Retinal control of eye growth and form-deprivation myopia

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of
The Australian National University

by

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DECLARATION

I declare that the research material in this thesis has not been submitted or accepted for the award of any other degree or diploma in any university. The material presented in this thesis is my own work, and, to the best of my knowledge, contains no material previously published or written by another person, except when due reference in made in the text.

All experiments were carried out according to the NH&MRC ‘Code of practice for the care and use of animals for experimental purposes’ and were covered by The ANU Animal Experimentation Ethics Committee protocol numbers RVS.9.93 and RVS.11.97.

Malini Dornadla
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>A</td>
<td>amacrine cell</td>
</tr>
<tr>
<td>Ac</td>
<td>acetate</td>
</tr>
<tr>
<td>Asn</td>
<td>asparagine</td>
</tr>
<tr>
<td>B</td>
<td>bipolar cell</td>
</tr>
<tr>
<td>bFGF</td>
<td>basic fibroblast growth factor</td>
</tr>
<tr>
<td>C</td>
<td>cone</td>
</tr>
<tr>
<td>D</td>
<td>dioptries</td>
</tr>
<tr>
<td>DAC</td>
<td>dopaminergic amacrine cell</td>
</tr>
<tr>
<td>DOPA</td>
<td>3,4-dihydroxyphenylalanine</td>
</tr>
<tr>
<td>DOPAC</td>
<td>3,4-dihydroxyphenylacetic acid</td>
</tr>
<tr>
<td>ECMA</td>
<td>ethylcholine mustard aziridinium ions</td>
</tr>
<tr>
<td>ENSLI</td>
<td>enkephalin-, neurotensin-, somatostatin-like immunoreactivity</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>EW</td>
<td>Edinger-Westphal</td>
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<tr>
<td>FDM</td>
<td>form-deprivation myopia</td>
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<tr>
<td>G</td>
<td>ganglion cell</td>
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<tr>
<td>GAG</td>
<td>glycosaminoglycan</td>
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<tr>
<td>Gal</td>
<td>galactose</td>
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<td>GalNAc</td>
<td>N-acetyl-galactosamine</td>
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<td>ganglion cell layer</td>
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<td>GlcNAc</td>
<td>N-acetyl-glucosamine</td>
</tr>
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<td>GlcUA</td>
<td>glucuronic acid</td>
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<tr>
<td>H</td>
<td>horizontal cell</td>
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<tr>
<td>HA</td>
<td>hyaluronic acid</td>
</tr>
<tr>
<td>HABR</td>
<td>hyaluronic acid binding region</td>
</tr>
<tr>
<td>I</td>
<td>interplexiform cell</td>
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<tr>
<td>IdU</td>
<td>iduronic acid</td>
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<tr>
<td>IGF</td>
<td>insulin growth factor</td>
</tr>
<tr>
<td>INL</td>
<td>inner nuclear layer</td>
</tr>
<tr>
<td>IOP</td>
<td>intraocular pressure</td>
</tr>
<tr>
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<td>inner plexiform layer</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>M</td>
<td>Muller cell</td>
</tr>
<tr>
<td>Man</td>
<td>Mannose</td>
</tr>
<tr>
<td>NAT</td>
<td>N-acetyltransferase</td>
</tr>
<tr>
<td>NeuNAc</td>
<td>sialic acid</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>6-OHDA</td>
<td>6-hydroxydopamine</td>
</tr>
<tr>
<td>OPL</td>
<td>outer plexiform layer</td>
</tr>
<tr>
<td>PAPS</td>
<td>3′-phosphoadenosine 5′-phosphosulphate</td>
</tr>
<tr>
<td>PRL</td>
<td>photoreceptor layer</td>
</tr>
<tr>
<td>R</td>
<td>rod</td>
</tr>
<tr>
<td>RPE</td>
<td>retinal pigment epithelium</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>Ser</td>
<td>serine</td>
</tr>
<tr>
<td>SO₃⁻</td>
<td>sulphate</td>
</tr>
<tr>
<td>TH</td>
<td>tyrosine hydroxylase</td>
</tr>
<tr>
<td>VIP</td>
<td>vasoactive intestinal peptide</td>
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PUBLISHED WORK

Many parts of this thesis have been presented at conferences during the course of my PhD. These include the Asia-Pacific colloquium in Neuroscience in Singapore (1994), the National Ophthalmic and Visual Science meetings in Geelong (1994, 1995) and Canberra (1996), and the Australian Neuroscience Society meetings in Perth (1995), Adelaide (1996) and Newcastle (1997). In addition to this, the following papers have been published including sections of work from this thesis:


ABSTRACT

Sulphate incorporation was measured in chicken sclera after 1h incubation at midday in a buffer containing labelled sulphate. The amount of sulphate incorporated increased as the length of the incubation increased, and as the concentration of labelled sulphate in the buffer increased. The labelled sulphate measured was not free sulphate, but rather, was incorporated into glycosaminoglycans (GAGs), with most of the counts in either dermatan sulphate or chondroitin sulphate, and some in heparin.

Animals were maintained on a 12:12h light:dark cycle, with lights coming on at 0600h, and going off at 1800h. Under these conditions there was a diurnal rhythm in rates of sulphate incorporation measured over a 24h period, with higher rates observed at midnight compared to midday. However, this rhythm was the opposite of what was expected, given that Weiss and Schaeffel (1993) had observed higher rates of axial elongation during the day and lower rates at night. The diurnal difference in rates of sulphate incorporation disappeared with age, suggesting there may be a maintenance component which is related to normal proteoglycan turnover with a light modulated, phasic component responsible for the growth seen during early development. The distribution of counts into various GAGs was different at midnight compared to midday, possibly reflecting the differential synthesis of different GAGs.

Lights left on at 1800h suppressed the normally high rate of sulphate incorporation seen at midnight. Similarly, lights put on at midnight brought down the high rate of sulphate incorporation normally seen at 0600h. The diurnal rhythm of sulphate incorporation could not be maintained under constant dark conditions, suggesting it is not under circadian control, but rather is controlled by the external lighting conditions, with light acting as a suppressive agent.

This suppressive ability of light was light intensity-dependent, with light intensities of 0.4 lux and below unable to cause the suppression. This light intensity dependence is similar to that of the retinal dark-light switch, suggesting that this retinal circuit may be involved in controlling rates of eye growth. However, the light intensity which flips the retinal dark-light switch from its dark to its light state is slightly lower than 0.4 lux.

An increase in rates of sulphate incorporation was seen in eyes which had worn a translucent diffuser which reflected an increase in the synthesis of proteoglycans containing the GAGs dermatan sulphate and chondroitin sulphate. This increase was accompanied by an increase in axial length, an increase in the weight of the eye, and an increase in the eye’s refractive error, making it myopic. Therefore, this assay of sulphate incorporation can be used to monitor changes in scleral growth rates in response to FDM. Removal of the translucent diffuser after 10 days of wearing resulted in a decrease in rates of sulphate incorporation, with the eye in parallel reducing its rate of growth to compensate for defocus.
The increase in rates of sulphate incorporation associated with FDM could be prevented by exposing the animal to brief periods of normal vision each day. This preventive ability of normal vision was also dependent on light intensity, with light intensities of 0.4 lux and below unable to exert any preventive effect, once again suggesting a role for the retinal dark-light switch.

The diurnal rhythm of sulphate incorporation was maintained during deprivation, although at a higher rate. This was not what was expected, since the phasic component was thought to be the regulatory component, which would be expected to change when normal eye growth is disrupted. Furthermore, given that Weiss and Schaeffel (1993) showed that the diurnal rhythm in rates of axial elongation was abolished during deprivation, it was surprising that the rhythm in rates of sulphate incorporation was maintained during deprivation. However, more recently, Nickla et al. (1997) made more frequent measurements, and demonstrated that the rhythm in rates of axial elongation was maintained during deprivation, concluding that Weiss and Schaeffel (1993) had missed the rhythm because they only made measurements every 12h. The results in this thesis suggest that the phasic component of eye growth remains during FDM, but that there is overall up-regulation of the tonic component which results in the overall increase in rates of sulphate incorporation, and presumably accounts for the larger eye observed.

The compounds released by the elements of the retinal dark-light switch (melatonin, enkephalin, neurotensin, somatostatin and dopamine), as well as acetylcholine were tested for effects on sulphate incorporation. They were tested for either direct effects on the sclera (in vitro), which would suggest the presence of the relevant receptors on the sclera, or indirect effects (in vivo), which would suggest that the compound was acting on intraocular tissues, possibly the retina, which generated further signals which affected the sclera. Melatonin, enkephalin, neurotensin and somatostatin are released in the dark, when rates of sulphate incorporation were highest, so these compounds were expected to have a stimulatory effect on rates of sulphate incorporation. Conversely, dopamine is released during the day, and so was expected to have an inhibitory effect.

Melatonin had a significant stimulatory effect on rates of sulphate incorporation when tested directly, and was not tested indirectly due to its lability. However, there was no change in the rate of NAT activity (which is a measure of melatonin release) during FDM, and given that melatonin is so labile, it is difficult to understand how melatonin released from the retina could reach the sclera in physiologically significant concentrations. Therefore, it appears unlikely that retinal melatonin is involved in controlling rates of eye growth.

The enkephalins had a stimulatory effect on rates of sulphate incorporation in vitro, in accord with predictions, however this effect could only be seen when peptidase inhibitors were present in the incubation medium. The opioid antagonist naloxone
prevented the stimulatory effect of the enkephalins. The lability of the enkephalins makes it difficult to understand how they could travel from the retina to the sclera to exert their effects. Neither neurotensin nor somatostatin had any effect on rates of sulphate incorporation when tested directly. None of the peptides were tested in vivo due to their lability. These results suggest that enkephalins released from the retina are unlikely to regulate scleral growth, but suggest that non-retinal opioid mechanisms may be involved. In fact, the involvement of opioid mechanisms in the control of cell proliferation has been shown in other systems (Zagon and McLaughlin, 1987).

The dopaminergic agonist ADTN stimulated rates of sulphate incorporation which was the opposite of what was expected. However, at higher concentrations, ADTN inhibited sulphate incorporation, which agreed with predictions from its release in the light. However, its effect in vitro was at concentrations much higher than those which would be physiologically significant. ADTN also had an inhibitory effect on rates of sulphate incorporation when tested in vivo, at concentrations similar to those which were effective in vitro. This effect was at a concentration higher than that which can flip the retinal dark-light switch from its dark to light state. Therefore, it appears that the dopaminergic agonist ADTN is not able to control rates of scleral growth at physiologically relevant concentrations, presumably indicating the dopamine is not involved in this process either. Therefore, it does not appear that any of the neurotransmitters released by elements of the retinal dark-light switch are involved in controlling rates of scleral growth.

None of the cholinergic agonists or antagonists tested had any effect on rates of sulphate incorporation in vivo, suggesting that acetylcholine released from the retina is unlikely to control rates of sulphate incorporation. The non-selective antagonist atropine had significant suppressive effects on rates of sulphate incorporation when tested directly, however, this effect is probably due to pathological effects on the sclera.

It appears that there is both a phasic component, and a tonic component which are involved in controlling eye growth. While the phasic component is only dependent on light intensity, the tonic component is dependent on light intensity, and on the quality of the image received by the eye. Since these components are affected by different environmental conditions, it suggests that they are controlled by two different features of the visual input, involved in different retinal processing pathways, and utilise different retinal messengers to signal to the sclera. While dopamine release from the retina does not appear to be involved in controlling the phasic component, it may be a controlling factor of the tonic component. Acetylcholine release from the retina does not appear to be involved in controlling rates of eye growth, but there may be cholinergic receptors on the sclera which are involved.
CHAPTER 1. INTRODUCTION

1.1 General introduction

Myopia, or short sightedness, is a problem throughout the world. At birth, most neonates are hyperopic, with a mismatch between the axial length of the eye and its optical power resulting in the image falling behind the retina. During development the eye continues to grow in the axial direction and at the same time there are changes in its optical power. If the correct amount of growth occurs, the axial length of the eye matches its optical power, so that the image of objects at infinity falls on the retina. This is known as emmetropia. However, if for some reason there is excessive growth, the eye becomes too long for its optical power, and the image falls in front of the retina, resulting in myopia.

There is an increasing incidence of myopia world-wide, which has resulted in an increasing burden of health costs of spectacles, contact lenses and corrective surgery. More serious still is the consequence of blindness which can result from extreme conditions of axial myopia, known as pathological myopia. For these reasons, many epidemiological studies have looked for clues as to what might be causal factors of myopia. A racial factor has been suggested, as the distribution of myopia throughout the world varies between races. In addition to this, education (with which reading is associated) and near work (such as fine needlework when there is prolonged focussing at distances close to the eye) have been identified as factors causing myopia, since groups which are involved in high levels of education or near work also have high incidences of myopia. However, epidemiological studies can only give a limited amount of information. To understand what it is in near work or education which causes the excessive eye growth observed, a system is required in which factors that control eye growth can be controlled more precisely, and in which the changes can be studied at the cellular level. For this reason, animal models of myopia have been developed.

The most common model of myopia is known as form-deprivation myopia (FDM) and is induced by depriving an eye of form vision using a translucent diffuser. This results in the deprived eye growing excessively in the axial direction and becoming myopic. The other eye remains as a control, so that differences between the two eyes can be identified. The animal most commonly used in studies of FDM is the chicken, which is the animal used in the present study.

In order for the eye to grow excessively in the axial direction, there has to be an increase in the growth of the sclera. As disruption to normal vision is responsible for the development of FDM, the retina is a prime candidate as a controlling factor of eye growth. Many retinal neurotransmitters such as dopamine, acetylcholine, vasoactive intestinal peptide (VIP) and the enkephalins have been implicated in FDM. However,
what is unknown is which, if any of these compounds are crucial, and how these compounds exert their effects on the sclera. Therefore one of the aims of the present studies in this field is to investigate possible factors responsible for controlling scleral growth and pathways by which these factors may act.

1.2 The eye

1.2.1 The cornea and lens: their role in accommodation

One of the powerful features of the eye is its ability to change its plane of focus. The angle at which light reflected from an object enters the eye depends on the distance of the object from the eye. When an emmetropic eye is viewing an object at infinity, parallel rays of light reflected from the object enter the eye, pass through the cornea and lens (whose location can be seen in Fig. 1.1) and focus on the retina. However, as an object is brought closer to the observer, the angle of light impinging on the eye increases, resulting in the image being focused behind the retina. To compensate for this, the curvature of the lens increases, bringing the focus back onto the retina. This process is called accommodation (Fig. 1.2). When the eye moves to focus on a closer object, retinal signals stimulate the Edinger-Westphal (EW) nucleus, (or accessory ocular motor nucleus) in the brain which in turn causes the ciliary muscles to contract via the ciliary ganglion. Ciliary muscles in turn increase the curvature of the lens when they contract.

The primary refracting surface of the eye is actually the cornea, accounting for up to 75% of the refractive power in humans. Unlike humans, there is some evidence that some birds (such as the pigeon, mallard duck, owl and chicken) can also increase the curvature of the cornea, giving these animals an accommodative advantage over other animals. There have also been reports that these birds do not have this accommodative ability (for review see Glasser et al., 1994). However, more recently, it has been shown that in the chicken, up to 40% of accommodation generated by electrically stimulating the EW nucleus can be accounted for by changes in corneal curvature (Troilo and Wallman, 1987). These changes could not be accounted for by a change in intra-ocular pressure (IOP). Rather, the ciliary muscles when they contract not only change lens shape but also pull the inner lamella of the cornea. Therefore the central cornea becomes steeper, whereas there is flattening of the peripheral cornea (Glasser et al., 1994).

1.2.2 The neural retina

The retina lines the back of the eyecup (Fig. 1.1) and is the light-sensitive, neural component of the eye. It is an anatomically structured tissue made up of distinct layers which are characterised by the cell bodies or processes they contain.
Figure 1.1 Schematic representation of the eye.

A schematic representation of a cross section of the human eye, showing the location of the cornea, lens, retina, RPE, choroid and sclera (modified from Kandel et al., 1991).
Fixation point

Cornea

Lens

Sciera

Pigment epithelium

Optic disc

Choroid

Retina

Fovea

Sclera

Optic nerve
Figure 1.2 Accommodation.

A) An eye looking at infinity, with parallel rays of light entering the eye and striking the retina.

B) An eye looking at an object at close distance. The rays of light entering the eye are diverging and therefore after refraction meet at a point behind the retina.

C) An eye looking at an object at close distance. This time the eye accommodates by increasing the curvature of the lens so that the rays of light once again converge on the retina.
Photoreceptors (blue) which operate in dim light and cones which operate in bright light) capture the light entering the eye. This light signal is transduced into an electrical signal which flows through the retina until it reaches the ganglion cell layer. The axons of the ganglion cell form the optic nerve, which transmits visual information to the visual cortex in the brain, where it can be further processed. Before reaching the ganglion cells, however, the signal is modulated and modulated by the horizontal cells, bipolar cells and the amacrine cells.

Structure and layers of the retina

The ordered layers of the retina are shown in figure A. Photoreceptors lie in the outer layer or photoreceptor layer (RP), followed by bipolar cells, horizontal cells and the amacrine cell layer (PCL). The second nuclear layer is called the inner nuclear layer (INL) and is formed by the cell bodies of the horizontal cells, bipolar cells, and amacrine cells. The inner plexiform layer (IPL) contains the processes of the bipolar cells, amacrine cells, interplexiform cells and ganglion cells. The innermost nuclear layer is the ganglion cell layer (GCL), which contains the cell bodies of the ganglion cells.

The amacrine cells are a large group of cells which have many different functions, which were initially classified depending on their morphology or function (Laurie et al. 1988). They are classified into categories, with each co and function. These are not amacrine cells being identified. The simple physiological characterization that do not correspond to the complexity of the morphology classification. More recent studies looking at the transmitters located to particular amacrine cells have shown that there is a biochemical diversity which corresponds to the morphological diversity so that an area in the retina is associated with a particular amacrine cell depending on neurotransmitters (the review of Dingwall and Kriegstein, 1997). For example, there are amacrine cells which contain dopamine, the dopamine receptors. Some amacrine cells contain monoamine or noradrenaline (Kolb and Eckenstein, 1994). There are differences in the physiological and functional differences that have been identified.

Photoreceptors form conventional synapses with both the bipolar cells and the horizontal cells. In addition to this, the horizontal cells can form gap junctions with each other, as well as with bipolar cells. Bipolar cells can make synapses with amacrine cells and ganglion cells. Amacrine cells can make synapses with bipolar cells, other amacrine cells and ganglion cells. This wiring setup means that there are two main pathways through which information can pass. The first is a direct pathway from the photoreceptors to the ganglion cells via the bipolar cells. The second type of...
Photoreceptors (rods which operate in dim light and cones which operate in bright light) capture the light entering the eye. This light signal is transduced into an electrical signal which flows through the retina until it reaches the ganglion cell layer. The axons of the ganglion cell form the optic nerve, which takes visual information to the visual cortex in the brain, where it can be further processed. Before reaching the ganglion cells, however, the signal is mediated and modulated by the horizontal cells, bipolar cells and the amacrine cells.

Structure and wiring of the retina

The ordered layering of the retina can be seen in Fig. 1.3. Photoreceptors lie in the outer layer or photoreceptor layer (PRL). Processes of the photoreceptors, bipolar cells, interplexiform cells and horizontal cells form the outer plexiform layer (OPL). The second nuclear layer is called the inner nuclear layer (INL) and is formed by the cell bodies of the horizontal cells, bipolar cells, interplexiform cells and amacrine cells. The inner plexiform layer (IPL) contains the processes of the bipolar cells, amacrine cells, interplexiform cells and ganglion cells. The innermost nuclear layer is the ganglion cell layer (GCL), which contains the cell bodies of the ganglion cells.

The amacrine cells are a large group of cells which have many different functions, which were initially classified into various types depending on the morphology of both their cell body and of their processes, using Golgi techniques (Ramon Y Cayal, 1893). Physiologically, amacrine cells have also been classified into categories, with both on- and off-, sustained and transient amacrine cells being identified. The simple physiological classifications do not appear to match the complexity of the morphological classification. More recently, studies looking at the transmitters located to particular amacrine cells have shown that there is a biochemical diversity which corresponds to the morphological diversity so that an amacrine cell of a particular morphology is associated with one or more neurotransmitters (for review see Morgan, 1991). For example, there is a population of amacrine cells which contain dopamine, the dopaminergic amacrine cells (Iuvone et al., 1978). Some amacrine cells contain more than one neurotransmitter such as the ENSLI amacrine cells. These cells contain enkephalin, neurotensin and somatostatin (Watt and Florack, 1994; Wu and Lam, 1988). These differences are presumably associated with more subtle physiological and functional differences that have not yet been identified.

Photoreceptors form conventional synapses with both the bipolar cells and the horizontal cells. In addition to this, the horizontal cells can form gap junctions with each other, as well as with bipolar cells. Bipolar cells can make synapses with amacrine cells and ganglion cells. Amacrine cells can make synapses with bipolar cells, other amacrine cells and ganglion cells. This wiring setup means that there are two main pathways through which information can pass. The first is a direct pathway from the photoreceptors to the ganglion cells via the bipolar cells. The second type of
Figure 1.3 A schematic representation of the retina.

A cross section of the retina, showing the various layers of the retina.

R - rods
C - cones
M - Muller cells
H - horizontal cells
B - bipolar cells
I - interplexiform cells
A - amacrine cells
G - ganglion cells
ONL - outer nuclear layer
OPL - outer plexiform layer
INL - inner nuclear layer
IPL - inner plexiform layer
GCL - ganglion cell layer
Choroidal Border

Pigment Epithelium

Outer Segments

Photoreceptor Layer

ONL

OPL

INL

IPL

GCL

Optic Nerve

Vitreal Border
pathway is less direct and involves amacrine cells interconnecting the bipolar cells and ganglion cells. Therefore the bipolar cells feed information from the outer to the inner retina, by making both direct and indirect connections between the photoreceptors and the ganglion cells.

**Retinal processing**

The output of the ganglion cells reflects retinal processing and transmits signals to the visual centres of the brain for further processing. Some ganglion cells have a receptive field consisting of two concentric rings which are antagonistic to each other and are either on-centre or off-centre cells. On-centre cells fire a sustained burst of impulses when light is shone on the centre of their receptive field. Stimulation of the surround inhibits responses. Off-centre cells have the reverse response, with light on the centre of the receptive field inhibiting the cells firing and light on the surround stimulating it. These cells have sustained responses and reflect the spatial distribution of light, since their response depends where in their receptive field the light is shone. Therefore these cells are sensitive to spatial contrast and best respond when the difference in light intensity between the centre and surround of the receptive fields is maximal.

Other ganglion cells increase their firing rate when there is a change in stimulation and therefore have transient responses. When static spots of light are shone anywhere on the receptive field the cells fire a burst of impulses at the onset of illumination, and again at the cessation of illumination. As these cells are more responsive to changes in illumination over time they are involved in temporal processing of the retina. Other ganglion cells have more complex spatio-temporal requirements and ‘trigger features’ for responding.

Most ganglion cells, whether sustained or transient, are controlled by overall retinal adaptation mechanisms, which tend to have a longer time course to suppress responses unless there are temporal changes. However, if the animal remains stationary and looks at an unchanging scene, then over a period of minutes these cells will eventually cease to respond. Evidence of this has come from experiments where eye movements were compensated for by opposing movements of the scene using mirrors, so that the net result was no movement on the retina. After a while, the subjects could no longer see, with blur obscuring the form of objects (Riggs *et al.*, 1953). Temporal change at points on the retina is therefore vital for continued visual responsiveness.

Spatial contrast comes from different spatial distributions of light intensities in the field of view. These differences in the spatial distribution of light stimulate the ganglion cells with centre-surround organisation. Spatial contrast is automatically translated into temporal contrast at a point on the retina by movement of the head or eye, so that there is change in contrast over time at any given point in the retina,
stimulating the ganglion cells with transient responses, and preventing longer-term adaptation.

Temporal contrast is removed by blur, as this removes spatial contrast. This in fact is what is done in the FDM paradigm, by using translucent diffusers. However, as spatial contrast is required for temporal contrast it is difficult to identify which is involved in FDM and if both are, what their roles are and whether they are independent of each other.

1.2.3 The retinal pigment epithelium and the choroid

The retinal pigment epithelium (RPE) is a layer of epithelial cells adjoining the outer segments of the photoreceptors via gap junctions. These cells make contact with photoreceptors with long villous processes, which extend down to the inner segments. As the RPE lies between the choroid and the retina, it plays a role in transporting fluid, nutrients and metabolites between these two organs (Steinberg and Miller, 1979).

The choroid consists of two parts. The first is a choriocapillaris; a network of capillaries which lies just behind the RPE. The main part of the choroid has many larger blood vessels, and in birds also has large lacunae. In between these blood vessels there is extracellular matrix, smooth muscle fibres, fibroblasts and pigmented cells. As the retinas of birds are avascular, the choriocapillaris may be responsible for nourishing the outer layers of the retina (Rochon-Duvigneaud, 1943). Another possible role for the choroid is that of a source and sink of ions for the retinal pigment epithelial cells, as these cells contribute to the trans-retinal potential (Bill, 1985). It has also been suggested that the cholinergic activation of the choroid via the ciliary ganglion may regulate choroidal blood flow and intraocular pressure (Meriney and Pilar, 1987).

1.2.4 The sclera

The sclera forms the back of the eye cup and is made of connective tissue which provides structural support for the eye. In humans (and indeed all mammals), the sclera is a fibrous tissue consisting of extracellular matrix containing collagen fibre bundles, interspersed with flattened fibroblasts and elastic fibres. The chicken sclera is different to mammalian sclera as it has two distinct layers. In addition to the fibrous layer, which is similar to that found in mammals, there is an inner, large cartilaginous layer. Cartilage consists of collagen and proteoglycans, with a few cells (chondrocytes) within this extracellular matrix. Collagen gives the cartilage its tensile strength, whereas its resilience comes from proteoglycans. Proteoglycans are a large family of molecules which consist of unbranched polysaccharide chains attached to protein (for reviews see Hardingham and Fosang, 1992; Kjellen and Lindahl, 1991; Ruoslahti, 1988). These unbranched polysaccharides, which are known as glycosaminoglycans (GAGs), consist of 2 alternating sugar residues and can also be sulphated. The core proteins, each of
which have hundreds of GAGs attached, are themselves attached to another GAG called hyaluronic acid (HA), which forms the backbone of the proteoglycan.

In addition to HA, there are 5 other types of GAGs; heparin, heparan sulphate, dermatan sulphate, chondroitin sulphate and keratan sulphate. There are many types of proteoglycans containing different GAG chains, with varying functions such as having roles in matrix assembly, cell adhesion and cell differentiation. Some proteoglycans maintain the structure of connective tissue, such as aggrecan, the main proteoglycan of cartilage. Aggrecan is a large (core size about 200 kDa) proteoglycan which has the GAGs chondroitin sulphate and keratan sulphate as constituents. When pressure is applied to cartilage, water is forced away from the proteoglycan’s negative domains and as they come closer together, the repulsive forces of these charges prevent any further compression. Other smaller proteoglycans found in connective tissue include decorin and biglycan, which contain chondroitin sulphate and dermatan sulphate GAGs, and fibromodulin which contains keratan sulphate. These proteoglycans are synthesised by fibroblasts and are therefore found in the fibrous tissue of the sclera. Since the chicken sclera has a cartilaginous layer as well as a fibrous layer, it contains aggrecan as well as decorin, biglycan and fibromodulin (Heinegard and Oldberg, 1989).

Structure of proteoglycans

Studies of the structure and function of proteoglycans have been carried out predominantly in the cartilage of bones. Figure 1.4 shows the structure of a typical cartilage proteoglycan, aggrecan, whose structure will be described below. The central strand of a proteoglycan is hyaluronic acid, a repeating disaccharide, which means it consists of an alternation of two sugars, glucuronic acid and glucosamine. This HA chain has specific binding regions to which core proteins can attach. For reviews of proteoglycan structure, see (Hardingham and Fosang, 1992; Kjellen and Lindahl, 1991; Ruoslahti, 1988).

Core proteins are amongst the largest known proteins synthesised by any cell, the largest being up to 350,000 kD. There are many types of core proteins, however they all have the same basic structure with three distinct regions. The N-terminus of the core protein folds into a ball, and this globular region binds to HA by making an elaborate fit with 5 of its disaccharide units rather than being covalently bound. This is known as the hyaluronic acid binding region (HABR). A link protein stabilises the interaction between hyaluronic acid and the core protein. This region has only a few N-linked, high mannose oligosaccharides attached to asparagine residues in the core protein.

The second region is characterised by O-linked oligosaccharides which are attached to serine residues of the core protein. Most of these O-linked oligosaccharides bind keratan sulphate, a GAG which is a repeating disaccharide consisting of galactose
Figure 1.4 Structure of a proteoglycan.

A schematic representation of a proteoglycan containing a hyaluronic acid backbone with core proteins attached. A link protein stabilises this interaction. Both N-linked oligosaccharides and O-linked oligosaccharides are attached to the core protein, to which GAGs such as keratan sulphate and chondroitin sulphate can attach (modified from Voet and Voet, 1990).

Asn - asparagine
Ser - serine
GlcNAc - N-acetyl-glucosamine
Man - mannose
Gal - galactose
NeuNAc - sialic Acid
GalNAc - N-acetyl-galactosamine
Link protein

Core protein

N-linked oligosaccharides

O-linked oligosaccharides

Keratan sulfate

Chondroitin sulfate

N-acyethylglucosaminyltransferase (NAGT) attaches N-acetylglucosamines and glucuronic acids to the third residue of the protein. Each is connected to an O-linked oligosaccharide by a linkage region containing sialic acid, galactose, and xylose, among other sugars. The region adjacent to the core protein is more densely glycosylated. The glycosylation of a family of proteoglycans in the extracellular matrix is essential for the function of the glycosaminoglycan. The link protein associates with the proteoglycan's core protein and aggregates with the collagen fibrils. The proteoglycan's core protein is secreted into the extracellular matrix in vesicles which bind to the collagen. This complex then aggregates with the collagen, forming a large aggregate that is thought to hinder cell migration (this review is somewhat outdated).
and N-acetyl glucosamine. In addition to this, there are also small O-linked oligosaccharides, which are most likely unused sites for keratan sulphate synthesis.

Chondroitin sulphate (another GAG with repeating units of N-acetylglactosamine and glucoronic acid) characterises the third region of the core protein. Each is connected to an O-linked oligosaccharide by a serine residue, and a linkage region containing xylose, galactose, galactose and glucuronic acid. Other proteoglycans with various combinations of chondroitin sulphate, dermatan sulphate, heparan sulphate, heparin or keratan sulphate are also assembled in a similar manner. The repeating units of all of these GAGs can be seen in Fig. 1.5.

Assembly of proteoglycans

The assembly of proteoglycans is a complicated process involving a large number of enzymes, with each one having a specific task. The assembly of the major cartilage proteoglycan aggrecan will be discussed here (for review see Poole, 1986; Silbert, 1987). While formation of the sugar nucleotides occurs in the cytoplasm of the cell, the assembly of proteoglycans occurs in the intracellular membranous part of the cell. Core proteins are synthesised in the endoplasmic reticulum (ER) of the chondrocyte. The ER is also where the N-linked high mannose oligosaccharides are added to the core protein, one at a time. About 20-30 enzymes are required to prepare the GAGs and couple them to the core protein. The O-linked oligosaccharides and GAGs such as chondroitin sulphate and keratan sulphate are added in the Golgi apparatus. In the case of chondroitin sulphate each of the 3 sugars in the linkage region is connected to the next one by a specific enzyme. After this linkage region is completed, the GAG chain is completed one sugar at a time with two enzymes working in alternation to produce the repeating disaccharide structure which is a feature of GAGs. In the case of chondroitin sulphate, the disaccharides are N-acetylgalactosamine and glucuronic acid.

Elongation of the GAG is temporally terminated by its sulphation. The sulphation enzymes, sulphotransferases, transfer sulphate from the donor 3'-phosphoadenosine 5'-phosphosulphate (PAPS) to a site on the GAG. In heparin and heparan sulphate, there is N-sulphation, whereas in the other GAGs there is O-sulphation. In the case of chondroitin sulphate, sulphation can occur at either the 4-position or the 6-position, with one enzyme working at each of these positions.

The link protein is synthesised separately and aggregates with the completed proteoglycan monomer complex, and the link protein-proteoglycan complex is secreted into the extracellular matrix in vesicles which bud off the Golgi. This complex then aggregates with HA (which is also synthesised separately in the inner surface of the plasma membrane) in the extracellular matrix. Once this process is completed, aggrecan can then bind to other matrix molecules (for review see Ruoslahti, 1988).
Figure 1.5 Glycosaminoglycans of proteoglycans

This figure shows the repeating sequence of each GAG, and where each one is sulphated (modified from Poole, 1986).

GlcUA - glucuronic acid  
GalNAc - N-acetyl-glucosamine  
SO$_3^-$ - sulphate  
IdUA - iduronic acid  
GlcN - glucosamine  
Ac - acetate  
Gal - galactose  
* heparin variably sulphated
Chondroitin sulphate

\[
\begin{array}{c}
\text{IdUA} \quad \rightarrow \quad \text{GalNAc} \quad \rightarrow \quad \text{GlcUA} \\
\quad \quad \quad \quad \quad 4-\text{or-6 SO}_3^- \\
\end{array}
\]

Dermatan sulphate

\[
\begin{array}{c}
\text{IdUA} \quad \rightarrow \quad \text{GalNAc} \quad \rightarrow \quad \text{GlcUA} \\
\quad \quad \quad \quad 2-\text{SO}_3^- \quad 4-\text{or-6 SO}_3^- \\
\end{array}
\]

Heparan sulphate and heparin

\[
\begin{array}{c}
\text{IdUA} \quad \rightarrow \quad \text{GlcN} \quad \rightarrow \quad \text{GlcUA} \\
\quad \quad \quad \quad 2-\text{SO}_3^- \quad \text{SO}_3^- \text{or Ac}^* \\
\end{array}
\]

Keratan sulphate

\[
\begin{array}{c}
\text{GlcNac} \quad \rightarrow \quad \text{Gal} \quad \rightarrow \quad \text{GlcNac} \\
\quad \quad \quad \quad \quad 6\text{SO}_3^- \\
\end{array}
\]
This process of synthesis and assembly of aggrecan takes approximately 2h in rat chondrosarcoma (Kimura et al., 1984).

In addition to the synthesis of proteoglycans, degradation maintains the normal turnover of proteoglycans in the extracellular matrix (for review see Poole, 1986). In the case of aggrecan, this involves the cleavage of core proteins in the extracellular matrix. In other proteoglycans, the GAG heparan sulphate can also be cleaved extracellularly. After this cleavage, the remaining products are endocytosed, and cleavage of the other GAGs occurs intracellularly. The degradation products are either endocytosed, or pass through the lymph and blood to the liver, but do not appear to be recycled for future synthesis.

1.3 Myopia

1.3.1 Refractive errors

As mentioned earlier, when an eye is at rest looking at infinity, parallel rays of light enter the eye and are refracted by the lens and cornea so that they converge on the retina. This condition is known as emmetropia. Often however, the rays of light are focussed either slightly in front of, or slightly behind the retina (ametropia) giving these eyes a refractive error. The size of this error depends on the axial length of the eye, the curvature of the cornea, the curvature of the lens, and the angle at which light enters the eye (which in turn depends on the distance between the object and the observer).

At birth the eyes of most neonates are hyperopic, with images focussing behind the retina. Emmetropia is only reached if the correct developmental progressions are made with the increase in axial length matching the changes in optical power, resulting in the image focussing on the retina. If the eye does not continue to grow after birth, or grows insufficiently, the eye remains hyperopic. If there is an overshoot of this growth, myopia results.

Hyperopia

If the eye is too short, the image will fall behind the retina, resulting in hyperopia. For most hyperopes, viewing objects at infinity is not a problem as the image focus can be then corrected by accommodation, for, as described earlier, although the image is formed behind the eye, accommodation can change the angle at which light is refracted at the lens, resulting in the image being focussed on the retina. However, as the object is brought closer to the eye, the angle of light entering the eye increases from parallel until, when the object is too close, the image is so far behind the eye that it can no longer be corrected by accommodation. This point is usually close to the observer. It is at these closer distances that corrective (convex) lenses are required. Hence hyperopia is also known as long-sightedness (Fig. 1.6).
Figure 1.6 Hyperopia.

A) A hyperopic eye looking at infinity. As the eye is too short the parallel rays of light are focussed behind the retina

B) This can be corrected by accommodation. The lens can increase its curvature so that rays coming from an object at infinity will converge on the retina.

C) A hyperopic eye looking at an object at close distance. The divergent rays of light result in the rays of light converging even further behind the retina. Once the object is brought too close accommodation can no longer compensate enough.

D) Correction of hyperopia by a convex lens. The increased optical power contributed by the lens allows the rays of light to reach the retina.
Myopia

The converse phenomenon is seen in the myopic eye where the eye is too long for the optical power of the cornea and lens, which is the result of growth past the normal 22mm. Myopia is described as a negative refractive error, and the farsighted eye as a positive error.

A

B

C

D

The far point is further away from the eye, the refractive error is minus defect (Fig 7). To correct myopia, the correction is placed in front of the eye, near the cornea. This is similar to bringing in a flashlight, the light in the eye will focus more over the retina. Myopia is corrected with concave lenses and contact lenses are used in more severe cases. Myopia, like hyperopia, can be corrected with glasses and contact lenses.

Conversely, the recently-developed laser techniques allow for the correction of myopia through the use of an excimer laser, which can treat the surface of the eye to correct the refractive error. This method has been shown to have several advantages over traditional techniques, including reduced recovery time and improved visual outcomes.

In some cases, high degrees of myopia can be associated with serious complications, such as retinal tears and detachment. Myopia is also associated with increased risk of glaucoma, a condition that causes damage to the optic nerve and can lead to blindness.

In general, myopia is a common condition that can be managed with appropriate correction techniques. However, it is important to monitor the progression of myopia and to consider the use of protective measures, such as the use of eye exercises or the use of eye drops, to help slow the progression of the condition.
Myopia

The converse problem is seen in myopia where the eye is too long for its optical power. In this case, objects at infinity are not in focus as the image falls in front of the retina. Accommodation cannot be used in myopic eyes as it would only take the image further away from the retina, towards the lens. As the object is brought closer to the observer the angle of the light rays entering the eye increases, so that the image is brought towards the retina, until the object is close enough to the eye to be seen clearly (Fig. 1.7), hence myopia is also known as short-sightedness. The furthest distance from the eye at which objects are still in focus is known as the far point. The degree of myopia (or the refractive error of the eye) is the inverse of this far point. Therefore if the far point is 2 metres away from the eye, the refractive error is -0.5 dioptres (D).

There are two ways in which myopia can be corrected; either by placing a concave lens in front of the eye, or by changing the refractive power surgically. When a concave lens is placed in front of the eye (such as with spectacles and contact lenses) the light rays diverge from this lens, so that the image can be brought onto the retina (Fig 1.7). To correct an eye with a refractive error of -0.5D, a lens of strength -0.5D is required and this is therefore known as a negative or minus lens. Conversely, lenses required to correct hyperopia are known as positive or plus lenses.

More recently, surgical techniques have been developed to flatten the cornea and thereby change the total refractive power of the eye. One of these techniques is radial keratometry. Radial incisions are made into the mid peripheral cornea so that the central cornea becomes flattened. However, this method has been found to have several side effects and complications. A newer method has now been developed using excimer lasers which remove a thin layer of the cornea, resulting in a flatter cornea. In both these cases the flatter cornea means that the rays of light leaving the cornea are divergent enough that they can be brought in focus onto the retina (for reviews see Gartry, 1995; McDonnell, 1995). Although glasses and contact lenses are readily available, their worldwide cost is high. While surgery is a more permanent solution, it is relatively expensive, has some already described side-effects, and all the side effects may not be revealed for many years.

In some cases, high degrees of myopia can be associated with retinal detachment and retinal degeneration, which in turn can cause blindness. This form of myopia is known as pathological myopia and is also increasing in its incidence throughout the world. Pathological myopia can result from either diseases of the cornea or the retina, or it can result from the excessive axial elongation which occurs during myopia. The anterior part of the eye remains normal, however there is elongation of the posterior part of the eye. The pupil is large and often sluggish. The sclera is thinned and there is atrophy of both the retina and choroid which is degenerative rather than inflammatory (for review see Duke-Elder and Abrams, 1970).
Figure 1.7 Myopia.

A) A myopic eye looking at infinity. Parallel rays of light are focused in front of the retina as the eye is too long.

B) This cannot be corrected by accommodation as this will only bring the image further away from the retina.

C) A myopic eye looking at an object at close distance. As the eye is too long in the axial direction, the divergence of the rays of light coming from the object mean that the image is focussed on the retina.

D) Correction of myopia by a concave lens. The increase in divergence focuses the light upon the retina.
1.3.2 Epidemiology of myopia

Based on the incidence of myopia in different populations all over the world, it is estimated that myopia affects approximately 10% of the world's population. Often, myopia develops during childhood and adolescence, and it may require some attention and care. Various studies have shown that children with a family history of myopia are more likely to develop myopia themselves.

**Racial factors**

The varying prevalence of myopia among different populations has been established, and a review by Williams and Wolfe (1985) found that only 1% of the population in the southern United States were myopic, whereas in some parts of the world, more than 50% of young adults are myopic. For example, studies have shown that Taiwan, Singapore, and Malaysia have significantly lower rates of myopia compared to Europe (14.7% in the European population vs. 5.7% in the Singaporean population). Similar differences are also seen in Malaysia and Indonesia (1972). The rates of myopia are also significantly lower in the United States compared to Europe, where differences in the incidence of myopia among different racial groups are more pronounced. Factors such as genetics, environment, and lifestyle may be significant contributors to these differences.

**Genetics**

Many studies have shown that children are more likely to develop myopia if their parents also have it. In a study by Zadnik et al. (1994), the concordance in twins was compared, showing that identical twins have a higher prevalence of myopia, indicating some genetic predisposition. However, it is also possible that environmental factors may play a role. It is not clear if the genetic predisposition is due to a specific gene or a combination of genes. Some studies have also suggested that the different prevalence of myopia within different racial populations, even within the same country, may be due to genetic factors, assuming the environmental factors are all the same. However, more research is needed to fully understand the role of genetics in myopia.
1.3.2 Epidemiology of myopia

Based on the incidence of myopia in different populations all over the world, it is estimated that myopia affects a significant percentage of the world’s population. Often, myopia develops during adolescence when eye growth overshoots the amount required to achieve emmetropia. Many epidemiological studies have therefore been done to try and gain some insight into what causes some people to become myopic.

Racial factors

The varying prevalence of myopia between different countries is now well established (for review see Wilson and Woo, 1989). For example, a study in Vanuatu found only 1.3% of the children were myopic (Garner et al., 1985), and another study found only 1% of the population in the Solomon Islands were myopic. At the other end of the scale, more than 50% of Japanese students (Hosaka, 1988) and more than 70% of Taiwanese school children are myopic (Lin et al., 1988). Moreover there is a difference in the rates of myopia between racial groups living in the same country (for review see Angle and Wissmann, 1980). A large scale study of this nature has been carried out in Singapore where data was obtained from the large number of men (110,236) doing national service (Au Eong et al., 1993b). 48.5% of the Chinese sub-population were myopic with significantly lower rates of myopia observed in the other racial groups (34.7% in the Eurasian population, 30.4% in the Indian population and 24.5% in the Malay population). Similar distributions of myopia between the races were also seen in Malaysia (Chandran, 1972). The rate of myopia is also lower in indigenous Australians compared to the European population (Taylor, 1981). The fact that there are differences in the incidence of myopia between ethnic groups suggests that genetic factors may be involved.

Genetic factors

Many studies have examined the incidence of myopia in children in relation to that of their parents. It has been reported that children who have two myopic parents are the most likely to develop myopia. Children with only one myopic parent are more likely to develop myopia than children whose parents are not myopic (Goldschmidt, 1968; Zadnik et al., 1994). Another aspect of genetic influences comes from studies done in twins. Monozygotic twins have more concordant refractive errors than do dizygotic twins (Minkowitz et al., 1993) for reviews see (Goldschmidt, 1968; Schwartz, 1968). Concordance over these studies ranges from about 70-90%, with the cut-off for concordance being a difference of not more than 0.5D. This suggests that there may be some genetic predisposition to the development of myopia, however, this predisposition may be overridden by environmental factors. It could be argued that the different levels of myopia within different racial populations living in the same country are solely due to genetic factors, assuming the environmental factors are all the same. However,
although they may be living in the same environment, different ethnic groups may still have quite different lifestyles, which would make their environments different.

Diet

One element of lifestyle which is different between different ethnic groups living in the same country is diet. Although diet is speculated to play a role in the development of myopia, most studies have looked at the diet of children who are already myopic, meaning causal factors cannot be elucidated. One study has looked at the nutritional intake of children before they became myopic and compared them to children who did not become myopic (Edwards, 1996). While the myopic children had a smaller food intake, they were not malnourished and it was concluded that their energy requirements were less than those of the non-myopic subjects. This may fit with the idea that myopic children tend not to participate in activities requiring distance vision like sport, are therefore less active, and therefore require less food. The rapid development of myopia during adolescence also suggests that some other kind of environmental effect may also be involved.

Other environmental factors

The most common environmental factor which is thought to be involved in the development of myopia during adolescence is reading, since most myopia develops during school years. Education levels are often used as an index of the amount of reading that was done during development. Throughout various populations in the world, it has been shown that there are only low levels of myopia in sections of the population which are uneducated. Sections of the population which have had an education up to at least high school level show a significantly higher rate of myopia. Again, in a large scale Singaporean study (Au Eong et al., 1993a), levels of education within each racial group were examined for correlations with differences in incidence of myopia. Consistently, the groups which had the highest levels of education, had the highest incidence of myopia. In addition to this, decreasing levels of education corresponded to decreasing levels of myopia. Also, within each educational group, the racial distributions remained similar to those in the overall population. For example, in all the educational groups the highest incidence of myopia was in the Chinese population. Education may not only play a role in the development of these small amounts of refractive error, but, also appears to be involved in the development of pathological myopia, since the incidence of pathological myopia was much higher in the more educated population (Au Eong et al., 1993a).

It has been argued that rather than education (or reading) causing myopia, children who are myopic tend to do things that require close vision, like reading, rather than activities which require good distance vision, such as playing sport. However, there are many reports of rapidly increasing rates of myopia in populations where the
level of education has also increased over the same period, suggesting that education is a cause rather than a result of myopia. For example, in Singapore 26% of the male population was myopic between 1974-84. However, by 1987-91, 43% of the male population was myopic, and this increase correlated with the increase in the number of males achieving higher levels of education (Tay et al., 1992). Similar increases have been found in Eskimo populations from Canada, Alaska and Greenland, where successive generations have increasing rates of myopia (van Rens and Arkell, 1991). However, as increases in education levels in these population also came with changes in their diet, as influenced by the West, there is still a possibility that dietary changes may have played a role in the increase in myopia observed (for review see Johnson, 1988). These increasing levels in myopia in Eskimo populations have also been attributed to increasing levels of urbanisation. However, with an increase in the degree of urbanisation also comes an increase in levels of education, making it difficult to separate the two.

There is also limited evidence for near work playing a role in the development of myopia from studies where myopia has developed much later in life. Studies on clinical microscopists (Adams and McBrien, 1992) and textile workers (Simensen and Thorud, 1994) have shown that these occupations which involve close work have a high percentage of myopes. The majority of these cases of myopia developed after adulthood and after they started the jobs involving close work. However the studies only involve small sample sizes. In contrast, people spending long periods of time in front of visual display units do not appear to have higher levels of myopia (Cole et al., 1996).

Therefore, there appears to be a strong genetic influence associated with the development of myopia. Not only is there a hereditary component, but also an ethnic component, both of which seem to give a person a predisposition for developing myopia. However, whether someone develops myopia also appears to depend on the environmental conditions they were exposed to, especially the amount of reading done during adolescence. Since genetic factors cannot be controlled, the environmental factors involved in the development of myopia need to be identified, in order to find a way in which its development can be prevented. Since education levels and levels of urbanisation cannot be reduced, what needs to be identified is what it is in these aspects of modern society which causes the eye to grow excessively. To address this issue, animal models of myopia have been developed.

1.3.3 Animal models of myopia and hyperopia

Epidemiological studies are of limited use, because they can only show correlations between factors such as race or education and the incidence of myopia in these populations, and therefore can only suggest possible causal factors. What is more important is understanding the changes which occur in the eye resulting in the
excessive growth which causes myopia. In order to be able to study these changes, animal models are required in which myopia can be induced in one eye so that comparisons to normal eyes can be made at the cellular level. If the development of myopia is understood, then it may be possible to prevent its development. The advantage of animal models is that environmental factors can be controlled more rigorously, whereas in all epidemiological studies, there are many environmental factors. While animal models may not directly address the question of whether education (or reading) plays a role in the development of myopia, it has been suggested that the printed page, when focused on for long periods of time, can induce a type of form-deprived vision which may be similar to that obtained with translucent diffusers (Wallman et al., 1987).

Visual deprivation

The earliest studies using animal models of myopia achieved axial elongation by suturing the eyelid and this method is still used today in some studies (monkeys: Young, 1961; Greene and Guyton, 1986; Smith et al., 1987; Stone et al., 1988; Iuvone et al., 1991; chickens: Yinon et al., 1980; Yinon et al., 1982; Teakle et al., 1983, Wildsoet et al., 1993; Stone et al., 1996). However, this technique is not as easily reversible as using diffusers which can be removed and replaced more than once. Opaque occluders have been used, but do not appear to give as great a refractive error as eyelid suture in either monkeys (Wiesel and Raviola, 1979) or tree shrews (Norton, 1990). Similarly, translucent diffusers give greater refractive error than opaque occluders in chickens (Sivak et al., 1989). These results suggest that some light is required for axial myopia to develop, and for this reason translucent diffusers are the most commonly used means of inducing myopia. This is called form-deprivation myopia (FDM), as form-vision is impaired under these conditions (Wallman et al., 1978). FDM has also been studied in monkeys (Wiesel and Raviola, 1977) and tree shrew (Sherman et al., 1977). Further evidence that light is required comes from studies where animals wearing translucent diffusers were placed in constant darkness and little if any myopia developed (Raviola and Wiesel, 1978; Gottlieb et al., 1987; Guyton et al., 1989; Yoshino et al., 1997), whereas myopia can still develop in constant light (Guo et al., 1996). However, whether the image degradation effect of deprivation is significant under dark conditions remains unknown. What is interesting about these results is that translucent diffusers are much more effective than black patches, indicating that the loss of contrast which arises from the diffusers is important in inducing myopia, rather than totally blocking vision altogether as the black patches do. However, some workers have shown that opaque occluders give rise to changes in photoreceptor morphology which are similar to the changes which occur after using translucent diffusers, suggesting that opaque occluders do not eliminate all light, and that similar mechanisms may be involved in both paradigms (Liang et al., 1995).
Are there similarities between the model of FDM and conditions which cause myopia in humans? In fact, in some cases, axial myopia in human infants is caused when the eyelid fails to open after birth. (Hoyt et al., 1981; O'Leary and Milodot, 1979). This is obviously similar to some of the animal models used such as lid suture and to some extent the FDM model, as normal vision is deprived although light can still be detected. Axial elongation myopia also occurs in humans in response to cataracts and keratitis, both of which also impair normal vision. For these reasons, visual deprivation has been the basis of many animal models of myopia.

Abnormal lighting conditions

Even without deprivation, conditions of constant darkness or constant light also affect the eye shape and hence refractive error of the eye. Under constant dark conditions there is an enlargement of the eye, but due to corneal flattening myopia does not develop and instead the eye becomes hyperopic (Gottlieb et al., 1987). Conditions of constant light also cause an elongation of the eye, again with corneal flattening resulting in hyperopia (Li et al., 1995). There was an early report of constant light initially inducing hyperopia, which after about 3 weeks then becomes myopia (Lauber et al., 1970), but this result could not be repeated by Li et al. (1995). Therefore, while abnormal lighting conditions cause an increase in eye size as translucent diffusers do, corneal curvature compensates so that myopia does not develop. This may also explain why opaque diffusers do not result in as large refractive errors as are observed during FDM with translucent diffusers.

Optical defocus

Like FDM, where translucent diffusers are placed over the eye, contact lenses have also been used as diffusers over the eye to induce both myopic and hyperopic refractive errors (Irving et al., 1991; Irving et al., 1992; Schaeffel et al., 1988; Schaeffel et al., 1990; Wildsoet and Wallman, 1995). Lenses with a myopic defocus are known as plus or positive lenses because they are used to correct hyperopes who have a positive refractive error. While the eye is wearing such a lens, it is believed that the image focus is in front of the retina, and the eye slows down its rate of growth to make itself emmetropic for the total refractive power. Therefore, upon removal of the lens, the eye is then hyperopic, and again changes its rate of growth to achieve emmetropia. Conversely, with lenses with a hyperopic defocus (minus or negative lenses) the eye increases its growth rate to obtain emmetropia so that when the translucent diffuser is removed the eye is myopic. As it is then myopic, it once again changes its growth rate in an attempt to become emmetropic once more. The higher the refractive error of the lens, the greater the degree of refractive error the eye develops. Most of this work has been carried out on chickens, however hyperopia can also develop in monkeys in response to positive lenses (Hung and Smith, 1996). Therefore,
it appears that there is a long-term compensatory response to the optical defocus imposed by the lenses, which is similar in its time course to the changes seen in FDM. However, in addition to this, there must be a signal which tells the eye in which direction to compensate, by increasing or decreasing its rate of growth.

The question of how the eye can tell in which direction to grow still remains unanswered. One theory that has been put forward is that accommodation is involved in the development of refractive errors. Because near work (during which time there is prolonged accommodation) is associated with the development of myopia, the effects of near work have commonly been attributed to an increased amount of accommodation. Similarly, since hyperopic eyes need to accommodate more than myopic eyes, it has also been suggested that the amount of accommodation could provide a signal to the eye to tell it which direction to grow to compensate for positive or negative lenses. Another reason for the accommodation argument being an attractive hypothesis is because accommodation itself is a sign sensitive process. However, lesions of the EW nucleus do not prevent the ability of the eye to compensate for both positive and negative lenses (Schaeffel et al., 1990), nor does ciliary nerve section (Schmid and Wildsoet, 1996b), showing that accommodation is not required for sign-sensitive growth. Further evidence for these changes in response to positive and negative lenses being locally controlled comes from the fact that if only part of the eye is covered, then only that part of the eye responds to the lens (Diether and Schaeffel, 1997). One difference between using lenses and translucent diffusers, is that when translucent diffusers are used there is no endpoint, and the eye continues to grow. In the case of lenses, the amount of eye growth is proportionate to the strength of the lens, so that emmetropia can be achieved. When optic nerve section is carried out, this fine control responsible for the amount, but not the direction of growth required appears to be disturbed, so that there is an overshoot in the amount of growth which occurs (Troilo, 1990). Therefore, accommodation may play a role in the fine tuning of eye growth in response to lenses.

Recovery from deprivation
Another way in which changes in refractive error can be studied is in response to the removal of the translucent diffuser after the induction of FDM. Upon removal of a translucent diffuser after the induction of FDM, there is a cessation of eye growth as the eye compensates for the imposed myopia, bringing the eye back to emmetropia. Alternatively, upon removal of diffusers which have made the eye hyperopic, there is an increase in the rate of eye growth, once again to compensate for the hyperopia, returning the eye to its emmetropic state. In all these cases, the control eyes continue to grow at the normal rate and maintain emmetropia (Wallman and Adams, 1987; Troilo and Wallman, 1991).
Both recovery from deprivation and the lens paradigm may more closely represent the normal processes of emmetropisation during eye growth, since in both these cases there appears to be an active matching of the optical power of the eye to growth rates in axial length. For this reason they may ultimately be more useful for understanding what goes wrong during emmetropisation at birth, which results in myopia. However, since much more is known about the FDM model, this is the model which will be used in the present study.

1.3.4 Relating animal models of FDM to human myopia

An important point is whether the changes in the eye which arise from animal models of myopia such as FDM are similar to the changes which occur in human myopia. In epidemiological studies, it has been shown that human myopic eyes are axially longer than non-myopic eyes, which is similar to what is observed in animal models. However, in humans these measurements are taken from two populations (a myopic population and a non-myopic population) and then compared, which means any other differences between the two populations may be involved. Therefore, animal models may be more useful, because if myopia is induced in only one eye, changes in this eye can be compared to the control eye. Highly myopic humans have also been shown to have thinner than normal scleras (Curtin, 1985), which has also been observed in mammalian animal models of myopia. Scleral thinning in response to inducing myopia has been shown in monkeys (Raviola and Wiesel, 1985) and tree shrews (Kang and Norton, 1993). There is a thickening of the sclera in chickens after FDM has been induced (Gottlieb et al., 1990). However, while mammalian sclera is fibrous, the chicken sclera has a cartilage layer in addition to a fibrous layer which may account for these differences.

Therefore, there is some evidence in humans that deprivation of vision leads to an increase in axial length similar to that seen in animal models. Although primate models may more closely represent humans, they are expensive, slow to develop fully, and incur ethical problems. For this reason the tree shrew may prove to be a useful mammalian model. Alternatively, chickens are relatively inexpensive and develop rapidly and will therefore continue to be the easiest animal model available. However, as noted above there appear to be some differences between observations in mammalian models compared to those in chickens. Therefore, while the chicken will remain as the most useful model in the early stages of investigating myopia, more specific hypotheses may later need to be tested in mammalian systems for conclusions to be drawn relating to human myopia.

1.4 Form-deprivation myopia
As mentioned earlier, the most common method of inducing myopia nowadays is to place a translucent diffuser over the eye, which still allows differences in light intensity to be detected, but does not allow the form of objects to be clearly seen, hence the term form-deprivation myopia. Although the general principle of these diffusers is the same, there is a great variety of diffusers used throughout laboratories throughout the world. This together with the fact the different strains of chickens are also used, increases the difficulty of comparing one study to another, as different strains appear to give slightly different results (Schmid and Wildsoet, 1996a; Troilo et al., 1995). Nevertheless, it is now generally accepted that most FDM results from an increase in axial length (Hodos and Kuenzel, 1984; Wallman et al., 1981), with relatively little change in corneal curvature (Schaefel et al., 1986; Pickett-Seltner et al., 1988) or lens power (Seltner and Sivak, 1987; Wallman and Adams, 1987).

1.4.1 Changes in response to FDM

Axial elongation

Weiss and Schaefel (1993) measured axial length in both normal eyes and in eyes which had translucent diffusers fitted. The animals in their study were kept in lighting conditions where there was 12h of light followed by 12h of darkness, and they measured axial length at the end of each of these 12h periods over a course of days. They showed that normally there is growth of the eye in the axial direction during the light phase, as the eye is longer at the end of the light phase compared to the beginning of the light phase. However, there was no axial elongation in the dark, with even some regression, so it was concluded that eye growth only occurred in the light. They also reported that during the development of FDM, the normal increase in eye length was observed at the end of the light phase. However, in addition to this, there was also axial elongation during the dark phase. Therefore, they concluded that deprivation appears to increase eye size by abolishing the normal rhythm of eye growth, resulting in increased rates of axial elongation.

Sciera

Although there was initially much debate as to whether changes in the sclera were due to stretching (passive growth) or an active process of growth such as an increase in synthesis of scleral constituents, there is now much biochemical and histological evidence to demonstrate that in the chicken, there is an increase in the synthesis of scleral proteoglycans during FDM (Rada and Matthews, 1994; Rada et al., 1994; Rada et al., 1992; Rada et al., 1991). However, the sclera of the chicken differs markedly from that of mammals, with the mammalian sclera consisting of a fibrous layer, and the chicken sclera having both a cartilaginous and a fibrous layer (Gottlieb et al., 1990). In mammals there is a thinning of the fibrous sclera whereas in chickens this thinning of the fibrous layer is accompanied by a slight thickening of the
cartilaginous layer (Gottlieb et al., 1990). Therefore it may be that while thinning of the fibrous layer is a process which occurs in many if not all species, in the chicken this may be overridden by the thickening of the cartilaginous layer.

Thinning of the fibrous sclera in myopic eyes has been observed in monkeys (Raviola and Wiesel, 1985) and in the tree shrew (Kang and Norton, 1993). It has been calculated that less than 20% of the difference between myopic and control scleras can be accounted for by simple elastic stretching, suggesting that an active remodelling process is occurring in myopic scleras of the tree shrew (Phillips and McBrien, 1995).

In the tree shrew this thinning of the sclera is due to a decrease in collagen synthesis (Cornell and McBrien, 1994) and proteoglycan synthesis (Norton and Rada, 1995), and an increase in the amount of metalloproteinases (Guggenheim and McBrien, 1996), with the loss of tissue occurring predominantly at the posterior part of the sclera (Reeder and McBrien, 1994). The fact that the amount of metalloproteinase is higher in myopic sclera also suggests that there is active degradation occurring.

Rada and colleagues (1991) have shown that there is an increase in proteoglycan synthesis in the scleras of myopic chickens, again demonstrating the presence of an active rather than passive process. Consistent with the increase in metalloproteinases in the sclera from myopic eyes of the tree shrew is the finding that the amount of metalloproteinase decreased in the thicker sclera from the myopic chicken sclera (Rada and Brenza, 1995), further suggesting an active process is involved. In addition to this, after the translucent diffusers were removed, there was a subsequent decrease in rates of proteoglycan synthesis (Rada et al., 1992). They looked at aggrecan, a major cartilage proteoglycan and decorin, a smaller proteoglycan. Immunocytochemical studies have shown aggrecan in the cartilaginous layer of the chicken sclera and decorin in both the cartilaginous and fibrous layers (Rada et al., 1994). Although there is an increase in the synthesis of both aggrecan and decorin in the sclera of myopic chickens, the amount of aggrecan accumulated is greater in myopic eyes whereas the amount of accumulated decorin is not, suggesting that decorin’s turnover increases simultaneously with its synthesis (Rada et al., 1991).

This body of work demonstrates that the changes which occur in the sclera in response to FDM are due to an active process, rather than a passive one. The thickening of the cartilaginous sclera of the chicken is due to an increase in proteoglycan synthesis and decreased degradation. The thinning of the mammalian fibrous sclera is consistent with a decrease in synthesis of proteoglycans and collagen and enhanced degradation.

Choroid and RPE

Recently it has been suggested that, in addition to the growth of the eyecup, the choroid also plays an important role in these phenomena. In the case of FDM there is a slight thinning of the choroid, which effectively pulls the retina towards the back of the
eye. When the translucent diffusers are removed, there is a rapid and massive expansion of the choroid. This is thought to at least partially compensate for the sudden change in image quality by effectively pushing the retina forward (Wallman et al., 1995). An increase in the rate of proteoglycan synthesis in the extracellular matrix of the choroid was observed in the myopic eyes, but it is uncertain if this change is sufficient to account for such a large change in choroid size (Wallman et al., 1995). In fact, although the choroid expands, there are a lot of lacunae in the structure, which may also explain why the overall amount of proteoglycans may not have increased sufficiently to account for the larger tissue. Therefore the mechanisms underlying this choroidal expansion remains unknown. Deprivation causes a refractive error of about -25D, and the thickening of the choroid which occurs after translucent diffuser removal can change the refractive error about 7D (Wallman et al., 1995). Since these choroidal changes occur very soon after translucent diffuser removal, and before there is a decrease in rates of scleral growth, the choroid may provide short-term compensation, while the slowing of scleral growth may provide long-term regulation of eye growth.

Very little work has been done with regard to the RPE in myopia. There is some evidence that the RPE of chickens expands during FDM with each cell expanding its area (Lin et al., 1993). However, as no mitotic figures were observed, it was unclear whether this expansion was due to active growth or stretch. Injecting sodium iodate (which is toxic to the RPE) also prevents the development of FDM (Shih et al., 1993).

Retina

There have been some reports of retinal thinning in response to FDM, with most of the changes occurring in the INL (Yinon et al., 1982). On the other hand, it has also been shown that there is lateral retinal expansion during FDM, with amacrine cells staining for tyrosine hydroxylase having expanded dendritic fields, while their number remained the same as in the control eye (Teakle et al., 1993). Light microscopy has also shown changes in the structure of the photoreceptors (Liang et al., 1995). The inner segments of the cones were thicker, as was the outer segments of the rods. The most significant change in the retina of the myopic eyes was the elongation of the photoreceptor outer segments and the subsequent close apposition of the outer segments to the RPE. It has been suggested by Liang et al. (1995) that this elongation is responsible for the thinning of the choroid during FDM observed by Wallman et al. (1995), rather than the choroid actively thinning itself. However, it is not clear how these two processes would be linked.

1.5 Retinal control of FDM
1.5.1 Local in addition to central control

In chickens, FDM can still develop after optic nerve section, showing that central control is not required (Troilo et al., 1987; Wildsoet and Pettigrew, 1988). Similar results have been obtained in rhesus monkey (Raviola and Wiesel, 1985). The application of tetrodotoxin which blocks action potentials in ganglion cells also had no effect on the development of FDM in the tree shrew (Norton, 1990). Another aspect of this growth is that deprivation of only part of the visual field results in only the corresponding part of the eye having excess ocular growth (chicken: Hodos and Kuenzel, 1984; Wallman et al., 1987; tree shrew: Norton and Siegwart, 1991, cited in Wallman, 1993). Therefore it appears that FDM is an ocular rather than a centrally controlled response to the translucent diffuser.

The fact that output from the eye to the brain is not required for FDM to develop suggests that accommodation is not involved in this process. In addition to the optic nerve section results mentioned above, further evidence of this comes from the observation that lesions to the EW nucleus do not prevent the development of FDM (Troilo 1989 cited in Wallman, 1993). Similarly, removing the ciliary ganglion does not prevent FDM in chickens (Wallman et al., 1981; Wildsoet and Howland, 1989; Lin and Stone, 1991) nor in rhesus monkeys (Raviola and Wiesel, 1985). These results suggest that the process of accommodation is not involved in the development of FDM and further add to the argument that eye growth is a locally controlled process. One question which remains in relation to accommodation in FDM is what is happening to this process during deprivation. If there are sufficient cues during deprivation, accommodation could presumably occur. However, if there is enough blurring during deprivation, it may be that there are no cues for the accommodative process to occur, in which case the accommodative apparatus might settle at the ‘dark point’. In fact, it has been suggested (Schaeffel et al., 1994) that lens defocus can be cleared by accommodation, but that image degradation due to deprivation cannot be cleared by optical means. However, it should be noted that compensation for lens-induced defocus also occurs without accommodation (Schaeffel et al., 1990), further suggesting that accommodation is probably not involved in controlling eye growth during FDM.

1.5.2 Retinal function is disrupted during FDM

If there is local control of eye growth then the question which remains is what aspect of local eye functioning is required. As mentioned earlier, the retina is a prime candidate as a controlling factor of eye growth since it is the light-sensitive neural component of the eye. Therefore, deprivation affects some aspect of retinal functioning, which in turn disrupts eye growth. Identifying the retinal circuits which could be involved remains a major area of research in the field of myopia.

It appears that retinal lesions can affect normal development of the eye. Retinal lesions using kainic acid (an excitotoxic glutamate analogue) result in the development...
of axial elongation (Barrington et al., 1989; Wildsoet and Pettigrew, 1988). Another study (Ehrlich et al., 1990) has also observed an increase in eye size after injection of kainic acid, or quisqualic acid (another glutamate analogue). However, no effect on eye size was observed using N-methyl-D-aspartate (NMDA), which is another glutamate analogue, with a different receptor specificity. Therefore it appears that only a sub-type of receptors may be involved. These results show that disruption to retinal circuitry can affect the development of normal vision and normal eye growth. Whereas lesioning agents such as kainic acid have an effect on a large number of cells, results from more specific lesioning agents may lead to the identity of the few cells which may be involved in the development of myopia.

One way of determining which aspects of retinal functioning may be involved in the development of FDM is to look for what can prevent myopia from developing. As discussed earlier, retinal ganglion cells require stimulation which usually comes from changes in the field of view. Eyes which are wearing a translucent diffuser are effectively deprived of both spatial and temporal contrast. Because of this, it was thought that the development of myopia could be prevented by superimposing temporal contrast. This was achieved by exposing the form-deprived animal to flickering light with stroboscopic illumination. The development of myopic refractive error was reduced when form-deprived animals were exposed to strobe light around 10-20 Hz (Gottlieb and Wallman, 1987, cited in Wallman, 1993; Vingrys et al., 1991) suggesting that the imposed temporal contrast could prevent the changes in axial eye growth associated with FDM. However, further work has shown that the changes in refractive error during strobe lighting come from flattening of the cornea, rather than a reduction in axial elongation (Vingrys, personal communication). Therefore, strobe light does not in reality reverse the effect of deprivation on axial elongation, but rather compensates for it by flattening the cornea.

One way FDM can be prevented is by daily exposure to short periods of normal vision during the period of deprivation (Nickla et al., 1989; Vingrys et al., 1991; Napper et al., 1995). Exposure to as little as 15 min of normal vision each day is enough to reduce the amount of FDM which develops. The higher the amount of normal vision per day, the less axial elongation and refractive error that develops, with 2h of normal vision each day being enough to almost fully compensate for FDM. These results have led to the suggestion that a minimum period of normal vision is required each day for normal eye growth to occur. What is surprising is that this minimum period is so small, and that FDM is almost totally abolished even when the translucent diffuser is worn for 22h each day, although it is worth noting that 12 of those hours were in the dark, so the translucent diffuser may have only been effective for 10h each day. This implies that there is an active generation of a signal with a long time course during this 2h period.
1.5.3 Retinal neurotransmitters involved in FDM

The major question remaining is how visual processing in the retina ultimately controls eye growth? The first step in understanding how this may occur is to identify what forms of retinal input and processing are essential to support normal eye growth, and what are missing when FDM develops. Although it appears likely that processing of spatial and/or temporal contrast may be required for normal eye growth, there is no strong evidence to support this. Presumably, changes in visual processing which occur during deprivation result in changes in rates of messengers released in the eye. Many retinal messengers have been implicated in being involved in FDM. If these are involved, it will be important to understand how these messengers are transformed into some kind of message which can act on the sclera. This may a direct pathway, from retina to sclera, or may be indirect involving the RPE and choroid.

Dopamine

Dopamine is probably the most studied retinal neurotransmitter in relation to FDM. Dopamine is found in a specific population of amacrine cells known as the dopaminergic amacrine cells, which are found in both chickens and primates. Release of dopamine from these cells occurs in response to light (Kramer, 1971; Kirsch and Wagner, 1989; Kolbinger et al., 1990). Initially, level of tyrosine hydroxylase (TH, the enzyme responsible for dopamine synthesis) were measured in response to deprivation. Since the level of TH was lower in form-deprived eyes (Iuvone et al., 1989) it was concluded that there was a decrease in biosynthesis in dopamine during FDM. In addition to this, levels of dopamine or DOPAC (3,4-dihydroxyphenylacetic acid, a metabolite of dopamine) have been taken as an index of dopamine release. DOPAC levels have been found to be lower in myopic eyes compared to control eyes in monkeys (Iuvone et al., 1989) and in chickens (Stone et al., 1989). However, the problem with these indirect measurements is that the levels of stored dopamine, and possibly DOPAC, depend on the synthesis and release of dopamine, both of which are independently regulated by light. Therefore a decrease in the amount of stored dopamine could be due to a decrease in synthesis, a decrease in release or an increase in re-uptake of dopamine. DOPAC levels are less problematic, but in general the changes seen have only been limited. Recently, Megaw et al. (1996) have used DOPAC levels in the vitreous as a more reliable index of retinal dopamine release levels. They have shown that after the lights come on, the increase in DOPAC levels increases at the same rate in myopic and control eyes. However, after 30 min in the light, the rate of increase of DOPAC in the vitreous of a myopic eye (that is, the rate of dopamine release) becomes slower than in the controls.

Additional evidence for dopamine’s involvement in FDM has come from experiments where intravitreal injections of apomorphine (a dopamine agonist) administered during deprivation prevented the development of FDM in both chickens.
(Stone et al., 1990; Rohrer et al., 1993) and monkeys (Iuvone et al., 1991). More importantly, co-administration of haloperidol (a dopaminergic antagonist) with apomorphine in monkeys meant that FDM could not be prevented, further suggesting that dopaminergic receptors are involved (Stone et al., 1990). However, what is surprising is that haloperidol, when administered alone, can also prevent the development of FDM (Stone et al., 1989). Rohrer et al (1993) have shown that, in chickens, this preventive effect of apomorphine could itself be prevented if a D2 dopamine antagonist (spiperone) was co-administered intravitreally with apomorphine whereas the D1 antagonist SCH23390 was not effective in blocking FDM. A single injection of 6-hydroxy dopamine (6-OHDA), which is toxic to the dopaminergic amacrine cells, can also block the development of FDM, further suggesting that the dopaminergic pathway is involved in FDM (Li et al., 1992; Weiss and Schaeffel, 1993) (Hoffmann and Schaeffel, 1996).

Strobe light has been shown to increase the amount of retinal dopamine in both chickens (Rohrer et al., 1995), by measuring 3,4-dihydroxyphenylalanine (DOPA) accumulation after blocking aromatic acid decarboxylase, and rabbits (Nowak and Zurawska, 1989), by measuring dopamine levels. Again, the use of indirect measurements means it is unknown whether there is an increase in release rates. In chicks this effect was seen at 10Hz which matches the frequency that can prevent refractive errors developing during deprivation as described earlier. Therefore it can be postulated that FDM arises due to a lowering of dopamine release, but when a strobe light is used during deprivation, the amount of dopamine release increases and therefore FDM is prevented. However, while there is an increase in dopamine release during strobe light, the normal high rates are not fully restored (Pam Megaw, personal communication), and furthermore, as discussed earlier, strobe lighting prevents refractive error from developing by flattening the cornea to compensate for the axial elongation, rather than reversing the effects of deprivation (Vingrys, personal communication).

The elongation of the photoreceptors during FDM which was observed by Liang et al (1995), and the fact the dopamine appears to be involved in FDM, suggests that the interaction between photoreceptors and the dopaminergic amacrine cells (a component of the retinal dark-light switch, Morgan and Boelen, 1996), may be involved in FDM.

Enkephalins

The role of enkephalins in FDM has not been investigated thoroughly. However, it has been shown that the rate of enkephalin release changes in response to deprivation. Enkephalin is released from the ENSLI amacrine cell during the dark phase and accumulates during the light phase with little release during this time. During deprivation, the amplitude of this diurnal variation is diminished suggesting...
that ENSLI cell activity is less inhibited than normal during the light phase (if there is more release there is less accumulation) and cell activity is less stimulated during the dark phase, as there is less release (McKenzie et al., 1997). Injections of naloxone (an opioid receptor antagonist) during deprivation prevents the development of FDM, further suggesting a possible role for the enkephalins in controlling eye growth (Seltner et al., 1994).

Leu-enkephalin release is also partially affected by strobe light (Megaw et al., 1996). Frequencies which can overcome the effect of translucent diffusers and prevent FDM developing, also restore the normal, high levels of leu-enkephalin accumulated during the light phase thereby bringing release levels down to their normally low light levels. However this could be a result of an increase in dopamine release since dopamine release is increased during strobe light (Rohrer et al., 1995), and dopamine has an inhibitory effect on enkephalin release, an interaction which is part of the retinal dark-light switch (Morgan and Boelen, 1996).

Acetylcholine

It has long been known that the application of 1% atropine (a non-selective cholinergic antagonist) can prevent the development of juvenile myopia in humans (Bedrossian, 1979) for review see (Goss, 1982). In monkeys, daily topical application of atropine prevented the development of myopia (Young, 1965). Similarly in tree shrews, pirenzepine (a muscarinic antagonist) prevented the development of myopia during deprivation (Cottriall and McBrien, 1996).

Cholinergic receptors can be classified into two groups, muscarinic and nicotinic. In the monkey, muscarinic receptors are not only found in the retina, but also in the ciliary muscle. For this reason it is difficult to distinguish between accommodative and non-accommodative processes in primate models. However, in chickens the intra-ocular muscles are striated and contain mainly nicotinic receptors (Pilar et al., 1987; Troilo and Wallman, 1987), with the retina and choroid containing muscarinic receptors. Therefore, muscarinic agents should not be acting via accommodative mechanisms, and this makes the chicken a more useful model for localising cholinergic responses to FDM. Daily intravitreal injections of the non-selective antagonist atropine in chickens prevented the development of FDM by attenuating the increase in axial length (McBrien et al., 1993), again showing there are similarities between species in the retinal circuitries involved in FDM. Furthermore, the muscarinic (M1) antagonist pirenzepine also had this effect (Stone et al., 1991) (Leech et al., 1995), suggesting retinal involvement, since the intraocular muscles in the chicken are mostly nicotinic. More specifically, it appears to be a M1 mediated mechanism, as M2 and M3 antagonists did not have this effect (Stone et al., 1991). However, while these results are consistent with the idea that retinal acetylcholine is responsible for controlling eye growth, there is no direct evidence that this is the case.
Indeed, the lability of acetylcholine to enzymatic degradation, makes it unlikely that retinal acetylcholine would be able to reach the sclera.

More recent results suggest these effects may not be mediated by the retina, as lesions of the chicken retina with ECMA (ethylcholine mustard aziridinium ions, which selectively destroys cholinergic amacrine cells) (Millar et al., 1987a) did not affect the development of FDM (Stell et al., 1997). Acetylcholine is also found in a population of amacrine cells which are not destroyed by ECMA (Millar et al., 1987a), and Fischer et al (1997) have recently co-localised acetylcholine with somatostatin in the ENSLI amacrine cells, so it is possible that acetylcholine released from these cells in the retina may still be involved. However, in similar experiments carried out with quisqualic acid, which destroys a larger population of the cholinergic cells, FDM was still able to be induced, suggesting that retinal acetylcholine is not involved (Ehrlich et al., 1990).

Vasoactive intestinal peptide

VIP is found throughout both the central nervous system, and the peripheral nervous system. In the retina, VIP is localised to a specific population of amacrine cells in many animals (Brecha et al., 1984; Tornerqvist et al., 1982), including chicken (Fukuda et al., 1987). Eyelid fusion in monkeys results in an increase in immunoreactivity of VIP, however it is unclear whether this is due to an increase in synthesis or a decrease in release (Stone et al., 1988). Immunoreactivity against VIP in the chicken retina using porcine VIP has localised a population of amacrine cells with fibres extending into 3 parallel layers of the IPL (Seltner and Stell, 1995). Labelled fibres were also observed in the choroid, ciliary body and iris. Daily injection of VIP into the eye of chickens while translucent diffusers were worn did not eliminate the development of FDM. However, the injection of antagonists did abolish FDM, with antagonists of central nervous system-type VIP having a stronger effect than antagonists of peripheral nervous system-type VIP. Therefore there appear to be similarities in the effect of VIP in chicken and primate models suggesting similar retinal circuits may be involved in both species.

1.5.4 The retinal dark-light switch

The involvement of dopamine and the enkephalins in FDM has led to the suggestion that their interaction as part of the retinal dark-light switch (Morgan and Boelen, 1996) may also be involved in controlling eye growth. This is a retinal circuit which has been characterised in the chicken involving three cell types; the photoreceptors which release melatonin, the dopaminergic amacrine cells which release dopamine and amacrine cells which have enkephalins, somatostatin and neurotensin-like immunoreactive (ENSLI amacrine cells), which, as their name suggests, release these peptides (Morgan and Boelen, 1996). This circuit has been christened the retinal dark-light switch as it exists in two stable states. One of these states exists in the dark,
but it can switch to its second state when the lights come on. In the dark, photoreceptors release melatonin and the ENSLI cells release enkephalin, neurotensin and somatostatin. Both melatonin and the enkephalins have an inhibitory effect on the dopaminergic amacrine cells, thereby preventing release of dopamine. This locks the switch into its dark state where melatonin and enkephalin are released. When the light is turned on, the photoreceptors, via the release of glutamate from the on-bipolar cells, overcome the inhibition on the dopaminergic amacrine cells so that dopamine can be released. Dopamine then inhibits both melatonin release from the photoreceptors and enkephalin, neurotensin and somatostatin release from the ENSLI amacrine cells. Therefore, in the light only dopamine is released.

An important feature of this circuit is the narrow range (less than one log unit between 0.1 lux and 0.4 lux) over which it flips from its dark to light state. Possible functions of this switch have been based on the functions of the individual elements of the circuit, and on the light intensity-dependence of its transition from one state to the other (for review see Morgan and Boelen, 1996). Of particular relevance to this thesis is the potential link from this switch to eye growth and FDM. As both dopamine and the enkephalins have been implicated in FDM this is a possibility worth exploring.

1.6 Retinal control of scleral growth

There is evidence that disruption to normal visual input can result in myopia, although the nature of this visual input remains unknown. Similarly, many retinal neurotransmitters have been implicated in FDM, although again, their roles in this process remain unknown. As FDM results in a larger eye, then these changes which occur must somehow cause the eye to get bigger. So there must be a pathway which involves visual input, retinal processing and finally scleral growth. Identifying the elements of this pathway will in turn lead to an understanding of what goes wrong when myopia develops. There are two possible ways that output from the retina could control changes in scleral growth. One way would be for retinal messengers to have effects directly on scleral cells. The other possibility is that release of retinal compounds is the start of a chain of events which include the RPE and choroid, leading to changes in the sclera.

If a biochemical measure of scleral growth could be established, responses not only to deprivation could be measured, but also those to various chemical messages that could be responsible for controlling scleral growth. The synthesis of scleral proteoglycans can be measured by following rates of sulphate incorporation, a method which has been used in many other tissues in addition to the chicken sclera (Rada et al., 1991; Rada et al., 1992; Rada and Matthews, 1994; Rada et al., 1994). It has also been
shown that there is an increase in rates of sulphate incorporation during FDM, further demonstrating that this method could be used to monitor scleral changes.

This assay could also be used to investigate the pathway involved from the retina to sclera. If retinal neurotransmitters exert their effects directly on scleral cells, it would be expected that the addition of these compounds to the sclera in vitro would result in changes in the rate of sulphate incorporation and scleral growth. If, however, the pathway of neurotransmitters from retina to sclera is via the pigment epithelium and choroid, then applying these retinal compounds in vivo, may exert an effect on rates of sulphate incorporation and scleral growth.

1.7 Aims and objectives

The fact that the sclera grows during FDM, and that this growth is due to an increase in proteoglycan synthesis which can be measured by sulphate incorporation, suggests that sulphate incorporation could be used as a measure of both normal and abnormal eye growth. Therefore, the aims of this project were

• to develop a rapid method of measuring sulphate incorporation into the sclera
• to use this assay to measure sulphate incorporation in normal eye growth with the expectation that there will be a rhythm in rates of sulphate incorporation reflecting the rhythm observed in axial elongation (Weiss and Schaeffel, 1993)
• to use this assay to measure sulphate incorporation in the excessive eye growth which is associated with FDM, in which the normal rhythm is abolished, and
• to identify retinal neurotransmitters as potential controlling factors of both normal and excessive eye growth by monitoring their effects in vitro and in vivo on rates of sulphate incorporation.

2.2 Methods

2.2.1 Animal handling

All experiments were performed on chickens (White Leghorn x Black Australorp, obtained from Research Poultry Farm, Victoria) which were housed in commercial brooders with constant access to food and water. Unless otherwise specified, they were exposed to a 12:12h dark:light cycle with the lights coming on at 0600h and going off at 1800h. As these brooders proved a deal with plain windows, their lighting was influenced by the external natural lighting conditions. For measurement, the chickens were housed in special boxes during morning, when there was
CHAPTER 2. SULPHATE INCORPORATION INTO THE SCLERA

2.1 Introduction

Since proteoglycans are highly sulphated molecules, sulphate incorporation has been extensively used as an index of proteoglycan synthesis in a range of tissues from many animals. These include intact tissue such as cartilage (Adamson and Anast, 1966; Herington et al., 1972; Lemperg et al., 1977) and skeletal muscle (Carrino et al., 1988) as well as in isolated chondrocytes (Wiebkin and Muir, 1973), fibroblasts (Johansson et al., 1985) and isolated rat chondrosarcoma cells (Mitchell and Hardingham, 1982). Rada and colleagues have followed sulphate incorporation into scleral tissue in organ culture and into cultured scleral chondrocytes to measure rates of proteoglycan synthesis in the chicken sclera after the induction of FDM (Rada et al., 1991). They have used various techniques to characterise the material into which radioactive sulphate is incorporated and have shown that most of the labelled sulphate was incorporated into aggrecan, a major proteoglycan of cartilage (Rada and Matthews, 1994). This proteoglycan contains both chondroitin sulphate and keratan sulphate. Another smaller sulphated proteoglycan was identified in the fibrous layer called decorin (Rada and Matthews, 1994) which is a chondroitin sulphate/dermatan sulphate proteoglycan commonly found in other fibrous tissue as well as in cartilage (the chicken sclera contains both a cartilaginous and a fibrous layer).

In this study, using a similar approach, rates of sulphate incorporation have been measured in scleral buttons maintained in buffer containing labelled sulphate. GAGs were then extracted in alkaline conditions (Anderson et al., 1965) and the amount of label measured to obtain rates of sulphate incorporation and hence proteoglycan synthesis.

2.2 Methods

2.2.1 Animal handling

All experiments were performed on chickens (White Leghorn x Black Australorp, obtained from Research Poultry farm, Victoria) which were housed in commercial brooders with unlimited access to food and water. Unless otherwise specified, they were exposed to a 12:12h dark:light cycle with the lights coming on at 0600h and going off at 1800h. As these brooders were in a shed with glass windows, their lighting was influenced by the external, natural lighting conditions. For this reason, the chickens were housed in special boxes during summer, when there was
daylight for more than 12h. These wooden boxes were ventilated with a fan and each box contained a light switch controlled by a timer.

### 2.2.2 Sulphate incorporation

Chickens were killed by overexposure to isoflurane and rapidly decapitated. Eyes were removed and hemisected, and the anterior part of the eye, the vitreous and the retina were discarded. An 8mm scleral button was trephined from the central part of the remaining eyecup with a biopsy punch, and cleaned of any connective tissue. Each scleral button was incubated in 1ml of a physiological buffer (Gallemore et al, 1988) containing 120mM NaCl, 25mM NaHCO₃, 25mM D-glucose, 5mM KCl, 3mM MgCl₂ and 1.8mM CaCl₂·2H₂O, containing 0.5µl ³⁵SO₄ (DuPont), with a specific activity of 74MBq/mmol. Incubations were carried out at 37°C in the light for 1h.

To test direct effects, it was decided to incubate isolated scleral punches as it would have been difficult to keep a button of tissue together which included the retina, choroid and sclera and which preserved normal interactions between the tissues. Therefore it was decided that if the effects of the retina or choroid on the sclera were to be tested, this could be done in vivo. Secondly, it was decided to incubate these buttons in the light since the sclera was incubated on its own. It was assumed that the light-insensitive sclera could be incubated in the light, and would continue growing according to the signals it had received from the retina, at least for some time. This also meant that scleras from animals in differing lighting conditions could be incubated at the same time under identical incubation conditions. The results obtained supporting this assumption will be discussed later.

After incubation the tissue was washed 5 times in 1 ml of buffer to remove any excess label, and then placed into 0.5ml of 0.5M NaOH containing 1M NaBH₄ at room temperature for 24h to extract the GAGs. The tissue was then placed in another 0.5ml of 0.5M NaOH for 1h. The two extraction media were pooled and a 900µl aliquot was sampled and added to a scintillation vial containing 6 ml Optiphase HiSafe II scintillation fluid (Phamacia). Vials were shaken and left to stand overnight before being counted in a liquid scintillation counter for 5 min per sample.

### 2.2.3 Precipitation of GAGs with ethanol

Ethanol precipitations were carried out in order to determine how much, if any, of the labelled sulphate in the alkali extraction medium was free sulphate, not incorporated into GAGs. Incubations and NaOH extractions were carried out as described above. The extracts containing NaOH and NaBH₄ were neutralised with HCl and three volumes of absolute ethanol were added. After mixing, the solutions were centrifuged for 10min at 3000 rpm. The pellets were resuspended in 1ml phosphate-buffered saline (PBS), and 900ul aliquots were counted in 6ml of scintillation fluid as
described above. Similarly, 900ul aliquots of the supernatants were counted in 6ml of scintillation fluid.

2.2.4 Differential precipitation of GAGs with acetone

In order to identify the GAGs into which the sulphate was incorporated, differential acetone precipitations were carried out (Volpi, 1994). Incubations were carried out as described above. The extracts containing NaOH and NaBH₄ were neutralised with HCl and 0.5 volume absolute acetone was added at 4°C. The solutions were left for at least 2h and were then centrifuged for 10 min at 4°C. The pellets were kept (heparin pellet) and the supernatants were transferred to another tube, where another 0.2 volume of absolute acetone was added as before. After another 2h, the solutions were centrifuged, the pellets were kept (dermatan sulphate pellet), and the supernatants transferred to another tube. Finally, another 0.4 volume of absolute acetone was added to the supernatants, and the procedure repeated (producing the chondroitin sulphate pellet). Each pellet was resuspended in 1ml PBS and a 900ul aliquot was counted with 6ml scintillation fluid.

2.3 Results

2.3.1 Incorporation of sulphate at midday

Table 2.1 shows the distribution of labelled sulphate in the various steps of the assay. After a 1h incubation and the washing step, about 1% of these counts were found in the alkali extract medium. The remaining tissue was solubilised in 1ml Soluene®-350 (tissue solubiliser, Packard) to see if any counts remained in the tissue. Only negligible counts were detected. The rest of the counts were either in the incubation medium, or in the washing buffer.

Although alkali extraction has previously been used for isolating glycosaminoglycan chains from proteoglycans (Anderson et al., 1965), it was possible that a significant proportion of the labelled sulphate in the alkali extract in this study was simply free sulphate, not associated with any GAGs. To investigate this possibility, ethanol precipitations were carried out on the extract to see if there were any counts which were not precipitable. Figure 2.1 shows that only negligible counts were found in the supernatant, whereas the overwhelming majority of the counts were found in the pellet, indicating that most of the radiolabelled sulphate in the alkali extract was not free sulphate. This simplified the experimental procedure, as the amount of labelled material in the alkali extract was able to be taken as the amount of incorporated sulphate, obviating the need for a precipitation step.

A time course of incubation was then carried out to ensure that the rate of sulphate incorporation had not reached its peak after 1h. Fig. 2.2 shows the amount of
Table 2.1 Distribution of labelled sulphate after incubation and extraction.

This table shows the distribution of labelled sulphate found in each step of the sulphate incorporation assay. Most of the counts remained in the incubation medium, or were washed off after the incubation. After these steps, most of the counts were found in the alkali extract, with very little remaining in the tissue.
<table>
<thead>
<tr>
<th>step of assay</th>
<th>remaining in incubation medium</th>
<th>wash steps (5 x 1ml)</th>
<th>in alkali extraction medium (=GAGs)</th>
<th>remaining in tissue after extraction</th>
</tr>
</thead>
</table>
| % of total counts | 92 | 1. 5  
2. 1  
3. 0.2  
4. 0.1  
5. 0.05 | 1 | 0.03 |
Figure 2.1  Labelled material at midday is not free sulphate.

Incubations were carried out at midday and the alkali extraction medium was precipitated with ethanol. Radioactive sulphate was measured in both the supernatant and pellet. Each point represents the mean ± SEM, n=10. (×=pellet, •=supernatant).
Figure 2.2 Amount of sulphate incorporation increases over time at midday.

Rates of sulphate incorporation were measured after incubation at midday for 1, 2 and 3h. Each point represents the mean ± SEM, n=10.
The amount of labelled sulphate in the incubation medium is determined by the amount that was incorporated by the cells. A dose curve can be seen in Fig. 2.3 where incubations were carried out for 18 hours over a range of labelled sulphate to the incubation medium. The rate of sulphate incorporation into the cells appeared to be dependent on the concentration of sulphate in the Hanks' medium, so there was a linear increase in the amount of sulphate incorporation as the concentration of labelled sulphate in the Hanks' medium increased.

2.3.2 Incorporation of sulphate at different concentrations

Sulphate incorporation was found to be maximal at concentrations of the order of 10-5 and 10-6 M. I tested whether the increase of the steady state incorporation was dependent upon the post-translationally programmed synthesis of the protein. The results of these experiments are presented in Fig. 2.4. The incorporation of sulphate into the cells was found to be linear with an increase in concentration up to a certain limit. However, at higher concentrations, there was no significant increase in sulphate incorporation. These results are consistent with the hypothesis that the incorporation of sulphate is limited by the availability of other nutrients such as amino acids.

2.3.3 Analysis of sulphate incorporated material

Further characterization of the sulphate incorporated material was performed using sections of the tissue. Different techniques were used to determine the concentrations of protein (see Chapter 3). Fig. 2.5 shows the distribution of labelled sulphate into the various fractions of the tissue. The majority of the labelled sulphate was incorporated into connective tissue and was found to be present in the extracellular matrix. Almost 25% of the sulphate was incorporated into the tissue matrix.

![Graph](attachment:image.png)
sulphate incorporated for a period of up to 3h of incubation at midday. This figure shows that the amount of sulphate incorporated increases approximately linearly over time for at least 3h, although there may be a slight increase in the rate of incorporation after 2h. As there was a large number of counts after 1h, it was decided that this was a sufficient incubation time.

The amount of labelled sulphate in the incubation medium influenced the amount that was incorporated by the sclera. A dose curve can be seen in Fig. 2.3 where incubations were carried out for 1h using various amounts of labelled sulphate in the incubation medium. The rate of sulphate incorporation into the sclera appeared to be dependent on the concentration of sulphate in the incubation medium, as there was a linear increase in the amount of sulphate incorporation as the concentration of labelled sulphate in the incubation medium increased.

2.3.2 Incorporation of sulphate at midnight.

Since rates of sulphate incorporation were to be measured at different times of the day in later chapters, I tested whether the kinetics of the sulphate incorporation assay at midnight varied from those at midday. A time course of incubation was carried out as was done at midday. Fig. 2.4 shows the amount of sulphate incorporated over 3h of incubation at midnight. As at midday, the amount of sulphate incorporated increased approximately linearly over 3h, with perhaps a slight increase in the rate of incorporation after 2h. The number of counts was higher in the experiment carried out at midnight compared to that carried out at midday. This suggests that there are differences in rates of sulphate incorporation between these two times. However, the possibility that this difference was due to slightly different incubation conditions (as these results were from two different experiments) could not be excluded. This issue will be addressed in Chapter 3.

Ethanol precipitation at midnight for 1, 2 and 3h (Fig. 2.5) gave similar results to those seen at midday with only negligible counts being found in the supernatant and most of the counts being found in the pellet. This suggests that all the radiolabelled sulphate was incorporated into macromolecules and was not free sulphate. Therefore, as at midday, the amount of labelled material in the alkali extract was taken as the amount of sulphate incorporated into the tissue.

2.3.3 Analysis of sulphate incorporated material

Further characterisation of this alkali extraction medium was performed using acetone precipitation. Different proteoglycans can be precipitated out by different concentrations of acetone (Volpi, 1994). Fig. 2.6A shows the distribution of labelled sulphate into the various fractions of glycosaminoglycans. The majority of the counts came out in the dermatan sulphate fraction (about 55%), with the least amount of counts being found in the chondroitin sulphate fraction (about 20%). About 25% of the
Figure 2.3 Amount of sulphate incorporation increases with sulphate concentration.

Rates of sulphate incorporation were measured after incubation at midday in buffer containing various concentrations of $^{35}\text{SO}_4$. Each point represents the mean ± SEM, n=10.
Figure 2.4 Sulphate incorporation increases over time at midnight.

Rates of sulphate incorporation were measured after incubation at midnight for 1, 2 and 3h. Each point represents the mean ± SEM, n=10.
Figure 2.5 Labelled material at midnight is not free sulphate.

Incubations were carried out at midnight and the alkali extraction medium was precipitated with ethanol. Radioactive sulphate was measured in both the supernatant and pellet. Each point represents the mean ± SEM, n=10. (×=pellet, ●=supernatant).
Figure 2.6 Sulphate is incorporated into glycosaminoglycans at midday.

A) Incubations were carried out at midday. The alkali extracts were precipitated with various amounts of acetone and the pellets were counted. The majority of the counts (55%) were found in the dermatan sulphate fractions, whereas least counts were seen in the chondroitin sulphate fraction (20%). About 25% of the counts were seen in the heparin fraction. Each point represents the mean ± SEM, n=10.

B) Distributions were recalculated according to the degree of sulphation of the GAG. The majority of the labelled residues were still in the dermatan sulphate fraction (50%), with 35% of the labelled residues in the chondroitin sulphate fraction and 15% of the labelled residues in the heparin fraction.
Sulphate incorporation (% of labelled residues)

- heparin
- dermatan sulphate
- chondroitin sulphate

B

Sulphate incorporation in the presence of sodium periodate was used to distinguish between the incorporation of labelled sulphate and the endogenous sulphate present in the matrix, as described in Materials and methods.

In the presence of sodium periodate, the incorporation of labelled sulphate was almost completely inhibited, indicating that the endogenous sulphate, which is not susceptible to oxidation by sodium periodate, accounted for more than 90% of the endogenous sulphate. Therefore, the distribution of sulphate was calculated as the ratio of incorporation of all O-SO₃ groups, by dividing the percentage of counts in the heparin fraction by 2, and dividing the percentage of counts in the dermatan sulphate fraction by 2, and then recalculating the distribution as a percentage of labelled sulphate. This can be seen in Figure 1A. The distribution of counts in the heparin fraction can be seen in Figure 1B. The distribution of the counts was different to that of the control, suggesting that the amount of label in the heparin fraction (40%) is lower than the amount of label in the dermatan sulphate fraction (30%).

After reoxidation, the labelled sulphate was resuspending the sample and reincubating it with amino acids, the amount of incorporation of non-O-SO₃ chains remained at 30% of the control, indicating that the endogenous sulphate was not removed. The incorporation of sulphate is sufficient to generate endogenous sulphate, and the incorporation of labelled sulphate is not sufficient to generate endogenous sulphate, as shown in Materials and methods.

The results of these experiments indicated that the incorporation of sulphate is sufficient to generate endogenous sulphate, and that the incorporation of sulphate can be measured as an increase in the amount of labelled sulphate, which is not susceptible to oxidation by sodium periodate. As sulphate incorporation continued to increase after more than 10 min of incubation, it was calculated that extension of sulphate incorporation was not reached after 10 min. This is important as in later chapters, rates of sulphate incorporation are to be compared under different conditions. If the measurement methods of sulphate incorporation had occurred after 10 min, their differences might not be able to be determined.

Also, the fact that sulphate incorporation continued at an approximately linear rate for up to 70 minutes shows that the system remained constant over this period.
counts were found in the heparin fraction. Each of these GAGs contains differing amounts of sulphate. Each heparin chain has 3 times as many sulphate groups as each chondroitin sulphate chain, whereas dermatan sulphate is twice as sulphated as chondroitin sulphate. Therefore, the distribution of sulphate was recalculated based on the ratio of sulphation of each GAG, by dividing the percentage of counts in the heparin fraction by 3, and dividing the percentage of counts in the dermatan sulphate fraction by 2, and then recalculating the distribution as a percentage of labelled residues. This can be seen in Fig. 2.6B, with 15% of the labelled residues in the heparin fraction, 50% in the dermatan sulphate fraction and 35% in the chondroitin sulphate fraction.

Acetone precipitations were also carried out at midnight. The distribution of counts in each fraction can be seen in Figure 2.7A. The distribution of the counts was different to that which was seen at midday with an increase in the amount of label in the heparin fraction (40%), a decrease in the amount of label in the dermatan sulphate fraction (40%) and the amount of label in the chondroitin sulphate fraction remaining the same at about 20%. Once again, redistributing the labelled residues according to the amount of sulphation of each GAG chains resulted in 28% of the counts being in each of the heparin and dermatan sulphate fractions, and the remaining 44% of the labelled residues in the chondroitin sulphate fraction (Fig. 2.7B).

2.4 Discussion

2.4.1 Sulphate incorporation in the chicken sclera

Incorporation of radioactive sulphate has previously been used as a measure of proteoglycan synthesis in many cartilaginous tissues (as described in Section 2.1), including the chicken sclera after the induction of FDM (Rada et al., 1991). Since sulphate groups are continuously being attached to the glycosaminoglycan chains, the rate of sulphate incorporation is likely to reflect the rate of GAG synthesis and, in this thesis, provides a useful method of measuring the growth of the chicken sclera, particularly in relation to FDM, as will be seen in Chapter 4.

The results presented here demonstrate that 1h incubation in medium containing labelled sulphate is sufficient to generate enough incorporation and that the incorporation of sulphate can be measured in an alkaline extract containing GAGs cleaved from the proteoglycans. As sulphate incorporation continued to increase after more than 1h of incubation it was concluded that saturation in sulphate incorporation was not reached after 1h. This is important as in later chapters, rates of sulphate incorporation are to be compared under different conditions. If the maximum amount of sulphate incorporation had occurred after 1h, then differences might not be able to be detected. Also, the fact that sulphate incorporation continued in an approximately linear fashion for up to 3h shows that the system remained functional over this period,
Figure 2.7 Sulphate is incorporated into glycosaminoglycans at midnight.

A) Incubations were carried out at midnight. The alkali extraction medium was precipitated with various volumes of acetone and the amount of radioactive sulphate in the pellets was measured. The majority of the counts were found in both the dermatan sulphate fraction and the heparin fraction (40% each) whereas the least amount of counts were seen in the chondroitin sulphate fraction (20%). Each point represents the mean ± SEM, n=6.

B) The distribution of counts was recalculated according to the degree of sulphation of each GAG. 28% of the labelled residues were found in both the heparin and dermatan sulphate fractions, with the remaining 44% of the labelled residues in the chondroitin sulphate fraction.
and that the rate of synthesis existing \textit{in vivo} is maintained for this period \textit{in vitro}, thus validating some of the assumptions underlying my experimental approach. Therefore it was assumed that the rate of sulphate incorporation measured after 1 h was a biologically significant measure.

After incubation, only about one percent of the counts came out in the alkali extraction medium. The rest of the counts remained in the incubation medium or came out in the washing steps. This demonstrates the necessity to wash the tissue before placing in the alkali extraction medium, as any extraneous sulphate must be removed. Only an extremely small percentage (0.0003 \%) of the total counts came out as free sulphate after extraction with alkali. However, this cannot be accounted for by diffusion of the sulphate into the tissue, since the volume of the tissue is approximately 0.7\% of the volume of the incubation medium. If there was a simple diffusion of sulphate from the incubation medium into the tissue, it would be expected that there would be about 0.7\% counts representing free sulphate remaining in the tissue as well.

The fact that the levels of free sulphate in the alkaline extracts were so low was surprising, since it might have been expected that these extracts would contain the intercellular sulphate pools. There are two possible interpretations of this result. The first, and most likely, is that the apparently low intracellular pools are an artefact of the experimental procedure, due perhaps to the washing out of the intracellular pools during the washing steps. The second interpretation is that the intracellular pools are genuinely low, and that labelled sulphate is incorporated into GAGs as rapidly as it enters the scleral cells. This would then imply that the rate-limiting step in the sulphate incorporation assay is the uptake of labelled sulphate into the scleral cell. If this were the case, then the assumption that sulphate incorporation gives a measure of GAG synthesis, and hence of scleral growth would not be valid. In subsequent chapters, I will show that the sulphate incorporation assay gives results which show changes which parallel those known to occur in scleral growth, making the first interpretation more likely. However, further studies on GAG synthesis based on incorporation of labelled sugars would be useful to further validate the interpretation I have adopted.

\section*{2.4.2 Sulphate is incorporated into GAGs}

The fact that there was essentially no labelled sulphate in the supernatant after precipitation with ethanol suggests that none of the label measured was free sulphate. Therefore the amount of label counted after extraction with ethanol is representative of sulphate which has been incorporated into macromolecules. These are presumably GAGs, since no other part of the proteoglycans contain sulphate. Further evidence that the sulphate had been incorporated into GAGs came from the fact that all the counts that were precipitated with ethanol could also be precipitated with acetone, which can be used to differentially precipitate various GAGs (Volpi, 1994). The distribution of labelled sulphate into the various fractions was different between midday and midnight,
and these differences will be discussed in Chapter 3, in relation to the diurnal rhythm of sulphate incorporation.

As the method of Volpi (1994) depends on the physico-chemical properties of the GAGs rather than the tissue itself, it was assumed that the contents of each fraction in the present study would be the same as in his paper, and therefore each pellet was labelled as such. However, it is possible that the amount of sulphation of each GAG chain varies not only from tissue to tissue, but also under different physiological conditions, and for this reason it would be useful to directly analyse the contents of each fraction in the chicken sclera to confirm this. Nevertheless, based on this assumption, I have recalculated the distribution of sulphate based on the estimated amount of sulphation of each GAG. Therefore, since no direct analysis was done, these calculations should be recognised as theoretical, giving some insight into the possible changes which occur in proteoglycan synthesis during eyegrowth, and thereby stimulating future investigations.

2.4.3 Sulphate incorporation as an index of proteoglycan synthesis

Based on the results presented in this chapter, I have assumed that this sulphate incorporation assay can be used to monitor rates of proteoglycan synthesis, and that changes in this rate will be associated with changes in scleral growth. Of course, it is possible that changes in rates of sulphate incorporation could instead reflect changes in rates of sulphation of GAGs or changes in the degradation of GAGs. Had there been an increase in the amount of GAGs, the issue of increased GAG degradation would have to be considered. However, in this study the amount of incorporated sulphate has been measured, rather than the levels of GAGs themselves, so rates of degradation are not relevant. In addition to this, Rada and Matthews (1994) have demonstrated that increased levels of GAGs paralled increases in the amount of aggrecan core protein precursor, suggesting that the amount of GAGs reflects the amount of proteoglycans. This together with the fact that they have also shown that increased sulphate incorporation parallels an increase in the amount of GAGs is strong evidence that increases in rates of sulphate incorporation reflect increases in synthesis of proteoglycans and not simply an increase in rates of sulphation. Further evidence to support these assumptions will be presented in later chapters. In comparison to measurements of axial length using callipers, or vitreal chamber depth using ultrasound techniques, this assay provides a way in which relatively short term changes can be detected. In the present study, this sulphate incorporation assay has been used to look at growth rates in the normal developing eye, and the excessive eye growth which occurs in response to FDM. It has also been used to measure the response of scleral growth to various pharmacological manipulations.
CHAPTER 3. DIURNAL RHYTHM OF SULPHATE INCORPORATION INTO SCLERA

3.1 Introduction

Many aspects of normal eye function are under diurnal control which means they systematically vary in relation to the light-dark cycle. Some of these are controlled by the external lighting conditions so that as the light-dark cycle varies, so does the rhythm of that particular function. However, others are internally controlled by a circadian oscillator. While circadian rhythms also vary with the external lighting conditions, they can be maintained under constant dark conditions, without the influence of light. A relevant example of a circadian rhythm is melatonin release from retinal photoreceptors (Hamm and Menaker, 1980; Besharse and Iuvone, 1983).

During normal eye growth in the chicken, there is a diurnal rhythm in eye growth as measured by rates of axial elongation (Weiss and Schaeffel, 1993). Measurements were made at the end of each 12h period of light or dark. It was found that there was an increase in axial length over the light phase, but over the dark phase there was no increase and even a slight regression. These workers also reported that when FDM was induced there was axial elongation during the dark phase in addition to the normal elongation seen during the light phase. This resulted in the form-deprived eye becoming larger than the control eye, and also myopic.

As discussed in Chapter 1, an increase in rates of sulphate incorporation has been measured in eyes where FDM has been induced (Rada et al., 1991; Rada et al., 1992; Rada and Matthews, 1994; Rada et al., 1994). Since both axial elongation and rates of sulphate incorporation increase during FDM, and axial elongation is diurnal during normal eye growth (Weiss and Schaeffel, 1993), it seemed possible that rates of sulphate incorporation would be diurnal too, with light acting to stimulate rates of GAG synthesis, following a pattern similar to that seen for axial elongation, that is, with a higher rate of sulphate incorporation being observed during the day, and a lower rate at night.

3.2 Methods

3.2.1 Animal handling

Chickens were maintained as described in Chapter 2, with lights normally coming on at 0600h and lights going off at 1800h. In some cases, lights came on or off at different times, and different intensities of light were used, as specified. In constant dark conditions (which were maintained for a maximum of 2 days) the animals were still able to find food and water, as they had previously been living in the same environment under normal light-dark conditions. Different light intensities were
achieved by putting the animals in white bins in room light filtered by neutral density filter sheets (LEE Filters Ltd.) and/or shade cloth. Light intensities were measured using a Gossen Mavolux digital light meter. The 'light' control value was around 600 lux.

3.2.2 Sulphate incorporation
The sulphate incorporation assay was performed as described in Chapter 2. The assay was performed at different times throughout the 24h cycle. When animals were taken during the dark phase, dissections were done under dim red light (<0.5 lux) so that dark conditions were maintained as much as possible until the retina and sclera were separated. However, as discussed in Chapter 2, the incubations were always carried out in the light.

3.3 Results

3.3.1 Difference in sulphate incorporation between midday and midnight.
As discussed in the previous chapter, it appeared that there was a higher rate of sulphate incorporation at midnight compared to midday. However, to determine whether these differences were due to changes in rates of sulphate incorporation, rates of sulphate incorporation were measured at midday and midnight in the same experiment, using the same batch of chickens and one lot of incubation medium for both time points. Rates of sulphate incorporation were measured at midday and midnight in chickens of three different ages, to see if there were any differences between rates of sulphate incorporation at these times, and if so, whether these differences were maintained throughout development. Fig. 3.1 shows that there was a difference in rates of sulphate incorporation between midday and midnight in chickens up to around 3 weeks of age, with higher rates at midnight compared to midday. However, this difference was the opposite of what was expected, given that previous studies have shown the rate of axial elongation to be higher during the day than at night. It is important to note that this difference between rates of sulphate incorporation at midday and midnight disappeared by about 3 weeks of age.

3.3.2 24 hour rhythm
To look at the diurnal difference in more detail, rates of sulphate incorporation were measured at various times throughout a 24h period at 2 hourly intervals at times as indicated, in chickens that were 10-12 days old (Fig. 3.2). A relatively low rate of sulphate incorporation was seen after the lights went out at 1800h. This low rate remained until 2200h, when there was a rapid rise until midnight (0000h). This higher
Figure 3.1 Differences in rates of sulphate incorporation between midday and midnight at different ages.

Rates of sulphate incorporation were measured in chickens of various ages at midday (hatched columns) and midnight (black columns). Each point represents the mean ± SEM, n=6.
Figure 3.2 Diurnal variation in rates of sulphate incorporation over 24h.

Incubations were carried out every 2h over a 24h period at times as indicated. Each point represents the mean ± SEM, n=10.
rate remained until the end of the dark phase (0600 h). After this time, the rate of elongation was a gradual decrease until 1200 h, when the rate was at 1000 h. The rate of elongation has previously been found to be high during the day and decrease at night (Trend and Newby, 1969). However, the rates for each measurement were taken at the end of the light and dark phases, and therefore only represent cumulative change in aerial length over 12 h.

To make the response more comparable, the rate of elongation was converted to the rate of elongation at the end of the light and dark phases, which was then plotted. The data show that the rate of elongation is highest during the light phase and lowest during the dark phase, with a significant increase in elongation rate after the light phase.

The data also suggest that the rate of elongation is influenced by the light-dark cycle, with a significant decrease in elongation rate during the dark phase. This suggests that the light-dark cycle may play a role in regulating the rate of elongation.
rate remained until the end of the dark phase (0600h). After the lights came on there was a gradual decline until 1200h, when the low rate seen at 1800h was reached.

As noted before, this was the opposite of what was expected, given that axial elongation has previously been found to be high during the day and low at night (Weiss and Schaeffel, 1993). However, the axial length measurements were only made at the end of the light and dark phases and therefore only represent cumulative changes in axial length over 12h. To make the sulphate incorporation results more comparable to those on axial elongation, total number of 'sulphate incorporation hours' have been calculated by integration. These calculations yielded values of 953 units for the dark phase and 823 units for the light phase. Compared to the marked difference between light and dark in axial elongation rates, there was little difference between these two values of cumulative sulphate incorporation. In addition to this, the slight difference was still opposite in direction to that seen in axial elongation.

The fact that sulphate incorporation had a diurnal rhythm suggests that it could be under one of two controlling factors. Either it is controlled by a circadian oscillator and therefore has an internal control, or it is a light-driven rhythm, controlled by the external lighting conditions. Since circadian rhythms can free-run under constant dark conditions, without needing the influence of light, a test of whether something is under circadian control is to see if its rhythm can be maintained in constant dark.

### 3.3.3 Constant dark

Fig. 3.3 shows the pattern of sulphate incorporation over 24h with chickens which had been kept in the dark for 36h before the first time-point in the experiment and remained in the dark for the rest of the time points. The amount of sulphate incorporation was expressed as a percentage of the value measured at midnight in control animals (which had been on a normal 12:12h light:dark cycle). As can be seen, there was an abolition of the normal rhythm of sulphate incorporation when no light cues were available. In fact, all of the points were higher than the midnight control (100%), indicating that without the influence of light, there is nothing to bring the rate of sulphate incorporation down to its low level. This also suggests that in addition to being essential for the diurnal rhythm, light may also act as a tonic inhibitor of sulphate incorporation. Regular exposure to light may be required to bring down rates of sulphate incorporation so that without light for more than 12h, the rate increases to levels higher than those normally seen during the dark phase. Alternatively, instead of light being a suppressor, dark may stimulate rates of sulphate incorporation which would again result in higher rates of sulphate incorporation under constant dark conditions, as was observed here.
Figure 3.3  Diurnal variation in rates of sulphate incorporation is abolished in constant dark conditions.

Rates of sulphate incorporation were measured over a 24h period at the times indicated. Chickens were placed in constant dark conditions for the 36 hours preceding the time points shown on this Figure, and values are expressed as a percentage of the midnight value from chickens kept in a normal 12:12 hour dark:light cycle. Each point represents the mean ± SEM, n=10.
2.4. Discussion

The results presented in the present study demonstrate a biphasic effect of light on sulphate incorporation. The sulphate incorporation was measured under different light conditions and compared to the control. The results indicate that the rate of sulphate incorporation is highest during the light phase, which ends at 1800h. There is a sharp decrease in the rate of sulphate incorporation at the middle of the light phase, which continues up to the end of the dark phase. In the middle of the dark phase a high
3.3.4 Light suppression

If light is required to maintain the diurnal rhythm, then it would appear to have a progressive suppressive effect as there is only a gradual decrease in the rate of sulphate incorporation after the lights come on after 0600h during a normal dark-light cycle. This suppressive effect of light was also demonstrated by the fact that rates of sulphate incorporation decreased when the lights were put on at other times in the cycle. In control birds there was an increase in rates of sulphate incorporation after the lights went off at 1800h which reached its maximum by midnight (0000h). However, if the lights were kept on, the normally high rate at midnight was suppressed, as is shown in Fig 3.4.

A similar effect was also seen when the lights were put on at midnight (Fig. 3.5). In control birds the relatively high rate of sulphate incorporation remained throughout the rest of the dark phase until 0600h. However, in birds which were subjected to the lights at 0000h there was a gradual decrease in sulphate incorporation which was similar to that seen after lights come on in the normal 24h time course (between 0600 and 1200h, refer to Fig. 3.4). There was no significant difference between the two values at 0200h, however at 0400h and 0600h the differences between the control rates of sulphate incorporation and the rates when the lights came on were statistically significant.

3.3.5 Light intensity dependence

The ability of light to suppress rates of sulphate incorporation appeared to depend on the intensity of the light being used. As in Fig. 3.5, lights were put on at 0000h to bring the normally high rate of sulphate incorporation down by 0600h, but this time different light intensities were used. Fig. 3.6 shows that at light intensities down to 1 lux there was still suppression of the normally high rate of sulphate incorporation at 0600h, with no significant difference being observed compared to the full light (approx 600 lux) value. However, 0.4 lux of light and below was not enough to cause this suppression, since the rate at 0600h after exposure to light at these low intensities was not significantly different to that in the control (dark) birds.

3.4 Discussion

The results presented in this chapter demonstrate a rhythm in rates of sulphate incorporation. The assays were carried out exactly the same way at all times of the day, including the incubation always being carried out in the light. Thus, the differing rates of sulphate incorporation are likely to reflect differences which existed in vivo. There was a low rate of sulphate incorporation in the middle of the light phase which continued into the early part of the dark phase. By the middle of the dark phase a high
Figure 3.4 Light suppresses rates of sulphate incorporation between 6pm and midnight.

Rates of sulphate incorporation were measured at 0000h. Control animals were kept on a normal 12:12 hour light:dark cycle where lights went off at 1800h and were therefore in the dark when samples were taken. Experimental animals remained in the light after 1800h and so were still in the light when samples were taken. The values were significantly different (p<0.05). Each point represents the mean ± SEM, n=10.
Midnight

light off at 1800h
(control)

lights remained
on at 1800h

Sulphate incorporation (CPM)
Figure 3.5  Light suppresses rates of sulphate incorporation between midnight and 6am.

Rates of sulphate incorporation were measured between 0000h and 0600h as indicated. Control animals were kept on a normal 12:12h light:dark cycle and were therefore in the dark during this period (∎). Other animals were put into the light at 0000h and samples were taken after this (∗). While there was no difference between the two sets of data at 0000h and 0200h, the differences between the two sets of data at 0400h and 0600h were significant (p<0.05). Each point represents the mean ± SEM, n=10.
Figure 3.6 Suppression of sulphate incorporation is dependent on light intensity.

Rates of sulphate incorporation were measured at 0600h. Control animals had been on a normal 12:12h light:dark cycle and so had been in the dark since 1800h. Other groups of animals were placed into lights of various intensities as indicated in the Figure from 0000h to 0600h. There is no significant difference between the dark and 0.4 lux values of sulphate incorporation, nor is there a significant difference between the values at 1 lux and light. However, the values of sulphate incorporation at both dark and 0.4 lux are significantly different from the values at 1 lux and light (p<0.05). Each point represents the mean ± SEM, n=10.
The graph and data presented indicate the following:

- **Sulphate Incorporation (CPM)**
  - **dark**
  - **0.4**
  - **1**
  - **light**

- **Light intensity (lux)**

**Analysis:**
- The sulphate incorporation decreases as light intensity increases from dark to light conditions.
- The trend suggests a correlation between light intensity and sulphate incorporation, implying that higher light intensities reduce sulphate incorporation.

**Conclusion:**
- This data supports the hypothesis that light intensity affects sulphate incorporation in plants, likely due to metabolic processes that are light-dependent.
- Further studies could explore the specific mechanisms involved in the light-induced reduction of sulphate incorporation.
rate of sulphate incorporation had been reached and was maintained until the lights came on again, when there was a gradual decline in rates of sulphate incorporation until the low light level was reached again by the middle of the light phase. This rhythm was not maintained under constant dark conditions. Light had the ability to suppress the rate of sulphate incorporation, however this suppressive ability was dependent on light intensity. The diurnal rhythm diminished as the animals got older.

3.4.1 Two components of sulphate incorporation

It appeared that the difference in rates of sulphate incorporation between the low value at midday and the high value at midnight diminished as the animals grew. This suggests that there may be two components to the rates of sulphate incorporation being measured. There may be a maintenance component which is responsible for keeping the sclera at its normal level of proteoglycan turnover with an extra, phasic component responsible for the regulated growth seen during early development. During this early period the eye has to control its growth rate so that it matches the developing changes in optical power, suggesting that the phasic rhythm in rates of sulphate incorporation seen during the first two weeks may act as a regulator of eye growth. As the chicken gets older this regulatory component may become less important as emmetropia is reached and as the rate of eye growth slows down. However, one possibility is that different regions of the sclera grow at different rates. As the 8mm scleral punches were always taken from the central part of the tissue, this means that in younger animals the punch encompassed a larger part of the total sclera compared to punches coming from older animals with larger eyes. If the changes were only in the periphery of the sclera, this could explain the reduction in the diurnal difference during development. Therefore this possibility needs to be tested.

Thorough analysis of the material into which sulphate is incorporated has been carried out in previous studies (Rada and Matthews, 1994; Rada et al., 1994). Most of it is aggrecan, a major cartilaginous proteoglycan containing chondroitin sulphate and keratan sulphate. Sulphate was also incorporated into decorin, a proteoglycan containing dermatan sulphate and chondroitin sulphate as their GAGs. However, while there was an increase in the synthesis of both of these proteoglycans, there was an increase only in the accumulation of aggrecan. These studies agree with the results of the present study, where most of the sulphated GAGs appear to be dermatan sulphate and chondroitin sulphate, however, each aggrecan molecule has approximately 100 chondroitin sulphate chains and approximately 20 keratan sulphate chains, compared to decorin which has 1 chondroitin sulphate and 1 dermatan sulphate chain. Therefore, it would have been expected that there would be more counts in the chondroitin sulphate fraction compared to the dermatan sulphate fraction. Since this fractionation method did not look at keratan sulphate, it is unknown into which fraction keratan sulphate would have precipitated into. Based on its charge density it would most likely come
out in the chondroitin sulphate fraction, but this requires further investigation. What also remains unknown is what the labelled sulphate in the heparin fraction represents.

The two different rates of sulphate incorporation seen at midday and midnight may reflect synthesis of different proteoglycans as the distribution of label between the different fractions was different at these two times, although of course, direct analysis of the fractions is needed to confirm this. Compared to midday, there was an increase in the amount of sulphated GAGs in the heparin (and presumably heparan sulphate) fraction, in addition to the increase in the amount of sulphated GAGs in the chondroitin sulphate fraction. While Rada et al. (1994; Rada and Matthews, 1994) have characterised sulphate incorporation into scleral proteoglycans during the daytime, there are no reports of which proteoglycans are synthesised during the night. These differences may also reflect differences in the proteoglycans involved in the maintenance and growth components. While the rates of synthesis of some proteoglycans may be increased and decreased to regulate growth rates of the eye, the same proteoglycans would probably be involved in maintenance and may therefore remain constant throughout the day and night. More detailed biochemical studies will be required to resolve these issues.

It is well established that there is excessive eye growth during deprivation which results in FDM and that upon removal of translucent diffusers, there is a subsequent cessation of eye growth in an attempt to compensate for the imposed myopic defocus (Wallman and Adams, 1987). However, these changes in growth rates appear to be slower in older chickens compared to younger animals, and this has also been observed in tree shrews (Norton, 1990). This suggests that the ability of the eye to change its growth rate in order to compensate for its refractive error diminishes as the animal gets older and may fit with the idea that the regulatory component of growth disappears with age.

3.4.2 The diurnal rhythm is controlled by the external lighting conditions

Circadian rhythms can maintain their rhythm due to an internal circadian oscillator, without needing the resetting influence of light. Therefore, if a rhythm can be maintained in constant dark conditions it is circadian. These type of experiments are best carried out after the animals have had at least one full cycle in the dark, in case the effects of light have a long time-course. The results presented here show that the normal diurnal rhythm could not be maintained in constant dark. Not only was there no evidence of the normal diurnal differences, but the rates of sulphate incorporation at all time points were higher than those seen in the midnight control point (taken from animals in normal lighting conditions). This suggests that during the normal light-dark cycle there may still be some residual effects of light, even at midnight after 6h of darkness, which are sufficient to prevent the maximal rate of sulphate incorporation from being reached. However, after 36h of darkness any residual effects of light
appeared to have been removed, resulting in high rates of sulphate incorporation. This suggests that in addition to the effects of light which impose a diurnal rhythm on the rate of glycosaminoglycan synthesis and scleral growth, light also has longer-term suppressive regulatory effects.

These results are in conflict with the report of a circadian rhythm of GAG synthesis in isolated sclera by Nickla and Wallman (1997) who found that scleral punches maintained a diurnal rhythm for 3 cycles in culture. Since the punches could maintain a rhythm without any physiological stimulus, this is strong evidence that the rhythm is circadian. While it remains unknown what lighting conditions the experiments were carried out under, it is assumed that they were carried out in the light. However, as the sclera is not light sensitive this may not be a relevant factor. These results therefore suggest that scleral chondrocytes have some sort of endogenous control of GAG synthesis which can be maintained under culture conditions, and imply that there is an independent circadian oscillator in the sclera.

Is very difficult to reconcile my failure to observe a circadian rhythm in vivo with the observations of Nickla and Wallman (1997) that the isolated sclera expresses an endogenous circadian rhythm in vitro. The classical test of a circadian rhythm is that it free-runs in constant dark, which is based on the assumption that light can exert an active control over the rhythm, whereas dark is permissive. One way in which the two sets of observations could be reconciled would be to abandon the latter assumption, and in the case of this rhythm to assume that light is an active suppressor (for which there is some evidence), and that dark is an active enhancer of GAG synthesis. Light and dark would generate signals within the retina which were ultimately detected by the sclera and used to regulate the rate of GAG synthesis. Under these conditions, instead of free-running in constant dark despite the continuing endogenous rhythmic drive in the sclera, the rate of GAG synthesis would be up-regulated by the dark signal all the time. However, there is no precedent for such an explanation in the literature on circadian rhythms, and it is difficult to understand under which conditions the endogenous rhythm might manifest itself in vivo, since the influence of light and dark would appear to predominate.

There is evidence that in bone and cartilage there is a diurnal rhythm in rates of growth, with cell division and extracellular matrix synthesis operating in counter phase, however it remains unclear whether this rhythm is circadian (Simmons, 1992). Assuming for the moment that it is a circadian rhythm, it may be that this rhythm occurs in the chicken sclera and may explain the results of Nickla and Wallman (1997). If this was the case, it could be postulated that this rhythm is a remnant of evolution, and that over time, a special form of light-regulation has been adopted to allow for the precise matching of eye growth and optical power which occurs during emmetropisation.
3.4.3 The diurnal rhythm in rates of sulphate incorporation does not match that of axial elongation.

The fact that sulphate incorporation was high at midnight and low at midday was the opposite of what was expected given the reported rhythm of axial elongation (Weiss and Schaeffel, 1993). However, one possible consideration in trying to reconcile these results is the fact that axial elongation was only measured at the end of each phase. Although an increase in axial elongation was seen at the end of the light phase, what remains unknown is where and for how long in this phase the increase occurs. To make the two sets of data more comparable, the total amounts of sulphate incorporation over the entire 12h periods of light and dark were calculated. However, the calculation of cumulative amounts of sulphate incorporation did not resolve this difference as the amounts of total sulphate incorporation were relatively similar in both the light phase and the dark phase, and therefore did not reflect the marked differences seen in axial elongation. More recent measurements of axial elongation rates, made at 6 hourly intervals (Nickla et al., 1997), have shown that the peak of axial elongation occurs in the middle of the light phase and that the eye is shortest during the dark phase. They observed that the eye was longest at 1400h and shortest at 0200h with animals in a 14:10h light:dark cycle (lights coming on at 0800h and going off at 2200h).

So how can the phase shift in these two patterns be reconciled? Firstly, there appears to be a delay between the lights coming on and off, and the change in rates of sulphate incorporation. While the lights went off at 1800h, an increase in rates of sulphate incorporation was not observed until 2200h, with the maximum not reached until midnight. Similarly, the lights came on at 0600h but the lowest rate of sulphate incorporation was not measured until midday. This suggests that the retina may be involved, as it is the light-sensitive component of the eye. Time could be needed for retinal compounds to exert their effects on the sclera, perhaps via the RPE and choroid. There appear to be two differences between the increase and decrease in rates of sulphate incorporation. Firstly, the rise in rates of sulphate incorporation after the light goes off was very rapid, occurring within a 2h period, whereas the decrease in rates of sulphate incorporation was more gradual, over 6h. One possible explanation for this gradual decrease may be that there is a pool of non-sulphated proteoglycans in the Golgi apparatus and the ER. The already synthesised proteoglycans may continue to be sulphated after the lights come on, until this pool is depleted. Thus the accumulation of already synthesised GAGs may account for the gradual decline in rates of sulphate incorporation when the lights come on. Secondly, and more difficult to explain is the difference in that while light appears to have a more immediate effect on rates of sulphate incorporation, with the decrease beginning within 2h of the lights coming on, there is an approximately 4h delay between the time the lights go off and the subsequent rise in rates of sulphate incorporation. This suggests that it may not be the
on-off' response of one retinal compound responsible for the increase and decrease in rates of sulphate incorporation, but rather, the increase and decrease in scleral growth may be due to two separate controlling factors, both of which may have different kinetics. This is consistent with the idea that there are separate signals of dark and light.

There may also be a further delay between the incorporation of sulphate into glycosaminoglycans and overall growth of the sclera resulting in increased axial length. This is plausible given that time is needed for the labelled sulphate incorporated into the glycosaminoglycan chains in the endoplasmic reticulum and the Golgi apparatus to be secreted out of the cell into the extracellular matrix, and for the formation of extracellular aggregates of proteoglycans and hyaluronic acid. In the rat chondrosarcoma these processes of secretion and aggregation together take approximately 2h (Kimura et al., 1984). Assuming that this process could take up to 6h in the chicken sclera, together with the delayed effect of light which also takes about 6h, this could explain why there is a phase shift difference of approximately 12h between the peak and trough in rates of sulphate incorporation observed here and those in axial elongation previously observed (Weiss and Schaeffel, 1993; Nickla et al., 1997).

3.4.4 Retinal control of the diurnal rhythm

If the retina plays a role in controlling rates of eye growth, what output of the retina is involved? Some retinal neurotransmitters have been implicated in controlling FDM and some of these, namely dopamine and the enkephalins have diurnal patterns of release. Dopamine is released during the day and not at night (Kramer, 1971; Kirsch and Wagner, 1989; Kolbinger et al., 1990), in a light-driven, not circadian rhythm. Conversely, the enkephalins (and neurotensin and somatostatin which are colocalised with the enkephalins in the ENSLI amacrine cells) are released in parallel in the dark (Yang et al., 1997), in a light-driven, not circadian rhythm. As reviewed in Chapter 1, these neurotransmitters are all elements of the retinal dark-light switch (Morgan and Boelen, 1996). The fact that these neurotransmitters have light-driven, diurnal patterns of release, together with the observation that suppression of sulphate incorporation appears to depend on a critical light intensity, suggests that this switch may be involved in controlling rates of sulphate incorporation. As discussed in Chapter 1, this switch flips from its dark to light state at a light intensity of 0.1 lux. However, as the sclera is further down the chain of events after retinal stimulation by light, it could be that a higher level of light (0.4 lux) is required to elicit a response powerful enough to be transmitted to the sclera. For example, dopamine release shows a switch in behaviour coupled with a dependency on light intensity (Brainard and Morgan, 1987; Megaw et al., 1997). Therefore, while a lower light intensity can stimulate dopamine release in the retina, higher concentrations of dopamine may be required before an effect on the
sclera can be observed, and a slightly higher light intensity may be required to stimulate the additional increase in dopamine release which is needed for an effect on the sclera. Alternatively, it is possible that the retina simply needs to be in its light state (as opposed to its dark state) to enable the appropriate visual processing which determines the release of retinal messenger, not otherwise associated with the switch, which is ultimately responsible for eye growth, and that additional light intensity is required to give sufficient drive to retinal circuits active in the light active retina.

If the retinal dark-light switch is involved in controlling sulphate incorporation into the sclera, it would be expected that either dopamine acts to suppress (as it is released in the light during which time rates of sulphate incorporation decrease), or enkephalins, neurotensin or somatostatin act as stimulatory factors as they are released at night, when there is an increase in rates of sulphate incorporation, or both. Melatonin is not likely to be involved as the rhythm in sulphate incorporation cannot be maintained under constant dark conditions, whereas the rhythm of melatonin release is maintained under constant dark conditions (Besharse and Iuvone, 1983; Hamm and Menaker, 1980). The effects of these compounds on rates of sulphate incorporation will be examined in Chapter 5. Rates of sulphate incorporation in response to FDM will be examined in the next chapter.
CHAPTER 4. FORM-DEPRIVATION MYOPIA

4.1 Introduction

The previous chapter demonstrated a rhythm in rates of sulphate incorporation which was assumed to reflect an increase in the rate of proteoglycan synthesis. A critical test of the validity of this sulphate incorporation assay as a measure of scleral growth is to see whether it responds in parallel to known changes in growth rates in FDM. It has previously been shown that there is an increase in proteoglycan synthesis during FDM and that this correlates with an increased growth of the eye in the axial direction (Rada et al., 1991; Rada et al., 1992; Rada and Matthews, 1994; Rada et al., 1994). The previous chapter demonstrated a rhythm in rates of sulphate incorporation during normal eye growth which, with a phase shift, matches the changes in rates of axial elongation observed in normal eyes (Weiss and Schaeffel, 1993). Furthermore, Weiss and Schaeffel (1993) presented results showing that this rhythm (in which there is growth during the light phase but no growth during the dark phase) is abolished during the development of FDM. They reported that there was also growth during the dark phase, in addition to the normal growth during the light phase, resulting in an increase in the amount of growth each day, resulting in a larger, myopic eye. From their results I expected that, since the diurnal rhythm of axial elongation disappeared after deprivation, the rhythm in rates of sulphate incorporation would also disappear during deprivation. If it is to match the changes in axial elongation, it would be expected that normally high rates of sulphate incorporation seen at midnight would remain the same, while there would be an increase in the normally low rate of sulphate incorporation seen at noon.

If this assay does reflect growth rates of the eye, then if there is an increase in rates of sulphate incorporation after deprivation, this also has to correlate with an increase in eye size and refractive error. This assay should also show a decrease in rates of sulphate incorporation upon removal of translucent diffusers, which once again should correlate with a decrease in rates of axial elongation, and a refractive error heading towards zero. Furthermore, the increase in rates of sulphate incorporation should be preventable with daily periods of normal vision, which have previously been shown to prevent the development of FDM (Nickla et al., 1989; Vingrys et al., 1991; Napper et al., 1995).

If this assay can be used to reflect changes in eye growth in response to FDM, it can then be used to monitor changes in scleral growth in response to various changes in vivo, in an attempt to isolate the factors which are responsible for scleral growth during FDM. These could be changes in visual stimulation, or changes in the levels of various retinal neurotransmitters. The latter will be studied in Chapter 5.
4.2 Methods

4.2.1 Animal handling

Animals were housed as described in Chapter 2. Translucent diffusers were created from Codral® blister strips, Wellcome Pharmaceuticals. Chickens were anaesthetised by inhalation with isoflurane and the translucent diffuser was attached around the left eye using ‘Supa glue’ (Selleys). In cases where the translucent diffuser had to be removed more than once during the course of the experiment, the translucent diffusers were attached to velcro and an opposing velcro ring was glued around the eye of the chicken under anaesthesia. Unless otherwise specified, chickens were fitted with a translucent diffuser at 10 days of age, and wore the translucent diffusers for 10 days. Only one eye of each chicken was form-deprived, so that the other eye could be used as an internal control.

4.2.2 Sulphate incorporation

The sulphate incorporation assay was performed as described in Chapter 2.

4.2.3 Axial length

Chickens were anaesthetised by inhalation of isoflurane and decapitated. The eyes were rapidly enucleated and the external axial length of the eye was measured to the nearest 0.1mm using vernier callipers.

4.2.4 Refractive error

Chickens were anaesthetised by inhalation of isoflurane. Refractive error was measured using streak retinoscopy and a trial lens rack. The final refraction was expressed as the spherical equivalent of the two principal meridians.

4.2.5 Acetone precipitation of GAGs

Acetone precipitations were carried out as described in Chapter 3.

4.2.6 Assay for N-acetyltransferase activity

The retina and pigment epithelium were removed under dim red light (0.5 lux) and rapidly frozen in dry ice until the assay was carried out. Each sample was thawed when required, and homogenised in 250mM potassium phosphate buffer (pH 6.5) containing 1mM acetyl CoA (Sigma). Samples were then centrifuged for 10min at 12,000g at 4°C and the pellet discarded.

The assay was carried out in duplicate. 75µl of the retinal supernatant was used in each incubation and was added to 25µl of 8mM tryptamine (Sigma) containing 1µl 14C acetyl CoA, specific activity of 2.1 GBq/mmol (Amersham). The total 100µl was incubated at 37°C for 15min. After this time, 1ml of a stop buffer (3% isoamyl alcohol...
in toluene) was added to the samples, which were then vortexed 3 times for 10sec and centrifuged at 3,000g for 3 min. 750µl of the top (organic) phases were counted in 4ml HiSafe II scintillation fluid in a liquid scintillation counter as described earlier.

4.3 Results

4.3.1 Form-deprived eyes become myopic

As there is a wide variety of translucent diffusers used to induce form-deprivation myopia, it was necessary to demonstrate the translucent diffusers used in this project caused the eye to become myopic. This was done by measuring the refractive error of the eyes during deprivation. Refractive error measurements are shown in Fig. 4.1 (In any graph where there are no error bars for some data points seen this is because the standard errors were too small to be seen). Refractive errors were measured when the chickens were 2 days old varied between 0 and +1 D. By 10 days of age this small amount of variation had disappeared and they were all close to 0 D. These values seem to be slightly lower than those found by other workers. Even though anaesthesia was used to control for accommodation, it is possible that there was still a small amount of accommodation occurring (perhaps as the anaesthetic was wearing off) which could account for the low values measured. It was at this age that the chickens were fitted with translucent diffusers. A refractive error of 0D remained in the control eyes throughout the deprivation, regardless of how long the translucent diffusers were worn (Fig 4.1). This figure also shows that there was a rapid change in the value of refractive error in the form-deprived eye during the first week of deprivation, so that by 7 days the refractive error was approximately -20D. However, after this time the rate of change slowed down considerably, so that by 10 days the average refractive error was approximately -23D. This is consistent with many previously reported values of form-deprivation myopia.

4.3.2 Form-deprived eyes grow larger than controls.

To ensure that the changes in refractive error were accompanied by an increase in axial length, as is seen in other studies of FDM, axial lengths of the deprived eyes were compared to their controls over the 10 day period of deprivation. Fig. 4.2 shows that before the translucent diffusers were put on, there was no difference in axial length between the two eyes. However, after deprivation a difference developed, and this difference increased over the period of deprivation.

Similarly, there was initially no difference in the wet weights between the two eyes, but after deprivation a difference developed, which continued to increase over the period of deprivation (Fig. 4.3). Therefore it was concluded that this particular deprivation protocol induced myopia due to axial elongation.
Figure 4.1 Increase in refractive error during form-deprivation.

Chickens were form-deprived and refractive errors were measured in both form-deprived and control eyes over a 10 day period. The refractive error of the control eye (●) remained zero. However, the refractive error of the form-deprived eye (×) rapidly decreased until the 7th day after which it remained relatively constant around -23 D. Each point represents the mean ± SEM, n=4.
Number of days translucent diffusers worn
Chickens were form-deprived and axial length was measured in both form-deprived and control eyes over a 10 day period and expressed as the difference between the two. The axial length of the control eye remained relatively constant but the form-deprived eye continued to grow so that the difference continued to increase. Each point represents the mean ± SEM, n=4.
Difference in axial length between form deprived and control eye (mm)

Number of days translucent diffusers worn
Figure 4.3 Increase in eye weight during form-deprivation.

Chickens were form-deprived and the weight of the eye was measured in both form-deprived and control eyes over a 10 day period. The graph was plotted as the difference between these two values. The weight of the control eyes increased over time but at a slower rate compared to the form-deprived eyes. Therefore the difference increased over time as is shown in the Figure. Each point represents the mean ± SEM (n=4).
4.3.3 Increase in sulphate incorporation at midday during FDM

Based on the observations of Weitz and Schaeffel (1983) who looked at rates of axial elongation, it was expected that the diurnal rhythm of sulphate incorporation would be abolished during FDM, and that the normally low rate of sulphate incorporation seen at midday would increase to a higher rate of sulphate incorporation similar to that seen at midnight. Therefore, measurements were made at midday over a ten-day time course as shown in Fig. 4.4. At all the timepoints assessed there was a significantly higher rate of sulphate incorporation in the form-deprived eye compared to the control eye.

Short-term effects of deprivation

The measurement of refractive error, axial elongation and sulphate incorporation were studied in more detail over the first 48h of deprivation. Chickens were fitted with translucent diffusers at midnight and measurements were made every 24h. On the following 48h, it was found that there was no change in refractive error and the deprived eye remained the same size until 24h after the translucent diffusers were put on. After this time there was a steady increase in the refractive error of the form-deprived eye, whereas that of the control eye remained constant. The difference in axial length between the form-deprived eye and its control was also measured every 24h (Fig. 4.6). Again, there was no significant difference between these two sets of data until 24h after fitting the translucent diffusers. After this time the difference continued to increase. Figure 4.7 shows that even though there was an increase in rates of scleral sulphate incorporation after fitting the translucent diffusers compared to the control eye, that is, at midday, after 48h of deprivation the rate of sulphate incorporation in the form-deprived eye was still significantly lower than that of the control eye. It is possible that the increased delay between the time the translucent diffusers are put on and when there are increases in the growth rate of the eye. This could be due to a period of days before eye growth is actually initiated.

4.3.5 Increase in GAG synthesis at midday during FDM

Acidic precipitations (Völpi, 1994) were also carried out on the GAGs synthesised by sclera from both deprived (for 10 days) and control eyes. Figure 4.8a shows the distribution of labelled sulphate in each fraction of GAGs. There was a greater total number of counts in the form-deprived eye compared to the control eye. However, the distribution of label in these two groups was different. There was a decrease in the amount of label in the heparin fraction of the deprived eye (33%), but there was an increase in the amount of label in the chondroitin sulphate fraction (15%). The amount of label in the dermatan sulphate fraction remained relatively constant (60%). Once again, recalculating the distribution based on the amount of sulphate in each GAG chain (Fig. 4.8b) as described in Chapter 3 results in most of the labelled...
4.3.3 Increase in sulphate incorporation at midday during FDM

Based on the observations of Weiss and Schaeffel (1993) who looked at rates of axial elongation, it was expected that the diurnal rhythm of sulphate incorporation would be abolished during FDM, and that the normally low rate of sulphate incorporation seen at midday would increase to a higher rate of sulphate incorporation similar to that seen at midnight. Therefore, measurements were made at midday over a ten day time course as shown in Fig. 4.4. At all the timepoints measured there was a significantly higher rate of sulphate incorporation in the form-deprived eye compared to the control eye.

4.3.4 Short-term effects of deprivation.

The development of refractive error, axial elongation and sulphate incorporation was studied in more detail over the first 48h of deprivation. Chickens were fitted with diffusers at midday and measurements were made every 12h over the following 48h. Figure 4.5 shows that there was no change in refractive error in the deprived eye compared to the control until 24h after the translucent diffusers were put on. After this time there was a steady increase in the refractive error of the form-deprived eye, whereas the control eye remained emmetropic. The difference in axial length between the form-deprived eye and its control was also measured every 12h (Fig. 4.6). Again, there was no significant difference between these two sets of data until 24h after deprivation, and after this time the difference continued to increase. Figure 4.7 shows that even though there was an increase in rates of scleral sulphate incorporation after 12h in the form-deprived compared to the control eye, (that is, at midnight after 6h of visual-deprivation in the light and 6h in the dark), the difference was not statistically significant until 24h after deprivation. Therefore there appears to be a delay between when the translucent diffusers are put on, and when there are increases in the growth rate of the eye. This suggests that eye growth is under long-term regulation which requires a period of days before eye growth is fully up-regulated.

4.3.5 Increase in GAG synthesis at midday during FDM

Acetone precipitations (Volpi, 1994) were also carried out on the GAGs synthesised by sclera from both deprived (for 10 days) and control eyes. Figure 4.8a shows the distribution of labelled sulphate in each fraction of GAGs. There was a greater total number of counts in the form-deprived eye compared to the control eye. However, the distribution of label in these two groups was different. There was a decrease in the amount of label in the heparin fraction of the deprived eye (5%), but there was an increase in the amount of label in the chondroitin sulphate fraction (35%). The amount of label in the dermatan sulphate fraction remained relatively constant (60%). Once again, recalculating the distribution based on the amount of sulphate in each GAG chain (Fig. 4.8b) as described in Chapter 3 results in most of the labelled
Chickens were form-deprived and rates of sulphate incorporation were measured in both form-deprived and control eyes over a 10 day period. Rates of sulphate incorporation in the form-deprived eyes were expressed as a percentage of the rates measured in control eyes. All measurements were made at midday. Rates of sulphate incorporation in form-deprived eyes gradually increased in relation to that of control eyes until the 8th day of form-deprivation. From this time until the 10th day of form-deprivation the difference between form-deprived and control eyes remained constant. At each time point the rate of sulphate incorporation in the form-deprived eye was significantly higher than in the controls (*p<0.05). Each point represents the mean ± SEM (n=8).
Figure 4.5  Short-term increases in rates of refractive error during form-deprivation.

Chickens were goggled at midday and refractive error was measured in both form-deprived and control eyes every 12h over a 2 day period. The refractive error in the control eye remained constant at zero (●). There was no change in the refractive error of the form-deprived eye (×) until 24h after the translucent diffuser was put on. After this time the refractive error of the form-deprived eye continued to decrease and was always significantly different to the control (**p<0.01, ***p<0.005). Each point represents the mean ± SEM, n=4. The bar across the top of the graph represents day (white) and night (black).
Length of time translucent diffusers worn (h)
Figure 4.6 Short-term increases in rates of axial length during form-deprivation.

Chickens were form-deprived at midday and axial length was measured in both the form-deprived and control eyes every 12h over a 48h period, and the difference between the two was plotted. There was no significant difference in axial length between form-deprived and control eyes until 24h after the translucent diffusers were put on. After this, the difference between the two eyes continued to increase and became significant (**p<0.01, ***p<0.005). Each point represents the mean ± SEM, n=4. The bar across the top of the graph represents day (white) and night (black).
Figure 4.7 Short-term increases in rates of sulphate incorporation during form-deprivation.

Chickens were form-deprived at midday and rates of sulphate incorporation were measured every 12h over a 48h period. Rates of sulphate incorporation in the form-deprived eye were expressed as a percentage of the control eye. Although there may have been an increase after 12h, there was no significant difference in rates of sulphate incorporation between the control and form-deprived eye until 24h of form-deprivation (**p<0.01, ***p<0.005). Each point represents the mean ± SEM, n=4. The bar across the top of the graph represents day (white) and night (black).
Sulphate incorporation in goggled eye (% of control)

Length of time translucent diffusers worn (h)
Figure 4.8 Increase in glycosaminoglycan synthesis during form-deprivation.

A) Chickens were form-deprived for 10 days and rates of sulphate incorporation measured on the 10th day at midday. The alkali extraction medium was precipitated with varying amounts of acetone and radioactive sulphate was measured in the pellets. There were more counts in the FDM scleras compared to the controls, with the distribution also different. 12% of the counts were in the heparin fraction, 60% in the dermatan sulphate fraction and 28% in the chondroitin sulphate fraction. Each point represents the mean ± SEM, n=6.

B) The counts were redistributed according to the degree of sulphation of the GAGs. Barely any labelled residues were found in the heparin fraction (2%), with 45% of the counts in the dermatan sulphate fraction and 53% in the chondroitin sulphate fraction.
A

Sulphate incorporation (% of total counts)

heparin  dermatan sulphate  chondroitin sulphate
4.3.4 Effect of removing the ambient auditory after 14 days.

After 14 days of deprivation, the sound-reared animals were separated and ear-tagged for individual identification. The rate of sulphate incorporation was determined every 12 h over the following 72 h and was recorded from the aqueous humour. Fig. 4.13 shows the change of refractive index with the removal of the ambient auditory. The results were similar to those obtained with the previous experiment, as shown in Fig. 4.15. The rate of sulphate incorporation in the nonsound-reared eye was reduced to 25% of the control eye after 24 h and remained low thereafter. In contrast, the rate of sulphate incorporation in the sound-reared eye was not reduced to the same extent. After 24 h, the rate returned to near control levels and remained relatively constant for the remainder of the experiment.

As shown in Fig. 4.12, the increase in auditory activity at 500 h in the sound-reared eye was also observed in the control eye. This suggested that the increase in auditory activity was not the cause of the decrease in refractive index. However, the decrease in sulphate incorporation was associated with a decrease in refractive index when both results of sulphate incorporation were taken into account.
residues being in the dermatan sulphate (45%) and chondroitin sulphate (53%) fractions, with very few labelled residues in the heparin fraction (2%).

4.3.6 Effect of removing the translucent diffuser after 10 days.

After 10 days of deprivation, the translucent diffusers were removed and refractive error, axial length and rates of scleral sulphate incorporation were measured every 12h over the following 3 days. Fig. 4.9 shows the change in refractive error after the removal of the translucent diffusers. There was a gradual decrease in the refractive error of the previously form-deprived eye after the translucent diffuser was removed. The axial length of the formerly deprived eye compared to the control can be seen in Fig. 4.10. The difference between the formerly deprived eye and the control eye became smaller after translucent diffuser removal. Rates of sulphate incorporation in formerly deprived compared to control eyes are shown in Fig. 4.11. The formerly deprived eye still had a higher rate of sulphate incorporation 24h after removal of the translucent diffuser. However, after this time the rate of sulphate incorporation decreased until by 48h it was the same as the rate of sulphate incorporation in the control eye. By 72h after the translucent diffusers were removed, sclera from the formerly deprived eye had a lower rate of sulphate incorporation compared to those from the control eye.

4.3.7 Brief normal daily vision prevents the increase in sulphate incorporation during FDM

Previous studies have shown that the development of FDM can be prevented by giving the animal daily brief periods of normal vision (Nickla et al., 1989; Vingrys et al., 1991; Napper et al., 1995). One of these studies (Napper et al., 1995) showed that as little as 15 min of normal vision per day could reduce the amount of myopia which developed during deprivation, compared to the amount of myopia which developed during continuous deprivation. In addition to this, the longer this period of normal vision each day, the greater the reduction in the amount of refractive error in the form-deprived eye. While this study showed that 2h almost fully reduced the amount of myopia which developed, I decided to use 3h of normal vision each day, in order to ensure complete suppression of FDM. Translucent diffusers were removed from 1200h to 1500h each day for 8 days. During this time the animals were placed into the light. As shown in Fig. 4.12, the increase usually seen at midday in the rates of sulphate incorporation in the form-deprived eye was abolished. In fact, the rate of sulphate incorporation in the deprived eye was statistically significantly lower than that of the control eye. Refractive errors were also measured and confirmed that the large refractive error associated with FDM did not develop when brief periods of normal vision were given daily.
Chickens were form-deprived for 10 days. Then the translucent diffuser were removed at midday, and changes in refractive error were measured every 24h over the next three days. Refractive error remained at zero in the control eye (●). 24h after the translucent diffuser was removed, the refractive error in the form-deprived eye (×) remained at the same value as it had been when the translucent diffusers were worn. However, 48h after the removal the refractive error had begun to decrease and this trend continued over the next 24h. At all points, there was a significant difference (***p<0.005). Each point represents the mean ± SEM, n=4. The bar across the top of the graph represents day (white) and night (black).
Figure 4.10 Decrease in rates of axial elongation after removal of translucent diffusers.

Chickens were form-deprived for 10 days and then the translucent diffusers were removed at midday. The axial length of both the form-deprived and control eyes was measured every 24h for 3 days, and the data was plotted as the difference between the goggled and control eye. The large difference seen between the form-deprived and control eye after 10 days of form-deprivation was also seen 24h after the translucent diffuser was removed. However, 48h after removal there was a decrease in the difference between the two sets of data and this difference continued to decrease over the following 24h. The differences remained significant at each time point (**p<0.05). Each point represents the mean ± SEM, n=4. The bar across the top of the graph represents day (white) and night (black).
Length of time after translucent diffuser removal (h)

Difference in axial length between form-deprived and control eye (mm)
Figure 4.11 Decrease in rates of sulphate incorporation after removal of translucent diffusers.

Chickens were form-deprived for 10 days and the translucent diffusers were then removed at midday before rates of sulphate incorporation were measured. Measurements were made 24, 48 and 72h after the translucent diffusers were removed. At 24h after removal of the translucent diffusers, the rate of sulphate incorporation in the previously form-deprived eye was still higher than in the control eye (*p<0.05). After 48 hours there was no difference in rates of sulphate incorporation between the previously form-deprived eye compared to the control eye. However, within 3 days of the translucent diffusers being removed, the rate of sulphate incorporation in the formally form-deprived eye was significantly lower than that in the control eye (*p<0.05). Each point represents the mean ± SEM, n=5. The bar across the top of the graph represents day (white) and night (black).
Sulphate incorporation in previously form-deprived eye (% of control)

Length of time after translucent diffuser removal (h)
Figure 4.12 A daily period of normal vision prevents the increase in rates of sulphate incorporation during form-deprivation.

Chickens were form-deprived for 10 days, but translucent diffusers were removed between 1200h and 1500h each day and kept in the light during this period. Rates of sulphate incorporation were measured at midday on the 10th day in these chickens and in those form-deprived continuously. While rates of sulphate incorporation were higher in eyes form-deprived continuously compared to their controls (p<0.01), there was also a significant difference between rates of sulphate incorporation in form-deprived and control eyes where the translucent diffusers were removed for 3 hours per day (p<0.05). However, in this case the form-deprived eye had lower rates of sulphate incorporation compared to the control. Dark shading represents the form-deprived eye and the lighter shading represents the control eye. Each point represents the mean ± SEM, n=8.
4.3.8 The preventive ability of normal vision is light-intensity dependent.

As discussed in Chapter 3, the possibility of the retinal dark-light switch being involved can be examined by testing the effect of different light intensities on the ability to suppress the development of FDM. Therefore, the above experiments were repeated using different light intensities during the periods of transcranial diffuse light removal (up between 1200h and 1500h). Fig. 4.13 shows that light intensity at 0.4 lux and below supports the development of FDM since sulphate incorporation in the deprived eye was higher than in the control (100%). However, light intensity above 0.4 lux suppressed FDM. At 1 lux and 10 lux there was no significant difference between rates of sulphate incorporation in the form-deprived eye compared to the control eye. The results in Fig. 4.12 show that full light caused the rate of sulphate incorporation in the form-deprived eye to be the same as in the control eye.

4.3.9 The sulphate incorporation is not abolished during FDM.

The absence of sulphate incorporation in form-deprived eyes is consistent with the expectation of an abolished sulphate incorporation in sulphate deprived eyes which matches that of visual deprivation. However, as shown in Chapter 3, whether the rate of sulphate incorporation was substantially abolished, rates of sulphate incorporation of the form-deprived eyes remained over a 24h period (Fig. 4.12). This suggests that there is a low rate of sulphate incorporation at midnight and a high rate of sulphate incorporation at midday at time points throughout the day, but no sulphate incorporation at any time point. This shows that the dark-light switch is working properly in the form-deprived eye.

4.3.10 Diurnal sulphate incorporation was maintained during deprivation.

The absence of sulphate incorporation was maintained in constant light conditions that mimicked the light-dark switching factors of normal growth, since the dark-light switch is under constant light and can be maintained under constant dark conditions. Since the diurnal variation in rates of sulphate incorporation was maintained during deprivation, it was interesting to investigate whether sulphate release pattern were maintained during deprivation. Fig. 4.15 shows that both the normally high rate of Na⁺ activity seen at midnight and the normally low rate of Na⁺ activity seen at midday were maintained during deprivation.

4.4 Discussion
4.3.8 *The preventive ability of normal vision is light-intensity dependent*

As discussed in Chapter 3, the possibility of the retinal dark-light switch being involved can be examined by testing the effect of different light intensities on the ability to suppress the development of FDM. Therefore the above experiment was repeated using different light intensities during the periods of translucent diffuser removal (ie between 1200h and 1500h). Fig. 4.13 shows that light intensities at 0.4 lux and below did not suppress the development of FDM since sulphate incorporation in the deprived eye was higher than in the control (100%). However, light intensities above 0.4 lux suppressed FDM. At 1 lux and 10 lux there was no significant difference between rates of sulphate incorporation in the form-deprived eye compared to the control eye. However, as in Fig. 4.12, full light caused the rate of sulphate incorporation to be lower than the control.

4.3.9 *The diurnal rhythm of sulphate incorporation is not abolished during FDM*

The observed increase in rates of sulphate incorporation in form-deprived compared to control eyes at midday fits with the expectation of an abolished diurnal rhythm in sulphate incorporation which matches that of axial elongation. However, to check whether the rhythm was actually abolished, rates of sulphate incorporation in myopic and control eyes were measured over a 24h period (Fig. 4.14). In control eyes there was a low rate of sulphate incorporation at midday and a high rate at midnight as was shown in Chapter 3. In myopic eyes there was a higher rate of sulphate incorporation compared to the rate of sulphate incorporation in the control eye at all time points. Therefore there was not just an increase in sulphate incorporation during the day, but rather, an overall upregulation of rates of sulphate incorporation over a 24h period. The diurnal rhythm was maintained, but at a higher level, which results in the larger eye observed.

4.3.10 *Diurnal differences in rates of melatonin release are maintained during FDM.*

The fact that the rhythm of rates in sulphate incorporation could not be maintained in constant dark suggested that melatonin was not a controlling factor of scleral growth, since melatonin release is under a circadian influence and can be maintained under constant dark conditions. Since the diurnal rhythm in rates of sulphate incorporation was maintained during deprivation, it was interesting to investigate whether melatonin release pattern were maintained during deprivation. Fig. 4.15 shows that both the normally high rate of NAT activity seen at midnight and the normally low rate of NAT activity seen at midday were maintained during deprivation.

4.4 *Discussion*
Chickens were form-deprived for 10 days, but translucent diffusers were removed between 1200h and 1500h each day. During this period, chickens were put into various light intensities as shown in the Figure. Rates of sulphate incorporation were measured at midday on the 10th day of form-deprivation, and rates in the form-deprived eyes were expressed as a percentage of that in the control eye. Light intensities of 0.4 lux and below could not prevent the development of FDM, hence there was higher rates of sulphate incorporation in the form-deprived eye compared to the control eye (*p<0.05). However, light intensities above 0.4 lux were able to bring down the high rate of sulphate incorporation so that at 1 lux there was no significant difference between rates of sulphate incorporation between the form-deprived and control eyes. However, as was shown in Fig. 4.12, high light intensities resulted in even lower rates of sulphate incorporation in the form-deprived eye compared to the control (*p<0.05) Each point represents the mean ± SEM, n=6.
Figure 4.14 The diurnal rhythm in rates of sulphate incorporation is maintained during form-deprivation.

Chickens were form-deprived for 10 days and rates of sulphate incorporation were measured over a 24h period. Control eyes (●) showed a rhythm in rates of sulphate incorporation over this time, with higher rates being measured at midnight compared to midday. This same rhythm was seen in form-deprived eyes ( ), but the rates of sulphate incorporation were higher at each time point compared to the control eyes at the same time. Each time point represents the mean ± SEM, n=8.
Figure 4.15 The normal rhythms of melatonin synthesis and release remain during form-deprivation.

Chickens were form-deprived for 10 days before NAT activity was measured in the retina at midday and midnight, in both form-deprived eyes (black bars) and control eyes (hatched bars). There was no significant difference between levels of NAT activity at midday between form-deprived and control eyes, nor was there a difference in levels of NAT activity between form-deprived and control eyes at midnight. However, the difference between levels of NAT activity between midday and midnight in both form-deprived and control eyes was highly significant (p<0.001).
NAT activity (nmol N-acetyltryptamine formed/retina/h)

midday | midnight
---|---

50 | 40
40 | 30
30 | 20
20 | 10
10 | 0

These results show a decrease in N-acetyltryptamine formation in the retina of the eye with age. The decrease in NAT activity is consistent across different age groups. The observed differences in NAT activity are thought to be associated with changes in gene expression and protein levels. This decrease in NAT activity may be linked to alterations in the regulation of neuronal activity and neurotransmission. Further studies are needed to investigate the underlying mechanisms. Overall, these findings suggest a potential role for NAT in age-related changes in the retina. 

4.4.1 In the current study, the observed decrease in NAT activity in the retina with age may be attributed to changes in gene expression. Various factors, including environmental and genetic factors, can influence gene expression and protein levels. It is possible that the decrease in NAT activity is a result of age-related changes in gene expression. Further research is needed to elucidate the specific mechanisms underlying these changes. 

In conclusion, the results of this study indicate a decrease in NAT activity in the retina of the eye with age. These findings suggest a potential role for NAT in age-related changes in the retina. Further research is needed to investigate the underlying mechanisms and their implications for neurological function.
These results demonstrate an increase in eye growth after the chicken has worn translucent diffusers. Not only did the eye increase in size in terms of axial length, but there was an accompanying change in refractive error resulting in myopia. In addition to this, rates of sulphate incorporation, as measured by the assay used in the previous chapters, were higher in the sclera from deprived eyes, compared to their contralateral controls. Removal of the translucent diffuser after myopia had developed, resulted in a subsequent decrease in the amount of sulphate incorporation in the eye which had been wearing the translucent diffuser over the few days following translucent diffuser removal. Development of the increase in rates of sulphate incorporation in the deprived eye could be prevented by giving the animals a period of normal vision each day during deprivation, which also prevented excessive eye growth. Thus changes in the rate of sulphate incorporation occurred under conditions which lead to increased scleral growth, but did not occur under closely related conditions in which changes in growth rates did not occur.

In addition to showing a strong correlation between sulphate incorporation and scleral growth, there were two new findings. Firstly, the preventive ability of daily normal vision was dependent on light intensity in the same way that the suppression of sulphate incorporation in normal eye development was, suggesting a possible role for the retinal dark-light switch. Secondly, the diurnal rhythm in sulphate incorporation which was observed earlier in normal eyes (Chapter 3) remained in eyes which had worn the translucent diffuser, but at overall higher rates of sulphate incorporation at all time points, which was not as predicted, given the previous findings of Weiss and Schaeffel (1993). Therefore, it appears that some aspect of the degraded image quality imposed by the translucent diffusers results in a tonic increase in scleral GAG synthesis rather than in a disruption of the phasic rhythm.

4.4.1 Is the increase in sulphate incorporation associated with FDM?

The observed increases in rates of sulphate incorporation while translucent diffusers were worn agree with previous observations (Rada et al., 1991, Rada et al., 1992, Rada et al., 1994; Rada and Matthews, 1994) of an increase in the production of aggrecan, a cartilage proteoglycan, and decorin, a fibrous proteoglycan. Rada and colleagues also measured rates of sulphate incorporation, but into cultured sclera or cultured chondrocytes, rather than intact scleral buttons in vitro as was done in this study. As discussed in Chapter 2, although the present study did not characterise the labelled product in the same way as Rada et al (1994; Rada and Matthews, 1994), sulphate incorporation was still used as an index of proteoglycan synthesis. There was an increase in the amount of labelled sulphate in the chondroitin sulphate and dermatan sulphate fractions of form-deprived eyes compared to control eyes at midday as shown in Chapter 3. Since chondroitin sulphate is a GAG of aggrecan, and chondroitin sulphate and dermatan sulphate are both GAGs of decorin, these findings are consistent
with previous results (Rada et al., 1991) which show there is an increase in the rates of synthesis of both aggrecan and decorin in FDM.

Although the total incorporation rate was greater, the distribution of sulphated GAGs at midday during deprivation was similar to that seen in midday in normal eyes, although there was less labelled sulphate in the heparin fraction. This suggests that during deprivation, the same GAGs are made at midday as in the control eye, but at a faster rate. As discussed in Chapter 3, different proteoglycans appear to be made at midday compared to at midnight in normal eyes, although until these GAGs are positively identified it is difficult to say what these differences are. As the diurnal rhythm was maintained during deprivation, and as it appears that similar proteoglycans were made at midday in both normal and form-deprived eyes, it would be interesting to see how similar the distribution of counts in the deprived eye at midnight is compared to the normal eye at midnight. If the distribution of labelled sulphate was similar, it would suggest that similar proteoglycans were made at midnight in both normal and form-deprived eyes. To date, significant increases in rates of proteoglycan synthesis have only been observed in aggrecan and decorin (Rada et al., 1991), but these measurements were only made during the day.

One surprising aspect of the distribution of labelled sulphate in the sclera of the form-deprived eye is that there was so much label in the dermatan sulphate fraction. Given that Rada et al. (1991) saw an increase in the synthesis of both aggrecan and decorin, it may be expected that there would be more counts in the chondroitin sulphate fraction and less in the dermatan sulphate fraction. Rada et al, (1991) also reported that while the amount of accumulated aggrecan (which contains chondroitin sulphate and keratan sulphate) increased, the amount of accumulated decorin (which contains chondroitin sulphate and dermatan sulphate) did not increase suggesting that the turnover rate of decorin increased as well. In addition to this, each aggrecan molecule has approximately 100 chondroitin sulphate chains, whereas decorin has only one chondroitin sulphate chain and one dermatan sulphate chain. Therefore, it is difficult to explain why so much label came out in the dermatan sulphate fraction and why there was less than expected in the chondroitin sulphate fraction. The source of sulphate in the heparin fraction remains unknown, and any keratan sulphate probably came out in the chondroitin sulphate fraction (as discussed in Chapter 3). Further work needs to be devoted to the nature of the proteoglycans and GAGs synthesised in normal and myopic sclera.

One possibility which needs to be considered is that the increase in sulphate incorporation observed during deprivation simply reflected the fact that there were more cells making more proteoglycans in the larger sclera of the form-deprived eye. However, it has previously been shown that while there is an increase in the number of cells in the myopic sclera, the cell density per cubic area is lower than in control scleras due to more extracellular matrix being made (Gottlieb et al., 1990). The present study
used scleral buttons of a fixed diameter. Since the overall thickness of this button is much the same for both control and deprived eyes, there would have been less cells in the scleral punch from the myopic eye compared to that from the control eye, since the cell density would have been less in the myopic punch. Therefore, the increase in sulphate incorporation is likely to be due to an increase in the rate of proteoglycan synthesis per cell. This has to be offset against the slight thickening of the cartilaginous layer, and the thinning of the fibrous layer. In acute experiments there was also less sulphate incorporation in scleras from eyes recovering from FDM (after translucent diffuser removal) which, because these eyes were larger than their contralateral controls, and the altered cartilaginous/fibrous ratio was still maintained, must mean that the rate of sulphate incorporation per cell is actually being slowed down.

The observed increase in sulphate incorporation seen during deprivation accompanied a change in refractive error, and since the deprived eye was larger in the axial direction compared to its contralateral control, it was assumed that axial elongation was the cause of the refractive error. Furthermore the change in refractive error did not occur until after there was an increase in axial elongation. The possibility that the changes in refractive error were due to changes in corneal curvature was not investigated because the equipment was not available. However, since eye growth occurs more in the axial direction than in any other direction (Wallman et al., 1981; Hodos and Kuenzel, 1984), and as there are many reports that corneal curvature does not change during FDM (Pickett-Seltner et al., 1988; Schaefel et al., 1986), axial elongation is believed to be the major contributor to refractive error in FDM (Wallman and Adams, 1987).

In addition to there being an increase in sulphate incorporation after a period of deprivation, the fact that removal of the translucent diffusers resulted in a decrease in rates of sulphate incorporation (which agrees with the results of a previous study by Rada et al., 1992), and also caused the refractive error to decrease, demonstrates that there is a correlation between refractive error and sulphate incorporation. This is also suggested by the fact that normal vision each day prevented not only the changes in refractive error from developing, but also prevented the increase in sulphate incorporation which was normally seen during FDM. Therefore this assay for sulphate incorporation may not only be a useful tool in determining the controlling factors of both normal eye growth and excessive growth during FDM, but may also be applicable to the emmetropisation paradigms, of recovery from FDM, and the responses to refractive errors imposed with lenses.

4.4.2 Tonic and phasic components of eye growth.

The fact that the diurnal rhythm in rates of sulphate incorporation was maintained during FDM was not as predicted, based on previous observations that the diurnal rhythm of axial elongation is abolished during FDM, and there is the same high
rate of axial elongation during day and night (Weiss and Schaeffel, 1993). However, more recently, Nickla et al (1997) have measured axial length in form-deprived eyes at 6 hourly intervals and have shown that the diurnal rhythm in axial elongation is maintained during deprivation. They found, as they found in normal eyes (discussed in Chapter 3), that the eye was longest at 1400h and shortest at 0200h. However, if they measured the eyes at 0800h and 2000h, there was no difference in the changes in axial lengths between 0800h and 2000h, and between 2000h and 0800h, which were the times at which Weiss and Schaeffel (1993) made their measurements. Therefore the apparent abolition of the diurnal rhythm in axial elongation during deprivation observed by Weiss and Schaeffel (1993) may have been due to their sampling times. The maintenance of a diurnal rhythm in axial elongation during deprivation as observed by Nickla et al (1997) is consistent with the observation in the present study where the rhythm in sulphate incorporation was still seen in form-deprived eyes.

Since the rhythm in rates of sulphate incorporation was maintained, this shows that the phasic component of eye growth remains intact during deprivation. As discussed in Chapter 3, this phasic component may be responsible for regulating the rate of eye growth in response to the eye’s optical power. However, what is surprising is that this regulatory growth component is still intact during deprivation, during which time there was an increase in the rate of eye growth. Therefore, it appears that this phasic component is not disturbed by the degraded image quality imposed by the translucent diffusers. Instead, it appears to depend purely on light-intensity, since, as was shown in Chapter 3, this diurnal rhythm of sulphate incorporation could not be maintained in constant dark conditions. Light was needed to bring down rates of sulphate incorporation, with the suppressive effect only occurring at light intensities above 0.4 lux.

Since the rates of sulphate incorporation at all time points were higher in form-deprived eyes compared to rates in control eyes, this suggests that there must be a disruption to the control of a tonic component of proteoglycan and GAG synthesis. Again, as discussed in Chapter 3, this tonic component may reflect the maintenance of normal proteoglycan turnover. In the case of this tonic component, it appears that the image quality is important as it may require regular exposure to a normal image in order to bring down the rate of sulphate incorporation. During deprivation the image quality is degraded and this may, over time, result in the loss of the inhibitory effect of light on rates of sulphate incorporation, which fits with the observation that the increase in rates of sulphate incorporation during FDM is not seen for a few days after the translucent diffusers are put on, consistent with previous findings (Rada et al., 1992). Similarly, after the removal of the translucent diffuser, the regular exposure to vision with spatial and temporal characteristics may once again gradually bring down the rate of sulphate incorporation, so that a decrease in rates of sulphate incorporation below control levels is not observed until a few days later. Therefore, exposing the eye to normal vision for
a few hours each day may be enough so that the image quality is not reduced enough over time which in turn may prevent the development of FDM. There is still some element of light-intensity dependency, since only light intensities above 0.4 lux used during the period of normal vision each day can prevent FDM. However, since only the tonic component of eye growth appears to be affected by the degraded image quality during deprivation, whereas the regulatory component is not, this suggests that there are two different controlling factors responsible for these two elements of eye growth.

Finally, it appears that there may in fact be a small circadian component of the 24h rhythm in sulphate incorporation during deprivation, as there was a decrease from the high night-time rate of sulphate incorporation before the lights come on at 0600h. Melatonin release, which is under circadian control, does not appear to play a role in sulphate incorporation. Nor does the normal rhythm in sulphate incorporation appear to be under circadian influence. Previous work (Hoffmann and Schaeffel, 1996) and results obtained during the present study demonstrate the maintenance of normal circadian rhythms in melatonin synthesis during FDM. If dopamine acts as an inhibitory factor in the light phase during normal eye growth, and the enkephalins, neurotensin and somatostatin act as stimulatory factors in the dark, their combined effects could override the endogenous rhythm of melatonin. However, during FDM when rates of dopamine release are depressed, as are the rates of peptide release, melatonin may be able to exert some discernible effect on rates of scleral growth. This apparent circadian component may be a small observation consistent with the observation by Nickla and Wallman (1997) that rate of GAG synthesis in cultured sclera is under circadian control as discussed in Chapter 3.

In summary, there appears to be a phasic component of eye growth which is reflected by a light intensity-dependent rhythm in rates of sulphate incorporation. Since this rhythm is maintained during deprivation, it does not appear to be dependent on the image quality received by the eye. However, while this phasic component is not involved in the development of FDM, it is not known whether it is involved in the emmetropisation process, which occurs upon translucent diffuser removal. Although there is a delay between lights coming on and off, and the effect of light on the sclera, these changes are acute, and probably represent short-term control of eye growth. In addition to this, there is also a more long-term tonic control of eye growth which increases in response to deprivation, and is therefore influenced by image quality. The long-term up-regulation seen during deprivation is prevented when periods of normal vision are given each day, and this preventive ability is also dependent on light intensity. Although both these components are dependent on light intensity, only one is affected by the degraded image quality imposed by translucent diffusers, suggesting both the two components are controlled by different messengers.
4.4.3 Retinal control of FDM

As was discussed in Chapter 3, the fact that the light intensity needed to prevent FDM was higher than that which is needed for the retina to flip from its dark to light state may simply be due to the fact that, in the present study, changes in the sclera are being measured, compared to other studies where retinal functions were studied. Higher light intensities may be required before retinal changes can exert their effects on the sclera. Again, as was discussed in Chapter 3, this may not necessarily mean that elements of the retinal dark-light switch are directly involved. Instead, it could simply mean that the retina must be in its light state for normal control of eye growth. In its light state, the retinal dark-light switch could facilitate a different form of retinal processing through light-adapted circuits, stimulating the release of another compound in the retina, which may then go on to control rates of eye growth. However, since both dopamine and leu-enkephalin have been implicated in FDM, it is possible that they are involved in the changes in sulphate incorporation measured here.

Since dopamine is released during the light phase (Kramer, 1971; Kirsch and Wagner, 1989; Kolbinger et al., 1990), during which time there is a decrease in the rate of sulphate incorporation, it may be expected that dopamine could inhibit scleral growth. This hypothesis is also supported by the fact that the amount of retinal dopamine is depleted by deprivation, during which time there is an increase in scleral growth (Stone et al., 1989; Stone et al., 1990; Megaw et al., 1996). Since dopamine release is under diurnal control, it could be responsible for the phasic component of scleral growth. While dopamine release during the light phase is lower during deprivation, it is not totally abolished, but there is still no release during the dark phase. Therefore, a diurnal rhythm of dopamine release is still maintained during FDM, although at a lower level, and this could be why rates of sulphate incorporation maintain their rhythm during deprivation.
CHAPTER 5. RETINAL CONTROL OF SULPHATE INCORPORATION INTO THE SCLERA

5.1 Introduction

From results presented in the previous chapters, it appears that light can suppress the rate of sulphate incorporation, although with a phase delay. The fact that the retina is the light-sensitive part of the eye suggests that retinal compounds may be responsible for controlling eye growth. This may also account for the phase delay, given that time could be needed for these retinal compounds to act on the sclera. Light could only suppress rates of sulphate incorporation at intensities above 0.4 lux. The retinal dark-light switch (for review see Morgan and Boelen, 1996) flips from its dark to its light state at a similar, but slightly lower light intensity (0.1 lux). In addition to this, dopamine and the enkephalins (both elements of the retinal dark-light switch) have been implicated in the excessive eye growth associated with FDM. Therefore, messengers released from the retinal dark-light switch, namely melatonin, the enkephalins, neurotensin, somatostatin and dopamine, may be involved in the control of scleral growth, and this hypothesis has been tested in the present chapter.

Melatonin is released from the photoreceptors during the dark phase (Hamm and Menaker, 1980; Besharse and Iuvone, 1983), during which time there is an increase in sulphate incorporation. Therefore it was predicted that melatonin may be a stimulator of sulphate incorporation. Similarly, the enkephalins, neurotensin and somatostatin are released from the ENSLI amacrine cells in the dark (Boelen et al., 1989; Yang et al., 1997), again suggesting these compounds may act in a stimulatory manner. The potential stimulatory effects of these compounds can be examined by testing the effects of their agonists, which mimic the effect of particular compounds, on rates of sulphate incorporation at midday. Since at midday there are low extracellular concentrations of these compounds, and low rates of sulphate incorporation, any increase in rates of sulphate incorporation after the agonist was added would suggest that the compound being tested was a stimulator of sulphate incorporation. Alternatively, the effects of antagonists, which block the effects of these compounds, on rates of sulphate incorporation can be tested at midnight, when there are high extracellular concentrations of these compounds. If the high rate of sulphate incorporation at midnight was suppressed by an antagonist, then it would also suggest that the compound being tested was a stimulator of sulphate incorporation.

Dopamine, on the other hand, is released in the light (Kramer, 1971; Kirsch and Wagner, 1989; Kolbinger et al., 1990), during which time there is a decrease in the rate of sulphate incorporation. Therefore, dopamine could be an inhibitor of sulphate incorporation. The effect of dopaminergic agonists on rates of sulphate incorporation should be tested at midnight, when there is a low extracellular concentration of
dopamine, to see if the normally high rate of sulphate incorporation can be suppressed. If this rate was suppressed, it would suggest that dopamine is the compound responsible. Alternatively, the effect of dopaminergic antagonists could be tested at midday, when the extracellular concentration of dopamine is high, to see if the normally low rate of sulphate incorporation at midday could be increased.

Acetylcholine has also been suggested to be involved in eye growth during FDM since the cholinergic antagonist atropine has been used to prevent the development of myopia in both humans (Bedrossian, 1979; Goss, 1982), and in animal models (Young, 1965; McBrien et al., 1993). In the chicken retina there are three populations of cholinergic amacrine cells (Millar et al., 1987b). In one of these populations, acetylcholine has been co-localised with somatostatin (Buckerfield et al., 1981), and therefore may be co-localised to the ENSLI amacrine cells. If this is the case, then, like the peptides released from these cells, acetylcholine may be released at night. Using the same line of argument as described for the peptides released from the ENSLI cells, the prediction would then be that acetylcholine would be a stimulator of sulphate incorporation. The other two populations of amacrine cells are involved in controlling eye movement, which suggests that they would be active over a large range of light intensities. Therefore there are no predictions as to when acetylcholine would be released from these cells, and therefore no predictions as to whether it might act as a stimulator or inhibitor of scleral growth.

It should be noted that there is a general problem with the use of antagonists. While agonists mimic the effect of the compound by binding to the receptor, antagonists act by competing with the agonist for binding sites on the receptor, displacing the agonist from its binding site, and thereby blocking the effect of the agonist. Therefore, antagonists only have a physiologically significant effect when the agonist is present. For example, the cholinergic antagonists atropine (McBrien et al., 1993) and pirenzepine (Leech et al., 1995), have been used to prevent the development of FDM when applied during deprivation. Given that acetylcholine is a highly labile compound, it would appear unlikely that acetylcholine released from other parts of the eye would reach the sclera in physiological concentrations. Therefore, these effects of the cholinergic antagonists may not be physiologically significant. Similar considerations would apply to other labile messengers.

If retinal neurotransmitters are involved in controlling rates of sulphate incorporation, the question that remains is, by what pathway do they do so? There seem to be two options. Given that there is a delay between lights coming on and off and changes in rates of sulphate incorporation being detected, it would appear most likely that retinal neurotransmitters act on the sclera via intermediate sites, probably the RPE and choroid, before changes in the sclera occur.

The other possibility is that retinal neurotransmitters could act directly on the sclera. Intrinsically, this possibility seems less likely because of distance and
degradation, so that it would be difficult to explain how retinal compounds could reach the sclera in physiological conditions. The peptides released from the ENSLI amacrine cells, as well as acetylcholine are all highly labile and therefore it would not be expected that they could exert effects on the sclera. Melatonin is also unlikely to be able to act on the sclera as it is rapidly degraded. Therefore, dopamine remains as the most likely compound which could act on the sclera after being released from the retina. In fact, there is a little evidence that retinal compounds can act directly on the sclera. In cultured scleral chondrocytes, both the cholinerigc antagonist atropine (Marzani et al., 1994), and the dopaminergic agonist apomorphine (Seko et al., 1994a) can inhibit the rate of chondrocyte proliferation, suggesting possible roles for acetylcholine and dopamine in the control of scleral growth.

These two alternative mechanisms of how the retina may affect scleral growth can be tested using rates of sulphate incorporation as an endpoint of potential effects of retinal neurotransmitters. Neurotransmitters can be applied directly to the sclera in vitro, thereby testing their direct effects on the sclera. Any change to the rate of sulphate incorporation would indicate that these compounds somehow influence the rate of scleral growth. Alternatively, neurotransmitters can be injected into the eye, and after a period of time, scleras can be removed and incubated, therefore testing for indirect effects of the compounds.

5.2 Methods

5.2.1 Animal handling

Animals were housed as described in Chapter 2.

5.2.2 Sulphate incorporation

Effects of neurotransmitters in vitro

In experiments where direct effects of neurotransmitters were tested, scleral buttons were preincubated for 30min in buffer containing various concentrations of the agonist or antagonist being tested. Radioactive sulphate was then added to the incubation medium, and the assay was performed for 1h as described in Chapter 2. Control scleras were also preincubated for 30min in buffer before radioactive sulphate was added. These experiments were all carried out on sclera removed at midday, when rates of sulphate incorporation were low.

Effects of neurotransmitters in vivo

Indirect effects were measured by injecting the various antagonists and agonists into the eye. Chickens were lightly anaesthetised with isoflurane, and 10ul of the compound being tested was injected intravitreally into one eye. The other eye remained as a control. Chickens fully recovered from the anaesthetic after no longer than 5min.
Evidence previously obtained in this laboratory has shown that the concentrations of kainate required to lesion the retina when administered *in vivo* were approximately 100 times higher than those required when administered *in vitro*. Therefore, the concentrations of the compounds acting in the eye were assumed to be 1/100 of the concentration injected.

Two injections were given before the sulphate incorporation assay was carried out as described in Chapter 2. The time of the first injection was either at midday or midnight depending on the agonist or antagonist, and the diurnal rhythm in release of the messenger. For example, dopamine is released during the light phase. Therefore it would be best to inject dopaminergic agonists in the middle of the dark phase when the extracellular concentration of dopamine is at its lowest, to test if the injected compound had any inhibitory effect on the high rates of sulphate incorporation seen at midnight. Alternatively, dopaminergic antagonists would be best injected at midday, when the extracellular concentrations of dopamine are high, to see if the normally low rate of sulphate incorporation could be increased. Melatonin, the enkephalins, neurotensin and somatostatin are released during the dark phase, and would therefore be expected to stimulate rates of sulphate incorporation which increase during the dark phase. Hence, agonists to these compounds would be best injected at midday, when concentrations of these compounds are low, and when rates of sulphate incorporation are low, to test for any stimulatory effects. Alternatively, antagonists to these compounds would be best injected at midnight, to see if they could reduce the normally high rate of sulphate incorporation. Since it is unknown when acetylcholine is released in the retina with respect to the external lighting conditions, both agonists and antagonists to acetylcholine should be tested at either midday or midnight.

Therefore, two protocols were used. In the first, injections were carried out at midday and 1500h, with the sclera removed and the incubation performed at 1800h. In the second, injections were carried out at midnight and 0300h, with the incubation performed at 0600h. From the previous chapters, there is evidence that there is an approximately 6h delay from the time lights come on or off, and the subsequent decrease and increase in rates of sulphate incorporation. In addition to this, empirical pharmacology with other drugs has shown that a 3h interval between injections is sufficient to obtain maximal effects of the drug on the eye (Boelen *et al.*, 1993; Boelen *et al.*, 1991; Boelen *et al.*, 1994). For these reasons, two injections 3h apart were given. The times were chosen according to the 24h time course (Fig. 3.4) as there were no differences in rates of sulphate incorporation between 1200h and 1800h, nor between 0000h and 0600h. Therefore, any differences between the control and injected eyes at 1800h or 0600h can be attributed to the injection. To ensure that the effect is due to the agonist or antagonist being injected, rather than the effect of the injection itself, vehicle injections were carried out as well.
5.2.3 Assay for N-acetyltransferase activity

The assay for NAT activity was carried out as described in Chapter 4.

5.2.4 List of chemicals used and suppliers

The following chemicals (listed with their suppliers) were used in this chapter:
- melatonin (Sigma)
- leu-enkephalin (Sigma)
- met-enkephalin (Sigma)
- thiorphan (Sigma)
- captopril (Sigma)
- bestatin (Sigma)
- bacitracin (Sigma)
- naloxone (RBI)
- neurotensin (Sigma)
- somatostatin (Peninsula Laboratories)
- (±)-2-Amino-6,7-dihydoxy-1,2,3,4-tetra-hydronaphthalene hydrobromide (ADTN, RBI)
- (±)-1-phenyl-2,3,4,5-tetrahydro-(1H)-3-benzazepine-7,8-diol hydrochloride (SKF38393, RBI)
- quinpirole (RBI)
- clozapine (RBI)
- carbachol (Sigma)
- pilocarpine (RBI)
- atropine (RBI)
- pirenzepine (Dr Karl Thomae GMBH)

5.3 Results

5.3.1 Direct effects of compounds released by the retinal dark-light switch on rates of sulphate incorporation

Melatonin

The effect of melatonin on rates of sulphate incorporation in vitro can be seen in Fig. 5.1. Melatonin had a stimulatory effect on rates of sulphate incorporation at low concentrations below 1 µM, as expected. However, at higher concentrations (10 µM and 100 µM) there was a significant inhibition of rates of sulphate incorporation compared to the control, the opposite of what was expected.

Peptides of the ENSLI amacrine cells

In the absence of peptidase inhibitors, both leu-enkephalin (Fig. 5.2a) and met-enkephalin (Fig. 5.2b) had no effect on rates of sulphate incorporation in vitro. However, enkephalins are readily hydrolysed and therefore it is possible that they were degraded during the course of the incubation. To check whether this was indeed the case, the experiments were repeated using a cocktail of peptidase inhibitors (0.2 µM thiorphan, 1 µM captopril, 20 µM bestatin and 2 mM bacitracin) in the incubation medium to prevent hydrolysis of the enkephalins. These results can be seen in Fig. 5.2c (leu-enkephalin) and Fig. 5.2d (met-enkephalin). In both cases there was a stimulatory effect on the rate of sulphate incorporation, even at the low concentration of 10 µM. This stimulatory effect agrees with the expected result as enkephalins are released during the dark phase when there is a higher rate of sulphate incorporation.

In order to check that the effects of leu-enkephalin and met-enkephalin were exerted on opioid receptors, the non-specific opioid antagonist naloxone was used (Fig. 5.3). The stimulatory effect of both leu-enkephalin and met-enkephalin was prevented
Scleral buttons were incubated at midday for 1h with various concentrations of melatonin in the incubation medium. At concentrations below 1uM there was a significant increase in rates of sulphate incorporation. However, at concentrations above 1uM, there was a significant suppression of sulphate incorporation compared to the control (*p<0.05). Each point represents the mean ± SEM, n=10.
Figure 5.2 Direct effects of leu-enkephalin and met-enkephalin on sulphate incorporation.

A) Scleral buttons were incubated at midday for 1h with various concentrations of leu-enkephalin in the incubation medium. There were no significant differences in rates of sulphate incorporation at any of the concentrations of leu-enkephalin used compared to the control (p<0.05). Each point represents the mean ± SEM, n=10.

B) Scleral buttons were incubated at midday for 1h with various concentrations of met-enkephalin in the incubation medium. There were no significant differences in rates of sulphate incorporation at any of the concentrations of met-enkephalin used compared to the control (p<0.05). Each point represents the mean ± SEM, n=10.

C) Scleral buttons were incubated at midday for 1h with various concentrations of leu-enkephalin and a cocktail of protease inhibitors (described in section 4.2) in the incubation medium. There was a significant increase in rates of sulphate incorporation at all concentrations of leu-enkephalin used (**p<0.01, ***p<0.005). Each point represents the mean ± SEM, n=8.

D) Scleral buttons were incubated at midday for 1h with various concentrations of met-enkephalin and a cocktail of protease inhibitors (described in section 4.2) in the incubation medium. There was a significant increase in rates of sulphate incorporation at all concentrations of leu-enkephalin used (**p<0.01). Each point represents the mean ± SEM, n=8.
A

B

[leu-enkephalin]
B

Sulphate incorporation (% of control)

control  1uM  10uM  100uM

[met-enkephalin]
Figure C

**Sulphate incorporation (%) of control**

- Control
- 1uM
- 10uM
- 100uM

[leu-enkephalin]
D

![Graph showing sulphate incorporation (% of control) for different concentrations of met-enkephalin. The x-axis represents control, 1uM, 10uM, and 100uM, while the y-axis represents the percentage of control. The graph indicates a significant increase in sulphate incorporation with increasing concentrations of met-enkephalin.](image-url)

[met-enkephalin]
Figure 5.3 Direct effect of leu- and met-enkephalin on sulphate incorporation in the presence of an opioid antagonist.

Scleral buttons were incubated at midday for 1 h with 10 uM of either leu-or met-enkephalin in the incubation medium, with or without 10 uM naloxone, in the presence of a cocktail of protease inhibitors. While leu-enkephalin and met-enkephalin stimulated rates of sulphate incorporation, this rate was suppressed below the control when naloxone was present. However, when only naloxone was present there was no significant effect on rates of sulphate incorporation (*p<0.05). Each point represents the mean ± SEM, n=8.
In the presence of naloxone, in addition to their effect, there was no effect demonstrated by leu-enk or met-enk, confirming the idea of sulphate incorporation lower than the control. Naloxone receptors had no effect on rates of sulphate incorporation.

Controlled and spontaneous were also tested in the presence of the peptide and sulphate incorporation. However, as can be seen in Fig. 5A, neither of these compounds had any effect on 10μM. The concentration at which the compounds had no effect is the 10μM concentration of 10 μM. However, an increase in sulphate incorporation was observed in the presence of 10μM naloxone. This indicates that these compounds may have an effect on cancer growth.

Conclusion of the KNO/Leukemia cells

The precipitation, differentiation, and morphogenesis of cancer cells were shown to decrease or increase, respectively, in the presence of sulphotide inhibition. Experiments in which the release of these compounds was measured from the cultures showed that the compounds, not only reduced but also increased the formation of sulphotide inhibition in the absence of these compounds. Ongen et al. (1990) have shown that these products inhibit or block free radicals and causes a 10 μM level with no interference with the sulphate incorporation.
in the presence of naloxone. In addition to this, there was a further suppression by naloxone, resulting in rates of sulphate incorporation lower than in the controls. Naloxone on its own had no effect on rates of sulphate incorporation.

Neurotensin and somatostatin were also tested in the presence of the cocktail of peptidase inhibitors. However, as can be seen in Fig. 5.4, neither of these compounds had any effect at 10uM, the concentration at which the enkephalins had their effect. Higher concentrations were not tested.

**Dopamine**

Since dopamine is released in the light, when rates of sulphate incorporation decrease, dopamine was predicted to be an inhibitor of sulphate incorporation. Therefore, the dopaminergic agonist ADTN was tested, to see if it could suppress rates of sulphate incorporation. In fact, this is what was seen (Fig. 5.5), although a significant inhibition was not seen until a concentration of 1mM. However, concentrations of both 10uM and 100uM resulted in an increase in rates of sulphate incorporation, which was the opposite of what was expected. Dopaminergic agonists specific for D1 (SKF38393) and D2 receptors (quinpirole) were also tested at concentrations of 100uM and 1mM (Fig. 5.6). However, neither of these compounds had any significant effect on rates of sulphate incorporation at these concentrations.

With the prediction that it might act as a stimulator, the dopaminergic antagonist clozapine was tested on rates of sulphate incorporation. However, despite this prediction, Fig. 5.7 shows that there was in fact an inhibition of sulphate incorporation at midday in the presence of 100µM and 1mM clozapine.

### 5.3.2 Indirect effects of compounds released from the retinal dark-light switch on rates of sulphate incorporation

**Melatonin**

Melatonin was not tested for indirect effects, since melatonin is rapidly degraded in the retina (Grace and Besharse, 1993) and therefore, it was assumed that after injection into the eye it would be not be maintained at a sufficient concentration to exert an effect on scleral growth.

**Peptides of the ENS/LI amacrine cells**

The enkephalins, neurotensin and somatostatin could not be tested in vivo because, as was shown in the experiments testing their effects in vitro, they are degraded without the use of peptidase inhibitors. Experiments in which the release of these compounds was measured have shown that the amount which can be recovered is low without the presence of these peptidase inhibitors (Dowton et al, 1990). Injecting these peptidase inhibitors in vivo into intact eyes over a 6h period could interfere with
Scleral buttons were incubated at midday for 1h with either 10uM neurotensin or 10uM somatostatin in the incubation medium, in the presence of a cocktail of protease inhibitors. Neither peptide had a significant effect on rates of sulphate incorporation (p<0.05). Each point represents the mean ± SEM, n=10.
Figure 5.5  Direct effect of ADTN on sulphate incorporation.

Scleral buttons were incubated at midday for 1h with various concentrations of ADTN (a dopaminergic agonist) in the incubation medium. At concentrations of 10uM and 100uM there was a significant increase in rates of sulphate incorporation compared to the control. (**p<0.01). However, at a concentration of 1mM there was a significant inhibition of rates of sulphate incorporation (***p<0.005). Each point represents the mean ± SEM, n=10.
[ADTN]
Figure 5.6 Direct effect of D1-specific and D2-specific agonists on sulphate incorporation.

Scleral buttons were incubated at midday for 1h with various concentrations of quinpirole (a D2-specific agonist) or SKF38393 (a D1-specific agonist). Neither of these compounds had any significant effect on rates of sulphate incorporation (p>0.05). Each point represents the mean ± SEM, n=8.
Figure 5.7 Direct effect of clozapine on sulphate incorporation.

Scleral buttons were incubated at midday for 1h with various concentrations of clozapine (a dopaminergic antagonist) in the incubation medium. At 100µM and 1mM there were significant differences in rates of sulphate incorporation compared to the control. (**p<0.01, ***p<0.005). Each point represents the mean ± SEM, n=10.
2.4.3 Reversal with 10 μM Clozapine

Since unstimulated astrocytes can incorporate sulfate into their matrix, the addition of 10 μM clozapine reduced the incorporation to control levels. This suggests that clozapine was able to reverse the effects of the higher concentrations of HPIH.

2.4.4 Discussion

The results of this study indicate that HPIH can stimulate astrocyte proliferation in vitro. The incorporation of sulfate into the astrocyte matrix is a sensitive and specific marker for proliferation. The data show that HPIH can induce a significant increase in sulfate incorporation, which is dose-dependent. The addition of 10 μM clozapine was able to reverse the effects of the higher concentrations of HPIH, suggesting that clozapine may have a neuroprotective effect in astrocyte proliferation.

Further experiments are needed to determine the mechanism by which HPIH stimulates astrocyte proliferation and to investigate the potential therapeutic applications of this finding.
any number of other processes, so these compounds were not tested *in vivo*, even in combination with the peptidase inhibitors.

Dopamine

Injections of ADTN (a dopaminergic agonist) were carried out during the dark phase as described in the methods. Injections of 10ul of both 10mM and 100mM ADTN resulted in a suppression of NAT activity (and hence melatonin synthesis) within an hour (Fig. 5.8), suggesting that the retinal dark-light switch had been flipped from its dark to light state by the dopamine agonist. However, although the switch had been flipped at both concentrations of ADTN, there was no decrease in rates of sulphate incorporation at the lower dose of 10ul of 10mM (Fig. 5.9). After injection of 10ul of 100mM ADTN however, there was a significant decrease in the rate of sulphate incorporation compared to the control.

5.3.3 Direct effects of acetylcholine

Both agonists and antagonists of acetylcholine were tested. The non-specific agonist carbachol was used (Fig. 5.10), but no significant differences compared to the control were seen until 1mM. However, the more specific agonist (M1) pilocarpine had the opposite effect, with an increase in sulphate incorporation being observed from 10uM and above (Fig. 5.11).

The muscarinic antagonist atropine had striking inhibitory effects at and above 10uM (Fig. 5.12), but pirenzepine, an M1 cholinergic antagonist, had no effect at any of the concentrations tested, between 10uM and 1mM (Fig. 5.13).

5.3.4 Indirect effects of acetylcholine

Neither the cholinergic agonists pilocarpine, (an M1 agonist), nor carbachol, (a non-specific agonist) had an effect on rates of sulphate incorporation when 10ul of 100mM of either of these compounds was injected during the light phase. Similarly, there was no effect of the antagonists pirenzepine (an M1 antagonist) or atropine (a muscarinic antagonist) on rates of sulphate incorporation when 10ul of 100mM of either of these compounds was injected during the light phase (Fig. 5.14). These compounds were not tested at midnight.

5.4 Discussion

Since melatonin, and the peptides (the enkephalins, neurotensin and somatostatin) are released in the dark during which time rates of sulphate incorporation increase, it was assumed that they would stimulate the normally low rate of sulphate incorporation if applied during the day. In fact, melatonin did have a stimulatory effect on rates of sulphate incorporation at concentrations less than 1uM. At concentrations above this, melatonin had an inhibitory effect on rates of sulphate incorporation, but
Figure 5.8 Suppression of NAT activity by injections of ADTN

Chickens were intravitreally injected into one eye with ADTN (a dopaminergic agonist) at midnight, and after 1h levels of NAT were measured. 10µl injections of both 10mM and 100mM ADTN were able to bring down the high rate of NAT normally seen at midnight in the control eye (***p, <0.005). Each point represents the mean ± SEM, n=4.
NAT activity (nmol N-acetyltryptamine formed/retina/h)
Figure 5.9 Indirect effect of ADTN on sulphate incorporation.

Chickens were intravitreally injected into one eye with 10ul ADTN (a dopaminergic agonist) at midnight and 0300h and rates of sulphate incorporation were measured at 0600h and compared to the control eye. Injecting 10ul of 10mM ADTN had no significant effect on rates of sulphate incorporation compared to the control, however a significant suppressive effect was observed with injections of 10ul of 100mM ADTN (**p<0.01). Each point represents the mean ±SEM, n=8.
[ADTN]

Bar graph showing sulphate incorporation (% of control) for control, 10mM, and 100mM conditions.
Figure 5.10 Direct effect of carbachol on sulphate incorporation.

Scleral buttons were incubated at midday for 1h with various concentrations of carbachol (a non-specific cholinergic agonist) in the incubation medium. At 1mM there was a significant difference compared to the control (**p<0.005). Each point represents the mean ± SEM (n=10).
Sulphate incorporation (% of control)

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Sulphate Incorporation</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>100</td>
</tr>
<tr>
<td>10uM</td>
<td>100</td>
</tr>
<tr>
<td>100uM</td>
<td>100</td>
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<td>1mM</td>
<td>75</td>
</tr>
</tbody>
</table>

[carbachol]
Figure 5.11 Direct effect of pilocarpine on sulphate incorporation.

Scleral buttons were incubated at midday for 1h with various concentrations of pilocarpine (an M1 cholinergic agonist) in the incubation medium. At all concentrations there were significant differences compared to the control. (*p<0.05  **p<0.01, ***p<0.005). Each point represents the mean ± SEM (n=10).
### Sulphate Incorporation (% of control)

- **control**
- **10uM**
- **100uM**
- **1mM**

**[pilocarpine]**
Figure 5.12 Direct effect of atropine on sulphate incorporation.

Scleral buttons were incubated at midday for 1h with various concentrations of atropine (a muscarinic cholinergic antagonist) in the incubation medium. There were significant differences at concentrations of atropine above 1µM compared to the control (**p<0.01, ***p<0.005). Each point represents the mean ± SEM, n=10.
Sulphate incorporation (% of control)

- control
- 1μM
- 10μM
- 100μM
- 1mM

[atropine]
Figure 5.13 Direct effect of pirenzepine on sulphate incorporation.

Scleral buttons were incubated at midday for 1h with various concentrations of pirenzepine (an M1 cholinergic antagonist) in the incubation medium. There was no significant difference between any of the concentrations of pirenzepine used compared to the control (p<0.05). Each point represents the mean ± SEM (n=10).
Sulphate incorporation (% of control)

control  10uM  100uM  1mM

[pirenzepine]
Figure 5.14 Indirect effects of cholinergic compounds on sulphate incorporation.

Chickens were intravitreally injected in one eye with 10ul of 100mM of various cholinergic agonists and antagonists at midday and 1500h, and rates of sulphate incorporation were measured at 1800h and compared to the control eye. None of the injected compounds caused a statistically significant change in rates of sulphate incorporation compared to the control (p<0.05). Each point represents the mean ±SEM, n=8.
these concentrations were not physiologically significant. Melatonin was not tested in vivo due to its high lability. The enkephalins had a stimulatory effect on rates of sulphate incorporation at concentrations, which were possibly of greater physiological relevance. Neither neurotensin nor somatostatin had an effect on rates of sulphate incorporation at this concentration. Once again, these peptides could not be tested in vivo due their high lability. Since dopamine is released in the light, it was expected to act as an inhibitor of sulphate incorporation. The concentration at which the dopaminergic agonist ADTN had its inhibitory effect in vitro was higher than that which could have physiological significance, suggesting there are no dopaminergic receptors on the sclera. ADTN also inhibited rates of sulphate incorporation after being administered in vivo, however only at concentrations that were higher than those required to flip the retinal dark-light switch from its dark to light state. Finally, atropine inhibited rates of sulphate incorporation when applied in vitro.

5.4.1 Melatonin is not involved in controlling scleral growth

If melatonin were responsible for controlling rates of sulphate incorporation, it would be postulated to have an stimulatory influence as it is released at night, during which time there is an increase in the rate of sulphate incorporation. Therefore, by adding melatonin to the incubation medium at midday (when sulphate incorporation is low), an increase in sulphate incorporation would have suggested a role for melatonin in controlling sulphate incorporation. Indeed, concentrations of melatonin below 1uM did have a stimulatory effect on rates of sulphate incorporation. However, an inhibition of rates of sulphate incorporation was seen using concentrations of melatonin above 1uM. 10uM is much higher than the KD value of melatonin of about 400 pM (Dubocovich and Takahashi, 1987). Therefore this inhibitory effect is probably not physiologically significant.

What is difficult to understand, is how melatonin could reach the sclera from the retina in physiologically significant concentrations, given that it is rapidly degraded (Grace and Besharse, 1993). Also, the fact that sulphate incorporation could not be maintained under conditions of constant dark (as shown in Chapter 3) suggests that retinal melatonin is not an important controller of sulphate incorporation, as the pattern of melatonin release from photoreceptors is maintained under conditions of constant dark (Hamm and Menaker, 1980) (Besharse and Iuvone, 1983). In addition to this, melatonin release patterns do not change after the induction of FDM as reported previously (Hoffmann and Schaeffel, 1996), and in the present study (Chapter 4), further suggesting that it is not a controlling factor of the diurnal changes of normal scleral growth, and of the excessive growth seen during FDM. What remains is the possibility that rather than retinal melatonin being involved, circulatory melatonin may be involved in controlling rates of scleral growth, and this effect may be related to the
reported circadian rhythms in isolated sclera (Nickla and Wallman, 1997). However, these effects would be overridden by the light and dark signals.

5.4.2 Are the ENSLI amacrine cells involved?

Just as melatonin is released in the dark, so too the enkephalins, neurotensin and somatostatin (the peptides) are released in parallel from the ENSLI amacrine cells in the dark (Boelen et al., 1989; Yang et al., 1997). Therefore, they too would be expected to stimulate sulphate incorporation if they were involved in controlling scleral growth. While no effects were seen with the enkephalins alone, both leu- and met-enkephalin had a stimulatory effect when peptidase inhibitors were added to the incubation medium. This effect was only observed at concentrations higher than those of physiological significance (kD about 2nM, Simantov et al., 1978). While the stimulatory effect of the enkephalins were at concentrations which are higher than their kD value, they were only higher by a factor of $10^3$. The concentration of extracellular enkephalin in the eye, calculated from rates of release by the retina over a 1h period (taken from data in (Millar et al., 1984)) is approximately 0.3nM. Given that there is degradation of enkephalins, this would mean the extracellular concentration of enkephalin would be even less than 0.3nM. In addition to this, concentrations of enkephalins of 100nM can affect cyclic AMP levels in the retina (Sally Firth, personal communication). All this data suggests that the effect of the enkephalins on rates of sulphate incorporation was not physiologically significant, as an effect was not seen until a concentration of 10uM.

The fact that this effect could be blocked by the opioid antagonist naloxone, suggests that the stimulatory effect of the enkephalins may have been due to activation of the opioid receptors. However, given that such a high concentration of enkephalin was required before an effect on rates of sulphate incorporation was observed, it maybe that the receptors involved had a relatively low affinity for enkephalin. Naloxone is a non-specific antagonist of opioid receptors (Hennies et al., 1988), however the enkephalins have a higher affinity for the mu- and delta- opioid receptors, and a lower affinity for the kappa-opioid receptor (Corbett et al., 1984), so it may be that the kappa-opioid receptors are involved. More recently, another type of opioid receptor (zeta) has been characterised which is involved in controlling rates of cell proliferation (Zagon et al., 1989), so it is possible that this receptor may also be involved in the proliferation of scleral chondrocytes.

Another puzzling aspect of enkephalin’s stimulatory effect on rates of sulphate incorporation is how the enkephalins could exert their effects on the sclera after being released from the retina. The fact that the enkephalins could directly stimulate sulphate incorporation, as predicted, suggests that the enkephalins released from the retina could act on the sclera. However, given that the enkephalins are rapidly degraded, it appears unlikely that they could remain intact for the time it would take to get to the sclera from
the retina. The fact that the enkephalins are degraded so rapidly may also explain why a higher concentration compared to the kD value was required before an effect on rates of sulphate incorporation were seen. Therefore, these results with the enkephalins, together with the fact that neither neurotensin nor somatostatin had any effect on rates of sulphate incorporation in vitro, suggests that peptides released from the ENSLI amacrine cells in the retina are unlikely to have any direct effects on rates of scleral growth.

5.4.3 Dopaminergic agonists suppress sulphate incorporation

Since dopamine is released from the retina during the light phase (Kramer, 1971; Kirsch and Wagner, 1989; Kolbinger et al., 1990), and light inhibits rates of sulphate incorporation, dopamine was expected to inhibit sulphate incorporation. The non-specific dopaminergic agonist ADTN did in fact significantly inhibit rates of sulphate incorporation in vitro at 1mM. However, 1mM ADTN is a very high concentration compared to the kD value of 9nM (Makman et al., 1980). The fact that significant inhibition could only be observed at high concentrations suggests that these effects were not physiological. What remains unclear is why concentrations of ADTN lower than 1mM had a stimulatory effect on rates of sulphate incorporation, but these too were probably unphysiological, suggesting that ADTN may not have been interacting with dopaminergic receptors. Further support for this idea came from the fact that the dopaminergic agonists specific for the D1 receptors (SKF38393) and the D2 receptors (quinpirole) did not exert any effect on rates of sulphate incorporation at concentrations of 100μM, or 1mM, much higher than the kD values of 5.3nM (Furuta et al., 1990) and 2.3nM (Levant et al., 1992) respectively, although lower concentrations remain to be tested.

The dopaminergic antagonist clozapine had the opposite effect of what was expected, as it too inhibited sulphate incorporation like the agonists. However, one puzzling aspect of the evidence of the effect dopaminergic compounds involved in FDM is that both the agonist apomorphine and the antagonist haloperidol can prevent the development of FDM when injected into the eye daily during deprivation (Stone et al., 1989). Surprisingly, however, co-administering apomorphine and haloperidol nullified this effect. Therefore, the inhibitory effect of clozapine agrees with the preventive ability of the dopaminergic antagonist haloperidol. It is possible that both dopaminergic agonists and antagonists can inhibit scleral growth, and this may be why clozapine had an inhibitory effect on rates of sulphate incorporation. However, clozapine only exerted an effect at concentrations of 100μM, higher than its kD value of approximately 100nM, and therefore it too appeared to act unphysiologically.

The in vivo results using ADTN gave essentially the same results with suppression of sulphate incorporation. Injection of 10ul of both 10mM and 100mM ADTN at midnight is sufficient to suppress NAT activity within an hour. Dopamine
inhibits the release of melatonin from photoreceptors (which can be measured by NAT activity) and previous work in this lab has shown that 10mM ADTN is sufficient to cause this inhibitory effect, although it remains unknown whether lower concentrations of ADTN can also exert this inhibitory effect. Therefore 10mM ADTN is enough to switch the retinal dark-light switch from its dark to light state. In addition to inhibiting NAT activity, the increase in dopamine release (mimicked by injection of ADTN) would also suppress release of the enkephalins, neurotensin and somatostatin from the ENSLI amacrine cells. However, injecting this concentration of ADTN at midnight gave no suppression of sulphate incorporation by 0600h. A higher concentration of 10ul of 100mM ADTN was required before suppression of sulphate incorporation was observed. This gives a final concentration of ADTN in the eye of 1mM, which is much too high to be physiologically significant. Therefore, since no physiologically relevant effects on rates of sulphate incorporation could be seen by any of the dopaminergic compounds tested, these results suggest that dopamine is not ultimately responsible for controlling rates of sulphate incorporation, either directly, or by suppressing peptide release from the ENSLI amacrine cells.

Following up the work by Stone et al. (1989, 1990), on apomorphine, it has been shown (Rohrer et al., 1993) that daily injections of apomorphine during deprivation can prevent the development of FDM, and that this effect itself be prevented by the D2-specific antagonist spiperone, therefore suggesting that intraocular D2 receptors were involved. Rohrer et al. (1993) also concluded that apomorphine acted on retinal cells, rather than at the sclera, as the concentration of labelled spiperone (which they took to be equivalent to apomorphine) was the same at the retina after either intravitreal or subconjunctival injection, but the concentration at the sclera was much lower when injected intravitreally compared to subconjunctivally. The estimated concentrations of apomorphine in the retina which gave a 50% response was approximately 100-200pM which is close to the kD value of 1nM (Seeman et al., 1985).

Seko et al. (1994a) have shown that there was inhibition of cultured scleral chondrocyte proliferation by apomorphine. Apomorphine was a much more powerful inhibitor than ADTN, being effective at 20µM compared to the 1mM ADTN which was required in the present study. What remains to be done is to look at the effects of apomorphine on rates of sulphate incorporation, to see if the effects of apomorphine on rates of sulphate incorporation can also be seen at these low concentrations. A possible reason for the differences in effectiveness of apomorphine and ADTN could be because Seko et al. incubated the chondrocytes for 24h, compared to the present study where the incubation was carried out in the presence of ADTN for 90min. The longer incubation period may mean that there is more time for apomorphine to exert its effects and therefore changes may be detected at lower concentrations.

How can this be reconciled with the observations in the present study where effects on rates of sulphate incorporation using another dopaminergic agonist ADTN...
were only observed at concentrations which were not physiologically significant? One explanation could be that apomorphine acts at a sub-type of D2 receptor which had a high affinity for apomorphine, but a low affinity for ADTN. Alternatively, apomorphine may have been acting at a non-dopaminergic receptor, which may explain why ADTN had no physiologically significant effect. In fact, it has recently been shown that apomorphine can block nicotinic acetylcholine receptor channels when dopamine could not exert the same effect (Nakazawa et al., 1994), suggesting there are some common properties between the binding sites of dopaminergic and cholinergic receptors. Therefore it is possible that apomorphine may be exerting its effect by blocking cholinergic receptors in addition to dopaminergic receptors and this may be why such low concentrations of apomorphine can prevent the development of FDM.

However, since muscarinic receptors have been implicated in FDM, it will be worth investigating if apomorphine can exert its effects at these subtype of receptors too. These possibilities remain to be tested.

The fact that concentrations of ADTN higher than 10mM were required for inhibition of sulphate incorporation means that simply flipping the dark-light switch from its dark to light state was not enough to suppress sulphate incorporation. However, this light dependency is similar to that which was seen with suppression of sulphate incorporation by different light intensities (Chapter 3). Higher light intensities were required to suppress sulphate incorporation into the sclera, compared to those which can flip the retinal dark-light switch from its dark to light state. Similarly, the ability to prevent FDM by normal vision each day during deprivation was also light intensity dependent (Chapter 4). Since higher light intensities stimulate higher release rates of dopamine (Megaw et al., 1997), these results could be due to higher concentrations of dopamine being required to inhibit sulphate incorporation in the experiments in the present chapter. However, given that the concentration of ADTN required was 1mM, which is much higher than the kD value of 9nM, it appears unlikely that dopamine released from the retina controls rates of scleral growth.

In summary, the dopaminergic agonist ADTN stimulated rates of sulphate incorporation at low concentrations which was the opposite of its predicted effect. ADTN did not exert its predicted inhibitory effect on rates of sulphate incorporation until high concentrations (which were not physiologically significant) in vivo, nor in vitro, suggesting that dopamine is not involved in controlling rates of eye growth. However, these results are in conflict with previously reported results that another dopaminergic agonist, apomorphine, does affect rates of scleral growth both in vivo, and in vitro, by acting at D2 receptors. It may be that in these cases apomorphine was either acting at dopaminergic receptors which had a high affinity for apomorphine, but not for ADTN, or that apomorphine was acting at a non-dopaminergic receptor, perhaps on the sclera.
5.4.4 How is the retinal dark-light switch involved?

The results presented here suggest that retinal melatonin is not involved in controlling rates of sulphate incorporation. While the enkephalins stimulated rates of sulphate incorporation, they only did so at concentrations higher than those of physiologically significance. Neither neurotensin nor somatostatin had any effect on rates of sulphate incorporation. Although it stimulated rates of sulphate incorporation at lower concentrations, the dopaminergic agonist ADTN also inhibited rates of sulphate incorporation, but only at concentrations higher than those of physiological significance. Therefore it appears that none of the messengers released by elements of the retinal dark-light switch are involved in the phasic component of eye growth. If this is the case, then how can the similar light-intensity suppression of the rhythm of sulphate incorporation be explained? It appears that there must be a dissociation between the flipping of the retinal dark-light switch and scleral growth. For example, during deprivation the release rates of melatonin synthesis and release remain the same as in control eyes, and dopamine is still released during the light phase, although at a slightly lower level. Therefore the retinal dark-light switch can presumably still function, and can still be in its light state.

This may be why the regulatory, phasic component of eye growth is still maintained during deprivation. It could be that the retinal dark light-switch has to be in its light state in order to process sufficient spatial and temporal contrast to stimulate the release of another retinal neurotransmitter, which could then go on and regulate scleral growth. It would then be this other retinal neurotransmitter, whose release may be affected by the image degradation imposed by deprivation, which is responsible for the tonic component, which causes an increase in rates of sulphate incorporation and eye growth. In addition, for normal vision given each day to prevent the development of FDM, the switch appears to need to be in its light state. So far, only acute experiments have been done, so that they essentially have tested for the controlling factors of the phasic component of eye growth. Using the paradigm of normal vision each day, it may be possible to look for what is responsible for the long-term tonic up-regulation of the tonic component of eye growth, which could still be dopamine.

5.4.5 Cholinergic influences on sulphate incorporation

Acetylcholine has long been implicated in myopia with atropine being used to prevent its development in both animal models (Young, 1965; McBrien et al., 1993), and juvenile human myopia (for review see Goss, 1982). It was originally thought that atropine’s effect was on the ciliary muscles, involved in controlling accommodation. However this does not now appear to be the case, since muscarinic receptors appear to be involved in FDM (Stone et al., 1991), and the avian ciliary muscle contains nicotinic receptors (Pilar et al., 1987).
In addition to atropine, the muscarinic antagonist pirenzepine could also prevent the development of FDM when injected daily during deprivation (Leech et al., 1995). Since muscarinic receptors are found in the retina, it was assumed that retinal acetylcholine may be involved. However, Stell et al. (1997) have shown that destroying the cholinergic amacrine cells with ECMA still allows FDM to develop. Although there is still acetylcholine in another population of amacrine cells which are not destroyed by ECMA, this result suggests that retinal acetylcholine is probably not involved in controlling the development of FDM. The development of myopia was also unaffected by the injection of quisqualate (Ehrlich et al., 1990), meaning that those amacrine cells destroyed with the toxin (including all cholinergic amacrine cells) are also not involved in eye growth. These results agree with those obtained in the present study, where none of the cholinergic agonists or antagonists applied in vivo could exert an effect on rates of sulphate incorporation in the sclera. However, as only one dose was tested, it will be important to test a range of doses for each compound in future investigations. Together these results suggest that retinal acetylcholine may not be responsible for controlling scleral growth. As there are other tissues in the eye which are rich in acetylcholine (such as the RPE and choroid), these tissues may be the source of acetylcholine which acts on the sclera. However, it would seem unlikely that acetylcholine would travel to the sclera from other parts of the eye, given the labile nature of acetylcholine.

If retinal acetylcholine is not involved, then another possibility is that atropine acts directly on scleral cells to prevent the development of FDM. The inhibitory effect of 10uM atropine in vitro on rates of sulphate incorporation mirror results reported by other laboratories, although those studies were carried out on cultured chondrocytes or organ culture, rather than scleral buttons. Cultured cells were incubated with atropine for 24h (Marzani et al., 1994), which inhibited rates of chondrocyte proliferation at concentrations above 1uM. Scleral chondrocyte proliferation can also be inhibited by atropine in cultured human fibroblasts (Shih et al., 1997).

The fact that in the present study pirenzepine had no detectable effect on rates of sulphate incorporation disagrees with results from Leech et al. (1995) where pirenzepine injected daily during deprivation could prevent the development of FDM. The smallest amount of pirenzepine injected into the eye that gave statistically significant differences in refractive error was approximately 7ul of 67mM, which, based on our dilution factor and taking into account the volume used, gives a final concentration of approximately 400uM, however the highest concentration used which gave the most striking effect was a final concentration of approximately 1mM. Recent work has shown that at concentrations above 100uM, atropine may have a pathological effect on rates on cultured scleral chondrocytes from the chicken, by reducing cell number and viability (Lind et al., 1997). Lind et al. have also shown that pirenzepine at concentrations above 500uM may to be toxic to scleral chondrocytes, so there is still
the possibility that the concentrations used by Leech et al (1995) may have been toxic. Therefore, while cholinergic antagonists may prevent the development of FDM, they may do this by pathologically inhibiting scleral cells, hence preventing scleral growth.

The fact that higher concentrations of pirenzepine are required before toxic effects are observed, compared to atropine, may provide an explanation as to why in the present study, inhibition of sulphate incorporation was seen only using atropine and not when using pirenzepine. Assuming that the suppressive effect of atropine on rates of sulphate incorporation was due to toxicity, an even higher concentration of pirenzepine may be required to get the same effect on rates of sulphate incorporation.

Since the non-selective antagonist atropine had an inhibitory effect on rates of sulphate incorporation, it was expected that cholinergic agonists would stimulate rates of sulphate incorporation. The M1 agonist pilocarpine did in fact have a stimulatory effect at concentrations of 10uM and above. On the other hand, the non-specific agonist carbachol inhibited rates of sulphate incorporation just as the antagonist atropine did. However, this stimulatory effect of carbachol was not until a concentration of 1mM. Since Lind et al (1997) have shown that a range of cholinergic antagonists (pirenzepine, telenzepine, 4-DAMP) and the agonist McN-A-343 are toxic in the range of 500uM-1mM, it is possible that the inhibition of sulphate incorporation that is observed with carbachol is also due to toxicity.

Therefore, it appears that retinal acetylcholine is unlikely to be involved in controlling rates of scleral growth. Even if acetylcholine released from other parts of the eye were involved, its labile nature would probably mean that it would not reach the sclera in physiologically significant concentrations. The fact that cholinergic antagonists can reduce rates of eye growth during FDM suggests that these compounds may act directly on the sclera, and perhaps in a pathological manner.

In summary, the messengers released from the elements of the retinal dark-light switch did affect rates of sulphate incorporation in accord with the predictions which were based on when the messengers were released, and when rates of sulphate incorporation increased and decreased during the diurnal cycle. Melatonin and the enkephalins, which are released in the dark, stimulated rates of sulphate incorporation, which normally increase during the dark phase. Dopamine, which is released in the light, inhibited rates of sulphate incorporation, which normally decreases during the light phase. However, all these effects appeared to be unphysiological, since they only occurred when high concentrations of the messengers were used. Since retinal melatonin is probably not involved, it may be that circulatory melatonin is involved in controlling scleral growth. The effect of enkephalin may reflect an opioid mechanism which is not enkephalin-specific, which may be why higher concentrations of enkephalin were required before an effect could be detected. The fact that ADTN did not have a physiologically significant inhibition on rates of sulphate incorporation as another dopaminergic agonist apomorphine has been shown to have, suggests that
apomorphine may have been acting at an unusual receptor subtype, or through a non-
dopaminergic mechanism. It also appears that retinal acetylcholine is not involved in
controlling rates of scleral growth, but that the direct effect of the cholinergic antagonist
atropine may be due to toxicity.

Sulphate incorporation into GAGs has been used in due thesis to reflect rates of
ciliary growth. There was a rhythm in rates of sulphate incorporation, which was
controlled by the external lighting conditions, with light suppressing rates of sulphate
incorporation. This diurnal rhythm was also observed during deprivation, suggesting
that light levels during deprivation were sufficient to maintain the rhythm, and also
suggesting that the degraded image quality imposed by the translucent diurnalists did not
interfere with this rhythm. This diurnal rhythm may represent photocontrol of eye
growth. In addition to this phase control, there may also be a tonic control of eye
growth which increases during deprivation due to the loss of form-vision. This would
explain the increased rate of sulphate incorporation observed during FDM, and the
larger eye observed.

The ability of light to suppress rates of sulphate incorporation was dependent on
light intensity. Only intensities about 0.4 lux had this suppressive effect. The increase
in rates of sulphate incorporation during deprivation was prevented by 0.1% of normal
vision each day, but again, only at light intensities above 0.4 lux during the 5th period.
This light intensity dependence is similar to that of the retinal dark-light switch, which
also has a narrow range of light intensities over which it can flip from its dark to light
state. Although 0.4 lux is slightly higher than that light intensity which can flip the
switch (0.1 lux), this may be due to the fact that slightly higher light intensities are
required for changes in the retina to be manifested in the sclera.

Since the retinal dark-light switch was thought to be involved, neurotransmitters
released from the elements of this switch were tested, to see if they had any effect on
rates of sulphate incorporation. Both indirect and direct effects were tested. Neither
noradrenaline, the enkephalin, noradrenalin, acetylcholine, nor dopamine had any
physiologically significant effect on rates of sulphate incorporation. Therefore,
elements of the retinal dark-light switch may themselves not control scleral growth but
rather, the observed light intensity dependence may simply reflect the need for this
switch to be in its light state, in order for it to stimulate the release of another retinal
messenger which ultimately controls scleral growth. The cholinergic antagonist
atropine inhibited rates of sulphate incorporation in vitro, agreeing with previous
findings. However, whether this effect does not appear to involve retinal acetylcholine
and may be a direct pathological effect on the sclera.
CHAPTER 6. GENERAL DISCUSSION

6.1 Summary of results

Sulphate incorporation into GAGs has been used in this thesis to reflect rates of scleral growth. There was a rhythm in rates of sulphate incorporation, which was controlled by the external lighting conditions, with light suppressing rates of sulphate incorporation. This diurnal rhythm was also observed during deprivation, suggesting that light levels during deprivation were sufficient to maintain the rhythm, and also suggesting that the degraded image quality imposed by the translucent diffusers did not interfere with this rhythm. This diurnal rhythm may represent phasic control of eye growth. In addition to this phasic control, there may also be a tonic control of eye growth which increases during deprivation due to the loss of form-vision. This would explain the increased rate of sulphate incorporation observed during FDM, and the larger eye observed.

The ability of light to suppress rates of sulphate incorporation was dependent on light intensity. Only intensities about 0.4 lux had this suppressive effect. The increase in rates of sulphate incorporation during deprivation was prevented by 3h of normal vision each day, but again, only at light intensities above 0.4 lux during this 3h period. This light intensity dependence is similar to that of the retinal dark-light switch, which also has a narrow range of light intensities over which it can flip from its dark to light state. Although 0.4 lux is slightly higher than that light intensity which can flip the switch (0.1 lux), this may be due to the fact that slightly higher light intensities are required for changes in the retina to be manifested in the sclera.

Since the retinal dark-light switch was thought to be involved, neurotransmitters released from the elements of this switch were tested, to see if they had any effect on rates of sulphate incorporation. Both indirect and direct effects were tested. Neither melatonin, the enkephalins, neurotensin, somatostatin, nor dopamine had any physiologically significant effects on rates of sulphate incorporation. Therefore, elements of the retinal dark-light switch may themselves not control scleral growth, but rather, the observed light intensity dependence may simply reflect the need for this switch to be in its light state, in order for it to stimulate the release of another retinal messenger which ultimately controls scleral growth. The cholinergic antagonist atropine inhibited rates of sulphate incorporation in vitro, agreeing with previous findings, however, whether this effect does not appear to involve retinal acetylcholine, and may be a direct pathological effect on the sclera.
6.2 Sulphate incorporation as an index of scleral growth

The results in this study have shown that this sulphate incorporation assay can be used as an index of scleral growth. The rhythm in rates of sulphate incorporation matched the rhythm in rates of axial elongation (and hence eye growth) reported by Weiss and Schaeffel (1993), although with a phase shift. During deprivation in the present study, there was an increase in the rate of eye growth, which correlated with an increase in rates of sulphate incorporation. This result agrees with previous findings that there is an increase in the rate of proteoglycan synthesis (measured by sulphate incorporation) during FDM (Rada et al., 1991). Similarly, upon removal of translucent diffusers, the rate of eye growth (as measured by axial length) slowed down, as did the rate of sulphate incorporation. Again, this agrees with previous findings (Rada et al., 1992). The increased rate of eye growth which is seen during deprivation can be prevented by giving the animal 3h of normal vision each day (Nickla et al., 1989; Vingrys et al., 1991; Napper et al., 1995). In the present study, 3h of normal vision each day not only prevented an increase in rates of eye growth, but also prevented the increase in rates of sulphate incorporation normally observed during deprivation. Therefore, since changes in rates of sulphate incorporation parallel known changes in rates of eye growth, this assay is a good index of eye growth.

One finding that did not agree with previously published results is that the diurnal rhythm in rates of sulphate incorporation during normal eye growth was maintained during deprivation. Weiss and Schaeffel (1993) previously measured rates of axial elongation as a measure of eye growth and reported that the diurnal rhythm in normal eye growth disappeared after deprivation. Since the rhythm of axial elongation they measured in normal eyes was similar to the rhythm of sulphate incorporation measured in this study (although with a phase shift), it was expected that the rhythm of sulphate incorporation would also be abolished during deprivation. In fact, this was not the case. However, Weiss and Schaeffel (1993) only made measurements of axial length every 12h, whereas more recent results by Nickla et al. (1997), where axial length was measured every 6h, have shown that the rhythm of rates of axial elongation is in fact maintained after deprivation, but with peaks and troughs at times different to those measured by Weiss and Schaeffel (1993), which is why the latter did not see this effect. Therefore, this further demonstrates that there is a strong correlation between rates of axial elongation (and hence eye growth) and rates of sulphate incorporation, and that this assay enabled us to correct a previous error which arose from insufficient sampling.

The advantage of this assay is that differences between deprived and control eyes can be measured relatively early in the development of FDM. In addition to this, these differences are much greater than the small differences between the axial lengths of the eyes, and therefore are much easier to measure. The fact that the incubation only
takes an hour means that measurements can be made frequently so that detailed rhythms in eye growth can be measured. In this way, the dynamics of eye growth can be monitored. It was shown here that there are at least two components to eye growth. Firstly, there was a phasic component, which was reflected by the diurnal rhythm in rates of sulphate incorporation. Secondly, there was a tonic component which was disrupted by depriving the eye of normal vision. This assay could therefore measure short-term changes in the phasic component, and more long-term changes in the tonic component, in response to various manipulations. One example of this was in response to various light intensities. Both the tonic and phasic components of eye growth appeared to dependent on light intensity since rates of sulphate incorporation during the diurnal rhythm (phasic component) could only be suppressed above 0.4 lux, and since the tonic increase in rates of sulphate incorporation during deprivation could also only be suppressed when periods of normal vision at intensities above 0.4 lux were given. Changes in the two components of eye growth can also be measured in response to various pharmacological manipulations, although only the phasic component was looked at in this study.

6.3 Phasic and tonic components of eye growth

The phasic component of eye growth was postulated to represent the regulatory component of eye growth, since it involved increases and decreases in the rate of eye growth. This regulatory component disappeared as the animals got older, which may reflect the fact that the rate of eye growth slows down during development. If the phasic component of eye growth represents the regulatory component of eye growth, it would have been expected that this regulation would have been disrupted during deprivation, since normal rates of eye growth were disrupted. However, this was not the case, since the rhythm in rates of sulphate incorporation was maintained during FDM.

The tonic component of eye growth was thought to represent the maintenance component of eye growth, and was also thought to be a more long-term regulator. Since there was an increase in rates of sulphate incorporation during deprivation, this suggests that while the phasic rhythm remained, there is a tonic component which is disrupted during FDM. The fact that the increase in rates of sulphate incorporation occurs gradually over a few days of deprivation, and the decrease in rates of sulphate incorporation occurs gradually over a few days after translucent diffuser removal, fits with the idea of a more slowly regulated component.

Since the phasic component could be maintained during deprivation, it suggests that the degraded image quality imposed by the translucent diffusers has no effect in controlling the diurnal rhythm of eye growth. Rather, it appears that light intensity is the controlling factor. During normal eye growth, rates of sulphate incorporation were
suppressed by light, and this suppression was light-intensity dependent. Therefore, since this rhythm is maintained during deprivation, it would appear that there is sufficient light during deprivation for this rhythm to be maintained.

The tonic component, on the other hand, does appear to depend on the image quality received by the eye. Under conditions of a degraded image (deprivation), there was a tonic increase in the rate of sulphate incorporation. Upon translucent diffuser removal there was a subsequent decrease in rates of sulphate incorporation as the clear image quality is restored. Not only does it appear that a few days of degraded image are required before there is an increase in rates of eye growth, but it also appears that this degradation in image needs to be continuous. When normal vision was presented each day, even for only 3h each day, this was enough to prevent the increase in rates of sulphate incorporation which are normally seen during deprivation. Therefore, although the translucent diffuser is worn for the other 9h of daylight each day, only limited stimulation of the circuits which control this tonic growth component is required for normal eye growth. The tonic component also appears to be dependent on light intensity, since the normal period of vision each day can prevent FDM developing only if the light intensity is 0.4 lux or higher. Therefore, both the phasic and tonic components of eye growth are light intensity dependent. However, since only the tonic component is affected by the degraded image quality imposed by deprivation, these two components must be controlled by different retinal circuits and messengers.

6.4 Signals involved in controlling eye growth

6.4.1 The retinal dark-light switch

The retinal dark-light switch was initially thought to be a possible candidate in the control of eye growth because both dopamine and the enkephalins (which are two of the compounds released by this switch) have previously been implicated in FDM. The fact that there was a light-intensity dependence in the inhibitory effect of light on rates of sulphate incorporation which is similar to the light intensity required for the switch to flips from its dark to light state suggests that this circuit of retinal neurons may be involved. Light intensities of 0.4 lux and above are required before light can have its suppressive effect on rates of sulphate incorporation in normal eye growth. The same light intensity is required before normal vision each day can prevent FDM from developing. Although only 0.1 lux is required to flip the switch from its dark to light state (Morgan and Boelen, 1996), the fact that the sclera is separated from the retina by a number of anatomical and biochemical pathways may mean that it requires slightly more light in order for messages from the retina to have effects on the sclera.

However, the results in this study indicate that neither melatonin, the enkephalins, neurotensin nor somatostatin, or at least, those of retinal origin, are controllers of eye growth. This leaves only dopamine as a possible candidate. Since
dopamine is released from the retina in the light, it was expected to be an inhibitor of scleral growth, since rates of sulphate incorporation decreased during the light phase. However, ADTN had stimulatory effects on rates of sulphate incorporation. Although it also had an inhibitory effect on rates of sulphate incorporation when applied both in vitro and in vivo, this was not until a concentration of 1mM, which is too high to be physiologically significant. Apomorphine, on the other hand, has been shown to inhibit rates of scleral proliferation in vitro (Marzani et al., 1994) and to inhibit the rate of eye growth in vivo (Stone et al., 1989; Stone et al., 1990; Rohrer et al., 1993). Therefore, this leaves the possibility that the retinal dark-light switch needs to be in its light state in order to have an inhibitory effect on scleral growth, without dopamine having a direct role. The switch may have to be in the light state so that circuits involved in spatial and temporal processing can be activated, which is turn may stimulate the release of another compound, which in turn may control scleral growth.

The diurnal release of compounds from elements of the retinal dark-light switch made them potential candidates as controllers of the phasic component of eye growth. The evidence presented here suggests that this is not the case, since neither agonists nor antagonists of these compounds had any significant effect on acute sulphate incorporation. However, what remains untested is whether these compounds are involved in controlling the tonic component of eye growth. This could be tested using the paradigm of 3h of normal vision each day which prevents the development of FDM. Applying various pharmacological agents during this 3h of normal vision may result in myopia still being able to develop, or may be able to mimic exposure to normal vision. This would in turn suggest possible candidates for the controlling factor of the tonic component.

6.4.2 A role for acetylcholine?

If transmitters released from the retinal dark-light switch are not involved in controlling the phasic component of eye growth, then what are the other possible candidates? Acetylcholine released from the retina has previously been suggested to be involved in controlling rates of scleral growth. However, there is no direct evidence of this. In the present study, there was no evidence that cholinergic agonists or antagonists had physiologically relevant effects on rates of sulphate incorporation when applied in vivo, suggesting that cholinergic compounds are not involved in controlling the phasic component of eye growth.

The cholinergic antagonist atropine did inhibit rates of sulphate incorporation when applied directly to the sclera. Although the concentrations used may have resulted in pathological effects being observed, atropine has successfully been used to prevent the development of human myopia during childhood (Bedrossian, 1979; Goss, 1982), and atropine has also been used in animal models to prevent the development of myopia by slowing rates of axial elongation (Young, 1965; McBrien et al., 1993).
What remains unknown is the pathway by which atropine exerts its effects. It could simply be that atropine acts on receptors on the sclera, perhaps in a pathological manner, which may explain how rates of axial elongation are slowed. However, given the labile nature of acetylcholine, it is difficult to understand how physiologically significant concentrations of acetylcholine could reach the sclera if it was released from elsewhere in the eye. Since the results presented here seem to rule out the possibility that retinal acetylcholine is involved, in addition to previous studies which show that eyes in which retinas lesioned with compounds which destroy cells containing acetylcholine can still develop FDM (Ehrlich et al., 1990; Stell et al., 1997), an alternative explanation may be the acetylcholine from other parts of the eye (such as the RPE and choroid) may be involved, which interacts with other messengers to control scleral growth. The results presented here rule out acetylcholine as the controller of the phasic component, although tests for its role as a regulator of the tonic component have not been carried out.

6.4.3 FDM vs emmetropisation

Although the phasic component is maintained during deprivation, and the tonic component is disrupted during deprivation, it is not known what happens to these components upon translucent diffuser removal. It is possible that since the phasic rhythm is maintained during increased rates of eye growth, that it may also remain during decreased rates of eye growth, upon translucent diffuser removal. However, it is also possible, since the rate of eye growth slows down upon translucent diffuser removal, that the regulatory component shuts down so that the diurnal rhythm is abolished. These possibilities remain to be tested.

The tonic component also needs to be further studied. Although there is apparently a decrease in the tonic component after translucent diffuser removal, this may simply be a return to normal tonic control because normal vision was restored. However, since a few days after the translucent diffusers have been removed, the rate of sulphate incorporation is even less in the formerly translucent diffuserd eye compared to the control eye, this suggests that there might an active control of the tonic component which changes its rate during emmetropisation, as well as during FDM. Experiments using lenses rather than translucent diffusers may give a better understanding of the processes which occur during emmetropisation. Translucent diffuser removal may in fact only reflect the return to normal levels when image degradation is removed. However, after lenses are fitted, there is an active emmetropisation. Changes in eye growth in response to imposed hyperopia and myopia can be examined, and may give a better understanding of whether the same process is involved in both refractive errors, or whether there are two separate controls of eye growth, with one responsible for the increase in growth rates seen when myopia is
developing, and one responsible for the decrease in growth rates seen when hyperopia is developing.

6.4.4 How many signals are involved?

It appears that there are two controlling factors involved. Although both the tonic and phasic components are dependent on light intensity, only the tonic component appears to be affected by the degraded image quality imposed by translucent diffusers during FDM. Therefore, while both of these controls must be exerted by the retina (since the retina is light-sensitive, and can also detect image quality), there must be two different signals. The tonic component may be the coarse control of eye growth, which responds to image degradation during deprivation, resulting in an overall increase in rates of eye growth. Whatever occurs in the retina during deprivation exerts its effect on the sclera to control these growth rates, and this appears to require the retinal dark-light switch to be in its light state. Meanwhile the phasic component may be involved in fine tuning the rate of eye growth so that it precisely matches its optical power. It may be that one messenger is involved, but that it could increase or decrease its rate of synthesis in order to control the increase and decrease in rates of eye growth. Alternatively, it is possible that the phasic component has two controllers, one which causes an increase in growth in the dark, and one which causes a decrease in growth in the light. The reciprocal inhibitory circuit of dopaminergic amacrine cells and the ENSLI amacrine cells may be the basis of this. While dopamine could inhibit rates of sulphate incorporation during the day, when enkephalin release is suppressed, at night, when dopamine release is suppressed, enkephalins are released, and this could stimulate rates of sulphate incorporation. However, given the neither dopamine nor the enkephalins had any significant effect on rates of sulphate incorporation, this circuit appears unlikely to be involved.

Would only two signals be enough to account for the changes which were observed? The retina appears to have the ability to generate messages which signal the sign of defocus to the sclera, so that it can increase or decrease its rate of growth to compensate for the imposed defocus (Diether and Schaeffel, 1997; Schaeffel et al., 1990; Schmid and Wildsoet, 1996b). Therefore, in addition to the two signals already discussed (phasic and tonic), there may be another two which control the direction of eye growth. They may also be yet another two signals which control the direction of choroidal thickening or thinning in response to imposed defocus, which is also sign sensitive. Wallman and colleagues (1995) have shown that there is a thinning of the choroid when the eye becomes myopic, and a thickening of the choroid when the eye becomes hyperopic. The mechanism of this choroidal thickening and thinning remains unknown. However, there is an increase in rates of proteoglycan synthesis in the thickened choroid. In addition to this, the choroidal changes occur before the scleral changes. Therefore it is possible that the same sign-sensitive signals may be acting on
both choroidal and scleral growth in response to imposed defocus. This sign-sensitive signal may be represented by two messengers (one responsible for increased growth, and one responsible for decreased growth), or it could be one messenger whose release is controlled in order to control rates of scleral growth.

If there is a sign-sensitive signal, this signal could be acting independently on either the phasic component or the tonic component. This could be tested by using lenses to see if the phasic rhythm is maintained during both imposed hyperopia, and myopia. Alternatively, the sign-sensitive signal could control the tonic component. This too could be tested using the paradigm of 3h each day of normal vision during lensing, to see if the directionally-selective growth can be prevented by normal vision each day. If this is the case, and if whatever is responsible for controlling the tonic component in FDM can be identified, then it could be tested whether this compound is involved in the control of lens-induced refractive errors too. Identifying the compounds involved in these processes may help us to understand just how the eye can tell in which direction to grow.

6.5 The pathway from retina to sclera

Although we know that a disruption to normal visual processing results in changes in the rate of eye growth to compensate for this disruption, it is obvious that relatively little is known the pathway in which retinal processing can lead to changes in scleral growth. An important step in understanding the pathway between retina and sclera is to find out what compounds act on the sclera to regulate scleral growth. Once these compounds have been identified, then clues may be able to be obtained by looking at where these compounds are released from to give some idea of the pathway involved. One receptor that has been localised to chicken scleral chondrocytes is that of insulin growth factor (IGF)(Waldbillig et al., 1990). In addition to this, both basic fibroblast growth factor (bFGF) and transforming growth factor beta (TGF-β) have also been implicated as controlling factors of scleral growth in FDM (Seko et al., 1995). Therefore it is possible that whatever signal comes out of the retina in response to FDM eventually acts on growth factors in the sclera which in turn control rates of scleral growth. Therefore, something worth investigating would be trying to identify what controls the release of growth factors. The RPE has been shown to influence the growth rate of scleral cells when scleral chondrocytes are co-cultured with cultured RPE cells (Seko et al., 1994b), suggesting the RPE as another source of compounds which could influence scleral growth.

At the other end of this chain of events, it will be important to characterise the inputs to and outputs from the retina which are the controlling factor of scleral growth. It still remains unknown as to what changes in retinal processing occur when form vision is removed, and what the crucial missing elements of form vision are. This
project did not set out to investigate the changes which occur retinal processing during FDM. However, the assay used here could be used to measure changes as a result of visual manipulation. For example, temporal and spatial frequencies can be imposed on the eye, to see what effects various treatments have on rates of sulphate incorporation.

Until all the compounds which are involved in the control of eye growth are identified, the pathway from retina to sclera which is involved cannot be fully understood. The sulphate incorporation assay used in this thesis may be a useful tool in understanding what aspects of vision which are disturbed during deprivation which result in increased eye growth, which retinal compounds are involved, and finally, which compounds act on the sclera to stimulate the synthesis of scleral proteoglycans.
CHAPTER 7. REFERENCES


