findings in a number of species (Pévet *et al.*, 1980; Gern *et al.*, 1984; Nowak *et al.*, 1988; Falcón and Collin, 1991) and it is unlikely therefore that HIOMT contributes to the regulation of melatonin levels as a result of the effects of light. However, Eichler and Moore, (1975) have demonstrated in frog retina, that is, in an ectothermic species, that HIOMT activity exhibits a diurnal rhythm which is engendered by diurnal temperature variation. The findings of Delgado and Viven-Roels (1989) that a decrease in environmental temperature abolished the night-time ocular melatonin peak in the frog, lends support to this hypothesis.
1.5.4 TPH

NAT activity may not be the sole rate-limiting factor of retinal melatonin synthesis. Hamm and Menaker (1980) have demonstrated changes in chick retina melatonin content which were not correlated with changes in NAT activity. Rhythms in 5-HT exist and have been found to mirror those of melatonin, that is, with higher levels at night (chicken Cassone et al., 1986; Siuciak et al., 1992). Availability of 5-HT is a limiting factor for melatonin synthesis by Xenopus eyecups: inclusion of the monoamine oxidase inhibitor pargyline or the 5-HT precursor 5-hydroxytryptophan each stimulates melatonin production (Cahill and Besharse, 1990). The latter is a dose dependent effect and can be induced at all times of the circadian cycle thus it would appear that neither of the post 5-HT enzymes are normally saturated. Addition of the primary precursor tryptophan does not affect melatonin synthesis indicating that retinal TPH may be saturated and thus may be an additional rate-limiting factor. Activity of tryptophan hydroxylase in the chicken in vivo (Thomas and Iuvone, 1991) and TPH mRNA levels in cultured Xenopus eyecups (Green and Besharse, 1994; Green et al., 1995) display true circadian rhythms. Thus there appears to be a coordinated scheme of regulation for NAT and TPH which results in an increase in the amplitude of the melatonin rhythm.

The effect of light on melatonin synthesis is not mediated to any great extent through an inhibitory effect on TPH. In chicken retina, one hour of light exposure during the dark phase suppressed TH activity by only 30% compared with 82% for NAT (Thomas and Iuvone, 1991). Acute effects of light on TH mRNA levels are also not observed (Thomas and Iuvone, 1991).

1.5.5 Neurotransmitters

1.5.5.1 Dopamine

The interaction between melatonin and dopamine in the retina are complex (review, Besharse et al., 1988). Dopamine has been implicated with the mediation of the suppressive effect of light on melatonin synthesis (Zawilska and Dubocovich, 1991) and also the phase shifting effect of light on the melatonin rhythm (Cahill and Besharse, 1991). In addition, melatonin inhibits dopamine release (Dubocovich, 1983) (section 1.7.1.1). Figure 1.6 summarises the mutually antagonistic actions of the retinal melatonin and dopamine systems involved in the control of rhythmic ocular physiology.

1.5.5.2 Others

The retinal neurotransmitter gamma-aminobutyric acid (GABA) and its agonists increase NAT activity in light exposed Xenopus eyecups (Boatright and Iuvone, 1989a) and also in the chicken eye in vivo (Kazula, 1993). Conversely, the GABA antagonist picrotoxin suppresses the nocturnal surge in NAT activity (Boatright and Iuvone, 1989a). GABA may have its effect via the dopaminergic system (Boatright and Iuvone, 1989b).
Figure 1.6
Putative model of multiple interactions between ocular dopamine and melatonin systems in the control of rhythmic ocular physiology. In light, the dopaminergic system is active. Dopamine triggers light adaptive retinal responses. Simultaneously, light, acting via dopamine and/or independently, inhibits melatonin release. In darkness, melatonin release inhibits the dopaminergic system and the retina undergoes dark adaptation. Synthesis of both melatonin and dopamine may be under control of an endogenous ocular oscillator. Light entrains the oscillator. Light entrainment of the oscillator controlling the melatonin rhythm may be partially mediated via dopamine. Melatonin may participate in similar systems with other neurotransmitters in the course of mediating its paracrine effects. Dark arrows show active pathways.
Beta-adrenergic inhibitory control of melatonin synthesis has been reported in green frog eyecup culture (ibid. Delgado et al., 1996). Also, melatonin levels are decreased in rabbit retina, iris and iris-root ciliary body by the mu opiate agonist DAGO (D-ala²-N-methyl-phen²-gly²-ol) (Rohde et al., 1993). Endogenous opioid peptides may therefore contribute to the regulation of intraocular melatonin.

1.5.6 Autoregulation of melatonin

Studies with *Xenopus* retina have shown that imposition of cyclic variation in melatonin level via manipulation of precursor levels does not affect the subsequent timing of the melatonin rhythm (Cahill et al., 1991). Thus it would appear that the ocular circadian oscillator controlling the phase of melatonin synthesis is insensitive to melatonin, that is, there is no feedback of melatonin to the clock.

There is evidence, however, that the synthesis rate is regulated by melatonin, both from the pineal and that within the eye. In the rat, peak night-time retinal melatonin levels exhibit a compensatory increase 3-4 weeks following pinealectomy implying that normally pineal melatonin exerts some inhibitory effect on retinal melatonin, that is, a negative feedback system may be in operation (Yu et al., 1981; Reiter et al., 1983). The site of action of melatonin in this inhibitory capacity may be a synthetic enzyme. For example, rat retinal HIOMT activity increases after pinealectomy (Nagle et al., 1973).

In contrast Nowak and colleagues have demonstrated a stimulatory effect of melatonin on retinal melatonin. Both systemic and intraocular exogenous melatonin administration increase retinal NAT activity in a dose dependent manner in light-exposed chickens (Nowak et al. 1992). These authors propose that this action of melatonin is an indirect one, melatonin acting to directly inhibit the dopamine system, thus removing the inhibitory influence that the dopaminergic system has on NAT activity. There was no effect of systemically administered melatonin on retinal HIOMT activity in this study.

Melatonin may also exert a positive feedback on synthesis of its substrates. For example, in the chicken, retinal 5-HT is increased following administration of exogenous melatonin (Cassone et al., 1986).

In addition to the effects melatonin may have on its own synthesis, there is evidence that its biosynthetic intermediates may also participate in its regulation. Systemic administration of either NAS and 5-HT stimulates retinal NAT activity in light exposed chickens (Nowak et al., 1992). In contrast, NAS is able to inhibit retinal HIOMT activity in the chicken retina, although it has no effect in rat or rabbit retina (Nowak, 1990).

1.5.7 Ontogeny

Studies concerning the ontogeny of melatonin in the rat retina have shown that it first becomes detectable in the twenty day old foetus although significant amounts are not apparent until 2 days post partum (Bubenik et al., 1978). In the chicken, NAT activity is measurable as early as embryonic day 6 and maximal levels are reached on the day preceding hatching (E20) and maintained post hatching (Iuvone, 1990). A significant day/night difference in NAT activity is first observed at
E20. True circadian rhythmicity of retinal NAT activity also develops at or prior to hatching (Iuvone, 1990). The rat pineal melatonin rhythm only appears at ten days post partum (Deguchi, 1978), confirming that the immunoreactive melatonin found in the eye before this time is in fact of extrapineal origin. The ontogeny of HIOMT activity in the rat has also been investigated. Activity is apparent from the 17th day of gestation and subsequently increases to 30 days post partum where it reaches a plateau (Cardinali and Rosner, 1971a).

1.5.8 Season

The effect of season on ocular melatonin has received little attention to date. However, there is some evidence that suggests a relationship between the two. The diurnal rhythm of melatonin is attributable partly to the direct effects of light. Accordingly, as day length decreases towards winter, the duration of the night-time rise in retinal melatonin has been shown to increase (Delgado and Vivien-Roels, 1989). Steinlechner and colleagues (1995) have also recently reported that the profile of day/night melatonin levels in the hamster retina differs greatly with the time of year. In April, for example, a monophasic rhythm peaking at night was present whilst in October, melatonin levels were not rhythmic. This effect was specifically related to the duration of the light phase, as temperature (see below) was held constant throughout. Hence the atypical melatonin rhythm described by Reiter and co-workers (1983) (section 1.5.1.1) may in fact be of biological significance.

Cardinali et al. (1972) have demonstrated rat retina HIOMT activity to be increased after long term exposure to light. In addition, in the hamster retina, mean daily HIOMT activity was found to be highest in the summer season (Balemans et al., 1983). On the basis of these findings Cahill and co-workers (1991) have suggested that in contrast to the non-participation of HIOMT in the short term control of retinal melatonin production, HIOMT may be regulated by long-term lighting conditions to effect additional seasonal changes in melatonin levels.

Temperature may also contribute to the seasonal effects on ocular retinal melatonin. In the frog eye, for example, a melatonin rhythm is not observed in winter unless the environmental temperature is artificially increased and conversely, in summer the pronounced melatonin rhythm can be abolished by lowering the temperature (Balemans et al., 1983).

1.5.9 Sex

There is conflicting evidence concerning the effect of gender on retinal melatonin content. Bubenik and co-workers (1978) were unable to demonstrate a sex difference in the rat retina with the semi-quantitative methodology employed (immunocytochemistry). However, quantitative RIA of retinal tissue from the same species indicated an obliteration of the peak in melatonin content in male rats following gonadectomy (Reiter et al., 1983). This sex difference in retinal melatonin levels may be due to a stimulatory effect of testosterone on HIOMT activity. A significant sex difference in HIOMT activity in the rat retina has been reported, with activity being less in the female (Cardinali and Rosner, 1971a). Furthermore, Nagle and co-workers (1974) have reported a permanent decrease in retinal HIOMT activity.
in male rats 24-72 h after gonadectomy. Activity was able to be restored to pre-surgery levels by daily subcutaneous testosterone injections.

1.6 Ocular metabolism of melatonin

Once synthesised or taken up into the eye, melatonin is available to perform its function(s) until such time as it is rendered inactive by metabolism or removed in some other way from its site of action. The major metabolic pathway of melatonin within plasma, that is, that originating primarily from the pineal, is 6-hydroxylation (Kopin et al., 1960). This occurs within the liver and to a lesser extent the kidney. Subsequently it is conjugated with sulphate, and to a much lesser extent glucuronide, before excretion via the urine (Kopin et al., 1961 and Kveder and McIsaac, 1961). Additionally, in rats (Leone and Silman, 1984; Leone and Silman 1985) and humans (Young et al., 1985) melatonin has been found to be metabolised via demethylation to its synthetic precursor NAS in addition to 6-hydroxymelatonin. In the brain, two further minor metabolites of melatonin, the kynurenamines (= substituted kynuramines) N\(^2\)-acetyl-N\(^2\)-formyl-5-methoxykynurenamine (AFMK) and N\(^2\)-acetyl-5-methoxykynurenamine (AMK), are produced (Hirata et al., 1974). Formation of the first involves oxidative pyrrole ring cleavage the reaction being catalysed by indoleamine 2,3-dioxygenase. AMK is subsequently produced from AMK by the action of formamidase (Hirata et al., 1974). Another minor pathway for melatonin metabolism (1-2%) in the liver involving deacetylation and deamination produces the metabolites 5-methoxytryptamine (5-MT), 5-methoxyindoleacetic acid (5-MIAA) and 5-methoxytryptophol (5-ML) (Rogawski et al., 1979 and Kveder and McIsaac, 1961).

The available evidence suggests that unlike the pineal gland, the eye possesses the capacity for rapid, local metabolism of melatonin. Metabolism may therefore represent an additional regulatory factor controlling melatonin levels. Furthermore, in contrast to plasma melatonin, melatonin within the eye does not undergo hydroxylation as its major pathway of catabolism. The major impetus initiating studies on ocular melatonin degradation was the fact that, despite high nocturnal levels of both synthetic enzymes and melatonin in eye tissues of various species, relatively little actually enters the circulation (Reppert and Sagar, 1983). Based on ocular melatonin levels, they estimated that 50 % of the night-time plasma melatonin should be attributable to that produced in the eyes; whereas the actual value was 9 %. A similar situation has been observed in Xenopus eyecup culture in which NAT activity is again high (Besharse and Iuvone, 1983) but melatonin content in the eyecup and release into the incubation medium is low (Cahill and Besharse, 1989). In the quail, the eyes contribute less than 40 % of circulating melatonin, even though they contain more melatonin than the pineal (Underwood et al., 1984). These authors have attributed their findings to either some storage mechanism for melatonin or local metabolism.

Initial data concerning ocular melatonin metabolism were generated in Xenopus eyecup cultures. It was found that both endogenous and exogenous melatonin were metabolised to the same three compounds, namely 5-MT, 5-MIAA and 5-ML (Cahill and Besharse, 1989), that is, the same indoles produced in the mammalian liver as minor melatonin metabolites (Rogawski et al., 1979; Kveder and McIsaac, 1961).
The pathway of ocular melatonin degradation proposed by Cahill and Besharse (1989) is shown in Figure 1.7. Melatonin is first deacetylated to 5-MT by aryl acylamidase. This is deaminated by monoamine oxidase (MAO) producing 5-methoxyindoleacetaldehyde which is subsequently either reduced to 5-ML or oxidised to 5-MIAA. Cahill and Besharse (1989) have employed enzyme inhibitors to confirm participation of the enzymes in this pathway. The MAO inhibitor pargyline caused accumulation of 5-MT from tritiated melatonin and production of 5-ML and 5-MIAA to be blocked whereas inhibition of aryl acylamidase by eserine caused the suppression of all metabolite formation. In addition, night-time melatonin release from the eyecups was increased sevenfold in the presence of eserine. Fluctuations in deacetylase activity may make some contribution to the generation of the melatonin rhythm or be responsible for acute modulation of melatonin concentration at its sites of action (Cahill et al., 1991) but at present there are no data regarding this hypothesis.

Within the Xenopus eye, NR and choroid-retinal pigment epithelium (C-RPE) compartments have been investigated independently and both found to contain melatonin deacetylase activity (Grace et al., 1991). In addition, the occurrence of this degradative pathway for melatonin has been compared in the vertebrate classes (Grace et al., 1991). In addition to amphibians (Xenopus), it has been demonstrated in teleost fish (goldfish), reptile (Anolis carolinensis) and bird (chicken) eyecup preparations. Using similar methodology it appears to be absent however from mammals (rat eyecup and retinal homogenates, and pig retinal homogenates). It may be the case that levels in these species are very low and/or the experimental conditions employed were inappropriate. Furthermore, other mammalian species may yet be shown to possess such a degradative path.

Local metabolism may represent a mechanism for the rapid termination of melatonin’s actions within the eye and its existence might, therefore be indicative of a short acting role for melatonin, for example, in some neuromodulatory capacity. However, although melatonin itself may be metabolised rapidly and thus deactivated, the products of such transformations may in fact themselves possess biological activity. 5-MT has been shown to inhibit cAMP accumulation in a similar fashion to melatonin in chick NR cell culture (Iuvone et al., 1995). Binding sites for certain of the metabolites, for example 5-ML, have also been reported (Skene, 1991). Furthermore, 5-MT is reported to be an effective substrate for NAT (Zawilska and Nowak, 1991). Thus inactivation of melatonin within the eye by the proposed pathways would generate substrate for further melatonin synthesis. 5-MT also serves as a metabolic precursor for 6-methoxy-tetrahydro-β-carboline which has also been implicated in the control of retinal physiology (review Skene, 1991).

Other metabolic pathways for melatonin may exist within the eye but these have been little studied. The cytochrome P-450-catalysed mixed-function oxidation reaction catalyses the oxidation of many endogenous lipophilic molecules with the products subsequently being rendered water soluble for excretion by conjugation reactions. Several isoenzymes of the cytochrome P-450 family have been demonstrated within the eye (Shichi et al., 1975; Schwartzman et al., 1987; Zhao and Shichi, 1995). It is feasible therefore that they might make some contribution to the control of ocular melatonin levels.
Figure 1.7
Proposed pathway of melatonin metabolism in the eye
(From Cahill and Besharse, 1989)

aryl acylamidase ➔

monoamine oxidase (MAO) ➔

alcohol dehydrogenase ➔

aldehyde dehydrogenase ➔

melatonin ➔

5-methoxytryptamine ➔

5-methoxyindole-acetaldehyde ➔

5-methoxytryptophol ➔

5-methoxyindole-acetic acid ➔
1.7 Actions of ocular melatonin

Ocular melatonin may potentially either act locally within the eye or be released and disseminated via the systemic circulation to reach distant targets. Melatonin is unusual in that it does in fact mediate its functions in a multiplicity of ways, that is, as an endocrine, paracrine and autocrine factor. Its actions are many and diverse. Several are related either directly, or indirectly with some aspect of circadian physiology.

As described (section 1.6), in several species it is probable that most ocular melatonin is rapidly degraded and thus prevented from entering the circulatory system. Alternatively, local synthesis may be insufficient for melatonin to be released in significant amounts. There are instances however in which ocular melatonin contributes a significant proportion of that in the circulatory system. This is particularly true of some avian species and lower vertebrates. Following pinealectomy of the Japanese quail, for example, a significant rhythm in plasma melatonin persists (Underwood et al., 1984). The eyes of this species contribute about one-third of the dark-time melatonin levels (Underwood et al., 1984). Similarly, in the pigeon, the plasma melatonin rhythm is maintained following pinealectomy, with 17% of the night-time peak of blood-borne melatonin deriving from the retina (Foa and Menaker, 1988). In the chicken also, despite the presence of an active deacetylation system (Grace et al., 1991), ocular melatonin makes a small but significant contribution (9%) to plasma melatonin (Reppert and Sagar, 1983). The trout retina is also capable of releasing melatonin into the circulation (Gern and Ralph, 1979).

Thus it is possible that in some species the eye functions as a classical endocrine organ releasing melatonin into the circulation where it is assumed to function as does that of pineal origin (Steinlechner, 1991).

In the majority of species investigated, however, there appears to be little or no release of melatonin into the circulation from the eye. It is likely, therefore, that melatonin mediates local physiological events. This hypothesis has been confirmed by the demonstration of several, local actions of melatonin. However, in some instances, the relative contributions that pineal and ocular sources make to the melatonin pool which mediates these local actions is not always clear.

1.7.1 Effects of melatonin on ocular neurotransmitter systems

1.7.1.1 Dopamine

Melatonin, at picomolar concentrations, inhibits electrically stimulated, calcium dependent $^3$H-dopamine release in the rabbit retina (Dubocovich, 1983). No supporting data for this observation were immediately forthcoming but eventually the finding was confirmed (Nowak, 1988). A similar effect has also been observed in an amphibian retina (Boatright and Iuvone, 1989). Melatonin has also been demonstrated to have an inhibitory effect on light-induced dopamine release (Nowak, 1988) indicating the possible physiological relevance of this action of melatonin on dopamine (light stimulates dopamine release within the eye (Iuvone et al., 1978)). This action of melatonin on dopamine is, as yet, the only one which has been found to be specifically mediated by a receptor (section 1.10.1). Systemic
administration of exogenous melatonin decreases retinal dopamine in mice (Melamed et al., 1986) providing further evidence for a role of melatonin in the suppression of retinal dopamine activity.

As previously discussed (section 1.5.2.1.1) melatonin and dopamine participate in a double negative feedback loop in which the actions of each are mutually antagonistic. It has been proposed that some, perhaps all, of the actions of melatonin, in both lower vertebrates (Besharse et al., 1988) and mammals (Dubocovich, 1988a), are in fact brought about indirectly through an interaction with the dopamine system. It is suggested that the interactions between ocular melatonin and dopamine represent the neurohumoral correlate of the dawn and dusk signal (Rême et al., 1991). The multiple points of (possible) interaction of the melatonin and dopamine systems within the eye are indicated in Figure 1.6.

1.7.1.2 Acetylcholine

In addition to dopamine, melatonin may be involved in similar systems of reciprocal interaction with other neurotransmitters. In rabbit retina, for example, melatonin inhibits ACh release induced by a variety of stimuli including the physiologically appropriate stimuli potassium and flashing light (Mitchell and Redburn, 1991). These authors postulate that the interaction occurs via melatonin receptors located on cholinergic neurons although at present there is no direct evidence of a receptor.

1.7.2 Photomechanical changes

Many of the para-autocrine actions of melatonin specifically associated with the retinal compartment of the eye are associated with dark-adaptive mechanisms. Through the process of dark-adaptation the vertebrate eye is prepared for optimal function in conditions of darkness.

One aspect of dark-adaptation concerns the phenomenon of photomechanical changes (retinomotor movements). Amongst vertebrates, the most extensive photomechanical changes occur in the lower classes. At one time they were believed to be absent from mammals. Subsequently, however, they were reported to be operational in the guinea pig (Pang et al., 1978). Photomechanical changes involve two components; pigment migration and repositioning of the photoreceptor elements (review, Burnside and Nagle, 1983).

In conditions of light, pigment containing melanosomes disperse into the long apical projections of the RPE cells. Simultaneously, cone photoreceptors shorten and rods elongate. Consequently, cone outer segments are positioned first in line for light reception and pigment is able to shield the rod outer segments from damage by bright light. Relocation of pigment also serves to enhance photopic acuity by absorbing scattered light. Conversely, in darkness, pigment aggregates towards the base of the epithelium thus exposing the rod outer segments. Also, cones elongate and rods shorten and in doing so the rod outer segments are moved in from the cone segments thus the retina is primed for function in scotopic conditions. Both photoreceptor (Welsh and Osborn, 1937) and RPE (Douglas, 1982) components of these photomechanical changes are under the control of a circadian oscillator. Melatonin
has also been implicated in both aspects of photomechanical changes. Consequently, Besharse and colleagues (1988) have hypothesised that ocular melatonin may function as a dark-adaptive signal.

1.7.2.1 Pigment aggregation

By analogy to the effect of melatonin on pigment in *Xenopus laevis* dermal melanophores (Messenger and Warner, 1977), that is pigment aggregation, melatonin might be expected to have similar effects on ocular pigment. This has been verified. Intracocular injection of melatonin in both trout (Chéze and Ali, 1976) and guinea pig (Pang and Yew, 1979) causes melanosome aggregation in the RPE. In the latter, melatonin causes pigment aggregation in the choroid also.

1.7.2.2 Control of cone length

Quay and McLeod (1968) were first to report an effect of melatonin on cone photoreceptors. They showed cone contraction following melatonin treatment in *Xenopus laevis* larvae. These findings, published originally in abstract form, although much quoted, were never reproduced. Twenty years later Pierce and Besharse (1985) conclusively demonstrated the opposite, that is, in *Xenopus* eyecups prepared from constant light treated animals, melatonin was shown to cause cone elongation. Subsequently, it was also shown that, in *Xenopus* eyecups, melatonin can only mediate this effect under a particular set of experimental conditions as high light intensity overrides melatonin’s effects (Pierce and Besharse, 1987) and there is a diurnal rhythm of sensitivity to melatonin: melatonin is unable to cause cone elongation in the subject day (Besharse et al., 1988).

Melatonin may effect its actions on photomechanical changes directly. However given the interaction which occurs between melatonin and dopamine in the retina, another mechanism may be one which involves dopamine (Besharse et al., 1988). There is some evidence for this. For example, the effect of melatonin on cone elongation is inhibited by dopamine (Pierce and Besharse, 1985).

1.7.3 Effects on the electroretinogram and visual information processing

The electroretinogram (ERG) is a record of changes of potential that takes place across the retina in a radial direction when light falls upon it. Typically it consists of a cornea-negative a-wave, then a cornea-positive b-wave, a slower cornea-positive c-wave and in some animals a d-wave coincident with cessation of illumination (Berson, 1981). The a-wave arises from the photoreceptors whereas the b-wave is derived from cells proximal to the photoreceptors (probably Müller cells) with some contribution from rod and cone system activity. The c-wave is generated by the RPE from a potassium-dependent differential hyperpolarisation of its apical and basal membranes in response to light with some input from the NR. In many vertebrate species diurnal changes in the ERG are apparent. In some species, including man (Nozaki et al., 1983), chicken (Lu et al., 1995) and lizard (*Anolis carolinensis*: Shaw et al., 1993; *Gecko gecko* Fowlkes et al., 1987), but not all (for example, white perch and green sunfish (McMahon and Barlow, 1992), these are true
circadian rhythms. In general, the \( b \)-wave amplitude exhibits rhythmic fluctuations with the maximum being achieved around the early to middle part of the light phase (Nozaki \textit{et al}, 1983). In contrast, the amplitude of the \( a \)-wave remains fairly constant throughout the day although in \textit{Anolis carolinensis} (Shaw \textit{et al}, 1993) both \( a \)- and \( b \)-waves show circadian rhythms in the time taken for the peak of each component to be reached (\( a \)-wave longest at midnight and the \( b \)-wave at midday).

Effects of melatonin have been demonstrated on the ERG and other aspects of ocular electrophysiology in several species. In the rabbit, intraocular irrigation with melatonin causes a marked reduction of the \( c \)-wave amplitude and a slight peak in the \( b \)-wave (Textorius and Nilsson, 1987). Amplitude of the \( c \)-wave is similarly decreased in chick retina-RPE-choroid preparations treated with melatonin (Nao-I \textit{et al}, 1989). Textorius and Nilsson (1987) also found that melatonin had an effect on the standing potential of the eye (the potential difference between the basal and apical membranes of the RPE). A similar effect of melatonin on standing potential has been observed in chickens both \textit{in vivo} (Rudolf \textit{et al}, 1992) and when melatonin is applied to C-RPE preparations (Nao-I \textit{et al}, 1989). Thus melatonin has marked effects on the RPE. The route by which melatonin reaches the RPE is important with respect to the effect melatonin has: circulating melatonin which arrives via the basal membrane causes opposite effects to those produced above when it acts primarily on the apical membrane (Textorius and Nilsson, 1987). Furthermore, Nao-I and co-workers (1989) have shown that the effects of melatonin on RPE electrical activity may be mediated indirectly, that is, melatonin may act on retinal neurons to modulate the release of another agent which then affects the RPE.

Kurusu and co-workers (1993) have shown that melatonin may also have effects on the \( b \)-wave, implying therefore an effect on components of the NR. In the Japanese quail, they found that intravitreal injection of melatonin during the light phase significantly decreased the ratio of \( b \)- to \( a \)-wave such that the ERG more closely resembled that obtained during the night. Thus sensitivity to light was decreased. In contrast, in the chicken, intraocular injection of melatonin had no effect on the ERG \( b \)-wave (Lu \textit{et al}, 1995) although systemic administration of melatonin significantly decreased \( b \)-wave amplitude. In the human also, oral administration of melatonin causes significant reduction in \( b \)-wave amplitude, that is, a decrease in the sensitivity to light (Emser \textit{et al}, 1993).

In view of these data a role for melatonin in the generation of the circadian changes in the ERG is suggested. This may be a direct effect of ocular melatonin within the eye. However, there is evidence, at least in the chicken, that circulating melatonin may be mediating the effect via a central site (Lu \textit{et al}, 1995).

There is an apparently conflicting body of evidence, however, which proposes that melatonin may instead be able to increase retinal sensitivity. In salamander retina, for example, melatonin has been demonstrated to enhance the sensitivity of horizontal cells to light (Wiechmann \textit{et al}, 1988). Similarly, iontophoretic application of melatonin to guinea pig retina increases its sensitivity to light (Semm and Vollrath, 1982). Reuss and Kiefer (1989), investigating the effect of systemic melatonin application on the properties of cat visual cortex cells, demonstrated an improved signal-to-noise ratio in response to visual stimuli in the presence of melatonin. Their findings led them to suggest that in normal circumstances this action occurs at the level of the retina and is under the control of locally produced melatonin.
The exact nature of melatonin's role is uncertain in view of the opposing nature of some of its actions.

Dopamine is yet again implicated in some of the effects of melatonin on the ERG. Intraocular depletion of endogenous dopamine (by inhibitors of its synthesis), a procedure which increases melatonin, causes standing potential to be decreased as it is in the presence of exogenous melatonin (Rudolf et al., 1992). Furthermore, peak sensitivity in terms of b-wave amplitude occurs in the morning, that is at the time when dopamine synthesis is peaking and when retinal melatonin is declining. In light of these factors Emser and colleagues (1993) propose a dopaminergic mechanism involved with generation of the ERG b-wave, with the system subsequently modified by the actions of retinal melatonin.

1.7.4 Other effects on RPE

Two further effects of melatonin on the RPE have been reported. Physiological concentrations of melatonin have an antiproliferative effect on bovine retinal pigment cells maintained in vitro (Yu et al., 1993). Melatonin is also chemotactic for cultured chick RPE cells (Shirakawa and Ogino, 1987). The biological significance of these novel actions of melatonin is, at present, undetermined.

1.7.5 Control of intraocular pressure (IOP)

Melatonin may play a part in the regulation of intraocular pressure (IOP) although there is conflicting evidence as to its precise role. Like the photomechanical changes described in section 1.7.2, IOP exhibits circadian rhythmicity in several species including rabbit (Anjou, 1961) and human (Newell and Krill, 1965). Concomitant fluctuations in melatonin level may be responsible: melatonin has been demonstrated in both aqueous humor and the ciliary body (sections 1.3.2.2 and 1.3.2.3) and displays diurnal rhythms in these compartments.

Chiou and McLaughlin (1984) were the first to implicate melatonin in the control of IOP. Systemic administration of agents which indirectly increase or decrease melatonin synthesis (through effects on the melatonin precursor, 5-HT) were found to increase and decrease IOP, respectively. In the rabbit IOP is higher at night (Anjou, 1961), that is, at the same time that ciliary body melatonin is reported to be increased (Rohde et al., 1985). Melatonin applied via the vortex vein significantly increases IOP in the rabbit (Rohde et al., 1993). Ocular hypertension was also found to be induced by melatonin (Chiou et al., 1985). Light exposure, known to decrease ocular melatonin synthesis, decreases IOP in rabbits during the night (Lee et al., 1995). Pharmacological treatments, for example with opioids, which may alter melatonin levels (Rohde et al., 1993a) also affect IOP (Rohde et al., 1993b). Taken together these observations provide evidence both direct and indirect for a possible role of melatonin in the control of IOP. In contrast, Kiuchi and co-workers (1993) were unable to demonstrate an increase in rabbit IOP following administration of melatonin by any of four routes including intravitreal injection and topical application.

In humans there is also some evidence confirming an involvement of ocular melatonin in the control of IOP. Not all the data are consistent with this hypothesis, however. Human IOP is higher during the daytime than at night and its rhythm is
therefore out of phase with that of the rabbit (Newell and Krill, 1965). Samples and co-workers (1988) have reported IOP to be decreased by oral administration of melatonin consistent with a depressive effect of melatonin on IOP. Furthermore, bright light suppression of melatonin production attenuated the night-time fall in IOP. In contrast, Koskela and Brubaker (1991) were unable to demonstrate a suppressive effect on aqueous flow by bright light. Furthermore, oral melatonin given during the day to mimic possible physiological night-time rises in melatonin does not suppress aqueous flow (Viggiano et al., 1994).

The exact source of endogenous melatonin which participates in the control of IOP has not been definitely proven. It is feasible that it might be ocular melatonin. Intra-ocular application of melatonin in the cat, for example, has been reported to increase IOP via an effect on aqueous humor outflow (a determinant of IOP) (Rohde et al., 1985). However, Liu and Dacus (1991a) have shown in the rabbit that following unilateral transection of the cervical sympathetic trunk dark elevation of IOP of the decentralised eye is decreased despite melatonin content of the aqueous humor remaining high. This implies that ocular melatonin itself may not play a major, direct role in the elevation of IOP.

1.7.6 Activation of rod disc shedding

Photoreceptors exist in a dynamic state. In 1967, Young showed that the disc membranes of photoreceptor cell outer segments are continually renewed. Subsequently it was found that assembly of new discs is balanced by a process of disc shedding from the apex of the outer segment (review, Besharse, 1982 and Bok, 1985). Cone disc shedding occurs in a similar fashion to that of rods. The process appears to be rhythmic but there appears to be no rhythm common to all species. Rod disc shedding occurs as a true circadian rhythm (Goldman et al., 1980; LaVail, 1980) with most discs being detached at light onset or soon after. A dark phase at least 30 minutes, “dark priming”, and a light phase, “light induction”, are required for shedding to occur. Furthermore, all shedding is totally inhibited after the first 30 minutes of bright light.

A possible relationship between melatonin and photoreceptor disc shedding was first suggested by LaVail (1976) on the basis that both are subject to rhythmic control and because reserpine, which blocks pineal melatonin synthesis, also blocked disc shedding. Administration of exogenous melatonin increases disc shedding in rat retina further implicating melatonin in the control of disc shedding (White and Fisher, 1989). The available data indicates that disc shedding is an intrinsic function of the eye (Teirstein et al., 1980), as its properties are maintained in enucleated eyes from Xenopus laevis as they occur in the eye in situ (Flannery and Fisher, 1979 and 1984). Circulating and central factors are therefore not required. Dahl (1992) has suggested that the effects of light and dark on disc shedding are transduced by photoreceptors and then mediated indirectly through various paracrine factors, that is, disc shedding is under the control of intraocular factors. She has proposed a triadic model involving melatonin, dopamine and rostrophin as the paracrine agents involved in the control of the disc shedding process. In this model, melatonin, synthesised in darkness, acts as the dark priming agent. This role of melatonin in activating rod shedding has been demonstrated conclusively in Xenopus laevis eyecup preparations (Besharse and Dunis, 1983).
1.7.7 Inhibition of phagocytic activity

Once shed, photoreceptor discs are phagocytosed by the RPE cells and subsequently degraded (Young and Bok, 1969). Like the disc shedding process, processing of this phagocytic load is a rhythmic event. In some species, the RPE may display a circadian rhythm (for example, Besharse and Hollyfield, 1979). Rod photoreceptor disc phagocytosis is maximal in the morning, several hours after light onset and that of cones in early night. Light is essential for expression of the rhythm of autophagy (Remé et al., 1985). This process represents another possible point for melatonin involvement. Ogino and co-workers (1983) have shown that melatonin specifically inhibits phagocytic activity of cultured chick RPE cells (measured by phagocytosis of latex particles), and suggest that melatonin synthesised in photoreceptors carries the stop signal for phagocytosis to the epithelial cells.

1.7.8 Effects on the cytoskeleton

Melatonin is reported to affect cellular processes that involve microtubules and microfilaments. In various cell lines, for example, melatonin has been demonstrated to modify cytoskeletal organisation (Benitez-King et al., 1991a). Several of the ocular physiological processes that melatonin affects, involve the cytoskeleton. These include retinomotor movements (review, Burnside and Nagle, 1983) and rod disc shedding (review, Besharse, 1982). Hence melatonin may effect its ocular actions by this means. Melatonin may need to bind to calmodulin to bring about these effects on the cytoskeleton (section 1.10.3.2).

1.7.9 Gross effects of systemic melatonin on the eye

In addition to the specific actions that melatonin mediates within the eye, various effects of melatonin on gross ocular physiology have been reported. Four weeks of systemic administration of melatonin by subcutaneous injection caused a dose dependent increase in absolute and relative intact eye weight in the hamster (Quay, 1984) Part of this increase was as a result of increased lens weight (dry and wet). Furthermore, there was a net increase in ocular fluid volume. Similar gravimetric effects of melatonin on eye weights or their component parts were not observed in the finch 60 days after melatonin implantation (Quay, 1986) However, there were other effects of melatonin in this species. In birds maintained under 15h light:9 h darkness photoperiod conditions, pupillary diameter measured at both ~ 2 and ~ 11 hours after light onset was significantly decreased compared with the control birds. Interpalpebral diameter (perpendicular distance between edges of eyelids at point of maximal separation) was similarly decreased by melatonin. In both these studies the melatonin treatment levels were supra-physiological. It is possible however that ocular melatonin at physiological levels may contribute similar types of effect.
1.8 Ocular melatonin - implications for biological rhythmicity

Throughout the preceding discussions of ocular melatonin a common theme has been the obvious relevance of this melatonin, either directly or indirectly, to biological rhythmicity. Not only has its synthesis been shown to be under circadian control within a number of species, but also ocular melatonin, in turn, is known to exert influence over certain aspects of "circadian" ocular physiology. As will become apparent, the possible ramifications of certain of these influences may be profound. This section aims to bring together the several routes by which melatonin may "input" to the circadian system. The circadian system can be considered as a hierarchical organisation with the main pacemaker, the SCN in the case of mammals, being located at the top, and with the outward manifestation of circadian control, that is, the rhythms themselves, at the bottom. The routes by which melatonin exerts its influences input at several of these organisational levels. In this discussion, the effects of melatonin have been arbitrarily divided into two groups; those involving the eye itself and those outside the eye (Figure 1.7). It is apparent however that the two may overlap to a certain extent.

1.8.1 Local (ocular) circadian physiology

At the most basic level ocular melatonin synthesis is itself a rhythmic event in the vast majority of species that have been studied. This may be true circadian rhythmicity, which in some instances is under the control of an oscillator which resides within the eye itself (section 1.5.2).
Ocular melatonin - possible relationships

1 Local rhythmic physiology

2 Extra-ocular rhythmic physiology

Figure 1.8
1. Melatonin is produced rhythmically within the eye. It may be directly responsible for the rhythmic occurrence of local events or it may influence them via an effect on the intraocular clock.
2. Local melatonin affects extra-ocular rhythmic physiology via action either within the eye or through its endocrine release into the circulation.

"Modulation" refers to either reception of light or subsequent processing of the light signal
Melatonin in turn may influence other local rhythmic events; an idea first propounded by Besharse and Iuvone (1983). Various physiological processes in the eye exhibit diurnal/circadian rhythms. In toads and fishes, levels of mRNA for the photoreceptor protein opsin fluctuate in a circadian manner (Korenbrot and Fernald, 1989). In the Japanese quail (Sasaki et al., 1995) and chicken (Oishi, 1984) corneal mitotic rate displays a circadian rhythm as does phospholipid synthesis in rat rod photoreceptors (Dudley et al., 1984). Rod outer segment disc shedding, retinomotor movements, and intraocular pressure are also under circadian control (sections 1.7.2 and 1.7.5).

The circadian production of melatonin may be directly responsible for the generation of these rhythms such that in the absence of melatonin they do not exist. Alternatively, the ocular rhythms may themselves be products of a circadian clock(s) and ocular melatonin may serve to modulate them in some way via an action on the clock. In the quail eye, for example, melatonin is able to phase advance the rhythm in corneal mitotic rate (Sasaki, 1995). It may also be responsible for the characteristic night-time type ERG, as melatonin has been shown to significantly decrease b-wave amplitude (Kurusu et al., 1993).

Besharse and co-workers (1988) and Dubocovich (1988a) have proposed that by acting in this manner, ocular melatonin, in conjunction with the dopamine system, may serve to signal the dawn and dusk periods and hence may be functioning in the capacity of a local internal zeitgeber in an analogous situation to that which has been proposed for pineal melatonin by Armstrong (1989).

1.8.2 Extra-ocular circadian physiology

The relationships between ocular melatonin and circadian physiology are not restricted to the ocular interface. Ocular melatonin may also have influences on aspects of circadian physiology outside the eye and potentially therefore much further up the hierarchy of circadian control.

The effects to be considered can be divided broadly into two groups. Firstly, there are those that result from the endocrine release of melatonin from the eye. In a number of species ocular melatonin contributes a significant proportion of the circulating melatonin level (section 1.7). In one of these, the pigeon, there is evidence to suggest that this release of melatonin from the eye contributes to maintenance of circadian function. Bilateral enucleation in the pigeon results in diminution of the clarity of locomotor activity and temperature rhythms however these effects can be negated by daily administration of melatonin (Oshima et al., 1989). In the Japanese quail also, a hormonal output from the eye, presumed to be melatonin, is required for maintenance of the integrity of the central circadian system (Underwood, 1994). Thus it may be that melatonin’s primary action is to input to the central oscillator in these species.

The second effect of melatonin on circadian rhythmicity outside the eye may in fact result from some action of melatonin within the eye. Given that the synthesis of ocular melatonin is very much dependent upon both the ambient lighting conditions and the time of day, it is possible that it may function to modulate the transmission of information regarding these variables from the eye to the central oscillator. The RHT


represents a direct anatomical link between the eye and the SCN through which photic information integrated by the former reaches the latter (Moore-Ede et al., 1983) and is therefore a putative target site for ocular melatonin to exert its influence.

In addition to, or instead of, modulating the light signal once it has been received by the retina, melatonin could affect actual reception of the light signal. Melatonin may do this by simply altering the stimulus threshold, or it may even be capable of influencing the perceived intensity of a stimulus in some way. In either case the photic information which is eventually relayed to the SCN would not necessarily be accurately representative of environmental lighting conditions. The reduction in pupillary and interpupillary diameters caused by melatonin (Quay, 1986) may be of relevance in this context; both may reduce photopic input to the retina and thus affect subsequent SCN signalling. In addition, several actions of melatonin within the eye are involved with some aspect of visual sensitivity, for example effects of the ERG (section 1.7.3). Several other aspects of visual sensitivity are also known to be under rhythmic control. Humans display a daily fluctuation in visual threshold (Bassi and Powers, 1986) and in rats (Terman and Terman, 1985), there is a circadian rhythm of visual sensitivity. At present there is no evidence that ocular melatonin is involved with either of these but it is possible that it might. The rhythm of visual sensitivity, for example, is maintained following lesion of the SCN implying a separate pacemaker or other control system (Terman and Terman, 1985). Thus, if visual sensitivity can be modified by melatonin it is likely that light reception required for circadian control can also be. Visual information is known to input to the SCN (via the lateral geniculate nucleus) (Rusak, 1977). Therefore, one additional possibility is that those changes in visual sensitivity for which melatonin is responsible, subsequently also affect the central clock. The specific photoreceptors that mediate mammalian circadian responses to light have not been identified. Studies with retinally degenerate mice suggest that circadian photoreception might be maintained either by a very small number of photoreceptors or, by an unrecognised class of photoreceptive cell (Foster et al., 1991). The role of ocular melatonin in circadian photoreception is currently poorly defined. The potential repercussions of the scenario, if it is physiologically operational, in which melatonin acts locally within the eye to effect changes in light reception and consequently in the signal being transmitted to the central circadian clock, are considerable. One obvious example relates to those circadian timing experiments which require light of a specified intensity to be administered at a particular time (for example phase response curve experiments). Depending on the melatonin status within the eye, which itself is dependent on light exposure and circadian phase, the actual information subsequently relayed to the SCN may or may not be representative of the experimental lighting conditions employed. Thus the response of the organism may also not be a direct translation of these lighting conditions.
1.9 Ocular melatonin and disease - cause or cure?

1.9.1 Ocular health and disease

The physiological actions of melatonin within the eye may, under certain circumstances, be deleteriously exaggerated and consequently assume pathophysiological relevance. Thus, ocular melatonin may be of significance with respect to disease aetiology. Conversely, recent studies have also attributed various protective functions to ocular melatonin.

1.9.1.1 Intraocular pressure (IOP)

The hypothesised participation of melatonin in the control of IOP (section 1.7.5) gives rise to the possibility that melatonin may exacerbate, or indeed contribute to the pathogenesis of, conditions such as glaucoma. Melatonin levels in the iris and ciliary body of chickens with light-induced avian glaucoma are increased compared with normal controls, probably as a result of the increased NAT activity which is also observed (Aimoto et al., 1985). Furthermore, the time at which NAT increases is coincident with the onset of the decrease in aqueous outflow. Pharmacological manipulation of ocular melatonin levels may therefore be of some use in the treatment of conditions involving IOP. As previously described, the mu opiate agonist DAGO causes a reduction in melatonin content of iris and ciliary body (Rohde et al., 1993) and several other topically applied opioids also affect melatonin levels in these tissues (Rohde et al., 1993). Consequently, the opioid peptides have been proposed as potential drug treatments for glaucoma via an action on the melatonin system.

1.9.1.2 Retinal degeneration

Melatonin has been implicated with retinal pathologies. Chronic administration of melatonin increases the severity of retinal damage induced by continuous high intensity illumination in rats (Bubenik and Purtil, 1980; Leino et al., 1984a; Wiechmann and O'Stein, 1992). Wiechmann and O'Stein (1992) propose that in disease states such as retinitis pigmentosa, the presence of a dark signal (melatonin) during light exposure results in disruption of temporal cell function.

In Royal College of Surgeons (RCS) rats, photoreceptor disc shedding and phagocytosis is compromised leading to retinal and RPE degeneration (LaVail, 1976). In view of the fact that melatonin is inhibitory to the RPE phagocytic process (Ogino et al., 1983), Hawlina and colleagues investigated the melatonin contents of RCS retina (1992). Melatonin levels in dystrophic rats were found to be significantly higher than controls. The possible contribution of melatonin to the degeneration process may be an indirect one through its inhibitory effect on dopamine (Hankins and Ikeda, 1994).

1.9.1.3 Other diseases

Yu and co-workers (1993) have proposed that the antiproliferative effect of melatonin on RPE cells that they observed may be exploited in order to control
cellular responses accompanying pathological growth activation, for example, in the condition of proliferative vitreoretinopathy.

Myopia can be induced in newly hatched chickens by experimentally inducing retinal image degradation (deprivation myopia) (Wallman et al., 1978). The process is under local retinal control and involves dopamine (Weiss and Schaeffel, 1993) and D$_2$ receptors (Rohrer et al., 1993). In light of the evidence for the mutual antagonism of the dopaminergic and melatonin systems within the eye (sections 1.7.1.1 and 1.5.5.1), the role of melatonin in deprivation myopia has been investigated. It was concluded from these studies, however, that melatonin is not involved in the retinal signalling pathway translating visual experience to deprivation myopia (Hoffmann and Schaeffel, 1996).

1.9.1.4 Melatonin as a functional ocular antioxidant

Ocular melatonin is rarely considered outside the context of circadian physiology, and indeed on the basis of its effects described above there is ample reason to warrant such an approach, many of its effects being able to be interpreted with respect to some aspect of circadian physiology. However, in addition to its contribution to both local and whole body circadian physiology, ocular melatonin may be of significance within the eye in an alternative capacity, namely that of an antioxidative protection mechanism.

The antioxidant action of melatonin has recently gained support as a potentially important role of pineal melatonin (review, Reiter, 1995) and evidence exists for it mediating a similar function within the eye. The retina is characterised by an extremely active aerobic metabolism - its oxygen consumption is highest of all body tissues (Sickel, 1972). The retina is thus susceptible to oxidative damage, particularly peroxidation of lipids. Furthermore, because of the transparency of the eye, damaging free radicals can also be produced photochemically. The retina possesses several proven defences against this damage, for example antioxidative enzymes such as superoxide dismutase (Hall and Hall, 1975) and natural antioxidant molecules like ascorbate and vitamin E (Riis et al., 1981; Organisciak et al., 1985). Melatonin may represent an additional free radical scavenger. The molecule has been demonstrated to be an efficient scavenger of both peroxyl (Pieri et al., 1994) and hydroxyl radicals (Tan et al., 1993). In rat retinal homogenates, melatonin was found to provide protection against lipid peroxidation, even in the presence of Fe$^{2+}$, a potent stimulator of lipid peroxidation (Chen et al., 1995). In addition, daily systemic administration of melatonin inhibits cataract formation induced in newborn rats by glutathione depletion (Abe et al., 1994) (a process known to enhance oxidative stress (Calvin et al., 1986). Thus melatonin may also be able to reduce oxidative damage to protein.

Whether melatonin undertakes similar functions within the eye in vivo at physiological concentrations, is as yet undetermined. Positive identification of the retinal melatonin system as a functional antioxidative system might have profound implications for both the aetiology and treatment of several ocular disease states which have been associated with free radical damage, for example, senile cataract (Spector and Garner, 1981), primary open angle glaucoma (Babizhayev and Bunin, 1989) and possibly autoimmune uveitis (Ishimoto et al., 1996). The whole concept of melatonin as a physiologically significant antioxidant is however somewhat
controversial and much more extensive investigation of this potential role is necessary.

1.9.2 Ocular melatonin - implications for systemic dysfunction

1.9.2.1 Seasonal affective disorder (SAD)

Retinal melatonin, in combination with dopamine, has been proposed by one author as the primary causative factor of SAD. This condition occurs annually in the autumn and winter months in certain individuals and is characterised by depression plus a collection of other symptoms such as hypersomnia and dysphoria (Rosenthal et al., 1984). Oren (1991) hypothesises that in this condition, there is either excessive activity of the retinal melatonin system or a deficiency in retinal dopamine, the end result being increased retinal melatonin levels. Bright light treatment has been demonstrated to be an effective treatment for SAD, particularly its depressive aspects (Terman et al., 1989). According to Oren, this is therefore in agreement with his hypothesis that the causal agent of SAD depression is ocular melatonin: light suppresses its synthesis both directly, and indirectly through the stimulation of dopamine synthesis. Another mechanism suggested by Oren for the involvement of ocular melatonin in SAD is via its ability to regulate the putative intraocular clock. Lewy and colleagues (1986) have proposed the “phase shift hypothesis” of SAD in which there is a delayed onset of nocturnal melatonin secretion. Oren postulates that inappropriate melatonin activity within the eye affects the local clock such that its subsequent input to the central circadian clock is deleteriously altered.

The involvement of ocular melatonin in SAD is, as yet, only a hypothetical concept. Rigorous testing of these proposals is required before their possible physiological relevance can be accepted.

1.9.2.2 Affective disorders

Manic-depressive illness has been associated with abnormal phase relationships between normal physiological circadian rhythms (Hallonquist et al., 1986). It is postulated that this in turn may relate, at least in part, to a defect in the retinal melatonin system, as described above for SAD (Seggie et al., 1986; Seggie, 1988, and Steiner et al., 1987). These authors propose that rhythmic ocular melatonin synthesis is affected in manic-depressive disorders resulting in increased sensitivity of the retina to light. Consequently, through changes in light input to the SCN, the circadian rhythms become asynchronous. Lithium therapy employed in these conditions is believed to act via an effect on retinal melatonin (Seggie et al., 1986; Seggie, 1988).
1.10 Mechanism of action of melatonin within the eye

1.10.1 Receptors

In order to mediate the diverse actions of melatonin within the eye that have been described (section 1.7), some mechanism for the translation of its message into cellular activity must exist. Much effort has been devoted to establishing the precise mechanism(s) of action of both pineal and ocular melatonin. It has been proposed that the biochemical events in target cells are initiated via involvement of a specific receptor and indeed a large body of evidence for the existence of the putative melatonin receptor has been established. Within the eye, both cell surface and intracellular receptors for melatonin have been reported (Table 1.4).

1.10.1.1 Radioligands used in binding studies

Radiolabelled melatonin has long been available in the tritiated form, that is, as [2-aminooethyl-2-^H]melatonin or [methoxy-^H]melatonin (having specific activities of ~ 30-40 Ci/mmol and ~ 80 Ci/mmol respectively) and the earliest melatonin binding studies used this as the radioligand. Little progress was made however and the initial reports of specific, binding sites have been difficult to replicate. More recently radioiodinated melatonin (2-[I^125]iodomelatonin) was developed for use in RIA (Vakkuri et al., 1984a,b). Subsequently this radioligand was utilised as a probe for melatonin receptors. It has many advantages over the tritiated ligand as a pharmacological tool and consequently has rapidly become the ligand of choice for the investigation of melatonin binding sites. It has a much higher specific activity (up to 2186 Ci/mmol) thus allowing increased sensitivity of receptor detection (advantageous for the study of high affinity, low density receptors) and also the emission of both high energy β and γ radiation facilitates its detection by autoradiography. Despite the significant alteration, following iodination, the melatonin molecule retains its biological activity both outside (Weaver et al., 1988; Sugden 1989) and within the eye; 2-iodomelatonin is able to inhibit the calcium-dependent release of [^H]dopamine from chicken NR as effectively as melatonin (Dubocovich and Takahashi, 1987).

There is some debate over the appropriateness of the two ligands for use in melatonin binding studies. It has been suggested by a number of authors (Krause and Dubocovich, 1991; Kennaway et al., 1994; Sugden, 1994) that use of ^3H-melatonin is not a particularly suitable radioligand, in part because of its low specific activity and have attributed the lack of consistent, reproducible data generated in the early years to the radioligand.
<table>
<thead>
<tr>
<th>Class</th>
<th>Species</th>
<th>Technique/ Radioligand</th>
<th>Location</th>
<th>$K_d$ (fmol/mg protein)</th>
<th>$B_{max}$</th>
<th>Author</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amphibia</td>
<td>frog: Rana pipiens</td>
<td>RRA+autorad/$^3$H</td>
<td>neural retina: OPL, melanosomes of RPE-choroid, neural retina: IPL, ONL, INL and GCL</td>
<td>600 nM, 600 nM</td>
<td>nd</td>
<td>Wiechmann, et al., 1986</td>
</tr>
<tr>
<td>Amphibia</td>
<td>frog: Rana pipiens</td>
<td>RRA+autorad/$^{125}$I</td>
<td>neural retina</td>
<td>125 pM</td>
<td>0.138</td>
<td>Wiechmann and Wirsig-Wiechmann, 1991</td>
</tr>
<tr>
<td>Amphibia</td>
<td>frog: Rana esculenta</td>
<td>RRA/$^{125}$I</td>
<td>neural retina</td>
<td>58 ± 14 pM, 41 ± 6 pM</td>
<td>4.8 ± 0.6, 5.3 ± 0.3</td>
<td>Skene et al., 1993</td>
</tr>
<tr>
<td>Amphibia</td>
<td>frog: Xenopus laevis</td>
<td>RRA/$^{125}$I</td>
<td>neural retina</td>
<td>58 ± 14 pM, 41 ± 6 pM</td>
<td>4.8 ± 0.6, 5.3 ± 0.3</td>
<td>Skene et al., 1993</td>
</tr>
<tr>
<td>Aves</td>
<td>chicken</td>
<td>RRA/$^{125}$I</td>
<td>neural retina</td>
<td>434 ± 56 pM</td>
<td>74.0 ± 13.6</td>
<td>Dubecovich and Takahashi, 1987</td>
</tr>
<tr>
<td>Aves</td>
<td>chicken</td>
<td>autorad/$^{125}$I</td>
<td>neural retina: IPL</td>
<td>19.8 pM</td>
<td>96.7</td>
<td>Laitinen and Saavedra, 1990a</td>
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<td>Aves</td>
<td>chicken</td>
<td>RRA/$^{125}$I</td>
<td>neural retina: RPE</td>
<td>446 ± 55 pM, 34.1 ± 2.2 pM</td>
<td>25.4 ± 2.2, 59.5 ± 5.2</td>
<td>Chong and Sugden, 1991</td>
</tr>
<tr>
<td>Aves</td>
<td>chicken</td>
<td>in situ hybridisation of CKA (chick Mel$<em>{1a}$) and CKB (Mel$</em>{1c}$)</td>
<td>neural retina: INL, GCL</td>
<td>434 ± 56 pM</td>
<td>74.0 ± 13.6</td>
<td>Reppert et al., 1995b</td>
</tr>
</tbody>
</table>

Table 1.4 (I)
Occurrence and characteristics of melatonin binding sites within the eye.

Abbreviations:
ONL = outer nuclear layer; INL = inner nuclear layer; IPL = inner plexiform layer; GCL = ganglion cell layer; $^{125}$I = $2$-$[^{125}$I]iodomelatonin; $^3$H = $[^ {3}$H]melatonin; nd = not determined; RRA = membrane radioreceptor assay; autorad = autoradiography; CKA = structural homologue of mammalian Mel$_{1a}$; CKB = Mel$_{1c}$

RT-PCR = comparative reverse transcription-polymerase chain reaction analysis
1.10.1.2 High and low affinity sites

Binding studies using tritiated melatonin indicated existence of a low affinity melatonin binding site with an equilibrium dissociation constant value ($K_d$) in the nanomolar range in a variety of tissues including bovine brain membranes (Cardinali et al., 1979) and rodent gonadal cytoplasm (Cohen et al., 1978). These latter authors also reported existence of a cytoplasmic binding site in the hamster eye. However, as discussed above, these investigations were probably hindered by the inherent disadvantages of this radioligand and the findings have not been reproduced. Using 2-$[^{125}\text{I}]$iodomelatonin, high affinity melatonin binding sites were identified for the first time in 1987 by both in vitro autoradiography and membrane radioreceptor assay in rat brain (Vanecek, 1988). This radioligand was subsequently used extensively in the search for melatonin binding sites and has identified putative melatonin receptors in many vertebrate species in a variety of tissues (review, Morgan et al., 1994).

The majority of receptors revealed by 2-$[^{125}\text{I}]$iodomelatonin, including those within the eye, display similar pharmacological characteristics. They are of high affinity with a $K_d$ within the range 20 - 400 pM. Specific 2-$[^{125}\text{I}]$iodomelatonin binding is saturable indicating a finite number of receptors and the interaction between melatonin and the receptor is reversible. In addition, the receptor displays high selectivity for melatonin or closely related analogues. This high affinity melatonin binding site therefore fulfils many pharmacological criteria of a receptor.

There is much less evidence, however, that melatonin binding is associated with mediation of a biological function. One of the first actions of melatonin to be described was its ability to cause melanin pigment-containing melanosome aggregation in amphibian dermal melanophores, an action mediated through a high-affinity melatonin receptor (reviewed by Rollag, 1988). Within the eye only one action of melatonin, namely that of inhibition of retinal dopamine release, has been specifically attributed to interaction with a binding site. This has been confirmed in rabbit (Dubocovich, 1983; Nowak et al., 1989) and frog (Boatright and Iuvone, 1989) retina. In this case it has been postulated that the melatonin receptor is a presynaptic heteroreceptor. Within the eye there is also evidence that melatonin receptor activation triggers a variety of intracellular second messenger systems. Melatonin’s influence appears predominantly to involve the cAMP system (section 1.10.2.2).

1.10.1.3 Agonists and antagonists

Investigation of the relationships between melatonin structure and affinity/activity, was first considered in detail in 1975 (Heward and Hadley). Using one of the few functional bioassays available, namely in vitro condensation of pigment granules in frog skin melanophores, these authors reported that the N-acetyl side chain group and a 5-methoxy group on the indole ring were prerequisites for binding and receptor activation, respectively. These findings have generally been confirmed for retinal melatonin receptors (for example, Dubocovich, 1985; Dubocovich and Takahashi, 1987). More recent studies however indicate that, at least in the melanophore pigment aggregation assay, the 5-methoxy moiety is not essential for
biological activity (Garratt et al., 1994) but does in fact make a substantial contribution to binding affinity (Sugden and Chong, 1991).

Amongst indoleamines in general, halogenic substitution on C-2 of the indole ring produces compounds with the highest binding affinity amongst the sites described (for example, bromomelatonin (Duranti et al., 1992). There is evidence that the indole nucleus itself is not essential for binding or activation of biological responses. For example, it can be replaced with a naphthalene group with no adverse effect on either of these properties (Yous et al., 1992). It has been suggested, therefore, that the indole moiety serves to maintain the appropriate functional groups in the correct spatial orientation (Sugden, 1994).

On the basis of these facts the rank order of potency of compounds for the inhibition of specific 2-[^{125}I]iodomelatonin binding at the high affinity melatonin binding site is 2-iodomelatonin > melatonin ≳ 6-chloromelatonin > 6-hydroxymelatonin ≳ N-acetylserotonin ≳ 5-HT. The majority of ocular receptor characterisation studies have confirmed this order (for example, frog NR: Skene et al., 1993; chicken NR: Dubocovich and Takahashi, 1987; tree shrew NR-RPE: Lu et al., 1991).

Importantly, in support of the opinion that the melatonin binding site labelled by 2-[^{125}I]iodomelatonin is a functional melatonin receptor, within both the rabbit and chicken retina, a highly significant correlation between the potency of indoles to inhibit the calcium-dependent release of ^{3}H-dopamine and the potency to compete for 2-[^{125}I]iodomelatonin binding in chicken retinal homogenates has been demonstrated (Dubocovich 1985; Dubocovich and Takahashi, 1987).

Understanding of the precise role of melatonin in physiological processes has been hampered by a lack of potent and selective receptor antagonists. Early candidates such as N-(2,4-dinitrophenyl) 5-methoxytryptamine (Zisapel and Laudon, 1987) have been proven to have poor affinity and weak activity at the high affinity melatonin site (for example, Sugden, 1992). Luzindole (2-benzyl-N-acetyltryptamine) (Dubocovich, 1988b), another putative antagonist, displays definite antagonistic activity in both the eye (inhibition of calcium-dependent dopamine release in the rabbit retina with $K_d$ 20 nM (Dubocovich, 1988a) and inhibition of cAMP accumulation in chick retinal culture (Iuvone and Gan, 1994) and in other functional bioassays. However, there are systems in which luzindole lacks antagonistic action, for example, cAMP production in sheep pars tuberalis (Howell and Morgan, 1991). There is no commercial source of luzindole and this has in part prevented further exploitation of its antagonistic properties.

Dubocovich (1988c) has proposed a classification scheme which incorporates two 2-[^{125}I]iodomelatonin binding site subtypes. The first, ML-1 corresponds to a site with the pharmacological properties described above. In contrast, the second, ML-2, refers to a binding site described in hamster brain membranes which has distinctly different properties (Duncan et al., 1986, 1988). It is a low affinity site with a $K_d$ in the nM range. Furthermore, the pharmacological specificity of the site is not the same as that of the high affinity site: the ML-2 site recognises the melatonin synthetic precursor, N-acetylserotonin and melatonin itself with equal affinity. However, this schema has not gained wide acceptance for a number of reasons. Firstly, autoradiographic localisation of the putative ML-2 site has not been accomplished. More importantly, this site has never been associated with mediation of a functional biological response. The classification has now in part been superseded as a result of
the recent cloning of a family (encompassing 3 subtypes) of high affinity melatonin receptors (section 1.10.2.4).

1.10.2 Cell surface ocular melatonin receptors

1.10.2.1 Location

Using techniques such as radioreceptor assays and autoradiography, high affinity membrane bound melatonin binding sites have been detected in ocular tissue of species from all animal classes (Table 1.4 (I)). The retina and C-RPE have been investigated most extensively. In the retina, specific, saturable, melatonin binding sites were first identified by Wiechmann and co-workers (1986). Further studies with 2-[\(^{125}\)I]iodomelatonin indicated that within the retina, binding is consistently localised to the innerplexiform layer (IPL) (rat and chicken: Laitinen and Saavedra, 1990; frog: Wiechmann and Wirsig-Wiechmann, 1991; mouse and rabbit: Blazynski and Dubocovich, 1991). In view of the effects of melatonin on retinal dopamine release, the existence of melatonin binding sites in the IPL is appropriate given that a predominant component of this area is the dopaminergic amacrine cell. Additionally, melatonin binding sites have been localised to various areas of the outer retina (Wiechmann et al., 1986; Blazynski and Dubocovich, 1991; Wiechmann and Wirsig-Wiechmann, 1991) and the RPE (Chong and Sugden, 1991), again regions at which melatonin has known actions on, for example, photoreceptor disc shedding and phagocytosis (sections 1.7.6 and 1.7.7). One other site of melatonin receptors in the rabbit eye has recently been identified as the iris-ciliary body complex (Osborne and Chidlow, 1994). This finding may correlate with the reported effects of melatonin on IOP (section 1.7.5). However there are no other reports of specific melatonin binding in this location.

1.10.2.2 Receptor-mediated signal transduction

There is limited evidence associating occupation of ocular melatonin receptors with activation of biochemical and cellular responses. That which is available, supports the hypothesis that melatonin has an inhibitory effect on cAMP production via a guanine nucleotide-binding regulatory protein (G-protein). Such a mechanism is in fact common to many extra-ocular melatonin receptors, for example, those in the pars tuberalis of the pituitary (Morgan et al., 1990). At high micromolar concentrations melatonin was shown to elevate intracellular cAMP in rabbit retina (Blazynski et al., 1985), although Dubocovich (1988a) suggested that this effect may be mediated by a 5-HT receptor rather than a melatonin receptor. Subsequently melatonin has been found to inhibit cAMP accumulation in the retina via a receptor-mediated mechanism in both chick retinal cell cultures (Iuvone and Gan, 1994) and retinal membrane preparations (Niles et al., 1991). Similarly melatonin receptors in cultured human and rat RPE cells (Nash and Osborne, 1995) and in the iris-ciliary body of the rat (Osborne and Chidlow, 1994) appear also to be negatively linked to the stimulation of adenylate cyclase. In chick retinal cell cultures the inhibitory effect of melatonin on forskolin-stimulated cAMP is blocked with pertussis toxin suggesting that coupling of receptor and adenylate cyclase occurs through a pertussis toxin-sensitive G protein such as G\(_i\) (Iuvone and Gan, 1994). Laitinen and Saavedra
(1990a) also demonstrated that binding of melatonin to the chick retinal receptor is modulated by guanine nucleotides. In contrast, Chong and Sugden (1991) reported that whilst the chick RPE melatonin receptor is G-protein coupled, that in the NR is not. Dubocovich and co-workers (1989) also failed to demonstrate an effect of GTP on 2-[125I]iodomelatonin binding to chicken NR.

More recently evidence has been reported which suggest that, in addition to the inhibitory effect of melatonin on dopamine release, there exists a second receptor-related mechanism by which melatonin is able to modulate dopaminergic neurotransmission. In chick NR cell cultures, melatonin receptors have been shown to be coupled, via an inhibitory G protein, to dopamine receptor (D1)-regulated adenylate cyclase (Iuvone and Gan, 1995). Melatonin may therefore be able to inhibit the cAMP response to dopamine receptor activation with associated consequences.

1.10.2.3 Affinity states

As a result of its G-protein interaction, the high affinity melatonin binding sites can exist in two states (Sugden, 1994). In one, the high affinity state ($K_d \approx 40 \text{ pM}$), the binding site is coupled to the G-protein (for example, chick NR melatonin binding site, as assessed by Laitinen and Saavedra, 1990). In the other, the low affinity state ($K_d \approx 400 \text{ pM}$), the binding site and the G-protein are dissociated (for example, chick NR melatonin binding site as assessed by Chong and Sugden, 1991). GTP is able to uncouple the binding site and G-protein in the high affinity state. In contrast the low affinity state is insensitive to GTP. Under certain assay conditions the two sites may be differentially detected. It is also possible that a given membrane preparation may possess a mixture of the two affinity states. The low affinity state reflecting the G-protein uncoupled form of the high affinity state is distinct from the other low affinity sites which have been reported, for example that in the hamster brain (Duncan et al., 1986, 1988). No high affinity site of this latter has been identified.

1.10.2.4 Cloning of the melatonin receptor - multiple receptor subtypes

In 1994 the *Xenopus* dermal melanophore melatonin receptor was cloned using an expression cloning strategy (Ebisawa et al. 1994). Using a polymerase chain reaction (PCR) approach based on the sequence of the frog receptor, the corresponding sheep and human melatonin receptors were subsequently cloned (Reppert et al., 1994). These were designated Mel1a. In 1995, a second receptor in this family (Mel1b) was cloned from humans (Reppert et al., 1995a). Subsequently, a third subtype (Mel1c) (the structural homologue of the *Xenopus* receptor) was identified in the chicken (Reppert et al., 1995b). The three subtypes are widely distributed in the vertebrate classes with two exceptions: Mel1a has not yet been cloned in mammals and there is no evidence that Mel1b occurs in birds.

Despite their structural divergence the expressed receptors of each subtype exhibit similar binding properties. All are $G_i$ coupled and their pharmacological characteristics are similar to those of the endogenous 2-[125I]iodomelatonin receptor. Rank order of potencies of ligands for inhibiting 2-[125I]iodomelatonin are also the very similar in the three groups, that is melatonin≥6-chloromelatonin>NAS>>5-HT,
although there is one difference, namely that 6-chloromelatonin is 10-fold more potent in inhibiting specific 2-[\textsuperscript{125}I]iodomelatonin binding in Mel\textsubscript{1b} than in Mel\textsubscript{1a}.

Cloning of the melatonin receptor genes is expected to pave the way for rapid advances in the study of the melatonin receptor. In particular, it will be invaluable for the localisation of receptor expression and investigation of regulation, areas which have already benefited. Application of site directed mutagenesis will provide further means to study receptor structure and function.

1.10.3 Intracellular targets for melatonin

1.10.3.1 Intracellular melatonin binding sites

As previously mentioned, Cohen and co-workers detected low affinity cytoplasmic melatonin binding sites in a variety of tissues including the hamster eye in 1978 (Cohen \textit{et al.}, 1978). These data have not been confirmed, however, possibly because of the difficulties experienced with the use of tritiated melatonin (section 1.10.1.1.1). More recently, interest has arisen in the possibility of a nuclear signalling pathway for melatonin. High affinity ($K_d \sim 190$ pM) melatonin binding sites have been detected in homogenates of rat liver cell nuclei (Acuña-Costroviejo \textit{et al.}, 1993 and 1994) and within the eye itself, a significant proportion of 2-[\textsuperscript{125}I]iodomelatonin binding to chicken retinal membranes being in fact to the nuclear fraction (Dubocovich and Takahashi, 1987).

In addition, melatonin has been identified as a ligand of the orphan nuclear receptor retinoid Z receptor \(\beta\) (RZR\(\beta\)), melatonin both binding and activating it (Becker-André \textit{et al.}, 1994). Thus a nuclear signalling pathway may exist, that is, direct melatonin-induced control of target gene transcription. RZR\(\beta\) may represent one of the nuclear binding sites for melatonin that have been described. RZR\(\beta\) has been cloned and its mRNA located in various tissues including the eye. Within the rat retina highest expression was present in the INL, with lesser amounts in the outer nuclear and ganglion layers (Becker-André \textit{et al.}, 1994). The \(\alpha\)-subtype of RZR was also subsequently found to be a nuclear receptor for melatonin (Wiesenberg \textit{et al.}, 1995). If found to be expressed in the eye as is RZR\(\beta\) then this will potentially represent an additional route of action for melatonin.

The possible existence of these nuclear sites of melatonin action within the eye may be of some significance given the reports of nuclear localisation of melatonin in several tissues (Menendez-Pelaez \textit{et al.}, 1993). Menendez-Pelaez and Reiter (1993) propose that melatonin preferentially accumulates in the nucleus where it binds to specific binding sites in order to initiate various genomic effects. A similar system may be in operation within ocular tissues. The existence of this intracellular melatonin binding site is controversial however, as the findings have never been confirmed.

1.10.3.2 Calmodulin

The Ca\textsuperscript{2+} binding protein calmodulin may also be of importance in the mediation of melatonin's actions. Calmodulin has been shown to bind melatonin in a saturable, reversible and specific manner (Benitez-King \textit{et al.}, 1993) hence calmodulin may represent an additional intracellular binding site for melatonin. Alternatively,
calmodulin may in fact be responsible for the intracellular binding which has previously been observed, for example by Cohen and co-workers (1978), (Benitez-King et al., 1993). Benitez-King and co-workers (1993) propose that several aspects of cell physiology may potentially be affected following melatonin binding to calmodulin, for example inhibition of calmodulin-dependent phosphodiesterase activity (Benitez-King et al., 1991b) and cytoskeletal rearrangements (Benitez-King et al., 1991a). This calmodulin-melatonin interaction may be of relevance within the eye.

1.10.3.3 Non-receptor mediated mechanisms of action

The proposed antioxidant properties of melatonin (section 1.9.1.4) represent functions which do not require a receptor for their mediation. Some authors have also suggested that the actions of melatonin in the RPE, for example, are not receptor-mediated and may result from melanin binding melatonin non-specifically (Laitinen and Saavedra, 1990a).

1.11 Use of cell cultures for the study of the ocular melatonin system

Several research groups have made use of in vitro cell culture systems, either exclusively or in conjunction with other experimental models, in the study of various aspects of ocular melatonin. Some of these have been mentioned previously. For example, chick retinal cell cultures, generated from embryonic tissue, have been utilised to investigate the control mechanism of NAT (Iuvone et al., 1990), to locate the intra-ocular circadian oscillator (Cahill and Besharse, 1993) and to study melatonin receptors (Iuvone and Gan, 1994). All the cell culture systems described so far have represented secondary cell cultures, that is, those prepared directly from the healthy tissues. Increasing use, however, is being made of the continuous cell line Y79. This was established in 1974 from a human retinoblastoma (Reid et al., 1974). The cells are multipotential, that is, they are capable of differentiation into all major cell types of the retina depending on culture conditions (Kyritsis et al., 1987a). Y79 has been demonstrated to synthesise melatonin from both 5-hydroxytryptophan and 5-HT by some cAMP-dependent mechanism (Pierce et al., 1989; Deng et al., 1991). The cells are also capable of synthesising 5-HT (from tryptophan), the precursor of melatonin (Yorek et al., 1987). Both NAT (Wiechmann et al., 1990; Janavs et al., 1991) and HIOMT (Kyritsis et al., 1987b; Wiechmann et al., 1990; Bernard et al., 1995) activity is present in Y79 cells with cAMP stimulation of melatonin synthesis acting through an effect on NAT (Janavs et al., 1991). The reciprocal inhibition of dopamine and melatonin characteristic of the normal retina is retained by Y79 cells; melatonin inhibits dopamine release (Godley et al., 1989) and dopamine inhibits melatonin release (Deng et al., 1991). The neoplastic nature of these cells, however, calls into question their representativeness of the retinal situation as it is in vivo. As has been described, there are many similarities between the Y79 and normal with respect to the melatonin system, however there are also differences. For example, melatonin production in Y79 is not susceptible to inhibition of RNA synthesis (Janavs et al., 1994) in contrast to the situation in chicken retina (Iuvone et al., 1990). If its
limitations are acknowledged and accepted however, the Y79 cell line will represent another useful tool for the investigation of the retinal melatonin system.

1.12 Research aims

The field of research concerned with ocular melatonin is increasing in prominence. Melatonin has been demonstrated in various ocular tissues in a large proportion of the species which have been investigated and evidence for a functional synthetic capacity exists. It is likely that melatonin mediates several local actions.

Currently however, there is sparse information regarding melatonin in the human eye. As far as the author is aware, only three publications have thus far reported attempts to demonstrate and quantify melatonin in human ocular tissue (Table 1.1). Further, there is only limited evidence suggesting a capability of the human retina for melatonin synthesis. As is frequently the case, novel research is undertaken in a wide range of other species in preference to humans for obvious ethical and practical reasons, obtaining a tissue supply, for example. To a certain extent information can be extrapolated from other species to man. However, at some stage, predictions concerning human biochemistry, made on the basis of data from other species, need to be confirmed or disproved in human tissue itself. Hence one of the primary aims of this thesis was to address the issue of the presence of ocular melatonin in man using a source of post-mortem eyes. The existence of a functional melatonin system within the human eye is of potentially great significance.

Chapter 2 describes the development and appropriate validation of methodology for the quantification of melatonin in NR and choroid-retinal pigment epithelium (C-RPE) tissues from post-mortem human eye specimens. Subsequently these methods were employed for the analysis of 46 specimens in order to establish melatonin levels within the human eye and to identify possible relationships between content and various donor parameters such as time of donor death, donor age, etc. (Chapter 3).

The presence of human ocular melatonin synthesis coupled with the reported effects of melatonin in other species, raises questions concerning its possible mode(s) of action. The existence of membrane bound, specific, high affinity melatonin binding sites within the eyes of many species suggests that these actions are receptor mediated. Furthermore, there is evidence that melatonin binding sites mediate at least one functional response. Therefore, in conjunction with investigations concerning melatonin quantification, the existence of melatonin binding sites within the human eye was also investigated (Chapter 6).

Prior to use with human tissues, the methodology for investigation of melatonin binding sites was established using ocular tissues from the Japanese quail.

The quail, as a species, is of particular interest to the field of ocular melatonin in its own right. The eyes have been demonstrated to be essential for maintenance of circadian organisation in this species. Moreover, the quail eye displays a robust circadian rhythm of melatonin production, control of which is under a biological clock which resides in the eye itself (Underwood et al., 1990). It was of some relevance, therefore, to ascertain whether binding sites for melatonin exist within the quail eye. Furthermore, much evidence already exists for the presence of melatonin and its binding sites within the ocular tissues of avian species hence it was
likely that melatonin binding sites would also be found in the quail eye, as indeed was subsequently proven to be the case. Having identified specific binding sites in the quail eye the effect of photoperiod on binding parameters was also investigated. Photoperiod related changes in melatonin binding site density in the quail brain have previously been demonstrated (Panzica et al., 1994). The binding site studies conducted on quail tissue are reported in Chapter 5. The methods employed for melatonin binding site analysis, that is, membrane receptor assay and autoradiography, are described in Chapter 4.
CHAPTER 2

DEVELOPMENT AND VALIDATION OF METHODOLOGY FOR THE QUANTIFICATION OF OCULAR MELATONIN BY RADIOIMMUNOASSAY
2.1 Introduction

Three analytical techniques have previously been employed to quantify ocular melatonin: RIA, HPLC and GC-MS (section 1.3.1). This chapter describes the development and validation of an existing RIA method for the quantitative investigation of melatonin in human NR and C-RPE. The rationale for use of RIA as the method of choice was based for the most part on practical considerations. There is limited access to the more complex chromatographic techniques, that is, HPLC and GC-MS. Furthermore, the melatonin RIA is currently a working assay in the laboratory. In addition however, RIA per se confers several advantages as an analytical tool. Firstly, the technique is highly sensitive: with the particular protocol employed in these studies, 1 to 2 pg/assay tube was consistently detected. Secondly, the antibody component conveys high specificity for the ligand being measured. Finally, the precision obtained with RIA is comparable in most cases with levels obtained for other physico-chemical techniques.

The basis of RIA is competition between unlabelled antigen and a finite amount of the corresponding radiolabelled antigen for a limited number of antibody binding sites in a fixed amount of antiserum. The competing reactions that form the basis of RIA are shown in Figure 2.1. Upon attainment of equilibrium the distribution of antigen between free and bound pools is determined. There are various separation methods. In the RIA employed in these studies a solid phase-bound second antibody directed against the first was used to pellet the bound fraction. Subsequently, bound radiolabelled antigen was quantified and used to estimate the antigen of interest. As

![Figure 2.1](image_url)

The molecular basis of the melatonin RIA employed. Modified from Yalow (1978).

unlabelled antigen-antibody complex

 labelled antigen + specific antibody

 Ag* + Ab

 labelled antigen-antibody complex

 solid phase bound

 second antibody directed against Ab

 unlabelled antigen in known standard solutions or unknown samples

 free and bound antigen separated by centrifugation

 labelled antigen-antibody complex

 unlabelled antigen-antibody complex

 labelled antigen-antibody complex

 unlabelled antigen-antibody complex
the unlabelled antigen content increases less radiolabelled antigen is able to bind the antibody.

The RIA employed throughout these studies was that originally reported by English and co-workers (1993): a GC-MS validated method for the measurement of melatonin in saliva. There are obvious differences between this sample matrix and those under investigation. Thus, in order to enable accurate quantification of melatonin it was necessary to develop a method of sample processing for use prior to RIA which would negate any deleterious effects of tissue components on the assay. Further, it was necessary to fully validate processing and assay procedures.

Two sample processing methods were compared. In the first, tissue melatonin was extracted directly with dichloromethane prior to RIA. This procedure has previously been used successfully to quantify ocular melatonin in a number of species (Delgado and Vivien-Roels, 1989; Skene et al., 1991). Other authors have extracted with different solvents, for example, chloroform (Reppert and Sagar, 1983); methanol (Pang et al., 1983); and diethylether (Serino et al., 1993). For the second sample processing method an additional procedure - acid precipitation of proteins - was included in the protocol before dichloromethane extraction. This approach was adapted from that reported by Pang and co-workers (1980; 1982) for the quantification of melatonin in the mammalian eye.

Availability of human tissue was limited hence validation studies were undertaken in both human and chicken ocular tissues. Melatonin has frequently been reported in the latter (for example, Hamm and Menaker, 1980; Reppert and Sagar, 1983). Authenticity of the endogenous melatonin-like immunoreactivity quantified by the RIA was subsequently established by TLC.

2.2 Materials and chemicals

2.2.1 RIA

2-[^125]Ijiodomelatonin was obtained from Amersham International, Buckinghamshire, U.K. Rabbit anti-melatonin antiserum (Batch 19540 - 16876), prepared by Dr. J.P. Ravault (INRA, Tours, France) (Tillet et al., 1986) and cellulose-linked donkey anti-rabbit IgG (SAC-CEL, AASacl) (obtained from IDS, Boldon) were gifts from Stockgrand Limited, University of Surrey, Guildford. Routine laboratory chemicals (analytical grade) including NaCl, NaOH, KOH, HCLO₄ (perchloric acid), gelatine, tricine buffer (N-tris(hydroxymethyl)methyl-glycine), Brij 35, were obtained from Sigma, Poole or Fisons, Loughborough. Analytical reagent grade dichloromethane and absolute ethanol were from Fisons. N-Acetyl-5-methoxytryptamine (melatonin) standard was from Sigma. Original stock melatonin solution (1 mg/ml) was prepared in absolute ethanol and stored at 4 °C for up to 1 month. Subsequent dilutions (1 µg/ml and 10 ng/ml in water) and the final dilution (in RIA buffer (section 2.3.2)) were prepared on the day of the assay. Water used throughout was double-distilled in glass.
2.2.2 TLC

Tritiated melatonin ([O-methyl-\(^\text{3}\)-H]melatonin) was purchased from Amersham International, Buckinghamshire, U.K. Radiolabel was diluted with ethanol to \(\approx 10,000\) dpm/50 \(\mu\)l. TLC plates (20 cm \(\times\) 20 cm; silica gel adsorbent with fluorescent indicator) were from Kodak (No.13181). Analytical grade solvents (ethyl acetate, dichloromethane, methanol, ethanol; propan-2-ol; butan-1-ol; and chloroform), glacial acetic acid, toluene (low sulphur content) and PPO (2,5 diphenyloxazole, scintillation grade) were from Fisons. POPOP (1,4-bis (5-phenyloxazol-2-yl) benzene) was obtained from Lancaster synthesis, Morecambe. Ascorbic acid was from Sigma.

Standards: melatonin; 5-methoxytryptamine (5-MT); 5-methoxytryptophol (5-ML); 5-hydroxytryptophol; \(N\)-acetylserotonin (\(N\)-acetyl-5-HT, NAS); 5-hydroxytryptamine (serotonin, 5-HT); 6-hydroxymelatonin; 5-methoxyindole-3-acetic acid (5MIAA); 5-hydroxyindoleacetic acid (5HIAA); 5-methoxyindole; 5-methoxyindole-3-carboxyaldehyde-L-tryptophan; 5-methoxytryptophan; 5-hydroxytryptophan; \(N\)-acetyltryptophan; and 6-methoxy-1,2,3,4-tetrahydro-9H-pyrido-(3,4\(\beta\))indole (6MeOTHBC) were from Sigma. \(N\)-acetyltryptamine was from Aldrich, U.K. 6-sulphatoxy-[\(^{125}\)I]iodomelatonin was a gift from Stockgrand Limited and \(O\)-acetyl-5-methoxytryptophol (Smith and Francis, 1980) was a gift from Dr I. Smith (Cortauld Institute of Biochemistry, The Middlesex Hospital Medical School, London, U.K.).

2.2.3 Biological materials

Post-mortem human eyes were obtained from the Moorfields Eye Hospital, London. Upon collection, eyes were frozen on dry ice and subsequently stored at -80 °C until use.

Female chickens (breed Warrens) obtained from EDS, Surrey were used throughout. From hatching, birds were maintained under a 8.5:15.5 h light:dark photoperiod and water and food (Dalgetty chick crumbs) were available \(ad\ \text{libitum}\). Birds were sacrificed in the morning by inhalation of an increasing CO\(_2\) concentration followed by cervical dislocation. At the time of sacrifice, birds were 6-7 weeks old, weight range 500-600 g.

2.3 Methods

2.3.1 Tissue preparation

2.3.1.1 Human tissue

After thawing the eyes, eye cups (consisting of sclera, choroid, RPE and NR) were prepared. These were produced by cutting around the circumference of the eye in approximately the region of the ora serrata thus removing the anterior segment of the eye including any remaining corneal tissue and the iris if present (Figure 2.2). The lens and vitreous body were removed with blunt-ended forceps. Eye cups were subsequently cut in half in the vertical plane and NR removed. C-RPE segments were teased from the sclera. Tissues were each transferred immediately to sample tubes.
containing chilled RIA buffer (1 ml) (section 2.3.2) then sonicated (Ultrasonic Homogenizer - 4710 Series, Cole-Parmer Instruments, Illinois) for two separate 10 s periods with constant cooling to produce homogeneous suspensions. If necessary, tissues were sonicated for additional time to achieve this. Aliquots of tissue suspensions produced by this procedure are termed "homogenate" throughout. In some instances tissue "homogenate" was centrifuged (15 000 x g, 10 min, 4 °C). The resulting tissue supernatants are termed "supernatant" throughout.

Dissection and sonication procedures were carried out in Class II and Class I safety cabinets respectively, in accordance with School of Biological Sciences safety rules for working with human tissues. Working areas and instruments were disinfected after use with hypochlorite solution (20,000 ppm free chloride) and waste materials autoclaved (134 °C for 25 minutes) before disposal.

Tissue wet weights were obtained by weighing the sample tubes containing buffer before and after addition of the tissue. Measurements were made to 4 decimal places.

2.3.1.2 Chicken tissue

Chickens (n = 30) were sacrificed in three batches on separate days. On each occasion, the birds were sacrificed singly and the required tissues removed and dissected before the next bird was killed in order to minimise any deleterious effects of death on melatonin content. Collection of tissues took 2-5 h depending upon the number of birds. Throughout this time period tissue pools were chilled on ice. Eyes were dissected in the same way as human eyes (section 2.3.1.1), NR and C-RPE tissue compartments removed to chilled RIA buffer and tissue homogenates produced.

Six 500 µl aliquots of each tissue were removed from the homogenates produced from the first batch of birds. Of these, three were processed fresh according to Method 2 (section 2.3.3) and melatonin contents determined by RIA. The remaining aliquots were frozen (-80 °C) and assayed later by the same method in order to assess the effects of freezing on melatonin content. The remainder of the homogenate pools from this batch of birds were also frozen at -80 °C.

On completion of preparation of the last tissue batch (day 3), all previous NR and C-RPE tissue pools were thawed at low temperature. The three batches were then pooled and an aliquot of both tissues removed for use in RIA validation studies (parallelism). The remaining tissue was diluted with RIA buffer on the basis of data from the first RIA to yield tissue melatonin concentrations which upon assay would produce melatonin contents (pg/tube) within the centre range of the standard curve (approximate final concentrations of eye tissues were 1.5 NR/ml and 3.5 C-RPE/ml). Tissues were sonicated again to ensure thorough mixing and split into 500 µl aliquots which were stored at -80 °C until assay. All RIA and TLC validation work using chicken tissue was carried out on these tissue pools.

The chicken tissue used to compare homogenate and supernatant melatonin contents was obtained from a separate batch of birds the body tissues of which were used in other experiments. Birds were of the same breed, sex, etc., and were maintained, as described previously (section 2.2.3). Upon thawing, eyes were removed immediately after sacrifice and frozen whole in isopentane chilled to -40 °C.
with dry ice before storage at -80 °C. Eyes were dissected and NR and C-RPE homogenates and supernatants prepared as described for human tissues.

2.3.2 Melatonin RIA

Melatonin-like immunoreactivity of processed samples was quantified according to the method of English and co-workers (1993) for the measurement of salivary melatonin. Assay buffer used throughout, including as a diluent for antibody and radioligand, was 0.1 M tricine, containing 0.9 % NaCl and 0.2 % gelatine, prepared in water and adjusted to pH 8.0 with 1 M NaOH. Buffer was stored at 4 °C and used within 10 days of preparation. Duplicate standards (range: 1-100 pg melatonin/tube and a zero standard), unknowns and quality control samples (QCs) (500 µl) were incubated with 100 µl rabbit anti-melatonin antiserum (1:20,000) for 30 min at ambient temperature. Radioligand (100 µl 2-[125I]-iodomelatonin - approximately 8,000 - 10,000 cpm) was then added to all tubes including duplicate non-specific binding tubes (600 µl buffer) and the assay incubated for a further 15 - 18 h at 4 °C. Tubes were vortex mixed after each addition. At the end of this second incubation period a solid phase second antibody system was used to separate first antibody-bound ligand and free ligand. Cellulose-linked donkey anti-rabbit IgG (100 µl) was added and the tubes incubated for 1 h at ambient temperature with intermittent mixing. Subsequently, 1 ml saline wash solution containing Brij (0.2 % (v/v) Brij 35, 0.9 % (w/v) NaCl in H₂O) was added and the incubation mixtures centrifuged (1,500 x g for 10 min at 20 °C). Immediately after centrifugation, supernatants were decanted and discarded. Radioactivity of the remaining pellets was determined by a 1260 MultiGamma, gamma counter (LKB, Wallac). Unknowns and QC concentrations were determined from the standard curve data using a RIACalc in which the curve fitting algorithm was a smoothed spline. Non-specific binding was expressed as the proportion of radioligand bound directly to second antibody.

The assay limit of sensitivity was defined as the lowest standard for which specific binding was at least two standard deviations away from mean specific binding of the zero standard. Coefficient of variation data (% CV values) were calculated as: standard deviation/mean x 100.

Tissue volumes routinely processed for quantification of melatonin by RIA were 500 µl, thus approximately half the tissue from each eye was assayed per experiment. The remaining aliquot was used either individually or was combined to produce pools of NR or C-RPE tissue containing low or high amounts of melatonin-like immunoreactivity for use in subsequent RIA validation (section 2.4.1) or TLC identification studies (section 2.4.2).

Melatonin contents of individual human eyes as determined by RIA were expressed as pg/g wet weight tissue. Human specimens were occasionally incomplete or damaged thus precluding calculation of absolute tissue melatonin contents per eye. Melatonin contents of tissues which read less than the assay limit of sensitivity on the standard curve were quoted as "< least detectable melatonin level/tissue weight (g)". For example, if assay limit of sensitivity = 1 pg/tube and melatonin in the tube represents content of 50 mg tissue, then least detectable melatonin concentration = 1 pg melatonin/50 mg tissue = 20 pg/g tissue.
2.3.3 Sample preparation

Prior to RIA, unknown samples, standards and QCs were processed in a variety of ways.
Method 1: no treatment
Referred to as "unextracted" throughout, that is, unknowns, standards and QCs were assayed directly.
Method 2: dichloromethane extraction
Referred to as "extracted" throughout, that is, unknowns, standards and QCs were solvent extracted as described below (section 2.3.4).
Method 3: acid protein precipitation followed by dichloromethane extraction
Referred to as "acid extracted" throughout. Unknowns, standards and QCs were treated with an equal volume of 0.4 M perchloric acid and vortex mixed. The mixtures were then brought to pH 8 with 4 M KOH. (The exact volume used was that which was shown at the time of the experiment to bring a comparable volume of the same batches of perchloric acid and assay buffer (1:1) to pH 8.) Samples were centrifuged at 13,000 rpm for 3 min in an Eppendorf centrifuge. An aliquot of the resultant supernatant was then removed and extracted with dichloromethane as described below (section 2.3.4).

Within an RIA, unknowns, standards and QCs were always processed in exactly the same way. Unknown, standard and QC volumes were equalised with RIA buffer as necessary, before processing. The starting amounts of melatonin standard were increased proportionately to account for the different losses associated with each method such that the standard curve for each method covered the same final range of melatonin concentrations, that is 1-100 pg assay tube. For all methods, standards were always prepared in RIA buffer.

2.3.4 Dichloromethane extraction

Unknowns, standards and QCs were extracted with 5 ml dichloromethane. If necessary, aqueous volumes were equalised with RIA buffer prior to extraction. Following addition of the solvent, samples were vortex mixed for 10 seconds then the two phases allowed to separate by standing. Subsequently, the majority of the aqueous phase was removed by glass pipette. Sample bottles were then submerged in dry ice-chilled acetone in order to freeze the remaining aqueous phase thus preventing contamination of the organic phase. The latter (4 ml) was removed to large glass test tubes and the solvent evaporated to dryness in a water bath maintained at 40 °C under a stream of nitrogen gas. Samples were routinely reconstituted in 1.2 ml assay buffer with 10 seconds vortex mixing. Duplicate 500 μl aliquots were then removed for RIA as described (section 2.3.2).

2.3.5 Quality control samples

Low, medium and high QC samples were prepared from assay buffer spiked with melatonin standard. QCs were made in bulk and aliquots stored at -20 °C. Buffer was spiked to yield nominal melatonin concentrations of 22.5, 90 and 360 pg/ml (QCs) such that following extraction QCs covered the range of the standard curve (3.75, 15 and 60 pg/tube). Preparation of QCs from human eye tissue
homogenates (either containing endogenous melatonin or by addition of exogenous melatonin) although desirable was not feasible due to limitations in terms of tissue availability.

2.3.6 Identification of melatonin-like immunoreactivity by GC-MS

Initial attempts to quantify melatonin by GC-MS with the methodology of Skene and co-workers (1983) failed to achieve sufficient sensitivity with the in-house system utilising the existing column conditions, etc. (μg rather than picograms as required). Consequently the technique was not pursued.

2.3.7 Identification of melatonin-like immunoreactivity by TLC

TLC was subsequently chosen as the method of choice for melatonin identification. Although not sensitive alone, in conjunction with RIA (TLC/RIA) sufficient sensitivity could be achieved.

2.3.7.1 General TLC conditions

Prior to use TLC plates were divided into 10 tracks by narrow silica-free channels to prevent inter-sample contamination. They were then "washed" by running once in the appropriate mobile phase and sprayed with ascorbic acid solution (0.1 % w/v in methanol) to minimise indole oxidation. Samples were applied to the origin which was 1.5 cm from the plate edge. The tank (internal dimensions 21 x 6 x 22 cm) was filled with mobile phase (≈ 90 ml) at least 30 minutes before use and lined with filter paper to ensure saturation of the atmosphere with solvent throughout the run. Immediately before separation, the TLC tank was flooded with nitrogen gas to further prevent compound oxidation.

The mobile phase was either ethyl acetate (100 %) or chloroform/methanol/acetic acid (9/1/0.1 by volume) as specified in section 2.4.2 and figure legends. Plates were run either 3 times in ethyl acetate or once in the other solvent.

$R_f$ values were calculated thus:

$$R_f = \frac{\text{distance moved by solute (measured to top of spot)}}{\text{total distance moved by solvent from origin}}$$

Although absolute $R_f$ values varied slightly with ambient conditions the order of compound separation for each mobile phase remained the same between runs.

2.3.7.2 TLC of standards

The resolution of several potential antibody cross-reactants was investigated in the solvent systems. Standards (1 mg/ml) were prepared in ethanol and 10 μl aliquots applied to prepared plates for TLC. Compounds were visualised under ultraviolet light.
2.3.7.3 Sample preparation prior to TLC

Samples were treated by either of the following methods: Method 2: dichloromethane extraction ("extracted"), or Method 3: acid precipitation followed by dichloromethane extraction ("acid extracted") (section 2.3.3). The evaporated extracts produced were then reconstituted in 100 μl chloroform with 10 s vortex mixing. These were applied to the TLC plate in 5 × 20 μl aliquots and chromatographed in the conditions described above. Authentic melatonin was always run simultaneously (either applied directly to the plate or extracted from buffer spiked with ethanolic melatonin) to determine its \( R_f \) value and as an indicator of plate elution efficiency.

2.3.7.4 Quantification of melatonin-like immunoreactivity following TLC

After chromatography, individual tracks were cut into fractions. Fractions 1 (the origin fraction) and 19 were 1.5 cm in length, and the remaining fractions were each 1 cm. Fractions were transferred to glass vials for elution.

Two elution procedures were utilised. "Method a" was used for chicken tissue experiments. For the experiments with human tissue, which contained much less melatonin-like immunoreactivity than the chicken tissue, an alternative elution system with improved elution efficiency was sought. Various solvents were investigated for their ability to elute tritiated melatonin (Table 2.1). The solvent system selected and used subsequently in all human tissue experiments is described as "Method b".

Method a: referred to as "dichloromethane elution" throughout. Plate fractions were vortex mixed in dichloromethane (1 ml) for 10 s. Fractions were removed and the solvent evaporated to dryness in a water bath maintained at 40 °C under a stream of nitrogen gas. Samples were then reconstituted in 1.1 ml RIA buffer and duplicate 500 μl aliquots assayed by RIA (section 2.3.2).

Method b: referred to as "dichloromethane:butan-1-ol elution" throughout. Plate fractions were vortex mixed in dichloromethane:butan-1-ol (1:1) (3 ml) for 10 s and sample bottles allowed to stand at ambient temperature for 2.5 h. Plate fractions were then removed and the solvent evaporated as above. Samples were reconstituted in 1.3 ml RIA buffer, vortex mixed for 10 s then centrifuged (3,000 × g, 20 min at 4 °C). Duplicate 500 μl aliquots of the resultant supernatant were removed for RIA.

In the RIA, all plate eluent samples were read against unextracted standards, that is, standards were processed by Method 1 (section 2.3.3).

2.3.7.5 Quantification of tritiated melatonin standard following TLC

Standard tracks were fractionated and eluted by Method a for chicken tissues experiments or Method b for human tissues experiments (section 2.3.7.4). For determination of \(^3\text{H}\)-melatonin, evaporated eluents were re-dissolved in 4 ml scintillant (toluene containing 5g/l PPO and 0.3 g/l POPOP) and counted (Wallac 1410 Liquid scintillation counter).
<table>
<thead>
<tr>
<th>A</th>
<th>elution time</th>
<th>recovery %</th>
</tr>
</thead>
<tbody>
<tr>
<td>dichloromethane</td>
<td>30 s</td>
<td>2.8</td>
</tr>
<tr>
<td></td>
<td>1 h</td>
<td>7.1</td>
</tr>
<tr>
<td></td>
<td>4 h</td>
<td>6.2</td>
</tr>
<tr>
<td></td>
<td>8 h</td>
<td>5.9</td>
</tr>
<tr>
<td></td>
<td>18 h</td>
<td>6.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>B</th>
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<th>recovery %</th>
</tr>
</thead>
<tbody>
<tr>
<td>propan-2-ol</td>
<td>1 h</td>
<td>95.8</td>
</tr>
<tr>
<td>butan-1-ol</td>
<td>1 h</td>
<td>82.1</td>
</tr>
<tr>
<td>dichloromethane:methanol (1:1)</td>
<td>1 h</td>
<td>77.3</td>
</tr>
<tr>
<td>ethyl acetate</td>
<td>1 h</td>
<td>66.8</td>
</tr>
<tr>
<td>chloroform</td>
<td>1 h</td>
<td>58.7</td>
</tr>
<tr>
<td>dichloromethane</td>
<td>1 h</td>
<td>15.7</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>C</th>
<th>elution time</th>
<th>recovery %</th>
</tr>
</thead>
<tbody>
<tr>
<td>propan-2-ol</td>
<td>30 s</td>
<td>34.4</td>
</tr>
<tr>
<td></td>
<td>5 m</td>
<td>50.8</td>
</tr>
<tr>
<td></td>
<td>15 m</td>
<td>59.4</td>
</tr>
<tr>
<td></td>
<td>30 m</td>
<td>62.6</td>
</tr>
<tr>
<td></td>
<td>1 h</td>
<td>76.3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>D</th>
<th>elution time</th>
<th>recovery %</th>
</tr>
</thead>
<tbody>
<tr>
<td>methanol</td>
<td>2.5</td>
<td>59.3</td>
</tr>
<tr>
<td>dichloromethane:butan-1-ol (1:1)</td>
<td>2.5</td>
<td>92.7</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>E</th>
<th>elution time</th>
<th>recovery %</th>
</tr>
</thead>
<tbody>
<tr>
<td>dichloromethane:butan-1-ol (1:1)</td>
<td>2.5 h</td>
<td>90.2</td>
</tr>
<tr>
<td></td>
<td>1 h</td>
<td>79.6</td>
</tr>
</tbody>
</table>

Table 2.1 Efficiencies of various solvent systems for elution of $[^3]$H-melatonin from silica TLC plates.

$[^3]$H-melatonin (~10,000 dpm in 60 µl ethanol) was applied to 1 x 1 cm squares of silica TLC plate, the solvent allowed to evaporate and the squares transferred to various solvent systems (3 ml), vortex mixed for 10 s and melatonin allowed to elute. After the elution period, the TLC section was removed and the solvent evaporated under a stream of nitrogen gas in a water bath maintained at 40 °C. Evaporated eluents were reconstituted in scintillation fluid and counted. Data (dpm) were expressed in terms of recoveries as a percentage of $[^3]$H-melatonin (dpm) applied to the plate.

Increasing elution time with dichloromethane failed to improve recoveries (A). One hour elutions with various solvents indicated propan-2-ol to be very efficient (B). Recovery with propan-2-ol was dependent on elution time (C). The propan-2-ol system was not further investigated, however, as the elution residue (plate silica components) was subsequently discovered to cause displacement in the RIA. Additional elution solvents were therefore investigated (D). Dichloromethane:butan-1-ol was found to be very effective. Recovery was time dependent (E). The elution residue was again found to cause displacement in the RIA but unlike the propan-2-ol system, this effect could be negated by removal of the sediment by centrifugation prior to assay.
2.4 Results

2.4.1 RIA Validation

2.4.1.1 Identity of extracted and unextracted melatonin

The solvent extraction procedure employed in sample preparation Methods 2 and 3 (section 2.3.3) might potentially have introduced two types of non-identity; either the melatonin might have been physico-chemically altered, so that it no longer reacts in the same way with the antibody (for example, a proportion of the different binding sites on the ligand may be damaged), or, the extract may contain materials which interfere with the antibody-antigen interaction, that is, a non-specific chemical effect on the reaction. The most obvious result of non-identity is non-parallelism between unextracted and extracted melatonin standards.

Figure 2.3 shows unextracted (sample preparation Method 1) and extracted standard curves (sample preparation Method 2) to be parallel indicating no significant alteration of melatonin during dichloromethane extraction. Extracted buffer blank samples consistently read less than the lowest standard (1 pg/tube) against both unextracted and extracted standard curves. Standards in all subsequent assays (except those quantifying melatonin in TLC eluents) were extracted.

2.4.1.2 Tissue homogenate versus supernatant

In order to investigate whether sonication disrupted cellular material sufficiently to release melatonin from the tissue, melatonin contents of chicken tissue homogenates and supernatants derived from the same tissue were compared. NR supernatant and homogenate melatonin contents were similar (Table 2.2). However, in the C-RPE, 95 % more melatonin was measured in the homogenate than in the supernatant. Melatonin contents of the human tissues examined were generally too low to observe any supernatant-homogenate difference although in C-RPE from pool B, in contrast to the supernatant, there was sufficient melatonin in the homogenate to be quantified accurately. All subsequent measurements were thus made on tissue homogenates.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>supernatant</th>
<th>homogenate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chicken</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NR</td>
<td>269.7</td>
<td>264.9</td>
</tr>
<tr>
<td>C-RPE</td>
<td>449.3</td>
<td>874.3</td>
</tr>
<tr>
<td>Human tissue pool A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NR</td>
<td>&lt;4.1</td>
<td>&lt;4.1</td>
</tr>
<tr>
<td>C-RPE</td>
<td>nd</td>
<td>&lt;14.1</td>
</tr>
<tr>
<td>Human tissue pool B</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NR</td>
<td>&lt;7.5</td>
<td>&lt;7.5</td>
</tr>
<tr>
<td>C-RPE</td>
<td>&lt;16.2</td>
<td>32.3</td>
</tr>
</tbody>
</table>

Table 2.2
Comparison of tissue supernatant and homogenate melatonin contents. Values are pg/g wet weight tissue. Samples were dichloromethane extracted prior to assay. (nd not detectable)
Figure 2.3
Parallelism of unextracted (Δ) and extracted (■) standard curves prepared in buffer. Binding is expressed as a percentage of that observed at zero displacement (B0). Data are means ± s.e.m. (n=6). Error bars are masked by symbols.
2.4.1.3 Effect of freezing

Chicken tissue melatonin contents before and after freezing were 165.9 ± 4.1 and 168.3 ± 6.5 (NR) and 85.0 ± 3.2 and 80.2 ± 2.9 (C-RPE) pg/500 μl tissue aliquot (mean ± s.e.m. of 3 aliquots), respectively, implying no effect of freezing on chicken NR or C-RPE melatonin contents.

2.4.1.4 Comparison of sample preparation Methods 2 and 3 for the determination of melatonin-like immunoreactivity by RIA

2.4.1.4.1 Assay characteristics

Resultant standard curves were very similar for both extracted methods of sample processing (Figure 2.4). Non-specific binding (expressed as a percentage of total radioligand activity) was routinely less than 4 % for both assays. Intra- and inter-assay coefficients of variation (CV) for buffer QC samples are presented in Table 2.3.

<table>
<thead>
<tr>
<th>Method</th>
<th>QC Melatonin amount (pg/tube)</th>
<th>CV (%)</th>
<th>Intra-assay</th>
<th>Inter-assay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extracted</td>
<td>60</td>
<td>7.1</td>
<td>10.5 (11.8)</td>
<td>(n=8)</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>11.1</td>
<td>12.2 (14.2)</td>
<td>(n=12)</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>12.6</td>
<td>14.0 (14.8)</td>
<td>(n=20)</td>
</tr>
<tr>
<td>Acid extracted</td>
<td>70</td>
<td>3.7</td>
<td>11.4</td>
<td>(n=10)</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>6.1</td>
<td>12.8</td>
<td>(n=12)</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>10.1</td>
<td>14.1</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.3

Intra- and inter-assay CVs (%) for quality control samples prepared in buffer. n values refer to number of assays. For the extracted method two QC batches were used.
Figure 2.4
Parallelism of RIA buffer standard curves following treatment of standards by the two extraction methods. Method 2 (Δ): standards were extracted with dichloromethane, Method 3 (■): standards were acid precipitated prior to dichloromethane extraction. Specific binding is expressed as a percentage of that observed at zero displacement (Bo). Data are means ± s.e.m. (n=4). Where error bars are not visible they are masked by symbols.
2.4.1.4.2 Assay consistency

Intra-assay CVs for the quantification of endogenous melatonin in chicken tissues are given in Table 2.4. The most widely ranging values were obtained for C-RPE processed by the acid extracted method.

<table>
<thead>
<tr>
<th>Method</th>
<th>Tissue</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NR</td>
<td>C-RPE</td>
</tr>
<tr>
<td>extracted</td>
<td>6.3</td>
<td>4.7</td>
</tr>
<tr>
<td>acid extracted</td>
<td>8.0</td>
<td>18.3</td>
</tr>
</tbody>
</table>

**Table 2.4**

Intra-assay CVs (%) for the measurement of endogenous melatonin-like immunoreactivity in chicken tissue pools. Mean melatonin contents (pg/ tube) as determined by the extracted method were 69.2 ± 2.0 and 38.0 ± 0.8 for NR and C-RPE, respectively (mean ± s.e.m., n=5).
Inter-assay CVs for the quantification of melatonin in chicken tissues are given in Table 2.5. Melatonin contents (pg/500 µl tissue aliquot) are also shown. Mean NR melatonin contents as determined by extracted and acid extracted methods were very similar. In contrast, for C-RPE melatonin content, there was a statistically significant difference between the methods; the acid extracted method value was much lower than the extracted method (unpaired Student’s t-test, p < 0.01).

<table>
<thead>
<tr>
<th>Method</th>
<th>Tissue</th>
<th>NR</th>
<th>C-RPE</th>
</tr>
</thead>
<tbody>
<tr>
<td>extracted</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>207.6</td>
<td>114.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>198.5</td>
<td>125.0</td>
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<td></td>
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<td>184.4</td>
<td>107.3</td>
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<tr>
<td></td>
<td></td>
<td>186.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>194.3 ± 5.5</strong></td>
<td><strong>115.5 ± 5.1</strong></td>
</tr>
<tr>
<td>% CV</td>
<td></td>
<td>5.6</td>
<td>7.7</td>
</tr>
<tr>
<td>acid extracted</td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>162.5</td>
<td>38.8</td>
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<tr>
<td></td>
<td></td>
<td>200.0</td>
<td>70.0</td>
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<td></td>
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<td>202.5</td>
<td>73.0</td>
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<td>243.8</td>
<td>42.5</td>
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<tr>
<td></td>
<td></td>
<td>168.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>195.4 ± 14.5</strong></td>
<td><strong>56.1 ± 9.0</strong></td>
</tr>
<tr>
<td>% CV</td>
<td></td>
<td>16.6</td>
<td>31.9</td>
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</table>

Table 2.5
Comparison of extracted and acid extracted methods for the determination of melatonin contents of chicken NR and C-RPE pools. Data units are pg/500 µl tissue aliquot. Each value represents the mean of 2-5 aliquots measured in an individual assay. Figures in bold type represent the mean ± s.e.m. of the data from individual assays.
2.4.1.4.3 Recoveries

Melatonin added to NR homogenates prepared from either individual specimens or pooled specimens was quantitatively recovered by both methods (Table 2.6). However, although melatonin was quantitatively recovered from C-RPE using the extracted method, the proportion of melatonin recovered from C-RPE was significantly less with the acid extracted method (Mann-Whitney U test, p < 0.05).

<table>
<thead>
<tr>
<th>Method</th>
<th>Tissue</th>
<th>NR</th>
<th>C-RPE</th>
</tr>
</thead>
<tbody>
<tr>
<td>extracted</td>
<td>A</td>
<td>102.1</td>
<td>102.5</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>85.3</td>
<td>125.5</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>119.2</td>
<td>84.5</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>112.0</td>
<td>103.1</td>
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<td></td>
<td>E</td>
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<td>92.5</td>
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<td>G</td>
<td>84.7</td>
<td>98.9</td>
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<tr>
<td></td>
<td>H</td>
<td>99.7</td>
<td>107.2</td>
</tr>
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<td></td>
<td>I</td>
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<td>104.7</td>
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<tr>
<td></td>
<td></td>
<td>100.0 ± 3.7</td>
<td>101.3 ± 3.9</td>
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<td></td>
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<td>(mean ± s.e.m.)</td>
<td>(mean ± s.e.m.)</td>
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<tr>
<td>acid extracted</td>
<td>A</td>
<td>94.0</td>
<td>97.1</td>
</tr>
<tr>
<td></td>
<td>B</td>
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<tr>
<td></td>
<td></td>
<td>98.1 ± 4.5</td>
<td>83.7 ± 4.4</td>
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<td></td>
<td></td>
<td>(mean ± s.e.m.)</td>
<td>(mean ± s.e.m.)</td>
</tr>
</tbody>
</table>

Table 2.6
Recovery of melatonin (140-150 pg) from human NR and C-RPE tissue with two assay methods. Data are expressed as percentages of values obtained from spiking buffer in the same assay with the same amount in order to account for small inter-assay differences in spiking level. Tissues A-D were tissue pools, and E-L were individual specimens. Figures in bold type represent the mean ± s.e.m. of the data in each group.

Further studies with the extracted method investigated recovery of a range of melatonin amounts (1 - 100 pg/assay tube post-extraction) from human NR and C-RPE pools. Quantitative recovery of melatonin, from both tissues, was obtained over the entire range of concentrations studied (Figure 2.5). The correlation coefficient of the relationship between recovered and added amounts of melatonin for NR and C-
Figure 2.5
Recovery of exogenous melatonin from pooled human NR (A) and C-RPE (B) homogenates. Tissue aliquots were spiked with a range of melatonin amounts (3-300 pg pre-extraction). Tissue samples and standards were extracted prior to RIA.
RPE homogenates was 0.998 in each case. The equations of the corresponding least squares regression lines of the data were $y = 1.26x + 1.54$ and $y = 1.17x - 1.04$ for NR and C-RPE, respectively, where $x$ = melatonin added, and $y$ = melatonin recovered implying endogenous tissue melatonin contents of 4.6 and 0 pg/500 µl aliquot, respectively, for NR and C-RPE. The former value compares with 3.5 pg/500 µl aliquot as determined by assay of an unspiked aliquot of the same tissue pool. C-RPE homogenate assayed similarly, yielded a value below the assay detection limit.

2.4.1.4.4 Parallelism

Serial dilution of chicken NR and C-RPE homogenates containing endogenous melatonin-like immunoreactivity and processed by extraction produced binding inhibition curves parallel with the extracted melatonin standard curve (Figure 2.6). Similar results were obtained in extracted human tissues from a single specimen or pooled from several eye specimens (Figure 2.7) and acid extracted human tissue from a single specimen (Figure 2.8).
Figure 2.6
Parallelism of serial dilutions of chicken NR (A) and C-RPE (B) homogenates containing endogenous melatonin-like immunoreactivity (▲, ■) with buffer standard curves (●). Tissue samples and standards were extracted prior to RIA. Tissue volumes were made up to 500 µl with buffer prior to extraction. Specific binding is expressed as a percentage of that observed at zero displacement (B0).
Figure 2.7
Parallelism of serial dilations of human NR (A) and C-RPE (B) homogenates containing endogenous melatonin-like immunoreactivity (▲, ■) with buffer extracted standard curve (●). Tissue was either derived from a single eye specimen (■) or pooled from several eyes (▲). Tissue samples and standards were extracted prior to RIA. Tissue volumes were made up to 500 μl with buffer prior to extraction. Specific binding is expressed as a percentage of that observed at zero displacement (Bo).
Figure 2.7
Parallelism of serial dilations of human NR (A) and C-RPE (B) homogenates containing endogenous melatonin-like immunoreactivity (▲, ■) with buffer extracted standard curve (●). Tissue was either derived from a single eye specimen (■) or pooled from several eyes (▲). Tissue samples and standards were extracted prior to RIA. Tissue volumes were made up to 500 µl with buffer prior to extraction. Specific binding is expressed as a percentage of that observed at zero displacement (Bo).
Figure 2.8
Parallelism of serial dilutions of human NR (■) and C-RPE (▲) homogenates containing endogenous melatonin-like immunoreactivity with buffer acid extracted standard curve (●). Tissue samples were acid extracted prior to RIA. Tissue volumes were made up to 500 µl with buffer prior to extraction. Specific binding is expressed as a percentage of that observed at zero displacement (Bo).
2.4.2 Identification of melatonin-like immunoreactivity by TLC

2.4.2.1 TLC of standards

Adequate separation of those compounds investigated (potential antibody cross-reactants) was obtained with ethyl acetate as the mobile phase (Figure 2.9). Resolution of melatonin and NAS was improved in the second mobile phase (chloroform/methanol/acetic acid, 9/1/0.1 by volume). In this system, the $R_f$ values of melatonin and NAS were 0.65 and 0.87, respectively and on the TLC plates the compounds were always separated by at least $2 \times 1$ cm fractions.

2.4.2.2 Chicken NR

TLC data are plotted throughout in terms of melatonin-like immunoreactivity of each plate fraction rather than at $R_f$ values in order to indicate actual distances between compounds on the plate. All the data on each individual figure were derived from samples run simultaneously on the same TLC plate.

In order to determine whether the TLC experimental protocol itself generated compounds which cross-reacted with the antiserum, and which may therefore have falsely elevated the levels of melatonin-like immunoreactivity observed in tissue samples, various reagent blanks were prepared and subjected to the TLC/RIA procedure used with chicken NR. None were found to produce melatonin-like immunoreactivity (Figure 2.10A).

Following TLC, both extracted and acid extracted NR yielded single bands which were coincident with authentic melatonin standard (Figure 2.10B). Table 2.7 shows that for both methods of sample processing calculated tissue melatonin contents were similar when assessed by either RIA directly, or by RIA of the melatonin-like immunoreactivity peak coinciding with melatonin standard in TLC, implying that the melatonin-like immunoreactivity content of the tissue was contributed solely by melatonin.

<table>
<thead>
<tr>
<th>Sample preparation</th>
<th>RIA</th>
<th>TLC/RIA</th>
</tr>
</thead>
<tbody>
<tr>
<td>extracted</td>
<td>$194.3 \pm 5.5$</td>
<td>$185.8 \pm 57.2$</td>
</tr>
<tr>
<td></td>
<td>(n = 5)</td>
<td>(n = 2)</td>
</tr>
<tr>
<td>acid extracted</td>
<td>$195.4 \pm 14.5$</td>
<td>$201.8 \pm 28.2$</td>
</tr>
<tr>
<td></td>
<td>(n = 5)</td>
<td>(n = 2)</td>
</tr>
</tbody>
</table>

Table 2.7
Comparison of chicken NR homogenate melatonin contents as determined by direct RIA of the tissue or RIA of the melatonin peak coinciding with authentic melatonin standard in TLC (TLC/RIA). Data are means ± s.e.m. Units are pg/500 µl tissue aliquot.
Figure 2.9
$R_f$ values of indole standards separated by TLC with ethyl acetate as the mobile phase. Where bars are absent, $R_f$ values are too small to be visible.
Figure 2.10
(A) The plate elution system employed with chicken tissue did not create melatonin-like immunoreactivity in various reagent blanks following TLC: extracted buffer (■); acid extracted buffer (●); and chloroform (▲).
(B) Dichloromethane extracted (●) and acid extracted (▲) chicken NR containing endogenous melatonin-like immunoreactivity gave single peaks which were coincident with authentic melatonin standard (tritiated melatonin, bars; and cold melatonin visualised by UV (■■■)).
TLC mobile phase was ethyl acetate. Melatonin was eluted from the TLC plate with dichloromethane. Arrows indicate RIA limit of sensitivity.
2.4.2.3 Human tissues

The alternative, increased efficiency, elution system used with human tissues (section 2.3.7.4 Method b) was also established not to generate melatonin-like immunoreactivity in various reagent blanks (Figure 2.11A). Dichloromethane extracted and unextracted melatonin standards were shown to run together indicating no effect of the extraction procedure on the chromatographic properties of melatonin (Figure 2.11B). Subsequently all melatonin standards were extracted prior to chromatography.

The low endogenous melatonin-like immunoreactivity contents of human NR and C-RPE homogenates as determined by direct RIA were confirmed by TLC/RIA (Figure 2.12A and 2.13A). These same tissues spiked with melatonin showed peaks of immunoreactivity which co-eluted with authentic standard. Calculated melatonin contents of standard and spiked tissue were equivalent (NR spiked with melatonin 42.8 pg, melatonin standard 47.3 pg; C-RPE spiked with melatonin 40.4 pg, melatonin standard 40.4 pg). There was, therefore, no loss of melatonin as a result of tissue metabolism, etc.

TLC of NR and C-RPE containing high levels of endogenous melatonin-like immunoreactivity produced single melatonin peaks coincident with authentic melatonin (Figures 2.12B and 2.13B). Melatonin contents were similar when determined by direct RIA or by TLC/RIA methods (Figure 2.14).

These same tissue pools containing high levels of endogenous melatonin were subsequently extracted and chromatographed in a second mobile phase which was better able to resolve melatonin from NAS (section 2.4.2.1). In both NR and C-RPE tissues, single peaks of melatonin-like immunoreactivity which chromatographed with authentic melatonin standard were obtained (Figure 2.15). Tissue melatonin contents (melatonin-like immunoreactivity co-eluting with authentic melatonin) as determined by this TLC method (NR = 17.9 pg, C-RPE = 32.4 pg) were comparable with those obtained with the previous TLC method (NR = 14.8 pg, C-RPE = 33.3 pg) indicating that the melatonin-like immunoreactivity content of the tissues was not contributed by NAS.
Figure 2.11
(A) The plate elution system used with human tissues did not create melatonin-like immunoreactivity in various reagent blanks following TLC: extracted buffer (■); extracted buffer spiked with ethanol (●); and chloroform (▲).
(B) Dichloromethane extracted (■) and unextracted (●) melatonin standards were coincident.
TLC mobile phase was ethyl acetate. Melatonin was eluted from the TLC plate with butan-1-ol:dichloromethane (1:1). Arrows indicate RIA limit of sensitivity.
Figure 2.12

(A) The low melatonin-like immunoreactivity content of a pool of human NR homogenate as determined by direct RIA was confirmed by TLC/RIA (●). Spiking this same tissue with melatonin (▲) produced a single peak of melatonin-like immunoreactivity which was coincident with melatonin standard (bars).

(B) Endogenous melatonin-like immunoreactivity of human NR homogenate (♦) was coincident with authentic melatonin standard (bars).

All samples were extracted prior to TLC. TLC mobile phase was ethyl acetate. Melatonin was eluted from the TLC plate with butan-1-ol:dichloromethane (1:1). Arrows indicate RIA limit of sensitivity.
Figure 2.13
(A) The low melatonin-like immunoreactivity content of a pool of human C-RPE homogenate indicated by direct RIA was confirmed by TLC/RIA (●). Spiking this same tissue with melatonin (▲) produced a single peak of melatonin-like immunoreactivity which was coincident with melatonin standard (bars).
(B) Endogenous melatonin-like immunoreactivity of human C-RPE homogenate (♦) was coincident with authentic melatonin standard (bars).

All samples were extracted prior to TLC. TLC mobile phase was ethyl acetate. Melatonin was eluted from the TLC plate with butan-1-ol:dichloromethane (1:1). Arrows indicate RIA limit of sensitivity.
Figure 2.14
Melatonin contents of human NR and C-RPE tissues as determined by two methods. Low and high refer to the endogenous melatonin-like immunoreactivity level. For RIA (■) tissues were extracted then assayed for melatonin. For TLC/RIA (■) tissues were extracted then subjected to TLC and only the melatonin peak co-eluting with authentic standard quantified by RIA. TLC mobile phase was ethyl acetate and the elution system was butan-1-ol:dichloromethane.
Figure 2.15
Endogenous melatonin-like immunoreactivity contents of human NR (●) and C-RPE (▲) homogenates were coincident with authentic melatonin standard (bars). The expected position of NAS is indicated by the area of shading (□). NAS was chromatographed simultaneously under identical TLC conditions in a separate tank to avoid contamination of the tissue samples - its relative position with respect to that of melatonin standard and the tissue samples was calculated on the basis of its $R_F$ value.

All samples (except NAS standard) were extracted prior to TLC. TLC mobile phase was chloroform/methanol/acetic acid (9/1/0.1 by volume). Melatonin was eluted from the TLC plate with butan-1-ol:dichloromethane (1:1). The arrow indicates RIA limit of sensitivity.
2.5 Discussion

A series of validation studies has been undertaken in order to ascertain which of two possible sample extraction methodologies was most suitable for the quantification of melatonin in human NR and C-RPE homogenates by an existing RIA. Samples were either protein precipitated with acid then dichloromethane extracted (acid extracted method) or extracted directly with dichloromethane (extracted method). Both extraction methods were able to measure melatonin quality control samples prepared in buffer consistently. However, for the measurement of endogenous melatonin in tissue, although there was no difference between the methods when investigating NR, the acid extracted method was found not to be suitable for use with C-RPE as there were both intra- and inter-assay inconsistencies. Additionally, this acid extracted method measured significantly less melatonin in C-RPE than did the extracted method. Recovery of exogenous melatonin from C-RPE using the acid extracted method was also incomplete. None of these problems were encountered with the extracted method. Furthermore, serial dilution of NR and C-RPE tissues processed by the extraction method produced curves parallel to the melatonin standard curve. On the basis of these findings the extracted method (direct extraction of samples with dichloromethane) was selected for use in all subsequent melatonin quantification studies.

Throughout these investigations, standard curves should ideally have been constructed in the biological fluids under investigation, that is, NR and C-RPE homogenates. However the availability of human ocular tissue was limited. In addition, this would require that the tissue homogenates contained no melatonin which in turn would require artificial preparation of such a melatonin-free sample, for example, by adsorption of the hormone or by use of affinity chromatography. Neither approach is ideal. With the former method there may be secondary effects on other components of the fluid that would eliminate a non-specific blank at the same time, whereas the latter is time consuming. Preparation of the standard curve in low melatonin content pools is another option but this too has inherent disadvantages. For example, tissue composition would not be identical between different assays.

In previous studies, melatonin has been extracted and measured successfully in both tissue supernatants (Skene et al., 1991) and homogenates (for example, Reppert and Sagar, 1983; Delgado and Vivien-Roels, 1989). However, in the present studies, comparisons of extracted tissue homogenate and supernatant indicated greater melatonin contents in the former. The effect was more apparent in C-RPE tissue. This may be because melatonin, which is lipophilic, is able to become more intricately bound within the more structured tissue matrix of the choroid and pigment layers compared with the NR which consists solely of "soft" neuronal tissue. Brief sonication would then not be able to effectively release the melatonin from the tissue into the supernatant. There may also be a receptor bound source of melatonin which again is not released into the supernatant by sonication. Finally, melatonin may be tightly bound to melanin and thus unavailable for assay: melanin is known to bind various drugs and other molecules non-specifically (for example, Lindquist, 1973). This latter theory might explain the finding that the difference between melatonin contents of supernatant and homogenates was greater for C-RPE, that is, the tissue containing more melanin. Extraction of the whole homogenate (as performed in the present studies) would be expected to transfer these bound pools of melatonin into the
organic phase for assaying and thus allow for more accurate measurement of melatonin concentrations. The endogenous tissue melatonin-like immunoreactivity was subjected to TLC in order to verify its authenticity and was found to co-chromatograph with melatonin standard. However, there existed the possibility that the immunoreactivity was being contributed, either partially or totally, by an antibody cross reactant. Of the possible cross reactants investigated (melatonin metabolites and various generic indoles), NAS and N-acetylttryptamine were shown to run closest to melatonin in the TLC conditions employed. The latter has neither been reported in the eye nor recognised as a component of the melatonin synthetic or degradative pathways, hence it is unlikely that its cross reactivity with the antibody (0.08 %) (product data sheet, Stockgrand Ltd.) is of great importance. In contrast, NAS presented a particular problem for a number of reasons. Although it too has a relatively low cross reactivity with the antibody (0.084 %), (product data sheet, Stockgrand Ltd), NAS is the synthetic precursor of melatonin within the eye and in some species, its levels have been demonstrated to match, if not exceed, those of melatonin (Yu et al., 1982; Allen et al., 1991). A proportion of serum NAS does not derive from the pineal (its primary site of synthesis) providing additional evidence for some other active site of synthesis, for example, the eye (Yu et al., 1981a; Brown et al., 1984). Furthermore, NAS may also be a metabolite of circulating melatonin (Leone and Silman, 1984; Leone and Silman 1985; Young et al., 1985). Ocular melatonin may possibly have a similar metabolic fate which would further contribute to local NAS levels. On the basis of its physico-chemical properties it is unlikely that a large proportion of NAS would be extracted into the dichloromethane and thus made available for RIA. However, no NAS RIA was available to confirm this. Hence, the mobile phase of the TLC system was changed in order to improve the resolution of melatonin and NAS. It was subsequently demonstrated that the melatonin-like immunoreactivity measured was not being contributed by NAS. It is likely therefore that the melatonin-like immunoreactivity is melatonin and the validated extraction method RIA described, accurately measures melatonin concentrations in NR and C-RPE tissues. Unequivocal identification would, however, require mass spectrometric evidence. There is a small chance that the immunoreactivity could relate to some unknown antibody cross reactant which co-eluted with melatonin under the TLC conditions employed. Such a compound would need to be of a similar structure to melatonin. It would also have to be physiologically relevant, that is, a possible precursor or metabolite in the eye. All the known precursors and metabolites were checked in TLC and did not co-elute with melatonin. Hence the most likely conclusion is that the RIA measures melatonin.
CHAPTER 3

QUANTIFICATION OF MELATONIN IN POST-MORTEM HUMAN EYES
3.1 Introduction

The existence of ocular melatonin has been reported in a number of mammalian species with interest being centred predominantly upon the retinal compartment (Table 1.1). Immunohistological studies have indicated that within the mammalian retina of those species investigated, as is the case for other vertebrate classes, melatonin is localised predominantly to the outer nuclear layer (for example, Vivien-Roels et al., 1981; Mennenga, 1991). It is likely on the basis of a number of findings that this ocular melatonin is synthesised de novo locally rather than being taken up from the circulation. Firstly, several precursors and intermediate molecules have been identified within the tissues of the eye, for example 5-HT (Ehinger et al., 1981) and NAS (Pang et al., 1983). Secondly, the presence of a variety of synthetic enzymes including NAT and HIOMT, the final two enzymes in the metabolic pathway of melatonin, has been demonstrated within various components of the eye (Tables 1.2 and 1.3).

In contrast, information concerning melatonin and its biochemistry specifically within the human eye is fragmentary. Currently, to the author’s knowledge, there are only three reports which document attempts to quantify melatonin in human eye tissues. These studies all involved limited sample sizes. The procedures employed and the findings of these studies are summarised in Table 3.1. Two of the three groups measured melatonin in the NR. Of these, the investigation of Leino (1984) was methodologically more thorough employing GC-MS. In contrast, Osol and Schwartz (1984) utilised a RIA for the measurement of melatonin in chloroform-extracted tissue. A significant omission from their study concerned assay validation data. Although the method had been validated and utilised previously by another research group for the measurement of melatonin in retinal tissue, this was in fact for a different species (Pang et al., 1977): no validation data were provided for use of the RIA specifically with human tissues. The third study of human ocular melatonin utilised HPLC with electrochemical detection (EC) to investigate the aqueous humor compartment. The presence of melatonin was reported in all three studies.

In addition to quantitative melatonin data there is an increasing body of circumstantial evidence that points towards the likelihood of melatonin being synthesised within the human eye. Evidence concerning the existence of melatonin synthetic enzymes is of particular significance. HIOMT activity has been reported in NR (Wiechmann and Hollyfield, 1987) and ciliary body (Martin et al., 1992). HIOMT gene transcripts have also been detected in the retina (Rodriguez et al., 1994; Bernard et al., 1995). In addition, the recent cloning of the human NAT gene has enabled identification of human retinal NAT mRNA (Coon et al., 1996). The human ciliary body is also reported to possess NAT activity (Martin et al., 1992).

Ocular melatonin has been reported to mediate several actions on local physiological processes (section 1.7) including effects for example on retinomotor movements (Pierce and Besharse, 1985). None of these postulated actions for melatonin have yet been established in the human eye, but it is probable that at least some will be common to man. Ocular melatonin has been implicated as a causative factor in some disease states (section 1.9.1). Conversely it has also been attributed protective properties. It may function, for example, as a local antioxidant (Chen et
<table>
<thead>
<tr>
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</thead>
<tbody>
<tr>
<td><strong>Tissue</strong></td>
<td>Neural retina-retinal pigment epithelium</td>
<td>Neural retina</td>
<td>Aqueous humor</td>
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<tr>
<td><strong>Tissue source</strong></td>
<td>Post mortem donor eyes (n=6). Time between death and frozen</td>
<td>Live donor enucleations (n=9). Tissues</td>
<td>Cataract surgery patients (n = 10)</td>
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<tr>
<td></td>
<td>storage not stated</td>
<td>removed immediately after enucleation</td>
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</tr>
<tr>
<td><strong>Melatonin levels</strong></td>
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<td>750 - 10,100 pg/g wet weight</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>45 - 180 pg/ml (n=4);</td>
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<tr>
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<td></td>
<td></td>
<td>2,300 pg/ml (n = 1)</td>
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<td><strong>Donor details</strong></td>
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<td></td>
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<tr>
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<td>3d - 79 y</td>
<td>11 y - 83 y</td>
<td>23 y - 86 y</td>
</tr>
<tr>
<td>Sex</td>
<td>3M / 3F</td>
<td>No details provided</td>
<td>5M / 5F</td>
</tr>
<tr>
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</tr>
<tr>
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<td>Not applicable</td>
<td>Not applicable</td>
</tr>
<tr>
<td>Technique</td>
<td>RIA</td>
<td>GC-MS</td>
<td>HPLC-EC</td>
</tr>
<tr>
<td>Notes</td>
<td>No method validation studies described</td>
<td>Eyes and/or retina diseased</td>
<td>Samples taken between 08:10 and 14:15</td>
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**Table 3.1**
Comparison of the three reports documenting quantification of melatonin in human eye tissues.
Manipulation of the human ocular melatonin system may represent a future strategy for disease management.

In this study, the melatonin contents of post-mortem human NR and C-RPE were quantified. NR and C-RPE were obvious tissue choices for study based on findings from other species. In addition, various practical considerations indicated to a certain extent which tissues and tissue groupings were investigated. Embryologically the RPE belongs to the retina, however, whereas it is firmly attached to the basal lamina of the choroid, it is only loosely attached to the rods and cones, this latter plane corresponding to the embryonic optic vesicle. Consequently, when dissecting the eye, the natural cleavage plane is between the NR and the RPE, hence the RPE was always investigated in conjunction with the choroid rather than the NR. Anterior uveal tissues such as the iris and ciliary body were not investigated as they were frequently absent or badly damaged, particularly as a result of corneal removal for keratoplasty.

3.2 Specimen information

The source of experimental tissue was as previously described (section 2.2.3). In most instances corneal tissue had been removed for keratoplasty. At the time of specimen collection, various parameters regarding the donor were recorded. Information regarding each of the following was generally available:

1. donor age
2. donor sex
3. time of donor death (TOD)
4. date of donor death (DOD)
5. cause of donor death (COD)
6. time of removal of the specimen from the donor

From these, two additional parameters were calculated, namely post-mortem interval (= time between donor death and enucleation, PMI) and “specimen age” (= time between donor death and eventual storage of the tissue at -80 °C).

3.3 Methods

3.3.1 RIA

Melatonin contents of the NR and C-RPE tissues of 46 human post-mortem eyes were assessed by the validated method previously described, that is, RIA of dichloromethane tissue extracts (Chapter 2). Specimens were investigated individually in order that any relationships that existed between melatonin content and the donor parameters could be identified.
3.3.2 Statistical analysis

Basic statistical testing was carried out by the author. Other analyses (Mann-Whitney tests; Chi-squared tests; analysis of variance; \( t \)-tests; some Spearman's correlation tests; stepwise logistic regression; and stepwise multiple regression) were carried out by The Statistical Advisory Service, University of Surrey (SPSS program v 4.0).

For correlation analysis specimens were necessarily omitted if the parameter being investigated was not available for that specimen. For analysis purposes, times of deaths were converted to values representing minutes away from a theoretical melatonin maximum (03:00 h). Date of death was analysed as month of death (MOD): dates were converted to values representing the number of months away from June.

The level of significance was taken as \( p < 0.05 \) unless otherwise stated.

3.4 Results

The melatonin contents of eyes from 46 individuals were investigated (Figure 3.1): 17 contained quantifiable melatonin in both NR and C-RPE; 2 in NR only and 7 in C-RPE only. The donor parameters of these specimens are shown in Figures 3.2 to 3.4. Both tissues of a further 20 specimens contained melatonin levels below the assay limit of detection. In those eyes containing melatonin, levels were variable, ranging from 10.0 to 425.5 pg/g wet tissue weight (mean 106.5, \( n = 19 \)) for NR, and 34.4 to 486.8 pg/g wet tissue weight (mean 179.3, \( n = 24 \)) pg/g wet for C-RPE. The Pearson's correlation coefficient between specimen NR and C-RPE melatonin contents was 0.70 (\( n=46 \)). Excluding the pairs of NR and C-RPE data which contained undetectable melatonin levels in both tissues a correlation coefficient of 0.60 (\( n=26 \)) was found. In those specimens that contained melatonin in both tissues, C-RPE melatonin contents were significantly higher than NR contents (paired Student's \( t \)-test, \( n=17 \)). Similarly, in the eight eyes which contained high melatonin levels (> 200 pg/g) in at least one tissue, C-RPE melatonin contents were also significantly higher than in NR.
Figure 3.1
Melatonin contents of NR (■) and C-RPE (■) tissues of 46 post-mortem human eyes as determined by RIA. Data are pg/g wet tissue weight. Absence of bars indicates that the melatonin level was below the limit of detection of the assay. Donor parameters are indicated in Table 3.2.
Figure 3.2
Sex (A) and age (B) distributions of specimen donors. Hatched overlays indicate numbers of specimens containing detectable melatonin. Graphs on the left hand side show NR data and those on the right C-RPE data. Where the number of donors does not total 46, donor parameters were not available for the missing specimens.
Figure 3.3
Distributions of times (A) and months (B) of deaths of specimen donors. Hatched overlays indicate numbers of specimens containing detectable melatonin. Graphs on the left hand side show NR data and those on the right C-RPE data. Where the number of donors does not total 46, donor parameters were not available for the missing specimens.
Figure 3.4 Distributions of (A) specimen ages (time between donor death and freezing of specimen) and (B) post mortem intervals (PMI), time between donor death and enucleation. Hatched overlays indicate numbers of specimens containing detectable melatonin.

Graphs on the left hand side show NR data and those on the right C-RPE data. Where the number of donors does not total 46, donor parameters were not available for the missing specimens.
The associated donor parameters of the specimens are provided in Table 3.2. The presence of melatonin in either NR or C-RPE was not obviously related to any of the donor parameters, specimen age, or PMI (Figures 3.2 to 3.4). The eight eyes with high melatonin levels (> 200 pg/g) in at least one tissue also did not share a common parameter (Table 3.3).

A summary of the statistical analyses undertaken and their results is given in Table 3.4. There were no significant correlations between any of the variables and melatonin contents (all data) of either NR or C-RPE when assessed both parametrically (donor-age, -TOD, -MOD, specimen age, PMI: Pearson's correlation) and non-parametrically (donor-age, -TOD, -MOD, specimen age, PMI: Spearman's correlation; donor sex: Mann Whitney test). Stepwise logistic regression analysis, in which the melatonin data were treated as a binary variable (that is, melatonin either present or absent), failed to identify any relationships between the presence of melatonin in NR or C-RPE and the parameters donor-age, -sex, -TOD, -MOD, specimen age and PMI. Similarly there were no significant differences for either NR or C-RPE between those specimens containing melatonin and those without, in terms of donor-age, -TOD, -MOD, specimen age, PMI (Mann Whitney test) or donor sex (Chi-squared test).

Tissue weights obtained from individuals eyes varied depending on the specimen size and its condition. The ranges of weights were 19.5 mg to 1.2614 g, and 9.57 mg to 592.6 mg for NR and C-RPE, respectively. For each specimen, all the tissue retrieved was homogenised, hence, for low weight tissues, their melatonin concentration would have to be greater than for high weight tissues in order for the final melatonin content measured to be above the assay limit of sensitivity. There was no correlation between assay limit of detection and those melatonin levels above zero and nor was assay limit of detection correlated with PMI or specimen age.

Subsequently, those specimens containing no melatonin were excluded from further analysis in order to test whether for the positive melatonin data there were any relationships specifically between the level of melatonin in the specimens and any of the parameters. Scatter plots of these data are shown in Figure 3.5. For C-RPE, none of the variables were correlated with melatonin level (Pearson's correlation or Spearman's correlation). Similar analyses on NR identified one significant relationship, between melatonin level and PMI (Spearman's correlation, $r = -0.66$, $p < 0.01$). Stepwise multiple regression failed to reveal regression relationships between melatonin content and any of the variables. Analysis of melatonin contents in terms of season (analysis of variance) and time of death (night versus day [08:00 to 20:00 h]) (unpaired Student's $t$-test and Mann Whitney test) also indicated no significant differences for either NR or C-RPE.
<table>
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<th>Age (y)</th>
<th>TOD</th>
<th>DOD</th>
<th>COD</th>
<th>Specimen Age (h)</th>
<th>PMI (h)</th>
<th>Melatonin Content (pg/g)</th>
</tr>
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<td>72</td>
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<td>?</td>
<td>146.7</td>
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<tr>
<td>16</td>
<td>M</td>
<td>59</td>
<td>00:45</td>
<td>07/12/93</td>
<td>cancer: lung</td>
<td>40.4</td>
<td>?</td>
<td>&lt; 36.6</td>
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<tr>
<td>18</td>
<td>F</td>
<td>80</td>
<td>03:00</td>
<td>08/12/93</td>
<td>cancer: lung</td>
<td>34.7</td>
<td>?</td>
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<tr>
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Table 3.2

Donor parameters of 46 human eyes assayed for melatonin by RIA. Data values are pg/g wet weight tissue

Abbreviations and notes

M male; F female; T-, D-, COD time-, date-, cause of death; SAH subarachnoid haemorrhage; RTA road traffic accident; LF liver failure; RF renal failure; CVS cardiovascular system; specimen age = time between donor death and frozen storage of specimen; PMI, post mortem interval = time between donor death and enucleation; ? parameter unknown; < = melatonin content less than assay limit of detection; " = exact age unknown; * liver failure due to paracetamol overdose; Sp spring; Su summer; A autumn; W winter

Eye 169 contained a plastic replacement lens.
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<th>COD</th>
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<th>C-RPE</th>
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3.11
Table 3.3
Associated donor parameters of specimens containing "high" melatonin levels (>200 pg/g wet weight) in NR and/or C-RPE.

Abbreviations used are the same as those in the legend of Table 3.2
### Table 3.4

Summary of statistical analyses undertaken on NR and C-RPE melatonin contents of 46 post-mortem human eyes.

**Abbreviations**

TOD, time of death; MOD, month of death; PMI, post-mortem interval
Table 3.4 ...continued

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<th>Statistical test</th>
<th>Data analysed/format of analysis</th>
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<th>C-RPE</th>
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<td>Mann-Whitney test (U values)</td>
<td>melatonin values greater than 0</td>
<td>donor sex 59 not significant</td>
<td>donor sex 90 not significant</td>
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<td>Analysis of variance: data grouped into 4 seasons (March-May; June-August; September-November; December-February) (F values)</td>
<td>melatonin values greater than 0</td>
<td>0.59 not significant</td>
<td>0.58 not significant</td>
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<td>Unpaired Student’s t-test night v day (where night = 20:00 to 08:00) (t values)</td>
<td>melatonin values greater than 0</td>
<td>0.28 not significant</td>
<td>0.46 not significant</td>
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<td>Mann Whitney test for night v day (U values)</td>
<td>melatonin values greater than 0</td>
<td>82 not significant</td>
<td>74 not significant</td>
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<tr>
<td>Pearson product moment correlation coefficient (r) between assay limit of detection and: a) melatonin level b) specimen age c) PMI</td>
<td>all data including zeros</td>
<td>a) -0.16 b) 0.0 c) -0.10 not significant</td>
<td>a) -0.11 b) -0.11 c) -0.12 not significant</td>
</tr>
<tr>
<td>Correlation coefficient (r) between assay limit of detection and: melatonin level a) Pearson’s b) Spearman’s</td>
<td>melatonin values greater than 0</td>
<td>a) -0.03 b) 0.38 not significant</td>
<td>a) 0.22 b) 0.20 not significant</td>
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3.14
Figure 3.5
Scatter plots of NR (■) and C-RPE (△) melatonin contents, versus (A) donor age; (B) month of donor death; (C) time of donor death; (D) specimen age; and (E) PMI.
3.5 Discussion

Over half of the specimens investigated in this study were found to contain melatonin in either or both NR and C-RPE compartments. The RIA methodology employed was validated for use with both tissues and TLC indicated that the tissue melatonin-like immunoreactivity was coincident with authentic melatonin standard in two mobile phase systems (section 2.4.2.3). These facts, together with the previous mass spectrometric identification of melatonin within the human eye (Leino, 1984) indicate that the immunoreactivity measured in these studies was almost certainly true melatonin. These findings thus confirm previous reports of melatonin within the human eye (Osol and Schwartz, 1984; Leino, 1984; Martin et al., 1992).

It is likely that this melatonin is synthesised locally rather than being taken up from the circulation. In the bovine retina, for example, the movement of melatonin across the RPE component of the blood:retinal barrier has been shown to be passive (Pautler and Hall, 1987), that is, there is no mechanism for concentration, and in several species, retinal melatonin persists following pinealectomy (for example, Yu et al., 1981a, b; Reiter et al., 1983; Underwood et al., 1984). In addition there is increasing evidence for the local presence of the requisite biosynthetic enzymes. HIOMT activity has been demonstrated in human retina (Wiechmann and Hollyfield, 1987) as have HIOMT (Rodriguez et al., 1994; Bernard et al., 1995) and NAT gene transcripts (Coon et al., 1996). In the human, isolated choroid and RPE tissues have not been investigated either for the presence of melatonin or the synthetic enzymes. In other species however melatonin has specifically been demonstrated to exist in both RPE (Binkley et al., 1979; Hamm and Menaker, 1980) and choroid (Rohde et al., 1993). NAT and HIOMT have also been identified in RPE (Quay et al., 1969; Binkley et al., 1979; Hamm and Menaker, 1980) but not by all authors (Cardinali and Rosner, 1971a). C-RPE melatonin contents were significantly higher than those of NR. In contrast, Hamm and Menaker (1980) reported that in the chick eye, NR contained more melatonin RPE.

In both previous studies with human retina, all specimens investigated were found to contain melatonin. In contrast in this study, a sizeable proportion (20/46 eyes) contained no detectable melatonin in either tissue. There are a number of reasons why melatonin may not have been detected. Firstly, these particular donors may simply never have possessed ocular melatonin as a result of a lack of synthetic capacity: there are species in which ocular melatonin is reportedly at very low levels or undetectable, for example, the lizard, Anolis carolinensis (Underwood, 1985). Furthermore, although, as discussed above, there is some evidence for the presence of synthetic enzymes within the human eye, this is not conclusive. Wiechmann and Hollyfield’s demonstration of retinal HIOMT activity (1987) has not been confirmed. Recently, Bernard and co-workers (1995) were unable to detect either HIOMT activity or immunoreactive protein in human retina. In addition, whilst these authors did report the presence of retinal HIOMT mRNA transcripts, actual levels were extremely low (Bernard et al., 1995). Together these findings led Bernard and colleagues (1995) to question the capacity of the human retina for melatonin synthesis. However, the fact that melatonin was present in some human specimens in this study indicates that it is unlikely that the human eye lacks the requisite synthetic machinery. Instead, some form of differential expression of the synthetic enzymes may exist between individuals. Certain individuals display atypical pineal melatonin...
production, that is, circulating melatonin levels are much lower than usual or even undetectable (Arendt, 1985). This may be the result of a genetic abnormality involving, for example, HIOMT. If such an abnormality exists then it is feasible that such a defect might also affect ocular melatonin synthesis. Incidence of this condition in the population is, however, rare, hence it seems unlikely that this alone is responsible for such a large proportion of specimens as found here not to contain melatonin.

In addition to donor parameters, information regarding two related factors was available in most instances for the specimens; PMI and specimen age, the former relating to the time between death and enucleation and the latter relating to the total time period between death and freezing of the sample. In NR, melatonin content was significantly negatively correlated with PMI, indicating that the two are associated. A similar effect was not apparent in C-RPE however, nor were there any relationships between specimen age and NR or C-RPE melatonin content. Reppert and Sagar (1983) observed that melatonin content of the chick eye remained constant at 0 °C for up to three hours prior to dissection, implying that changes did in fact occur subsequently. Furthermore, retinal melatonin levels measured in freshly enucleated eyes from live donors (Leino, 1984) were reported to be considerably higher (up to 10 ng/g) than those measured in post-mortem tissues, both by Osol and Schwartz (1984) (350 to 2000 pg/g) and in this study (not detectable up to ~ 500 pg/g wet weight). Thus certain aspects of specimen storage prior to freezing may have been responsible for a reduction (partial or complete) in tissue melatonin content in this investigation. For example, some form of degradation of the melatonin molecule may have occurred during this period. The presence of a functional deacetylase (aryl acylamidase) has been demonstrated in retinal tissue from a number of species, for example chicken and *Xenopus laevis* (Grace et al., 1991). This enzyme degrades melatonin to 5-MT which is subsequently deaminated by monoamine oxidase producing 5-MIAA and 5-ML (Cahill and Besharse, 1989). In those mammals which have been investigated however, that is, rat and pig, existence of this pathway of deacetylation has not been demonstrated (Grace et al., 1991). The primary degradative route of circulating melatonin involves hydroxylation and sulphation forming 6-sulphatoxymelatonin for urinary excretion (Kopin et al., 1961 and Kveder and McIntsaac, 1961). This metabolic route has not been investigated in the eye. It is possible, however, that it is operative: members of the mixed function oxidase cytochrome P-450 family, for example, are present within the eye (Shichi et al., 1975; Schwartzman et al., 1987; Zhao and Shichi, 1995). Furthermore, quantifiable levels of 6-hydroxymelatonin have been demonstrated in human aqueous humor (Martin et al., 1992). In addition to this specific metabolic degradation, the melatonin molecule may be altered in the process of carrying out certain of its actions. Melatonin is reported to possess antioxidant capacity and this feature may be exploited functionally in the eye (Chen et al., 1995; Abe et al., 1994). Melatonin can react, both enzymatically and non-enzymatically with the superoxide anion via pyrrole ring cleavage to produce N'-acetyl-N'-formyl-5-methoxykynurenamine (AFMK). The former involves catalysis by the haem-containing enzyme indoleamine 2,3-dioxygenase (Hirata et al., 1977). This enzyme has been shown to be present in the eye: bovine iris/ciliary body, aqueous humor and retina all possess enzyme activity (Malina and Martin, 1993). Furthermore, in the ciliary body of this species, the enzyme has been shown to produce kynurenamines from melatonin (Malina and

3.17
Martin, 1992). Indoleamine 2,3-dioxygenase is distributed widely in human tissues (Yamazaki et al., 1985) and it is possible that it is present in the human eye. Melatonin is able directly to trap superoxide anions and hydroxyl radicals (particularly in the presence of the chemical hemin (Hardeland et al., 1993a)), that is, with no enzyme involvement (Hardeland et al., 1993b). Such oxidative degradation of melatonin may also, therefore have contributed some decrease in ocular melatonin contents of the human specimens between donor death and eventual frozen storage of the tissue in the studies undertaken here.

The human retina melatonin contents reported by Osol and Schwartz (1984) were higher than observed in the present investigation despite both studies having being undertaken on post-mortem human tissues. This may be because the specimen ages were less in the former study, however, no information regarding this parameter was provided so this cannot be confirmed. Furthermore, the method employed by these authors was not fully validated and the antibody used may have been measuring a cross reactant.

The occurrence of melatonin was not associated with any donor parameter (age; sex; time, month or cause of death), therefore additional analyses were performed on only those specimens which contained quantifiable melatonin in order to identify any relationships between the level of melatonin and the donor parameters. Again, none indicated concurrence. In contrast, in other species, melatonin levels within the eye do appear to be related to various biological factors. The most prominent is the relationship between melatonin level and time of day. In the majority of species which have been demonstrated to possess retinal melatonin, levels are in most cases substantially raised during the dark phase (Table 1.1). Such day/night variations are generated in part by the direct inhibitory effect of light on the melatonin synthetic process (for example, Hamm and Menaker, 1980; Hamm et al., 1983; Reppert and Sagar, 1983) with the remainder of the rhythmic component possibly being under the control of an endogenous oscillator (for example, Hamm and Menaker, 1980; Underwood et al., 1988; Cahill and Besharse, 1990; 1991). NAT is the key regulatory enzyme mediating the changes in melatonin resulting from both the effect of light (Binkley et al., 1979; Hamm et al., 1983) and the internal oscillator (Besharse and Iuvone, 1983). The controlling clock may be situated either centrally or locally. There is increasing evidence for the latter and recently, Tosini and Menaker (1996) have demonstrated that circadian melatonin production in the mammalian retina is under control of an intra-ocular clock. In this investigation, however, although time points throughout the 24 hour period were examined, time of death was not correlated with melatonin level in either NR or C-RPE tissues, nor was there an overall significant difference between night and day levels. A small number of studies have previously reported similar findings. In squirrel retina (Reiter et al., 1981); rabbit retina-choroid (Aimoto et al., 1985); and chicken RPE (Hamm and Menaker, 1980), the characteristic day night variation in melatonin level when determined by these authors was lacking. In the latter case (chicken RPE), there was a corresponding absence of rhythmic NAT activity (Hamm and Menaker, 1980). As previously mentioned however these cases represent the minority: commonly, ocular melatonin is higher at night.

Other parameters have also been reported to affect ocular melatonin levels. In the rat eye there appears to be a difference in melatonin content between the sexes: testosterone may have a stimulatory effect on retinal HIOMT activity (Cardinali and
Rosner, 1971a; Nagle et al., 1974) and retinal melatonin is decreased following castration (Reiter et al., 1983). Seasonal differences in ocular melatonin may also exist. Delgado and Vivien-Roels (1989) have shown in the frog eye that the night time melatonin rise is higher and of longer than duration when the animals are maintained under short period conditions. Steinlechner and colleagues (1995a) have also recently reported the profile of day/night melatonin levels in the hamster retina to differ greatly with the time of year. Cahill and co-workers (1991) have suggested that HIOMT may be regulated by long-term lighting conditions to effect seasonal changes in melatonin levels. In addition to light, season may also have effects via the associated temperature changes. In the frog eye, for example, the melatonin rhythm is only maintained in higher environmental temperatures (Balemans et al., 1983). In this study, however, for neither sex nor month/season of death was there any statistically significant relationship with melatonin content.

Similarly there were no obvious changes in specimen melatonin content associated with donor age. In other species, retinal melatonin and its synthetic enzymes become detectable during the foetal stage with the most dramatic changes in levels occurring in the period prior to adulthood (Cardinali and Rosner, 1971a; Bubenik et al., 1978; Iuvone, 1990). NAT activity in the chicken retina appears to reach a maximum one day prior to hatching (Iuvone, 1990) and in the rat retinal HIOMT activity plateaus at 30 days post partum (Cardinali and Rosner, 1971a). The changes which occur in ocular melatonin throughout later life once the adult maximum has been reached have been little reported in any species.

Although a comprehensive data set concerning the donor parameters, etc. of the specimens used in this study were available, there exist many more variables about which no information was available. In particular, almost certainly all of the donors had received some form of medication in the period immediately prior to death and this may have had effects on melatonin synthesis or metabolism - certain medication strategies, for example, beta-blockers are known to suppress pineal melatonin production (Arendt et al., 1985; Cowen et al., 1985). Also, no information was available regarding treatment of the specimens prior to enucleation. For example, cooling period and conditions of the cadaver would have varied greatly between specimens. Thus, two specimens having the same PMI, may be greatly different in terms of a range of other parameters, yet these samples would still be grouped together for analysis of relationships between melatonin content and PMI length. These "unknown factors" may also have influenced those variables about which information was available. For example, although the times of death were known, the lighting conditions immediately preceding death were not. Therefore, even if death occurred during the dark phase, for example, when melatonin would be expected to be high, if this was in the presence of bright light, then melatonin synthesis may have been inhibited. In addition, although melatonin levels may have the potential to be affected by season, modern man's lifestyle in terms of heating and lighting is such that the appropriate seasonal cues might be overridden. These examples represent just a few of many ways in which relationships, which almost certainly exist biologically in vivo, may in fact be masked in the data from a study such as this.

Other ocular tissues were not considered in this investigation. Previously, however, melatonin has been quantified in human aqueous humor obtained from live donors (undergoing cataract surgery (Martin et al., 1992). This melatonin may have been synthesised locally in ciliary body/iris compartments, but as it was only present in
five out of ten samples it is possible that it did in fact diffuse to the aqueous compartment from another active synthetic site such as the NR. Aqueous humor is essentially a plasma filtrate so it is also possible that the melatonin measured may have derived from the plasma. In favour of their hypothesis that melatonin is synthesised within the ciliary body, these same authors were also able to demonstrate NAT and HIOMT activity in human ciliary body although the findings have never been confirmed. In some other species there does appear to be NAT activity in these tissues, for example chicken iris and ciliary body (Aimoto et al., 1985), and rabbit iris-ciliary body (Rohde and Chiou, 1987). Importantly, however, HIOMT activity has never been reported in these ocular tissues by other authors in any species. Furthermore, Cardinali and Rosner (1971a) have specifically demonstrated HIOMT activity to be lacking from rat iris.

The present study provides definite evidence for the existence of melatonin within human NR and C-RPE. No role for this chemical in the human eye has yet been identified. Given, however, the many actions which it is reported to undertake in other species and its postulated role in certain eye diseases, extensive future investigation of the human ocular melatonin system is warranted.
CHAPTER 4

METHODODOLOGY EMPLOYED FOR THE ANALYSIS OF OCULAR 2-[^I^2^5]IODOMELATONIN BINDING SITES
4.1 Introduction

This chapter describes the methodology employed for analysis of 2-[^{125}I]iodomelatonin binding sites in ocular tissues from human and quail species. Findings of these studies are reported in subsequent chapters. Binding was investigated both in membrane preparations and by autoradiography. Also discussed are various methodological problems which were encountered.

4.2 Materials

4.2.1 Chemicals

Na\(^{125}\)I was obtained from ICN Biochemicals Ltd., Thame, U.K. or Amersham International, Little Chalfont, U.K. Iodogen (1,3,4,6-tetrachloro-3\(\alpha\),6\(\alpha\)-diphenylglycoursil), GTP (guanosine 5'-triphosphate, sodium salt) and ATP (adenosine 5'-triphosphate, calcium salt) were obtained from Sigma, Poole, U.K. Indoles used in displacement studies were also from Sigma with the exceptions of 6-chloromelatonin which was obtained from Eli Lilly Laboratories (Indianapolis, Indiana, U.S.A.); 2-iodomelatonin from RBI (Natick, Massachusetts, U.S.A); and 2-bromomelatonin and 2-phenylmelatonin (Spadoni et al., 1993) which were gifts from Dr B. Stankov (University of Milan, Department of Pharmacology, University of Milan, Italy). Other chemicals and general laboratory reagents (all analytical grade), including solvents, Folin and Ciocalteu's phenol reagent, eosin yellowish, Ehrlich's haematoxylin, chrome alum (chromium (III) potassium sulphate dodecahydrate) and bovine serum albumin were obtained from Sigma. Analytical grade hydrochloric acid and glacial acetic acid were purchased from Fisons, Loughborough, U.K.

4.2.2 Biological materials

Quail eyes were provided by Dr B. Stankov. For binding site characterisation and autoradiography studies, adult male quails (Coturnix japonica) were maintained in natural photoperiod conditions prior to sacrifice. For rearing purposes the birds were exposed to artificial light (50-60 lux) during the period of natural darkness. Birds were sacrificed between 11:00 and 12:00 h. For the studies investigating the effect of photoperiod on receptor density and affinity (section 5.2.2), adult male quails were maintained under either long day (16 h light : 8 h darkness, lights on at 06:00 h) or short day (8 h light : 16 h darkness, lights on at 09:00 h) photoperiods and sacrificed at intervals across the 24 hour period. Following enucleation, quail eyes were frozen at -80 °C and subsequently transported to the University of Surrey on dry ice.

Human eyes were obtained as described previously (section 2.2.3). Ovine eyes were obtained from a local abattoir on the day of slaughter (no longer than 5 h post-slaughter). No information regarding the sheep (sex, age, etc.) was available.

The source of chickens was as previously described (section 2.2.3). Birds were sacrificed by cervical dislocation. For routine membrane binding experiments, tissues were removed immediately and used for the production of membrane preparations.
To investigate whether the time period between sacrifice and membrane preparation had an effect on the quality of 2-[\(^{125}\)I]\(^{i}\)iodomelatonin binding, the following protocol was employed. Six chickens were sacrificed, one eye from each bird was removed and membranes prepared as detailed below (section 4.3.2.1) and frozen. The carcasses, each with one remaining eye in situ, were left at room temperature for 48 h. Subsequently, enucleations were performed and the eyes transferred to refrigerated storage (4 °C) for a further 48 h. After this period eyes were frozen on dry ice, left for 2 hr and finally stored at -80 °C. Membranes were prepared six days later and frozen.

4.3 Methods

4.3.1 Preparation of 2-[\(^{125}\)I]\(^{i}\)iodomelatonin radiolabel

The radiolabel used throughout was prepared according to the method of Vakkuri and co-workers (1984 a,b), that is, direct iodination involving the oxidation of \(I^-\) to \(I_2\) (IT) followed by electrophilic substitution of \(I^-\) into the indole nucleus of the melatonin molecule at carbon-2, the initial oxidation being effected by “Iodogen”.

Aliquots (10 \(\mu\)l) of Iodogen solution (0.1 mg/ml in chloroform) were transferred to Eppendorf tubes and the chloroform evaporated thus leaving 1 \(\mu\)g Iodogen per tube. Tubes were stored at 4 °C until use. The iodination procedure was carried out in one of these tubes; 10 \(\mu\)l of a 1mg/ml melatonin in 0.05 M sodium phosphate buffer, pH 6.0 was combined with 2 \(\mu\)l Na\(^{125}\)I solution (\(\approx\) 0.2 mCi) and vortex mixed. The reaction was quenched after 2 minutes by the addition of 100 \(\mu\)l chloroform. The mixture was then vortex mixed and incubated for ~ 24 hr at 4 °C.

Iodinated melatonin was subsequently purified by TLC. The TLC plates were those previously described (section 2.2.2). Plates were prepared for use by dividing them into tracks and washing with ethyl acetate (section 2.3.7.1). Melatonin standards (5 \(\mu\)l and 10 \(\mu\)l 1 mg/ml melatonin in 0.05 M sodium phosphate buffer, pH 6.0, and 5 \(\mu\)l 1 mg/ml melatonin in chloroform) were spotted on the end and centre tracks. The organic phase of the iodination mixture was applied to the remaining tracks and the plate run three times in an ethyl acetate (100 %) mobile phase as described in section 2.3.7.1.

Following separation, a single sample track was cut into 1 cm segments and each counted (1260 MultiGamma counter; LKB, Wallac, 79 % counting efficiency) to determine the position of iodinated melatonin. The position of cold melatonin was also determined (under UV light) to ensure adequate separation of labelled and cold melatonin. Iodinated melatonin was always separated from cold melatonin by at least 2 cm. Subsequently, those fractions of the TLC plate containing iodinated melatonin were cut into fine strips, transferred to a glass storage container and a known volume of propan-2-ol (~ 4 ml depending on label activity) added to elute the radiolabel. Following vortex mixing the product was stored at 4 °C. After 24 h a 10 \(\mu\)l aliquot was counted in order to determine actual label concentration.
4.3.2 Membrane radioreceptor binding studies

4.3.2.1 Dissection of tissues

4.3.2.1.1 NR and C-RPE

NR and C-RPE tissue components were dissected as described previously (section 2.3.1.1, Figure 2.2). With the sheep and human eyes, these two tissues were easily dissected with no obvious contamination in either fresh or frozen eyes. In contrast, in the chicken and quail eyes there was occasional contamination of NR with melanin pigment. With the fresh chicken eyes, the extent of the contamination was found to be very much affected by the time period between sacrifice and dissection; the longer this period the more practical difficulty in separating the NR from the C-RPE and therefore the greater the risk of contamination. For this reason chickens were always sacrificed sequentially and the required tissues from an individual bird dissected before sacrifice of the next. With the frozen chicken and quail eyes contamination worsened as the length of the thawing period increased therefore specimens were thawed for the minimum period necessary.

4.3.2.1.2 Iris muscles and ciliary body

Ovine and human iris sphincter pupillae, dilator pupillae and ciliary body tissue were dissected as illustrated in Figure 4.1. Sheep iris muscles and ciliary tissue were used for membrane preparation on the day of dissection. Human iris muscles were removed from specimens dissected for use in other studies, and refrozen at -80 °C until there were sufficient to pool for use.

4.3.2.2 Preparation of membrane homogenates

Dissected tissues were transferred to chilled buffer. Buffer used throughout including for binding experiments was 50 mM Tris-HCl, with 4 mM CaCl₂, pH 7.4. Tissues were sonicated (Ultrasonic Homogenizer - 4710 Series, Cole-Parmer Instruments, Illinois, U.S.A.) for two separate 30 s periods (1 x 30 s only for NR) with constant cooling. Membranes were subsequently ultracentrifuged at 50,000 x g for 20 min at 4 °C. The rotor and chamber were pre-chilled. Supernatant was discarded and the surface of the membrane pellet washed once with buffer. Membranes were then resuspended in buffer and washed by sonication and ultracentrifugation as previously. Pellets were again washed and the membranes finally resuspended in buffer by sonication. Throughout the procedure both membrane preparations and buffer were maintained at 4 °C. Final membrane pellets were resuspended in buffer to achieve desired working protein concentrations.

For quail NR and C-RPE membranes, 1 ml buffer usually contained tissue from 1 to 1.5 quail eyes. Chicken NR and C-RPE membrane suspensions were prepared at a concentration of 0.75 to 1 eye/ml. These concentrations routinely yielded 20 to 70 µg protein per assay tube. Sheep NR and C-RPE were pooled at 0.25 eyes/ml and 0.5 eyes/ml, respectively to give ~ 80 to 100 µg protein per assay tube. Human eyes were generally processed individually and each NR or C-RPE was prepared in ~ 5 ml buffer, hence incubation protein contents were variable (20 to 150 µg ).
Figure 4.1 Procedure for dissection of iris and ciliary body tissues

Corneal tissue was removed (1). The anterior section of the eye was cut off (2) and scleral tissue carefully peeled away from the underlying tissue to give (3). This was laid flat and the desired tissues cut out in ring form. Tissue innermost to the pupil (~ 1 mm) was taken for iris sphincter pupillae.
Human and sheep iris muscle studies were undertaken on tissue pooled from several specimens (~ 2 eyes/ml). These concentrations yielded 15 to 30 μg protein per assay tube.

Sheep ciliary body tissue was prepared at a concentration of approximately 1 eye/1 ml (~ 50 μg per assay tube).

Eye membrane preparations were usually used immediately after production, however those from quail, chicken and sheep NR and C-RPE were occasionally stored at -80 °C prior to use. In these instances membranes were resuspended in lesser buffer volumes and diluted for use upon thawing. Prepared eye tissues were stored for no longer than three weeks before assay.

Chicken brain membranes were resuspended at a final concentration of 1 brain/50 ml. Aliquots (1 ml) were frozen (-80 °C) and diluted for use (1/10, v/v). This produced an incubation protein content of ~ 90 μg per tube.

4.3.2.3 Removal of melanin

In some instances, initial tissue homogenates were subjected to an additional pre-centrifugation step (2,000 × g for 10 min at 4 °C) to remove melanin pigment. The resulting supernatants were then ultracentrifuged for the production of membranes as described (section 4.3.2.2).

4.3.2.4 Binding studies

4.3.2.4.1 General

The binding method employed was based on that previously reported by Vanecek (1988). Binding reactions were carried out in disposable glass tubes. Incubations for all binding studies contained 100 μl radioligand solution, 100 μl membrane suspension and 50 μl buffer, drug or drug vehicle (for displacement studies and receptor-effector coupling studies), that is 250 μl total volume. Non-specific binding was determined throughout in the presence of 1 nM cold melatonin. Specific binding was defined by subtraction of non-specific binding from total radioligand binding. Binding reactions were always initiated by addition of tissue. All incubations were carried out in a water bath maintained at 28 ± 0.5 °C, over a 40 min period (with the exception of kinetic studies in which the incubation time varied). Separation of bound and free radioligand was achieved by rapid filtration of the incubate through Whatman GF/B filter paper using a 24-channel Brandel cell harvester followed by three × 3 ml ice cold buffer washes. Filter bound radioactivity was subsequently determined using a 1260 MultiGamma counter (LKB, Wallac, 79 % counting efficiency). Generally, binding was investigated for each condition within an experiment in triplicate and at the least in duplicate.

4.3.2.4.2 Kinetic studies

Total and non-specific binding were determined at timed intervals over a 40 min period (association data). Addition of 1 μM cold melatonin (end concentration) was employed to study dissociation over the following 250 min period. Filter bound
radioactivity was subsequently determined and analysed by pseudo-first order and first order rate equations using the EBDA/LIGAND computer program v 4.0 (Munson and Rodbard, 1980; McPherson, 1985) to determine observed association and dissociation rate constants, respectively. The actual association rate constant was derived from the observed association rate constant by the following relationship; $k_{+1} = k_{obs} - k_{-1}/[L]$ where $[L]$ = radioligand concentration and $k_{-1}$ = dissociation rate constant.

4.3.2.4.3 Saturation studies

For saturation studies, binding was routinely measured over a range of radioligand concentrations, nominally 9 to 600 pM. Data, as disintegrations per minute, were analysed using the EBDA/LIGAND non-linear curve-fitting computer program using one-site model fits for the determination of receptor equilibrium dissociation constants ($K_d$) and number of receptors ($B_{max}$). Throughout the course of experiments saturation binding studies were undertaken at regular intervals with chicken brain membrane preparations as a quality control in order to confirm that the binding methodology was functional: $K_d = 31.2 \pm 1.8$ pM, $B_{max} = 7.94 \pm 0.42$ fmol/mg protein (mean ± s.e.m., n=12). New batches of 2-[125I]iodomelatonin were also tested in this same system prior to use in eye tissue studies.

4.3.2.4.4 Displacement studies

Incubations were carried out at a fixed concentration of radioligand in the presence of 50 µl displacing drug (to produce a range of final concentrations) or drug vehicle. Initial stock drug solutions ($5 \times 10^{-3}$ M) were prepared with the following proportions of ethanol: melatonin, 5-ML, 5-MT (10 %); 2-phenylmelatonin (40 %); 6-chloromelatonin, 2-iodomelatonin, NAS (50 %); 6-hydroxymelatonin (100 %); 2-bromomelatonin (45 %). 5-HT was prepared in 30 % 0.01 N HCl. Subsequent dilutions were with buffer or water (5-HT). Precautions were taken as necessary to minimise drug breakdown due to instability and light sensitivity. Radioligand concentration was between 100 and 108 pM. Competition curves were analysed with the EBDA computer program for the determination of $K_i$ values (equilibrium dissociation constant of the unlabelled competing ligand). Best fit curves including a blank were plotted using the GRAPHPAD INPLOT computer program. In a few instances the lowest drug concentration was used as a blank.

4.3.2.4.5 Effect of nucleotides on 2-[125I]iodomelatonin binding

Incubations were carried out as described for displacement studies (section 4.3.2.4.4) but with varying concentrations of GTP or ATP instead of displacing drug. The drug vehicle was buffer. $IC_{50}$ values (concentration of unlabelled ligand that inhibits 50 % of the specific binding of the radioligand) were obtained using the EBDA computer program.
4.3.2.5 Protein determinations

Protein contents of the membrane suspensions were determined according to the method of Lowry (Lowry et al. 1951). Standard curve tubes each contained 0 to 150 or 200 µg protein in 400 µl water (prepared from a 1 mg/ml bovine serum albumin stock solution) and 100 µl binding buffer. Tissue aliquots (100 µl) were made up to 500 µl with water. To all tubes were added 2.5 ml alkaline copper reagent (2 % (v/v) copper tartrate solution (0.5 g CuSO₄·5H₂O + 1 g Na₂citrate in 100 ml H₂O) in aqueous Na₂CO₃ (20 % w/v)/NaOH (4 % w/v) solution). Tubes were vortex mixed and allowed to stand at room temperature for 10 minutes. Folin-Ciocalteu's phenol reagent (250 µl) was then added to each tube and following mixing, colour was allowed to develop by standing at ambient temperature for a further 2 hours before reading absorbances at 720 nm (Kontron Uvikon 860 spectrophotometer). Protein contents of tissue samples (assayed at least in duplicate) were calculated from standard curve data (duplicate determinations per concentration) using the "Lowry" option of the EBDA/LIGAND program.

In some instances protein assay sample tubes were centrifuged (2000 x g, 10 min, 20 °C) after the 2 hr incubation period to remove melanin, and the resulting supernatant measured. Standards were unaffected by this procedure: standard curves constructed from absorbances measured on the same standards before and after this centrifugation step were superimposable.

4.3.2.6 Method development

4.3.2.6.1 Effect of melanin pigment on 2-[¹²⁵I]iodomelatonin binding

Initial saturation binding studies with quail tissues, particularly C-RPE showed non-specific binding levels to be consistently high. Studies by other workers have previously shown non-specific radiolabelled melatonin binding in choroid and/or RPE membranes to be higher than that in neural retina (Wiechmann et al., 1986; Chong and Sugden, 1991). Furthermore, the autoradiography studies of Wiechmann and Wirsig-Wiechmann (1991) and Laitinen and Saavedra (1990a) have indicated that within the eye, 2-[¹²⁵I]iodomelatonin may associate non-specifically with melanosomes. To investigate whether the presence of melanin was contributing to the non-specific 2-[¹²⁵I]iodomelatonin binding observed in the quail tissues the following protocol was employed. Tissues were sonicated and the resulting NR and C-RPE homogenates each divided into two portions. One portion was used to prepare membranes directly (section 4.3.2.2). The other was subjected to an additional low speed pre-centrifugation procedure to remove melanin (section 4.3.2.3) prior to preparation of membranes in exactly the same way. Cell nuclei would also have been removed by this pre-centrifugation process. The resultant membranes from both procedures were used in saturation binding studies. Figure 4.2 shows that removal of melanin decreased total 2-[¹²⁵I]iodomelatonin binding in both C-RPE and NR. This decrease could be largely attributed to loss of non-specific binding as specific binding remained at a similar level. Even though not naturally pigmented, pre-centrifugation also reduced the non-specific binding of NR. As described previously, quail NR was susceptible to contamination with melanin from other pigmented tissues during the
Figure 4.2
Effect of melanin pigment on 2-[¹²⁵I]iodomelatonin binding in quail NR (A) and C-RPE (B) membranes. Total binding (■); non-specific binding (O) and specific binding (●).
dissection procedure. It is likely therefore that the decreases in non-specific binding observed in NR resulted from loss of contaminating melanin.

On the basis of these observations the majority of subsequent quail studies were conducted using pre-centrifuged membrane preparations. Further details regarding this melanin issue in experiments using both quail and other species are provided where appropriate in subsequent chapters.

4.3.2.6.2 Effect of melanin on protein determinations

The method employed for determination of membrane protein content utilised the Folin phenol method as adapted by Lowry (1951), that is, a colorimetric reaction with absorbance being measured in the 700-750 nm wavelength range (section 4.3.2.5). It was considered important therefore to determine whether the presence of melanin pigment within the tissue samples, if not removed by pre-centrifugation during the membrane preparation procedure, interfered with the accurate determination of tissue protein contents. If there was an effect this would have implications for subsequent $B_{\text{max}}$ calculations; an overestimated protein content would lead to low $B_{\text{max}}$ values.

Low speed centrifugation of protein assay sample tubes prior to measurement of absorbances (section 4.3.2.5) caused an apparent decrease in protein content in quail and chicken NR and C-RPE tissues. This decrease was assumed to be as a result of removal of melanin, either endogenous (C-RPE) or contaminating (NR), rather than removal of other cellular components, as there was no effect on quail NR or C-RPE which had been centrifuged during membrane preparation, or on human NR tissue which was not initially contaminated with melanin and which had not been pre-centrifuged (Figure 4.3). Quality control samples (aqueous albumin standard) were similarly unaffected by centrifugation. Subsequently therefore, unless stated otherwise, for all those tissues not pre-spun to remove melanin during membrane preparation, tissue sample protein assay tubes were subjected to the additional low speed centrifugation procedure prior to measurement of absorbances.

4.3.3 In vitro autoradiography

4.3.3.1 Preparation of slides

Slides (glass) were sonicated in Decon detergent (~10 %) for 75 min then allowed to soak in the same solution overnight. Subsequently the slides were rinsed in running water until it formed an even film over the slide surface (at least 3 hr), washed in double distilled water (30 min) and allowed to air dry. Slides were subbed with gelatin solution (1 % gelatin (w/v) in double distilled water, containing 0.1 % chrome alum) and dried at ambient temperature in a dust free environment. Human sections were mounted on large slides (76 × 39 mm, Chance Propper Ltd., Waverley, U.K.). Sections from other species were mounted on standard slides (75 × 25 mm).

4.3.3.2 Specimen preparation and sectioning

Various problems are encountered when cryostat sectioning the eye. The existence of several tissues types each with different consistencies poses particular
Figure 4.3
Effect of low speed centrifugation of membrane protein assay samples on protein content. Samples were assayed for protein according to the method of Lowry (1951). Abs (720nm) was measured before ( ) and after ( ) a low speed centrifugation step. (A) quail membranes; (B) chicken membranes; (C) quail membranes (pre-centrifuged during membrane preparation); (D) human membranes; (E) albumin standard quality controls. Data are means ± s.e.m. (n = 3 samples). Where error bars are not visible they are masked.
problems. For example, the frozen lens is susceptible to cracking and whereas the neural retina can very easily be cut, the sclera is tough. In addition, if cutting whole cross sections of large eyes, as the vitreous body is cut the friction of the blade causes it to melt. Loss of this support results in the section structure breaking down. The following methods were eventually found to be most suitable for use with the species investigated.

Frozen whole eyes were cut using a saw into hemispheres along the plane of the visual axis, that is through the pupil and lens to the posterior of the eye in approximately the region of the fovea (Figure 4.4). The majority of vitreous and lens material was then scraped out. A small amount of vitreous material in contact with NR was not removed to prevent damage to the NR structure. All manipulations were carried out on dry ice to prevent thawing. The half eye cups so produced were then fully embedded in Tissue-Tek OCT compound (Miles Inc., Elkhart, Indiana, U.S.A.). Sections were produced from the cut face, therefore each tissue slice continued NR, C-RPE and iris tissue. In two instances unfrozen human eyes were obtained. For these, the anterior section of the eye was cut away to produce eye cups as detailed in section 2.3.1.1 and the vitreous body removed. Specimens were frozen in dry ice chilled isopentane at -30 °C. The whole eye cup was then embedded in OCT for sectioning.

Frozen, whole sheep eyes were prepared in a similar way to frozen human eyes except that the eyes were cut in half to produce anterior and posterior sections. The latter were then cut in half and NR and C-RPE studied in sections cut from this face (Figure 4.4).

Quail eyes were sufficiently small to be embedded whole in OCT and cut without further preparation (Figure 4.4).

Human sections were cut in a Bright cryostat. All other sections were cut in a Microm cryostat (HM505E). All sections were cut in some combination of the transverse and sagittal planes. The exact orientation was unknown as only enucleated eyes were ever obtained. Sections (20 μm thickness) were cut at -20 °C, thaw-mounted onto prepared slides and air-dried at ambient temperature. Dried sections were stored at -20 °C in airtight containers with silica gel desiccant. Human sections, cut at the Institute of Ophthalmology, London, were transported back to the University of Surrey in containers of desiccant, packed on dry ice and were then transferred to -20 °C storage. Sections were stored for no longer than two weeks before use.

Sections were cut in pairs, the first onto slide 1 for determination of total binding and the second onto slide 2 for non-specific binding. Depending on the section size each slide contained two to four slices cut in this manner. Further sections were cut onto additional slides in a similar fashion. Between each pair of sections, two slices were discarded.
Figure 4.4 Procedure for dissection of eyes for autoradiography.
4.3.3.3 Binding studies

The protocol employed was based on that previously reported by Laitinen and Saavedra (1990a). Sections were equilibrated to room temperature before incubation in buffer (50 mM Tris-HCl, with 4 mM CaCl$_2$, pH 7.4) for 15 min. Slides were shaken to remove excess moisture and then laid flat. For determination of total binding an aliquot of 2-[¹²⁵I]Iodomelatonin solution in buffer was applied to each section. Non-specific binding was determined in the presence of the same radiolabel solution containing 1 μM melatonin. Radioligand aliquot volume was dependent on section size (100 to 750 μl) but within an experiment for an individual species all sections were treated with the same volume. In all instances binding was investigated by single point analysis using a subsaturating concentration of radiolabel selected for each species on the basis of previous membrane saturation experiments (nominal concentrations: quail 100 pM, human and sheep 120 pM). After a 1 h incubation, slides were washed three times in ice cold buffer (5 min each), rinsed in chilled double distilled water (30 s) then allowed to air dry. Unless otherwise stated all solutions were maintained, and incubations carried out, at ambient temperature (18 to 22 °C).

Dried sections were apposed to X-ray film (Hyperfilm-$^3$H, Amersham International, Buckinghamshire) in cassettes (Hypercassettes, Amersham International) for variable time periods at ambient temperature. All the sections from an individual eye were always placed in the same cassette and apposed to a single piece of film. Total and non-specific slide pairs were always placed next to each other. Films were processed manually at ambient temperature with the “Photosol” system (Fuji Medical): films were developed (CD 18) for 3 min, fixed (CF 40) for 5 min and washed (water) for 10 min. All manipulations with film were performed under dim red light (15 W, Kodak Safelight Filter GBX-2).

After production of autoradiographs, all tissue sections were stained to reveal the histological organisation. Prior to staining, sections were fixed for 2 min in Clark’s solution (75 % absolute ethanol, 25 % glacial acetic acid (v/v)) then allowed to air dry. The following protocol for haematoxylin and eosin staining was employed. Incubation times are shown in brackets. Slides were agitated gently once during each step to ensure thorough mixing of the reagents with sections.

1. Stain in haematoxylin (15 min).
2. Rinse in running water (1 min)
3. Differentiate in acidified alcohol (70 % ethanol (v/v) containing 1 % conc. HCl (v/v)) (5 s).
4. Rinse in running tap water (10 min)
5. Stain in eosin (1 % in tap water (w/v)) (2 min).
6. Remove excess stain in water.
7. Dehydrate in an ethanol series; 85 %, 2 × 100 % (30 s each).
8. Clear in xylene series (2 × 30 s).

Coverslips were applied to sections with DPX (BDH, Poole, U.K.) as the mountant.

4.3.3.4 Analysis

Autoradiographs and stained sections were digitised and analysed with the MCID M4 (Microcomputer imaging device) image analysis system (Imaging Research Inc.) after acquisition of images through a CCD video camera (Model XC-77CE,
Sony). Autoradiographs were illuminated on a Northern Light lightbox (Model B 95). To correct for non-uniform illumination in the field of view, a shading error correction was acquired and automatically applied to subsequent images. A new shading error correction was always established following any modification of factors such as magnification which would affect the illumination pattern.

Once optimal lighting, magnification and focusing conditions were established all autoradiographs (and histology sections) from an individual experiment were digitised under the exact same conditions to enable valid comparison between them. Individual tissue areas were frequently visually indistinct on digitised images of the autoradiographs, for example, NR and RPE. Also, irregularity of the sections precluded subtraction of non-specific binding image from total binding image to ascertain extent of specific binding. To overcome these problems the following protocol for analysis was adopted. Each histology section was digitised and then the corresponding autoradiograph was aligned with this image and digitised. Histology and autoradiograph images were thus captured in different channels. During subsequent analysis the channels were linked. It was then possible to select appropriate tissues for analysis on the histology image whilst sampling from the corresponding area on the autoradiograph.

For quail NR and C-RPE analysis, the specimen magnification employed was such as to obtain an image of approximately half of the posterior portion of each section, that is, from the centre to the region of the ora serrata. For human and sheep NR and C-RPE analysis, about one-third of the whole posterior portion, from the centre outwards, was imaged. All NR or C-RPE in each image was sampled. For analysis of human iris/ciliary body tissues images of the anterior portion of the eye from posterior to the ciliary body were captured. All iris and ciliary tissue within the image was sampled. In addition, in some specimens, sections were of sufficient quality to allow sampling of ciliary muscle.

In all instances, data were automatically acquired as relative optical density (ROD) values:

\[
\text{ROD} = \log_{10} \frac{\text{maximum possible number of grey levels (256)}}{\text{observed grey levels}}
\]

ROD values for each segment of a particular tissue within an image were then used to generate a weighted mean ROD value for the tissue within that image. The contributions of individual ROD values to the mean were weighted on the basis of target area from which they were taken. One mean ROD value per tissue was thus derived from each tissue section. For each film analysed under a particular set of conditions, a ROD value for background was established by averaging ROD values obtained from a number of section-free film areas distributed across the film. This background value was subtracted from all mean tissue ROD values.

For each eye, for each tissue mean ROD values for total and non-specific binding were usually each obtained from at least five sections. Comparisons between the total and non-specific binding mean ROD values were made by the Student’s unpaired t-test. Pairing was such that consecutive sections were compared. This method also had the advantage of accounting for any effect of variation of optical density across the film caused by storage cassettes, etc. as pairs of slides were always exposed in the same
region of film. A significant difference ($p < 0.05$) between total and non-specific binding was taken as evidence of specific $2-[^{125}\text{T}]$iodomelatonin binding. Calibration standards ($^{125}\text{T}$) were not used therefore no inter-eye comparisons were made.
CHAPTER 5

ANALYSIS OF QUAIL EYE 2-[$^{125}$I]IODOMELATONIN BINDING SITES
5.1 Introduction

The eyes of the Japanese quail (Coturnix japonica) are increasingly being recognised as major contributors to the organisation of circadian physiology in this species. Their influence is multifaceted. It is likely that some of these effects are mediated through ocular melatonin.

Locomotor activity in the intact quail displays rhythmicity which persists in constant darkness, and is therefore circadian rhythmicity (Underwood and Siopes, 1984). Bilateral enucleation of quails results in disruption of the activity rhythm of birds maintained in both 24 hour light:dark cycles and continuous darkness implying an important role of the eyes in the circadian organisation of this species (Underwood and Siopes, 1984; Konishi et al., 1985). The rhythm of body temperature in the quail is also dependent on the eyes: there is complete loss of its circadian control following enucleation (Underwood, 1994).

The eyes are, however, but one component of the quail’s circadian system. Circadian organisation in this species is the product of a complex interaction of multiple oscillators, namely the eyes, the pineal and the SCN (Underwood and Edmonds, 1995). The involvement of the eyes in the circadian control of the quail is believed to be via their participation in an eye-SCN axis: output from the eyes appears to be a requirement for maintenance of normal SCN rhythmicity (Underwood, 1994).

Within the quail eye itself, as in many species, there is a diurnal rhythm in melatonin concentration (Underwood et al., 1984). This is a true circadian rhythm (Underwood and Siopes, 1985). Furthermore, the clock controlling this rhythm has been demonstrated to reside within the eye itself (Underwood et al., 1988; 1990b). Some melatonin is released from the eyes and enters the circulation. According to Underwood and co-workers (1984), about 33% of the night-time blood levels of melatonin are in fact contributed by the eyes. Initially, this release of melatonin was believed not to be the means of communication between the eyes and the other components of the circadian system. Rather, some form of neural message was assumed, as optic nerve sectioning was found to produce the same effects as blinding by enucleation on the circadian activity rhythm (Underwood et al., 1990a). Later, the effect of optic nerve section on the quail’s body temperature rhythm was investigated and it was discovered that unlike enucleation this procedure does not abolish its circadian rhythmicity (Underwood, 1994). Current opinion therefore attributes at least some part of the eyes’ influence on whole body circadian organisation to occur via an hormonal output, with melatonin as the obvious candidate (Underwood and Edmonds, 1995).

The significance of any possible local actions of ocular melatonin in the quail, either in relation to extra- or intraocular circadian physiology, has hardly been considered. Similarly, to date, no mechanism by which such an effect of melatonin could be mediated within the eye has been investigated. As has been discussed previously (section 1.7), several important local functions of ocular melatonin in other species, many of which have circadian implications, have been reported. It has been proposed that it serves as an internal zeitgeber to control the timing of local rhythmic events (Besharse and Iuvone, 1983). In the quail eye itself a circadian rhythm in corneal mitotic rate has been described (Sasaki et al., 1995). It is feasible therefore that this or other local rhythms might be under the control of ocular melatonin.
Given (1) the importance of the role the eyes play in the circadian system of the quail; 2) the presence of a robust ocular melatonin rhythm under the control of an intraocular clock; and (3) the existence of melatonin binding sites within the eyes of several species, some of which have been linked to a functional response, it was of interest to ascertain the presence or absence of melatonin binding sites within the quail eye.

The quail is a photoperiodic species, that is, its reproductive potential fluctuates seasonally according to the length of the prevailing daily light period (Follett et al., 1992). In the quail, as in other species, a role for melatonin as the link between environmental conditions and this aspect of physiology has been strongly implicated. The circulating level of melatonin is synchronised with day length: a direct relationship exists between the duration of night length and its secretion (Underwood and Siopes, 1985; Kumar and Follett, 1993) and immunological manipulation of plasma melatonin levels has been shown to induce gonadal growth (Ohta et al., 1989). In addition to its effects on reproduction, photoperiod has also been shown to influence the density of high affinity melatonin binding sites within certain areas of the quail brain (Panzica et al., 1994). These authors have suggested that this additional effect of photoperiod may also be a result of changes in the circulating melatonin profile. In the investigations described, ocular melatonin binding site characteristics (affinity and density) were investigated in order to determine whether an analogous system is in operation in the quail eye. That is, are changes in ocular melatonin binding sites concomitant with alteration of the photoperiod, an action known to influence ocular melatonin profiles (Underwood et al., 1984; Underwood and Siopes, 1985).

5.2 Methods

The occurrence and characteristics of high affinity 2-[\(^{125}\)I]iodomelatonin binding sites were investigated in NR and C-RPE tissue membranes by radioreceptor assay (sections 4.3.2). NR and C-RPE membrane binding site affinities and densities were also investigated at intervals across a 24 h period in birds which had previously been maintained under either long or short day photoperiods. Details of the quails used are described in section 4.2.2.

The presence of a binding site in both NR and C-RPE tissues was confirmed by autoradiography as described in section 4.3.3.

5.3 Results

5.3.1 Characterisation of 2-[\(^{125}\)I]iodomelatonin binding sites

Tissues used in all the studies except NR displacement studies were subjected to the additional centrifugation step during membrane preparation to remove melanin (section 4.3.2.3). This difference for the NR displacement studies was unlikely to affect the results as there was little visible contamination of the NR and subsequent repeats of this study using NR from which melanin was removed yielded identical
results for those compounds tested (limited tissues supplies prevented testing of the complete series of compounds).

5.3.1.1 Kinetic studies

Figure 5.1 shows rapid association of 2-[\textsuperscript{125}I]iodomelatonin with the binding site in both NR and C-RPE preparations at 28 °C over the first 40 minutes of the incubation period, equilibration being reached by the end of this time period. Binding was stable over the subsequent 250 min period in NR. In C-RPE, binding was stable for 150 min but at 250 min there was a significant decrease in binding. Binding of 2-[\textsuperscript{125}I]iodomelatonin was reversible upon the addition of 1 \textmu M cold melatonin, specific binding being reduced by 85 % and 95 % after 250 minutes in NR and C-RPE, respectively. Rate constants are shown in Table 5.1. Kinetic dissociation constants (K_d) derived from these parameters (k_1/k_-1) were 11.9 and 15.0 \textmu M for NR and C-RPE, respectively.

5.3.1.2 Saturation studies

Saturation binding data reported here were always obtained from freshly prepared tissue. In both NR and C-RPE membrane preparations, specific 2-[\textsuperscript{125}I]iodomelatonin binding increased with radioligand concentration over the range 7 - 150 \textmu M. Above approximately 150 \textmu M specific binding failed to increase significantly indicating attainment of saturation. Scatchard transformation of specific binding data from both preparations yielded linear plots. Hill coefficients (mean ± s.e.m., n=4) were 0.96 ± 0.06 and 0.94 ± 0.03 for NR and C-RPE, respectively. Calculated equilibrium dissociation constants (K_d) (mean ± s.e.m., n=4) were NR 50.8 ± 19.5 \textmu M and C-RPE 98.2 ± 35.4 \textmu M. Corresponding B_{max} values (maximum numbers of binding sites, mean ± s.e.m.) were 12.4 ± 2.7 fmol/mg protein and 21.5 ± 3.2 fmol/mg protein for NR and C-RPE, respectively. Representative examples of saturation studies are shown in Figure 5.2.

In one instance choroid and RPE tissues were separated. Saturation studies on these individual tissues provided evidence for the presence of specific, saturable 2-[\textsuperscript{125}I]iodomelatonin binding sites in each (Figure 5.3). As these were preliminary studies, melanin was neither removed during membrane preparation nor for protein determination, hence the B_{max} values quoted may not be wholly representative.

Specific binding as a proportion of total 2-[\textsuperscript{125}I]iodomelatonin binding for NR and C-RPE tissues was typically between 70 to 95 % in preparations from which contaminating melanin had been removed. If melanin was present, however, as previously discussed (section 4.3.2.6.1) the proportion of specific binding was much lower; over the radioligand concentration employed specific binding was only between 30 to 90 % (NR) or 5 to 30 % (C-RPE) of total. Full saturation studies performed on tissue containing melanin (Figure 5.4) gave similar K_d values (51.6 ± 2.5 \textmu M, and 102.4 ± 11.4 \textmu M for NR and C-RPE, respectively (values ± associated error using LIGAND) implying that the change in methodology to remove the melanin, had little effect on specific binding thus confirming the results of preliminary experiments (section 4.3.2.6.1, Figure 4.2).
### Table 5.1
Association and dissociation rate constants of 2-[\(^{125}\)I]iodomelatonin binding in the quail eye.

<table>
<thead>
<tr>
<th></th>
<th>Association ((M^{-1} \text{min}^{-1}))</th>
<th>Dissociation ((\text{min}^{-1}))</th>
<th>Kinetic dissociation constant (K_d) (pM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NR</td>
<td>(7.18 \times 10^6)</td>
<td>(8.55 \times 10^{-3})</td>
<td>11.9</td>
</tr>
<tr>
<td>C-RPE</td>
<td>(7.99 \times 10^5)</td>
<td>(1.20 \times 10^{-2})</td>
<td>15.0</td>
</tr>
</tbody>
</table>

![Image of Table 5.1](image.png)

**Figure 5.1**
Kinetic analysis of specific 2-[\(^{125}\)I]iodomelatonin binding to (A) NR and (B) C-RPE membranes at 28 °C. Binding was reversible upon the addition of 1 \(\mu M\) cold melatonin. Radioligand concentration was 104 pM in (A) and 112 pM (B).
Throughout, unless stated otherwise, $K_d$ and $B_{\text{max}}$ values are quoted ± approximate standard error values attributed by the LIGAND program. Values are estimates from single experiments only. This procedure has been employed in Figures 5.2-5.4, 5.6, 6.1-6.3 and 6.5 and Tables 6.2 and 6.3.
Figure 5.2
Saturation of specific 2-[\textsuperscript{125}I]iodomelatonin binding (O) in NR (A) and C-RPE (C). Non-specific binding (●) was determined in the presence of 1 \, \mu M melatonin; total binding (■). Data are means of at least duplicate determinations from a representative experiment. (B) and (D) are the Scatchard transformations of data from (A) and (C), respectively. In these examples for NR: $K_d = 101 \pm 10.4$ pM, $B_{max} = 20.2 \pm 1.3$ fmol/mg protein, and for C-RPE: $K_d = 21.8 \pm 2.4$ pM, $B_{max} = 15.6 \pm 0.7$ fmol/mg protein (data are values ± associated error using LIGAND).
Figure 5.3
Saturation of specific 2-[125I]iodomelatonin binding (O) in choroid (A) and RPE (C). Non-specific binding (●) was determined in the presence of 1 μM melatonin; total binding (■). (B) and (D) are the Scatchard transformations of data from (A) and (C), respectively. For choroid: \( K_d = 156.9 \pm 29.5 \) pM, \( B_{\text{max}} = 32.5 \pm 4.2 \) fmol/mg protein, and for RPE: \( K_d = 64.7 \pm 21.7 \) pM, \( B_{\text{max}} = 17.8 \pm 3.3 \) fmol/mg protein protein (data are values ± associated error using LIGAND).
Figure 5.4
Saturation of specific 2-[¹²⁵I]iodomelatonin binding (O) in NR (A) and C-RPE (B) membranes from which melanin pigment had not been removed. Non-specific binding (●) was determined in the presence of 1 μM melatonin; total binding (■). In this example, $K_d$ values for NR and C-RPE were 51.6 ± 2.5 pM and 102.4 ± 11.4 pM, respectively (data are values ± associated error using LIGAND).
5.3.1.3 Displacement studies

Several unlabelled indoles, including the melatonin analogues 2-bromomelatonin and 2-iodomelatonin, were assessed for their ability to displace specific 2-[^125]I]iodomelatonin binding. Monophasic displacement curves for all inhibitors were obtained in both tissues (Figure 5.5A, B). However for 6-chloromelatonin in C-RPE a two-site model produced a significantly better fit (K_i values for the two sites were 0.028 and 32 nM). Apart from this discrepancy, pharmacological profiles of the binding site in both NR and C-RPE preparations were very similar. Linear regression of logarithmically transformed K_i data (except 6-chloromelatonin) yielded a highly significant correlation between the binding sites of the two tissues (r=0.98; slope = 0.91; p<0.00001, n=9) (Figure 5.5C). In general, substituted N-acetyl-5-methoxytryptamine compounds were most effective in displacing 2[^125]I]iodomelatonin. The 5-hydroxyindole, 5-HT and the 5-methoxyindoles, 5-ML and 5-MT, had little effect. 2-Phenylmelatonin was a very effective inhibitor of 2[^125]I]iodomelatonin binding in both tissues.

5.3.1.4 Effect of nucleotides on 2[^125]I]iodomelatonin binding

In both NR and C-RPE membranes, GTP inhibited 2[^125]I]iodomelatonin binding in a dose dependent manner. The inhibitory effect of ATP was much less than that of GTP. Representative GTP and ATP IC_50 values were 140 and 2050 μM, respectively, for NR, and 16 and 1220 μM, respectively, for C-RPE.

5.3.2 Effect of photoperiod on NR and C-RPE 2[^125]I]iodomelatonin binding

Figure 5.6 shows the changes in binding site affinity and density across a 24 h period when quails were maintained in either long or short day photoperiods (see section 4.2.2 for photoperiod details). Eyes were dissected and membranes prepared fresh on the day of each saturation study. All tissue homogenates were pre-centrifuged to remove melanin. The same batch of radioligand was used throughout. Tissues from randomly selected time points were assayed on each occasion. Hill coefficients for all saturation experiments approached unity implying the presence of a single class of high affinity binding sites at each time point of the 24 h cycle. There was insufficient material to perform more than one Scatchard per time point therefore for statistical analysis, for each photoperiod, light phase data were combined and compared with combined dark phase data (Figure 5.7). Two factor analysis of variance was performed on the parameters of each tissue with photoperiod (short or long) and light phase (day or night) as the independent variables. There were no significant differences (p < 0.05) associated with the B_max or K_d values for NR, or the C-RPE B_max values. For C-RPE K_d values, however, there was a significant interaction between light phase and photoperiod. Subsequent one way analysis of variance indicated that in the day time, K_d was significantly higher in the long day photoperiod compared with the short day photoperiod (p <0.001), and in the long day photoperiod K_d was higher during the day than in the night (p <0.001).
Figure 5.5
Competition curves for inhibition of 2-[\(^{125}\)I]iodomelatonin binding by various indoles in NR (A) and C-RPE (B). Radioligand concentration was 100 to 108 pM in (A) and 101 to 106 pM in (B). (C) Correlation of compound \(K_i\) values between NR and C-RPE. Linear regression of logarithmically transformed data gave a slope of 0.91 and a correlation coefficient \((r)\) of 0.98 \((p<0.00001, n=9).\) 2-Bromomelatonin (□ c), 2-iodomelatonin (■ b), 2-phenylmelatonin (▲ a), melatonin (△ d), 6-chloromelatonin (○ e), 6-hydroxymelatonin (O f), N-acetylserotonin (● f), 5-methoxytryptophol (● g), 5-methoxytryptamine (× i), 5-hydroxytryptamine (★ h). All curves represent one-site models except 6-chloromelatonin in C-RPE (two-site).
Figure 5.6
Effect of photoperiod duration on quail NR and C-RPE melatonin binding site affinity and density over a 24 hour period. Quails were maintained under short (8L:16D) (■ full line) or long (16L:8D) (○ dashed line) photoperiods prior to sacrifice. Periods of darkness are indicated by shaded areas: short day dark period ■■■■, long day dark period ■■■■. Individual data points were derived from a single saturation binding experiment using membranes prepared from 4 eyes. In each graph the end point is double plotted. Saturation data for the 1 h C-RPE point could not be Scatchard transformed. Data are values ± associated standard error using LIGAND.
Figure 5.7
Effect of photoperiod (SP 8L:16D; LP 16L:8D) on mean day ( ) and mean night ( ■ ) 2-[125]iodomelatonin binding site affinity and density in quail NR and C-RPE membranes. Data are means ± s.e.m. (n values for NR and C-RPE: SP day (3), night (5); LP day (5), night (3); except C-RPE LP night (2)). * p < 0.001 compared with LP day
5.3.3 Localisation of specific 2-[\(^{125}\)I]iodomelatonin by autoradiography

Duplicate total and non-specific binding sections were exposed for a range of times (from 1 day to 6 weeks). Binding of 2-[\(^{125}\)I]iodomelatonin (nominal concentration 100 pM) in NR and C-RPE areas was apparent after 7 days of exposure. Preliminary visual inspection of the autoradiographs indicated that from this time onwards there was a difference in density between total and non-specific sections across the entire region of the NR, that is, throughout the circumference of the eye. Of the exposure times investigated, the difference appeared to be greatest at 3 weeks. Subsequently therefore, sections were routinely exposed for 3 weeks. Semi-quantitative densitometric analysis (section 4.3.3.4) of single eyes from 6 individual birds indicated that displaceable 2-[\(^{125}\)I]iodomelatonin binding was present in both the NR and C-RPE regions (Figure 5.8). For both NR and C-RPE there was a significant difference between total and non-specific binding (unpaired Student’s t-test). In agreement with previous membrane binding study findings (section 5.3.1.2), the proportion of specific binding was greater in NR than C-RPE. Representative autoradiographs are shown in Figure 5.9.
Figure 5.8
Analysis of 2-[125I]iodomelatonin binding in six individual bull eyes by autoradiography. Data (relative optical density (ROD) measurements) represent the mean ± s.e.m. of at least 6 tissue sections. Non-specific binding was defined with 1 µM melatonin. Radioligand concentration used was 102 to 135 pM.

5.13
Figure 5.9
 Autoradiographic localisation of 2-
iodomelatonin [3H] binding in the guinea pig eye.
(A) total binding, (B) non-specific binding, (C) non-
specific binding, (D) are the tissue sections
respectively after staining with haematoxylin and
eosin. Radioligand concentration was 108
pm. Non-specific binding was defined with 1 µM
melatonin.
5.4 Discussion

The present data provide definite evidence for the presence of melatonin binding sites within the quail eye. 2-[\textsuperscript{125}I]Iodomelatonin bound specifically to both NR and C-RPE membranes. In both tissue preparations there was a saturable population of binding sites. Transformation of specific binding data to yield linear Scatchard plots and Hill coefficients approaching unity suggest a single class of binding sites which interact with their ligand non-cooperatively. Specific binding of 2-[\textsuperscript{125}I]iodomelatonin was rapid initially and subsequently stable with time. Further, the binding was reversible. $K_d$ values indicate the binding to be of high affinity and $B_{max}$ values provide evidence for a binding site of low capacity. Equilibrium and kinetic dissociation constants were comparable. Thus several criteria required for definition of a binding site as a receptor have been satisfied.

Within the quail it seems that there are in fact populations of 2-[\textsuperscript{125}I]iodomelatonin binding sites in both NR and C-RPE tissue compartments although pharmacologically, they are very similar. This is consistent with results from studies performed on another avian species. Using a similar radioreceptor assay, Chong and Sugden (1991) demonstrated the presence of melatonin binding sites in both NR and RPE of the chicken eye. Prior to this melatonin receptors had been reported in chicken NR by Dubocovich and Takahashi (1987). Autoradiographic studies in the same species however showed melatonin receptors to be located exclusively within the inner plexiform layer of the NR with no saturable specific binding in the RPE (Laitinen and Saavedra, 1990a). In other species also the melatonin binding site appears to be localised predominantly to the NR (for example: mouse and rabbit, Blazynski and Dubocovich, 1991; and frog, Skene et al., 1993). Wiechmann and co-workers (1986) have demonstrated that the melanosomes within frog C-RPE bind melatonin specifically. However, in the present studies the C-RPE membrane preparation contained no melanosomes so this cannot explain the presence of binding sites in the C-RPE tissue investigated. In addition to the chicken (Chong and Sugden, 1991), specific, albeit low affinity, melatonin binding has only been identified in C-RPE tissues (cytosol fraction) from one other species; the trout (Gern et al., 1981).

With the dissection procedure employed, it was recognised that the potential for cross contamination between NR and C-RPE tissues existed and that the binding observed in both tissues may in fact be contributed by a single tissue only. Autoradiographical studies were therefore undertaken to ascertain the precise localisation of 2-[\textsuperscript{125}I]iodomelatonin binding. These studies confirmed the presence of specific binding in both NR and C-RPE tissues. In addition, preliminary radioreceptor binding assays indicated the possibility of a saturable, specific binding site in both choroid and RPE. Unfortunately, however, the autoradiography methodology employed did not provide sufficient resolution to enable discrimination of binding between RPE and choroid components. Melatonin binding specifically within RPE has been investigated by few groups. As previously mentioned, it is present in the chicken (Chong and Sugden, 1991) but is reported to be absent from Xenopus and Rana frog species (Skene et al., 1993). Recently, two distinct melatonin receptor cDNAs (CKA and CKB) have been cloned from chicken brain (Reppert et al., 1995b). CKA is the structural homologue of the mammalian Mel1a receptor and CKB
represents the third receptor subtype Mel₁e (Reppert et al., 1995b). Both CKA and CKB are expressed in the chick retina, however, although the proteins are structurally distinct, they cannot be distinguished with existing ligands (Reppert et al., 1995b).

The affinity of the melatonin binding site observed in the quail NR is higher than that of the chicken NR as determined by Dubocovich and Takahashi (1987) ($K_d$ 434 pM), (the binding site is also of lower capacity) and is in better agreement with the $K_d$ estimate of Laitinen and Saavedra (1990a) ($K_d$ 19.8 pM). Affinity data from tree shrew retina (Lu et al., 1991) were comparable to that of the quail although binding capacity of the tree shrew was much lower (1.97 fmol/mg protein). Differences between species, binding assay methodologies (temperature of incubation and method of tissue preparation, for example) and animal housing conditions, could explain these differences in $B_{max}$ values.

In both NR and C-RPE tissues binding was highly specific, presence of a methoxy group at position C5 and an N-acetyl group on the indole nucleus of the inhibiting compound conferring highest potency for competition for 2-$[^{125}\text{I}]$iodomelatonin binding. Both the chicken pharmacological profile (NR and RPE) derived by Chong and Sugden (1991) and that of the frog NR, derived by Skene and colleagues (1993), are identical with that obtained in the quail NR with the exception of 6-chloromelatonin. Pharmacological profiles obtained in the quail were also broadly similar to the NR melatonin binding sites previously characterised in the chicken (Dubocovich and Takahashi, 1987), rabbit (Dubocovich, 1985) and tree shrew (Lu et al., 1991) although there were slight differences notably within the melatonin, 2-iodomelatonin, 6-chloromelatonin group of compounds.

With regards to the signal transduction mechanism of the melatonin binding site in the quail eye, involvement of a G protein seems likely on the basis of present data. A large proportion of the melatonin binding sites investigated to date for G protein coupling have yielded positive results, for example ovine pars tuberalis (Morgan et al., 1989), rat SCN (Laitinen and Saavedra, 1990b) and European hamster pars tuberalis (Skene et al., 1992b). In addition, data concerning G protein linkage of melatonin retinal receptors in another avian species, the chicken, is consistent with this hypothesis. Laitinen and Saavedra (1990a) demonstrated the regulation of 2-$[^{125}\text{I}]$iodomelatonin binding by guanine nucleotides in the NR of this species. Chong and Sugden (1991) report similar findings in RPE although coupling of the binding site to a G protein was not observed in NR. In contrast however, some workers have failed to show an effect of GTP on 2-$[^{125}\text{I}]$iodomelatonin binding in chick NR (Dubocovich et al., 1989). G-protein coupled melatonin binding sites have also been demonstrated in frog NR (Skene et al., 1993).

Melatonin binding sites have previously been investigated in the quail brain (Yuan and Pang, 1990; Cozzi et al., 1993). The binding site described by Cozzi et al. (1993) displays marked similarities with those investigated in the present study. These include comparable affinities, sensitivity to GTP and almost identical pharmacological profiles. Such similarities between retina and brain melatonin receptors are also apparent in the chicken (Dubocovich et al., 1989). The recent demonstration of the expression of the CKA and CKB melatonin receptor subtypes in both chicken brain and retina may explain the reported similarities between the brain and retina binding sites in avian species.
In chick brain (Brooks and Cassone, 1992) and rat SCN and pars tuberalis (Gauer \textit{et al}., 1993) there exists a diurnal variation in melatonin binding site density. Other tissues also display diurnal variations in 2-[\textsuperscript{125}I]iodomelatonin melatonin binding, for example, rat thymus (Martin-Cacao \textit{et al}., 1995). Frequently the highest density is observed during the light phase, however Laitinen and co-workers (1988) have reported the reverse in rat SCN. Importantly, with respect to the studies reported here, within the quail brain, the number of binding sites is reported to be over 40% higher at mid-light in birds maintained under a 12L:12D photoperiod (Yuan and Pang, 1990). Furthermore, in the quail, melatonin receptor density during the light phase is dependent upon the prevailing photoperiod (Panzica \textit{et al}., 1994). The variation in melatonin binding site density is proposed to be melatonin-related, that is homologous regulation, resulting either from fluctuations in the level of circulating melatonin (Yuan and Pang, 1990; Gauer \textit{et al}., 1993; Martin-Cacao \textit{et al}., 1995), or in the case of the photoperiod effects, changes in the melatonin profile (Panzica \textit{et al}., 1994). In contrast, in this study, melatonin binding site density in the quail eye in both NR and C-RPE tissues remained constant between light and dark phases irrespective of photoperiod despite the fact that the ocular melatonin level is reportedly influenced in a similar fashion to that of the plasma level (Underwood \textit{et al}., 1984; Underwood and Slopes, 1985). There are other tissues within the quail however that do not display variations in melatonin binding, for example, the testes (Wang \textit{et al}., 1992 1906).

The reported day/night fluctuations in melatonin receptor number occur without changes in affinity (for example, Yuan and Pang, 1992; Gauer \textit{et al}., 1993) and in the quail NR, in addition to the lack of effect on $B_{\text{max}}$, there was also no effect of the light/dark phase or photoperiod on $K_{\text{d}}$ values. In contrast, in C-RPE there were statistically significant differences in affinity. Given the magnitude of these differences however, that is, less than twofold, their physiological significance is questionable. The differences cannot be a result of G-protein coupling as a shift from the G-protein coupled to uncoupled form of the binding site results in a low affinity state with $K_{\text{d}} \sim 400$ pM (Sugden, 1994).

No attempt was made in these studies to elucidate the functional role of the melatonin binding site described. Within the quail eye these binding sites might potentially contribute to any of the following:

1) Mediation of local physiological functions.

Within chicken and rabbit NR, for example, there is clear evidence for melatonin binding sites mediating inhibition of dopamine release (Dubocovich, 1983,1985; Dubocovich and Takahashi, 1987). Many other actions of ocular melatonin on NR or C-RPE tissue components have also been reported, but few have yet been associated with receptor binding (section 1.7). These may also be of significance in the quail.

2) Manipulation of an intraocular clock, for example in the role of entraining agent.

The ocular melatonin-generating system itself is controlled by an intraocular clock (Underwood \textit{et al}., 1990b). This clock may serve to regulate other local circadian events. Melatonin binding sites represent a possible route by which melatonin could exert an influence on the ocular clock.

3) Modulatory input to the whole body clock mechanism.

This might be either indirectly through effects on the intraocular clock and the contribution it subsequently makes to whole body circadian organisation, or directly
via modification of information being sent from the eye to the centres of circadian control. The eyes are of great importance with respect to maintenance of circadian integrity within the quail. Endocrine release of retinal melatonin contributes part of the signal through which the eyes influence the rest of the circadian system (Underwood and Edmonds, 1995). In addition there is evidence for involvement of a neural pathway (Underwood et al., 1990a). One possibility therefore is that melatonin, at the receptor level, may exert some modulatory influence over the message being relayed via this neural pathway.
CHAPTER 6

INVESTIGATION OF 2-[\textsuperscript{125}I]IODOMELATONIN BINDING SITES IN POST-MORTEM HUMAN EYES
6.1 Introduction

There is substantial evidence for the existence of high affinity melatonin binding sites within the vertebrate eye including that of several mammals (Table 1.4). The NR component of the eye has been most often studied and in accordance with the findings in other animal classes, there are several reports documenting the existence of high affinity binding sites in this tissue in mammals. Within the mammalian retina the highest density of binding sites appears to be located within the inner areas of the retina, particularly the inner plexiform layer where the integrating neurons and the retinal ganglion cells synapse (Laitinen and Saavedra, 1990a; Blazynski and Dubocovich, 1991).

Studies have concentrated mainly upon various laboratory species. Until relatively recently there were no reports concerning the investigation of melatonin binding sites within the human eye, although specific melatonin binding had been demonstrated in a lower primate, the tree shrew (Lu et al., 1991). The restricted availability of appropriate human tissue sources, that is, non-pathological specimens from live donors, has undoubtedly been one of the major factors responsible for this paucity of data. In 1994, Capsoni and co-workers (1994) reported, in abstract form, for the first time, results of a study investigating melatonin binding sites in human tissue. Little information regarding the methodology employed and the specimens investigated, for example, donor details, was provided, thus making evaluation of their findings difficult. High affinity, reversible binding was observed. Scatchard analysis and indole displacement studies revealed the existence of two, picomolar affinity sites. The autoradiographic methodology employed (high resolution liquid emulsion type) allowed localisation of the binding site to the C-RPE complex. To date however, these findings have not been reported in an extended form and their validity may therefore, be questioned.

Of much greater significance is a subsequent paper detailing the cloning of a human gene encoding a melatonin receptor (Mel₁₈) which is expressed predominantly in retina (Reppert et al., 1995a). The Mel₁₈ receptor (Reppert et al., 1994) has also been found to be expressed in human retina, albeit at much lower levels (Reppert et al., 1995a). Both of these recombinant receptors are functionally coupled to the inhibition of adenylyl cyclase: melatonin prevents forskolin induced cAMP accumulation.

Of all species the human melatonin binding site is, at least in terms of its potential for future therapeutic exploitation, perhaps the most worthy of study. Ocular melatonin is increasingly viewed as being of local physiological relevance. The reported actions of melatonin within the mammalian eye are now many. In rabbit retina, melatonin inhibits the calcium-dependent ^3H-dopamine release, elicited by depolarisation with either electrical field stimulation or high potassium, from prelabelled tissue (Dubocovich, 1983). This action of melatonin has been unequivocally established to be receptor-mediated (Dubocovich, 1985) and it is likely that at least some of its other actions will eventually be attributed to receptor activation. Interest is growing in melatonin as a contributory factor in retinal disease. In the Royal College of Surgeons rat, for example, the endogenous defect leading to photoreceptor degeneration is believed to be related to abnormalities associated with the retinal dopamine-melatonin system (Hankins and Ikeda, 1994). Other authors have linked melatonin, in conjunction with the damaging effects of bright light, with
ocular disease states such as macular degeneration (Wiechmann and O'Steen, 1992) and retinitis pigmentosa (Bubenik and Purtill, 1980). Thus identification and thorough characterisation of a human ocular melatonin receptor would facilitate design of specific agonists and antagonists and enable pharmacological intervention for the possible treatment of melatonin-related ocular disease. Melatonin receptor defects may also be of aetiological significance and represent additional therapeutic targets.

In these studies, high affinity melatonin binding sites were investigated in post-mortem human eyes. Given the known sites of action of melatonin within the eye and the reported distribution of ocular melatonin binding sites within other species, primary consideration was given to NR and C-RPE tissues. The methodology previously developed (section 4.3.2) and used successfully for the identification and characterisation of melatonin binding sites in the quail eye (Chapter 5) was employed, that is, membrane radioreceptor assay and autoradiography with 2-[^125]Ijodomelatonin as the ligand. Specimens were investigated individually in order that any relationships existing between the resulting binding data and the donor parameters could be identified.

6.2 Methods

Membrane radioreceptor assays and autoradiography were carried out as described in sections 4.3.2 and 4.3.3, respectively. The human tissue source was the same as reported in section 2.2.3. Sheep and chicken tissue sources were as described in section 4.2.2.

6.3 Results

6.3.1. Human membrane radioreceptor assays

6.3.1.1 NR and C-RPE

6.3.1.1.1 Saturation studies

Saturation studies were undertaken on NR and C-RPE membranes from 18 human eyes investigated individually. The donor parameters of the specimens are provided in Table 6.1. In one eye (eye 4) saturable, specific 2-[^125]Ijodomelatonin binding was present in both NR and C-RPE components (Figure 6.1). In two additional eyes binding was present in either NR or C-RPE, only (Figure 6.2). In all cases a single site was suggested on the basis that Scatchard transformation of the data yielded linear plots, with Hill coefficients approaching unity. The maximum level of specific binding observed in these instances was low (14-20 % total binding) but consistent. Binding parameters are shown in Table 6.2. These three specimens shared a common donor parameter with respect to time of death; all deaths had occurred in the daytime. However, five other specimens from day time donor deaths lacked a melatonin binding site.
<table>
<thead>
<tr>
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<th>Age</th>
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<th>DOD</th>
<th>COD</th>
<th>Specimen age (h)</th>
<th>PMI binding (h)</th>
<th>Saturable binding</th>
<th>Pre-centrifugation</th>
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Table 6.1
Donor parameters of human eyes investigated for the presence of 2-[125I]iodomelatonin binding in NR and C-RPE membranes. Presence of saturable, specific binding is indicated by (+). In some instances tissues were precentrifuged during membrane preparation to remove melanin ($\S$).

Abbreviations and notes
M male; F female; D day; N night; T-, D-, COD time-, date-, cause of death; MI, myocardial infarction; RTA road traffic accident; LF liver failure; RF renal failure specimen age = time between donor death and frozen storage of specimen; PMI, post mortem interval = time between donor death and enucleation; ? parameter unknown; * due to paracetamol overdose; CVS cardiovascular system; Sp, spring; Su, summer; A, autumn; W, winter.
Figure 6.1
Saturation of specific 2-[\textsuperscript{125}I]iodomelanotonin binding (○) in human eye 4 NR (A) and C-RPE (C). Non-specific binding (●) was determined in the presence of 1 μM melatonin; total binding (■). (B) and (D) are the Scatchard transformations of data from (A) and (C), respectively. In NR, \( K_d = 236.2 \pm 58.1 \text{ pM} \) and \( B_{\text{max}} = 2.1 \pm 0.4 \text{ fmol/mg protein} \); and in C-RPE \( K_d = 208.4 \pm 53.6 \text{ pM} \) and \( B_{\text{max}} = 5.0 \pm 1.0 \text{ fmol/mg protein} \). Data represent values ± associated standard error using LIGAND.
Figure 6.2
Saturation of specific 2-[125I]iodomelatonin binding (O) in human eye 122 NR (A) and 104 C-RPE (C). Non-specific binding (●) was determined in the presence of 1 μM melatonin; total binding (■). (B) and (D) are the Scatchard transformations of data from (A) and (C), respectively. In 122 NR, $K_d = 202.4 \pm 36.0$ pM and $B_{max} = 1.2 \pm 0.1$ fmol/mg protein; and in 104 C-RPE $K_d = 377.7 \pm 47.3$ pM and $B_{max} = 10.2 \pm 1.3$ fmol/mg protein. Data represent values ± associated standard error using LIGAND.
In all other instances (NR and C-RPE of 15/18 eyes) specific binding was either not observed, or, was present (at most, although not all radioligand concentrations) but could not be Scatchard transformed.

<table>
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<tr>
<th>Eye ID</th>
<th>Tissue</th>
<th>Protein (µg)</th>
<th>$K_d$ (pM)</th>
<th>$B_{max}$ (fmol/mg protein)</th>
<th>Hill coefficient</th>
</tr>
</thead>
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<td>236.2 ± 58.1</td>
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<td>C-RPE</td>
<td>69.9</td>
<td>208.4 ± 53.6</td>
<td>5.0 ± 1.0</td>
<td>0.97</td>
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<td>377.7 ± 47.3</td>
<td>nd</td>
<td>1.10</td>
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<td></td>
<td>C-RPE</td>
<td>29.0</td>
<td>202.4 ± 36.0</td>
<td>1.2 ± 0.1</td>
<td>1.04</td>
</tr>
<tr>
<td>122</td>
<td>NR</td>
<td>110.8</td>
<td>219.3 ± 16.9</td>
<td>1.6 ± 0.5</td>
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<td>C-RPE</td>
<td>32.2</td>
<td>293.1 ± 84.7</td>
<td>7.6 ± 2.6</td>
<td>1.04 ± 0.07</td>
</tr>
</tbody>
</table>

Table 6.2
Binding parameters obtained in 2-[125I]iodomelatonin saturation studies in individual human eyes. Data are values ± associated standard error using LIGAND. nd, specific binding not detectable, or present but not able to be Scatchard transformed.

6.3.1.1.2 Effect of melanin

On the basis of studies undertaken in the quail which showed that melanin increased the level of non-specific binding (section 4.3.2.4.1), in some instances (as indicated in Table 6.1) NR and C-RPE tissue homogenates were pre-centrifuged before membrane preparation to remove melanin (section 4.3.2.3). This was to determine if the absence of binding in the initial human studies was because specific binding was masked by high levels of non-specific binding. As expected, in NR, which was never visibly contaminated with melanin, this pre-centrifugation did not change the results. A similar lack of effect was observed with C-RPE despite removal of large amounts of melanin.

6.3.1.1.3 Effect of protein

Saturation studies were performed on tissues from individual specimens therefore there were limits on the maximum protein content of each sample. For NR the range protein contents was 23.7 to 149.9 µg and for C-RPE, 22.7 to 101.3 µg. Occurrence of specific binding was not obviously related to higher protein concentrations (Table 6.2). Increasing the protein concentration by pooling a pair of eyes from the same donor (NR 170 µg, C-RPE 370 µg) and by pooling several NR (specimens for which there were no accompanying donor data) (255 µg) also failed to reveal evidence of specific binding.
6.3.1.4 Displacement studies

Displacement studies with cold melatonin \((10^{-12}-10^{-7} \text{ M})\), radioligand concentration = 134-145 pM) were performed on membranes (with or without melanin) from two individual eyes and a pool of eyes. There was insufficient specific binding in either NR or C-RPE tissues to observe an effect of the displacing drug. Incubation protein contents ranged from 81 to 257 µg for NR and 53 to 121 µg for C-RPE samples.

6.3.1.2 Human iris

Preliminary isolated organ experiments conducted concurrently in another laboratory indicated a possible contractile effect of melatonin on sheep iris sphincter muscles (personal communication, Dr. S.M.O. Hourani, University of Surrey). Binding studies were therefore carried out on this ovine tissue (section 6.3.3). The contractile effect observed also stimulated interest in the possibility of human iris melatonin binding sites and therefore these were also investigated.

6.3.1.2.1 Saturation studies

Pooled human iris sphincter muscle showed evidence of specific 2-\([^{125}\text{I}]\)iodomelatonin binding in saturation studies. In 2/2 cases (pools I and II, Table 6.3) specific binding was observed over the radioligand concentration range examined and Scatchard transformation of the data indicated the presence of a single high affinity site (Figure 6.3). Binding parameters are shown in Table 6.3. From Figure 6.3 it can be seen that non-specific binding was reduced in pool I as a result of removing melanin, in agreement therefore with the quail findings (section 4.3.2.6.1). The corresponding radial muscles were also investigated however these binding data could not be Scatchard transformed.

<table>
<thead>
<tr>
<th></th>
<th>(K_d) (pM)</th>
<th>(B_{\text{max}}) (fmol/mg protein)</th>
<th>Hill coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>293.8 ± 83.6</td>
<td>20.7 ± 3.6</td>
<td>1.01</td>
</tr>
<tr>
<td>II</td>
<td>147.2 ± 37.2</td>
<td>13.6 ± 3.6</td>
<td>0.91</td>
</tr>
</tbody>
</table>

**Table 6.3**

Binding parameters from human iris sphincter muscle 2-\([^{125}\text{I}]\)iodomelatonin saturation binding experiments. In I, tissues from 6 eyes were pooled and in II tissues from 4 eyes were pooled. Incubation protein contents for I and II were 12.9 and 19.5 µg, respectively. Data are values ± associated standard error using LIGAND.
Figure 6.3
Saturation of specific 2-[\textsuperscript{125}I]iodomelatonin binding (O) in human iris sphincter muscle pools (A and C). Non-specific binding (■) was determined in the presence of 1 µM melatonin; total binding (□). (B) and (D) are the Scatchard transformations of data from (A) and (C), respectively. Melanin was removed during membrane preparation for (A) only. In (B), \( K_d = 293.8 \pm 83.6 \) pm and \( B_{max} = 20.7 \pm 3.6 \) fmol/mg protein; and in (D) \( K_d = 147.2 \pm 37.2 \) pm and \( B_{max} = 13.6 \pm 3.6 \) fmol/mg protein. Data represent values ± associated standard error using LIGAND.
6.3.1.2.2 Displacement studies

A displacement study with cold melatonin (10^{-11} to 10^{-7} M) did not provide definite evidence for displaceable specific binding in pooled sphincter or radial membranes prepared from 5 irises (radioligand concentration 144 pM; melanin removed by pre-centrifugation). There was some effect (30 % displacement) at 10^{-7} M in sphincter muscle but at lower concentrations there was little apparent effect and consistency of the replicates was poor. With radial muscle there was little specific binding and no consistent displacing effect of melatonin. Incubation protein contents were 17.4 and 19.3 µg for sphincter and radial muscles, respectively.

6.3.2 Effect of tissue storage on 2-[^{125}I]iodomelatonin binding

An investigation was undertaken to ascertain the effect of a prolonged pre-frieezing storage period on the “quality” of specific 2-[^{125}I]iodomelatonin binding subsequently observed in radioreceptor assays. The chicken was chosen as the experimental model as there is much evidence for melatonin receptors within the eye of this species (for example, Dubocovich and Takahashi, 1987 and Chong and Sugden, 1991). The exact experimental protocol employed has been described (section 4.2.2). In brief, 2-[^{125}I]iodomelatonin binding studies (saturation experiments) were performed in NR and C-RPE membranes prepared from eyes of freshly sacrificed animals and from “aged” eyes. The latter were subjected to a rigorous protocol involving a sequence of various storage states in order to simulate the worst case scenario that a human eye might be subjected to prior to analysis.

Preliminary saturation experiments were undertaken to confirm the presence of 2-[^{125}I]iodomelatonin binding in the particular supply of birds to be used. The binding parameters obtained were, NR: \( K_d = 81.2 \pm 4.3 \) pM, \( B_{max} = 16.8 \pm 0.4 \) fmol/mg protein, and C-RPE; \( K_d = 55.7 \pm 3.6 \) pM \( B_{max} = 3.9 \pm 0.5 \) fmol/mg protein (all values ± associated standard error using LIGAND). Scatchard transformation of binding data from fresh eye NR and C-RPE membranes yielded similar \( K_d \) and \( B_{max} \) values (NR: \( K_d = 72.1 \pm 5.5 \) pM, \( B_{max} = 14.2 \pm 1.1 \) fmol/mg protein, and C-RPE \( K_d = 66.1 \pm 4.1 \) pM \( B_{max} = 4.8 \pm 0.3 \) fmol/mg protein). In contrast, data from the aged eye membranes (NR and C-RPE) could not be Scatchard transformed. Figure 6.4 shows that in both tissues, at all radioligand concentrations the level of specific binding was greatly decreased by the storage process and additionally, at some concentrations there was no specific binding.
Figure 6.4

Effect of a prolonged pre-storage period on specific $2\cdot{[^{125}I]}$iodomelatonin binding in chicken NR and C-RPE membranes. For "fresh" membranes, birds were sacrificed and tissue membranes prepared immediately. For "aged" membranes, eyes were subjected to an extended period of storage prior to membrane preparation (section 4.2.2). All binding studies were performed on the same day under the same experimental conditions. Only saturation data from fresh NR and C-RPE membranes were able to be Scatchard transformed.
6.3.3 Ovine ocular 2-[\textsuperscript{125}I]iodomelatonin binding sites

As described in section 6.3.1.2, isolated organ experiments with sheep iris sphincter indicated a possible contractile effect of melatonin. This, coupled with the fact that the sheep is a highly photoperiodic species (Arendt, 1995) indicated that investigation of 2-[\textsuperscript{125}I]iodomelatonin binding in membrane preparations from a variety of tissues within the ovine eye, including the sphincter muscle, might be of interest.

6.3.3.1 Membrane radioreceptor assays

Various ocular tissue membrane preparations from sheep were initially screened for the presence of 2-[\textsuperscript{125}I]iodomelatonin binding (Table 6.4). Saturable specific binding was observed in NR only (Figure 6.5). Binding was not consistently demonstrated in this tissue, however. Data from only 3 out of 5 membrane preparations were able to be Scatchard transformed. The membrane preparations used derived from 5 separate batches of eyes obtained on different occasions. The absence of NR binding in some instances was not related to differences in storage as positive binding was obtained in both fresh and frozen membranes.

<table>
<thead>
<tr>
<th>Table 6.4</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Saturation studies</strong></td>
</tr>
<tr>
<td>NR</td>
</tr>
<tr>
<td>$K_d$ 90.6 ± 31.9 pM; $B_{max}$ 1.5 ± 0.5 fmol/mg protein;</td>
</tr>
<tr>
<td>Hill coefficient 0.95 ± 0.04 (mean ± s.e.m., n=3)</td>
</tr>
<tr>
<td>C-RPE</td>
</tr>
<tr>
<td>Iris</td>
</tr>
<tr>
<td>sphincter pupillae</td>
</tr>
<tr>
<td>dilator pupillae</td>
</tr>
<tr>
<td>Ciliary body</td>
</tr>
</tbody>
</table>

Frequency of saturable, specific 2-[\textsuperscript{125}I]iodomelatonin binding in various ovine ocular tissues. Each saturation study investigated 7 (NR and C-RPE) or 4-7 (iris and ciliary body) radioligand concentrations over the range 9 to 800 pM.

The iris and ciliary body tissues investigated were taken from batches of eyes which had previously been demonstrated to possess saturable, specific binding in NR. Removal of melanin as described in section 4.3.2.3 had no effect on specific binding in any of the tissues investigated.

The ovine NR melatonin binding site was subsequently utilised in various methodological investigations. Firstly, because the human membrane studies had used low protein contents, ovine NR membranes were employed to determine the effect of protein content on the level of specific binding. Specific 2-[\textsuperscript{125}I]iodomelatonin binding at two radioligand concentrations was investigated (Figure 6.6). At the lowest
Representative 2-[\textsuperscript{125}I]iodomelatonin binding saturation data in ovine NR membranes (A). Total binding (■), non-specific binding (determined in the presence of 1 µM melatonin (●), specific binding (O). Scatchard transformation of the data is also shown (B). In this instance $K_d = 81.2 \pm 16.0$ pM, $B_{\text{max}} = 1.8 \pm 0.2$ fmol/mg protein. Data represent values ± associated standard error using LIGAND.
Figure 6.6

The dependence of ovine NR membrane specific $2-[^{125}\text{I}]$iodomelatonin binding (fmol) on protein concentration. Two radioligand concentrations were investigated; 48 (■) and 188 (O) pM.
protein content (~ 10 µg), specific binding was only observed at the lower radioligand concentration. Specific binding at both label concentrations increased linearly between 25 and 175 µg protein.

6.3.3.2 Autoradiography

 Autoradiographic studies were subsequently performed on sheep NR and C-RPE. On the basis of previous saturation studies, other tissues, for example iris, were not investigated. Each of the eyes investigated for the presence of specific binding by autoradiography was taken from a batch of eyes which had previously been demonstrated to possess saturable, specific binding in the saturation studies.

 Preliminary studies to ascertain exposure times indicated that whereas total binding in the C-RPE region became apparent at ~ 2 days (127 pM 2-[\(^{125}\)I]iodomelatonin), in the NR, staining was only visible from ~ 3 weeks. At 3 weeks there was no obvious difference between total and non-specific binding sections in either tissue region upon preliminary visual inspection. Densitometric analysis confirmed the absence of a statistical difference between total and non-specific binding in this and two additional eyes (Figure 6.7). Increasing the exposure time (up to 12 weeks) in two of these eyes also failed to reveal a statistical difference between total and non-specific binding. Similarly, increasing the buffer pre-incubation time from 15 min to 1 h also did not reveal significant differences between total and non-specific binding (Figure 6.7).
Figure 6.7
Effect of increased exposure time (eyes A, B and C) and increased pre-incubation period (eye C) on 2-[125I]iodomelatonin binding in ovine NR (N) and C-RPE (R). Non-specific binding was defined with 1 μM melatonin. Data (relative optical density (ROD) measurements) represent the mean ± s.e.m. of 3 to 5 tissue sections. Radioligand concentration used was 124 to 150 pM.
6.3.4 Human autoradiography

Autoradiographic studies were undertaken with human eye tissues in order to confirm the NR and C-RPE melatonin binding site data from radioreceptor assays, and to further investigate the possibility of a melatonin binding site in the iris.

Duplicate total and non-specific sections of a single eye were exposed for variable periods of time (1 day to 4 weeks). Binding in the C-RPE region was apparent after 1 day. NR was faintly visible from 8 days and clearly visible from 2 weeks. All subsequent sections were exposed for ~2 weeks. For each experiment, after 2 weeks of exposure, 2 test total and non-specific sections were developed to ensure that NR was visible before developing the remainder. If not, exposure was continued. (Autoradiographs of the test sections were not included in the densitometric analysis.)

Representative examples of the autoradiograms obtained with the corresponding tissues sections are shown in Figures 6.8 and 6.9. It is apparent that 2-[^125]Iiodomelatonin bound extensively throughout both total and non-specific sections. Pigmented structures, in particular, were intensely labelled. Visual inspection indicated no apparent differences in the staining density between total and non-specific sections.

Eleven human eyes were investigated for the presence of specific 2-[^125]Iiodomelatonin in NR and C-RPE tissues (Figure 6.10A). The donor parameters of these specimens are shown in Table 6.5. Specific binding was only observed in NR of one specimen (eye 165). However the difference between total and non-specific binding sections (= specific binding) was not noticeable to the naked eye. To ensure that the results obtained in the autoradiographic studies were not artefactual, tissue sections from 6 eyes, including eye 165 which displayed specific binding, were re-imaged under different lighting and focus conditions, etc. and the densitometric analysis repeated. In all cases, for both NR and C-RPE tissues, the results were identical, that is, specific binding was again only observed in NR of eye 165.

Iris-ciliary body (ICB) tissues were present in sections from 8 of the 11 eyes studied. The two components were not easily distinguishable, partly because of the high level of staining associated with the pigmented areas of each. They were therefore analysed together as a unit. There was no evidence for specific 2-[^125]Iiodomelatonin binding in ICB of any of the 8 eyes (Figure 6.10B). In 5 of these 8 eyes, ciliary muscle (M) was distinguishable. Analysis of this tissue individually similarly provided no evidence of specific 2-[^125]Iiodomelatonin binding (Figure 6.10B).

Increasing the exposure period of sections of eye 173 (up to 16 weeks) failed to reveal any significant differences between total and non-specific binding in NR, C-RPE or iris-ciliary body components (Figure 6.11).
Figure 6.8
Autoradiographic localisation of 2-[^125]Iiodomelatonin binding in human eye 134. (A) total binding, (C) non-specific binding. (B) and (D) are the tissue sections from (A) and (C), respectively after staining with haematoxylin and eosin. Radioligand concentration was 108 pM. Non-specific binding was defined with 1 μM melatonin.
Figure 6.9
Autoradiographic localisation of 2-[\textsuperscript{125}I]iodomelatonin binding in human eye 168. (A) total binding, (C) non-specific binding. (B) and (D) are the tissue sections from (A) and (C), respectively after staining with haematoxylin and eosin. Radioligand concentration was 108 pM. Non-specific binding was defined with 1 \textmu M melatonin.
Figure 6.10
Analysis of 2-[125I]iodomelatonin binding in human eyes: (A) NR and C-RPE; (B) iris-ciliary body (ICB) and ciliary muscle (M). Non-specific binding was defined with 1 μM melatonin. Specific binding was only observed in eye 165 NR (* unpaired t-test, p < 0.01). Data (relative optical density (ROD) measurements) represent the mean ± s.e.m. of 5 to 12 tissue sections. Radioligand concentration used was 125 to 150 pM.
<table>
<thead>
<tr>
<th>Eye ID</th>
<th>Sex</th>
<th>Age</th>
<th>TOD</th>
<th>DOD</th>
<th>COD</th>
<th>Specimen age (h)</th>
<th>PMI (h)</th>
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<td>12/04/94</td>
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<td>3.5</td>
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<tr>
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<td>5 D</td>
<td>2 Sp, 2 Su</td>
<td>3 cancer, 2 SAH, 2 CVS</td>
<td>34.8 ± 9.0</td>
<td>11.9 ± 6.4</td>
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<td></td>
</tr>
<tr>
<td>4 F</td>
<td>6 N</td>
<td>3 A, 4 W</td>
<td>2 CVS</td>
<td>mean ± sd</td>
<td>mean ± sd</td>
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</table>

Table 6.5
Donor parameters of the human eye specimens investigated for specific 2-[\(^{125}\)I]iodomelatonin binding by autoradiography.

**Abbreviations and notes**
M male; F female; D day; N night; T-, D-, COD time-, date-, cause of death; MI, myocardial infarction; SAH, subarachnoid haemorrhage; specimen age = time between donor death and frozen storage of specimen; PMI, post mortem interval = time between donor death and enucleation; ?, parameter unknown; CVS cardiovascular system; Sp, spring; Su, summer; A, autumn; W, winter.
Specimens 173 and 177 were obtained unfrozen, the remainder were frozen.
Figure 6.11
Effect of time of exposure on 2-[\textsuperscript{125}I]iodomelatonin binding in human eye: NR (N), C-RPE (R) and iris-ciliary body (ICB). Data (relative optical density (ROD) measurements) represent the mean ± s.e.m. of 4 tissue sections. Non-specific binding was defined with 1 μM melatonin. Radioligand concentration was 150 pM.
### 6.3.5 Human eye data summary

A summary of the binding studies undertaken with human eyes is provided in Table 6.6

<table>
<thead>
<tr>
<th>Membrane radioreceptor assay</th>
<th>Autoradiography</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saturation studies</td>
<td>Displacement studies</td>
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<td>NR</td>
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</tr>
<tr>
<td>C-RPE</td>
<td>2/18</td>
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<td>Iris:</td>
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<td>dilator pupillae</td>
<td>0/2</td>
</tr>
<tr>
<td>Iris-ciliary body</td>
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<tr>
<td>Ciliary muscle</td>
<td>NA</td>
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</tbody>
</table>

Table 6.6

2-[^125]I]iodomelatonin binding studies conducted in human ocular tissues. Data refer to frequency of saturable, specific binding in saturation studies; frequency of displaceable binding in displacement studies; and frequency of specific binding in autoradiography. NA not assayed
6.4 Discussion

Evidence is steadily accumulating for the existence of a melatonin binding site within the mammalian eye. Furthermore, in the rabbit this site represents a true functional receptor which mediates the inhibitory effect of melatonin on retinal \(^{3}\)H-dopamine release (Dubocovich, 1983; 1985). Within the human eye itself melatonin receptor genes have been found to be expressed in the retina (Mel\(_{1b}\), and to a lesser extent Mel\(_{1a}\)) (Reppert et al., 1995a) but what has been lacking to date is a comprehensive pharmacological investigation of the binding site itself in situ.

In these studies saturable, specific high affinity 2-[\(^{125}\)I]iodomelatonin binding was demonstrated in both NR and C-RPE membranes from post-mortem human eyes. Linear Scatchard plots and Hill coefficients approaching unity in all cases indicated the presence of a single site. The affinity of the sites for 2-[\(^{125}\)I]iodomelatonin (mean \(K_d \sim 250\) pM) was much less than that of the ocular binding sites described in, for example, chicken (Laitinen and Saavedra, 1990a), tree shrew (Lu et al., 1991) and frog (Skene et al., 1993) and was more in keeping with that in rabbit retina (Dubocovich, 1988; Blazynski and Dubocovich, 1991) and the chicken NR data of Dubocovich and Takahashi (1987) and Chong and Sugden (1991). The observed \(K_d\) values were also in the same region as that of the cloned Mel\(_{1b}\) receptor rather than the higher affinity Mel\(_{1a}\) receptor (Reppert et al., 1995a). Capsoni and co-workers (1994) have reported preliminary evidence for two binding sites in the human eye; one similar to that described here (\(K_d 340\) pM) and the other with much higher affinity (\(K_d 15\) pM). The observed density of binding sites in human C-RPE was greater than in NR and both densities were several fold less (over 35-fold for C-RPE) than that reported for the low affinity state site in C-RPE (Capsoni et al., 1994) (Table 1.4 (I)).

Although clear evidence for a specific 2-[\(^{125}\)I]iodomelatonin binding site was obtained with the membrane radioreceptor methodology employed, there was, however, only a very low occurrence of binding. 3/18 eyes displayed saturable binding and the binding was not consistently observed in both eye tissues (NR and C-RPE). One reason for these findings is that the data accurately reflect the biological situation, that is, perhaps under normal circumstances only a small proportion of individuals possess NR and/ or C-RPE melatonin binding sites. This may be as a result of some common donor parameter: either a known parameter, or some combination of parameters, but which was not apparent in these studies due to the low frequency of melatonin binding, or some other factor about which no information was available. Donors of the three specimens which contained a melatonin binding site had all died during the day, that is, when ocular and plasma melatonin levels are low. These donors were also over 60 years of age, that is, in the age group in which pineal melatonin synthesis is known to decrease (Touitou et al., 1981). If the melatonin binding site was being regulated by melatonin itself, then this may partly explain the occurrence of binding sites in these specimens. In the rat pars tuberalis and SCN, for example, (Gauer et al., 1993), melatonin binding site density is increased during the light phase when plasma melatonin is low. In the present human studies it is apparent that not all the specimens from donors which had died during the day and were aged contained melatonin binding sites, however, thus there must be other factors involved.

It is possible that the membrane melatonin binding site in the eye is actually of little significance, and represents, for example, some form of vestigial receptor with
the main functions of melatonin instead being mediated by some form of intracellular receptor. The studies of Cohen and co-workers (1978), although never confirmed, indicated the presence of some form of cytoplasm binding site in the hamster eye and more recently melatonin has been established as a ligand of the orphan nuclear receptor RZRP (Becker-André et al., 1994) which is also expressed in NR.

Alternatively, if the assumption is made that under normal circumstances human NR and C-RPE do possess melatonin binding sites, and increasingly the evidence points towards this, then the question must be asked why were they not consistently apparent in these studies. It is unlikely that the negative findings resulted from an inherent problem with the method as it had previously been used successfully to establish the existence of a melatonin binding site in the quail eye (sections 5.3.1 and 5.3.3). However other methodological factors may have contributed. For example, in the quail, removal of tissue melanosomes by centrifugation prior to membrane preparation was found to reduce non-specific binding. It is possible then that in the human C-RPE specific binding was present but was masked by a much larger amount of non-specific binding. However, when melanosomes were removed no increased binding was evident in the human tissues. Additionally, this procedure indicated that because specific binding was still present in C-RPE from which melanosomes had been removed (unlike frog C-RPE (Wiechmann et al., 1986)), they were not responsible for all observed binding.

Throughout the human studies, incubation protein contents were low and this would have decreased the levels of specific binding observed. However, increasing the protein content of the human incubation system failed to reveal an increased incidence of specific binding although coupled with possible low binding site densities these protein increases may still have been insufficient to reveal specific binding. The studies undertaken with sheep NR indicated that, as expected, protein was an important factor in determining binding and that specific binding was not observed below a certain level of protein. Furthermore, other authors describing ocular melatonin binding sites with particularly low densities have reported using membrane preparations with high protein contents. For example, Vanecek described a binding site in rat NR with $B_{\text{max}}$ 0.35 fmol/mg protein and reported using incubation protein contents of 60 to 160 mg compared to the low microgram level used in these studies. With the membrane receptor assay it was possible that specific binding was present but highly localised in discrete areas and therefore not detected when diluted with other areas of ocular tissue not containing the binding site. However, autoradiography studies confirmed the low incidence of specific $^{125}$Iiodomelatonin binding in NR and C-RPE tissues. Serial sections across the entire eye were not investigated but this was justified as each tissue section was a cross section of the entire eye. Although sections were cut from the middle of the eye, each always included NR and C-RPE tissue from the periphery. In addition, binding in NR has been demonstrated to exist throughout the entire circumference of the eyeball in other species (Laitinen and Saavedra, 1990a; Wiechmann and Wirsig-Wiechmann, 1991; quail, Chapter 5). Furthermore, there are no known reasons why the occurrence of melatonin binding sites should be restricted to specific areas.

Binding was not observed in the sheep NR autoradiography studies although it was found in the membrane assays. This may have been because the binding site was only present in some eyes, and hence could be demonstrated in membranes pooled from several eyes but not in the individual eyes studied for autoradiography.
Alternatively it is possible that endogenous ligand, that is melatonin, might be blocking the binding site and thus affecting the autoradiography binding assay. In rabbit NR and RPE a small increase in specific binding was observed following constant light treatment of the animals prior to sacrifice (Blazynski and Dubocovich, 1991). However, in chick brain neither treating tissue sections with melatonin prior to assay nor increasing the buffer pre-incubation temperature had any effect on the resultant binding (Brooks and Cassone, 1992). Similarly, increasing the buffer pre-incubation period in this study failed to reveal specific binding in sheep NR. In the human autoradiography studies it was unlikely therefore that endogenous melatonin was responsible for the low frequency of binding observed. Another possible reason for the apparent lack of 2-[\(^{125}\)I]iodomelatonin binding in both sheep and human autoradiography studies was related to the low density of binding sites in these tissues. Laitinen and Saavedra (1990a) reported that prolonged exposure time was necessary to visualise the low level of specific 2-[\(^{125}\)I]iodomelatonin binding in rat retina. However, increasing film exposure time, even to 3 months, did not reveal specific binding in either human or sheep studies. In contrast, several authors have reported that low density specific binding (\(B_{\text{max}} \leq 5 \, \text{fmol/mg protein}\)) is visible in autoradiography after 3 weeks or less exposure (Blazynski and Dubocovich, 1991; Wiechmann and Wirsig-Wiechmann, 1991). Thus it seems unlikely that specific 2-[\(^{125}\)I]iodomelatonin binding was not demonstrated in human and sheep tissues in the present studies simply because of its low density.

Having eliminated these factors as possible causes of the low frequency of binding observed in human NR and C-RPE, there remained one in particular over which there was no control, that is, the post-mortem nature of the specimens. The time period between donor death and frozen storage of the specimen ("specimen age") was never less than \(~20\) hours and extended to a maximum of \(~68\) hours in one instance. Furthermore the time between donor death and the enucleation itself was also long for several specimens (up to \(~22\) hours). The majority of specimens were used as tissue sources for corneal transplant. Presumably, therefore, after enucleation they were placed in refrigerated storage. Prior to enucleation no information regarding the storage conditions was available but obviously at least for a proportion of this time period the specimens would not have been chilled. Corneal tissue is physiologically robust in part because, lacking its own blood supply, it is able to utilise atmospheric oxygen. Hence, prior to their removal for transplant the eye specimens are not treated with the same degree of care, particularly in terms of storage period, that would be afforded other more delicate tissues (personal communication, Dr G. Jeffrey, Institute of Ophthalmology, London).

In these studies, the presence of 2-[\(^{125}\)I]iodomelatonin binding appeared not to be associated with shorter pre-freezing specimen storage periods, however the possible existence of a relationship between binding and length of non-refrigerated storage period, an essentially unknown variable, could not be precluded. Saturation experiments conducted on membranes prepared from "fresh" and "aged" chicken eyes indicated a substantial deleterious effect of storage on specific 2-[\(^{125}\)I]iodomelatonin binding. The pattern of specific binding demonstrated in saturation studies in the aged chicken eye tissues was very similar to that frequently obtained with those human specimens that did not possess a binding site, that is, irregular specific binding that could not be Scatchard transformed. Therefore it is likely that storage of the human eyes prior to freezing could be at least partially responsible for the frequent lack of
specific binding. Amongst the various processes which occur upon cell death, two are potentially highly significant with respect to receptors: enzymatic digestion and protein denaturation. Enzymatic digestion results from the release and activation of intracellular hydrolytic enzymes which would normally be strictly contained within the cell in lysosomes. The effects of enzyme digestion coupled with protein denaturation on a protein such as a membrane bound receptor are potentially highly significant, therefore. It may be then that the melatonin receptor in certain human specimens has undergone irreversible damage by the time of assay such that it is no longer able to bind radioligand in the same way. It is unlikely that this hypothesis alone accounts for the negative data obtained however as melatonin receptors have been found in human SCN 9 to 24 hours post-mortem (Reppert et al., 1988) and in cerebellum 8 to 20 hours post-mortem (Fauteck et al., 1994) suggesting that the melatonin binding site is not particularly unstable. Other membrane receptors have been successfully studied in human post-mortem ocular tissues. For example, D_2-dopamine receptors have been characterised in NR membranes from specimens up to 24 hours post-mortem (McGonigle et al., 1988) and by autoradiography in specimens up to 6 hours old (Denis et al., 1990). However these latter authors have attributed certain discrepancies in their data compared with those obtained previously to the time elapsed between death and enucleation. Beta_2-adrenergic binding sites have also been observed in human eyes 4 to 8 hours post-mortem (Elena et al., 1990).

In addition to retinal and choroid components these studies investigated other ocular tissues, namely the iris muscles and ciliary body. Despite the contractile effect of melatonin previously observed in isolated organ experiments, membrane radioreceptor studies with ovine sphincter pupillae did not identify a 2-[^125]Ijiodomelatonin binding site. In contrast, preliminary investigations with human sphincter pupillae membrane preparations did indicate the possible existence of a melatonin binding site in this region. Binding was saturable and Scatchard transformation indicated a single high affinity site (mean K_d 220 pM) although a cold melatonin displacement study did not provide clear evidence of displaceable 2-[^125]Ijiodomelatonin binding. Recently, Osborne and Chidlow (1994) have reported the presence of melatonin receptors in the iris-ciliary processes of the rabbit which are negatively linked to the adenylyl cyclase system. In this study, discrimination of binding between the iris and ciliary body was not possible. Similarly, in the human autoradiography studies conducted here, the macroscopic resolving power of the methodology employed was not sufficient to analyse human iris sphincter pupillae, or even iris and ciliary tissues, separately and these latter two were, therefore, by necessity, analysed together. This may at least partly explain why autoradiography was not able to confirm the membrane assay data. In addition, the posterior face of the iris is heavily pigmented leading to very high non-specific staining density in the region of quantification which may have obscured a small amount of specific binding.

Evidence for the presence of melatonin binding sites on smooth muscle has already been provided in another location, namely that within the arterial wall. High affinity sites (K_d 35-40 pM) have been described in the rat in both caudal and anterior cerebral arteries (Viswanathan et al., 1990; Sugden, 1994). In these tissues melatonin acts as a direct vasoconstrictor (Evans et al., 1992). It is feasible therefore that melatonin does have a contractile effect on the smooth muscle of the iris. The findings of Quay (1986) lend support to this hypothesis. Quay showed that in finches implanted with melatonin, pupillary diameter was significantly decreased compared
with control birds and attributed this effect to an action of melatonin within the central nervous system. The possibility exists however that under normal circumstances a similar effect is mediated through locally produced melatonin, or indeed circulating pineal melatonin, acting via a local, that is sphincter pupillae, receptor.

The biological function of a contractile action of melatonin on sphincter muscle, is difficult to imagine given that in darkness, that is, in the period when melatonin is present, the pupil is in a state of dilation, in order to maximise light reception. Also, Liu and co-workers (1996) have reported a circadian rhythm in pupil diameter in the rabbit with peak pupil size occurring in the subjective light phase, implying that if there is a causal relationship between melatonin and pupil size then it is unlikely that melatonin is contributing a sphincter contractile effect. At present, however there is no convincing evidence for a direct contractile effect and nor even can the possibility of melatonin as an agent of relaxation be discounted. Moreover, melatonin may exert these effects via a non-receptor mediated mechanism. It is essential, therefore, that the putative sphincter melatonin binding site within the human iris, described herein, is investigated in much greater detail before its physiological relevance can be established. The small amount of iris tissue provided by each eye specimen imposed a severe limitation on the studies carried out and precludes further use of membrane radioreceptor assays. The difficulty of dissecting the sphincter pupillae completely from the dilator muscle was also a problem. It is likely that for future studies it will be necessary to use the higher resolution liquid emulsion-type autoradiography to reveal, if it exists, the exact site of the melatonin binding site within the anterior uveal tissues.
CHAPTER 7

GENERAL DISCUSSION
Since its identification in 1959, the indoleamine melatonin has been established as being of immense importance with respect to the phenomenon of biological rhythmicity. Melatonin was first isolated from the pineal gland of the cow but subsequently found to be present in this organ in all species that have since been studied. The possible existence of additional sites of melatonin synthesis was initially not widely accepted. In 1965, however, Quay (1965) demonstrated that, in several lower vertebrates, HIOMT, the crucial end enzyme of melatonin biosynthesis was in fact present within retinal tissue. In the 30 years since this discovery, interest in ocular melatonin and its physiological relevance has grown considerably.

The presence of melatonin or melatonin-like immunoreactivity has been confirmed in various eye tissues of a large number of species (Table 1.1). The biochemical pathway of local melatonin synthesis has been elucidated and found to parallel that of the pineal, that is, it involves the sequential action of NAT and HIOMT enzymes with 5-HT as the initial substrate (Figure 1.5). Synthesis is rhythmic such that melatonin levels are generally higher at night and, in many species, production represents a true circadian rhythm. Moreover, in an increasing number of species, control of the ocular melatonin rhythm is recognised as being under the control of an oscillator which resides within the eye itself. In addition to synthesis, studies have also considered the potential importance and routes of local degradation of ocular melatonin. Not surprisingly, a great deal of the interest in ocular melatonin has centred on its actions and their possible physiological relevance. The actual mechanisms by which these actions are mediated have attracted similar attention.

This thesis has considered two aspects of ocular melatonin: its presence and quantification, and the existence of melatonin binding sites. Two species were of primary interest, quail and man.

Despite the existence of a now considerable number of publications concerned with ocular melatonin, information regarding melatonin in the human eye remains scant. At the commencement of experimental work for this thesis, few systematic attempts had been made to measure melatonin within the human eye and there were no reported human ocular melatonin binding site studies. Ironically, it is information such as this that would have the greatest potential for future application. In a medical context, ocular melatonin has been attributed both pathogenic and protective roles (section 1.9). Manipulation of the ocular melatonin system in some way, for example, via pharmacological means, may represent an alternative therapeutic strategy for the treatment or prevention of some eye diseases.

A principal aim of the research described herein was, therefore, to maximally exploit an available source of human eye specimens in order to firstly ascertain melatonin levels and secondly to probe the tissues for melatonin binding sites. In both cases interest was centred predominantly upon NR and the C-RPE, that is, those tissues which, in other species, had previously been shown to contain melatonin and its binding site.

Quantification of melatonin was undertaken by RIA using methodology which was validated specifically for use with human NR and C-RPE tissues. Authenticity of the endogenous melatonin-like immunoreactivity was verified by TLC/RIA. Melatonin was found to be present in both NR and C-RPE compartments thus confirming the existence of melatonin within the human eye. Levels were variable ranging from about 10 to 490 pg/g wet tissue weight for both NR and C-RPE. Overall, these contents were lower than previously reported (Leino, 1984; Osol and
Schwartz, 1984). There were no statistically significant relationships between these melatonin contents and any of the known specimen donor parameters, that is, sex, age, or month or time of death. On the basis of data from other species these findings for sex, age, and month of death were to a certain extent expected - no definitive associations between these factors and ocular melatonin content have yet been proven (sections 1.5.2.4 to 1.5.2.6). In contrast it was anticipated that melatonin levels would vary in accordance with time of death as a clear diurnal variation in ocular melatonin has been reported in most species studied with higher melatonin levels occurring at night. Frequently this day/night rhythm has been shown to be a true circadian rhythm in lower vertebrates and very recently, this rhythm has been shown to be under the control of a local oscillator in the mammalian retina (Tosini and Menaker, 1996). In this human study however, melatonin contents were not significantly increased when donor death occurred during the night.

The significance of human ocular melatonin has not yet been addressed experimentally; neither in these studies nor by previous authors. It is anticipated, however, that it will be found to fulfil at least some of those actions previously reported for melatonin in other species (section 1.7). The distribution of melatonin, that is, within both the NR and the C-RPE compartments, corresponds to the site of many of these actions.

In the present study, not all specimens investigated possessed demonstrable levels of melatonin in both tissues and a large proportion of specimens (almost 45 %) did not contain melatonin in either tissue. The occurrence of melatonin was not apparently related to any donor parameter. Within the time course of this thesis, preliminary evidence has been provided which suggests that the human eye, particularly the retina, possesses the capacity for melatonin synthesis. Gene transcripts of both the essential enzymes, NAT (Coon et al., 1996) and HIOMT (Rodriguez et al., 1994; Bernard et al., 1995), have been identified in human retina. This, coupled with the fact that there is abundant evidence for melatonin synthesis within ocular tissues of several other mammalian species implies that it is unlikely that a biological factor was responsible for the absence of melatonin in such a large proportion of the specimens. Instead, it is hypothesised that both this finding and the depressed levels of melatonin indicated in the remaining specimens (as compared with the human retinal levels reported by Leino (1984) are in fact attributable to the post-mortem nature of the tissues. In support of this theory, NR melatonin levels were found to be significantly negatively correlated with post-mortem interval (PMI). There was no correlation between C-RPE melatonin levels and PMI however, and neither were melatonin contents related to specimen ages, thus suggesting that the effects of storage are not simply a direct function of time. It is probable that additional factors relating to the fate of the specimen prior to being frozen are important determinants of melatonin content. Storage temperature is an obvious candidate. Information regarding other aspects of the donor prior to death was also lacking. The possible implications of medication and environmental lighting conditions, for example, cannot be ignored. If such factors are important, then this might partly explain the absence of the expected relationship between time of donor death and melatonin content.

In conjunction with the investigation of ocular melatonin contents, an additional group of human eye specimens was probed for melatonin binding sites. The relevant methodology was initially established using quail ocular tissue as the model. This
species is of particular significance with respect to the field of ocular melatonin research in its own right, however, as not only is the quail eye essential for maintenance of overall circadian organisation (Underwood and Siopes, 1984), but also it exhibits a rhythm of melatonin synthesis which is under circadian control of a local oscillator (Underwood et al., 1990b). It was of interest therefore to probe the quail eye for a high affinity melatonin binding site, as the mechanism(s) through which this ocular melatonin may act locally have not yet been established. Both NR and C-RPE membranes were found to contain 2-[125I]iodomelatonin binding sites, and this tissue distribution was confirmed by autoradiography. Subsequent characterisation of the binding sites revealed single sites in both tissues which were pharmacologically very similar and which fulfilled several criteria of a melatonin receptor, that is, binding was saturable, reversible and specific. This 2-[125I]iodomelatonin binding site shares many similarities with the ocular melatonin binding sites previously described in other species including affinity and pharmacological profile. An additional study investigated the effect of maintaining quails under long and short photoperiods on ocular 2-[125I]iodomelatonin binding. Neither binding site density nor affinity appeared to be affected by the photoperiodic conditions. Possible roles for the binding site described in the quail eye have been outlined previously (section 5.3).

Having provided definite evidence for the existence of melatonin binding sites within the quail eye it is essential that they are associated with a function before they can be termed receptors. Consideration should perhaps be first given to the inhibitory effect of melatonin on retinal dopamine release, that is, the action already proven to be receptor mediated in the chicken and rabbit (Dubocovich, 1983; 1985). Specific cellular localisation of the melatonin receptor within the retina, for example, by liquid emulsion type autoradiography, may also be of assistance in determining the functions of melatonin in the quail.

Having established the methodology for analysis of ocular melatonin binding sites in the quail eye, this was used to probe a group of human eye specimens for melatonin binding sites. Saturation studies with 2-[125I]iodomelatonin indicated the presence of saturable, specific binding in NR and C-RPE membranes, although not always both in the same specimen. Linear Scatchard plots and Hill coefficients approaching unity indicated a single class of site with high affinity ($K_d \sim 250 \text{ pm}$) and low density ($B_{\text{max}}$ less than $\sim 10 \text{ fmol/mg protein}$). This site may represent a mechanism through which melatonin mediates its actions within the eye. Its occurrence in both NR and C-RPE is in agreement with the distribution of melatonin shown in the quantification studies, and also with its postulated actions. The data are reinforced by the findings of Capsoni and co-workers (1994) who also reported the existence of melatonin binding sites in C-RPE. In addition, gene transcripts of the cloned melatonin receptors Mel1a and Mel1b have been identified in human retina implying that the molecules are expressed in the eye (Reppert et al., 1995b), although the precise localisation within NR and/or C-RPE has not been reported.

Only a very small proportion of the human specimens investigated however (3/18), demonstrated specific 2-[125I]iodomelatonin binding in membrane assays, a finding confirmed by autoradiography. This low occurrence precluded further pharmacological characterisation of the binding site. A study undertaken with chicken NR and C-RPE tissues indicated that an extended storage period prior to freezing had a deleterious effect on 2-[125I]iodomelatonin binding in both tissues. Consequently it is proposed that the human binding site investigations with NR and C-RPE were
compromised, as was the melatonin quantification study, by the post-mortem nature of the tissue and this may explain why binding was observed in such a limited number of specimens. Because of the proteinous nature of the melatonin receptor molecule, it is likely that it would be more susceptible to damage during the post-mortem interval than would the melatonin molecule itself and this ties in with the experimental findings in that a greater proportion of specimens lacked melatonin binding sites than lacked detectable melatonin.

Melatonin was previously observed to effect contraction of sheep sphincter pupillae in isolated organ experiments (personal communication, Dr S.M.O. Hourani, University of Surrey) therefore membranes prepared from both this tissue and a number of other sheep ocular tissues were also probed for 2-[125I]iodomelatonin binding sites. No evidence was found for the presence of a melatonin binding site in ovine sphincter muscle. Saturable, specific binding was demonstrated only in NR. Human iris tissues were subsequently also investigated. In contrast with the ovine findings, studies with human iris muscle membranes provided weak evidence for a 2-[125I]iodomelatonin binding site that was specific to the sphincter pupillae. The iris studies reported in this thesis were intended to be preliminary only. The iris has attracted little, if any, attention previously with respect to melatonin binding site investigations. The possible existence of a functional melatonin receptor in this structure raises several questions regarding its physiological significance. For example, if it is found to mediate an effect on pupil diameter what are the implications, for circadian photoreception?

If, because of the post-mortem nature of the specimens, the findings of the studies described do not accurately represent the human ocular melatonin system in vivo, further use of the tissues in similar investigations will not be justified. Ideally, future studies should be undertaken on freshly enucleated specimens from live donors. These specimens are rarely available however. Given the extreme difficulty in obtaining such tissues maximum use has to be made of those tissue sources that are available, that is, post-mortem specimens. Hence it would be prudent for future investigations to address the human ocular melatonin system from alternative angles, that is, to study instead those aspects which may be less affected by the post-mortem nature of specimens. Firstly, it will perhaps be of benefit to investigate its generating system, that is, the synthetic enzymes NAT and HIOMT. This could be done by either assessing their catalytic activity or by molecular biology techniques. As previously mentioned, gene transcripts of both NAT and HIOMT have recently been located in human retina. It is accepted, however, that neither enzyme studies nor molecular techniques would escape the detrimental affects of tissue death. In conjunction with investigations of the synthetic system, pathways of metabolic degradation should also be assessed as these may be partially responsible for the low melatonin levels observed. The deacetylase route is believed to be absent in mammals (Grace et al., 1991), but it has not been investigated specifically in the human eye. The possible regulation of ocular melatonin levels by a cytochrome P-450 mediated mechanism is also of potential significance given that this enzyme is present within the eye (Shichi et al., 1975; Schwartzman et al., 1987; Zhao and Shichi, 1995). With respect to the deacetylase route, identification of an active metabolic system such as this may be indicative of a means for rapid termination of melatonin's actions. Alternatively, it may represent a route by which other biologically active molecules are produced. Hence, it may be of interest to widen the investigation of human
melatonin to encompass additional methoxyindoles such as 5-methoxytryptamine and 5-methoxytryptophol. The action(s) of melatonin within the eye also remain to be considered. There is no reason to believe that these would be dissimilar to those established in other mammalian species (section 1.7), therefore the starting point will be to search for evidence of any of these.

With respect to future study of the melatonin binding sites in human NR and C-RPE, it is unlikely that continued direct investigation as was undertaken in these studies will reveal much additional information, given the low proportion of specimens which appear to contain the binding site. Following the recent cloning of the melatonin receptor, availability of probes for the three receptor subtypes will allow gene expression to be studied instead. Reppert and co-workers (1994) have suggested that post-mortem loss of Mel₁₆ mRNA may be responsible for some of the difficulty in detecting it in human brain. Consequently this is a possibility when investigating the eye, although HIOMT mRNA has been detected (albeit at low levels) in human retina up to 13 hours post-mortem (Bernard et al., 1995).

Further study of the putative iris 2-[^125]I]iodomelatonin binding site described is essential. The small amounts of tissue provided by each eye specimen necessarily precludes full characterisation of the site(s) in membrane preparations. The molecular methods described above could also be applied to the iris tissues. Given that there are no existing reports concerning melatonin binding sites in the iris it will be worthwhile for the search to be extended to other species in the first instance, in order that post-mortem effects are eliminated. If binding was found, this species could be used as an experimental model for method development prior to further human iris studies.

Cellular responses following melatonin binding were not investigated in the human studies. The binding sites described therefore cannot strictly be referred to as receptors. Hence, it is also necessary that functional studies are undertaken. Whether the human tissues are capable of such responses is questionable given their post-mortem nature. Taking into account all existing animal evidence relating to the ocular melatonin system, however, future identification of the human ocular melatonin binding site as a true receptor is probable, in the appropriate tissue source, that is freshly enucleated human eyes, or post-mortem specimens frozen immediately following donor death.

In the absence of freshly enucleated specimens from live donors, human cell cultures represent an alternative model for investigation of several aspects of the human ocular melatonin system. The Y79 cell line, derived from a human retinoblastoma, is increasingly being used for this purpose (section 1.11). These cells possess the requisite synthetic enzymes and are capable of melatonin synthesis (Pierce et al., 1989; Deng et al., 1991). Use of an immortal cell line such as this has its limitations however, as cellular physiology may deviate from that of normal NR. Useful information concerning the RPE and its relationship with melatonin may also be gained from culture systems. Human RPE cultures have previously been utilised in a number of research areas. Successful primary or secondary cultures can be prepared from relatively old post-mortem specimens (up to 72 hours) (Boulton et al., 1983; Miceli and Newsome, 1991; Osborne et al., 1991) hence the human eye source currently available would be ideal for generation of such cultures. One RPE cell line (K-1034) has also been established (Kigasawa et al., 1994) and this may represent another model for investigation.
The human ocular melatonin system is deserving of study for a number of reasons, however, there still exists very little information about it. Limited availability of appropriate tissue sources for investigation, that is freshly enucleated eyes from live donors, has undoubtedly been partly responsible for this lack of data. The present studies aimed to quantify melatonin and investigate melatonin binding sites in post-mortem human eyes. Evidence is provided for both the existence of melatonin and its binding sites in NR and C-RPE tissues thus further justifying the need for continued investigation in this area of research.
Publications and presentations resulting from this thesis

Publications


Presentations

Oral:


Poster:


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"The progress of true science, which is the experimental kind, is necessarily slow."

M. de Mairan, 1729