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ROBERT NAM KANG

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
AFIT Student Attending:
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A LIGHT AND ELECTRONMICROSCOPIC STUDY OF TREE SHREW SCLERA DURING NORMAL DEVELOPMENT, INDUCED MYOPIA AND RECOVERY

by

ROBERT NAM KANG

A DISSERTATION

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Physiological Optics in the Graduate School, The University of Alabama at Birmingham

BIRMINGHAM, ALABAMA

1994

ABSTRACT

In the developing eye of humans and other animals, it appears that an "emmetropization" mechanism guides most eyes toward emmetropia, a condition where there is no refractive error. This mechanism appears to control axial length of the eye so that the plane of retina gradually matches the plane of focus. Control of axial length during development may involve controlling the development of the sclera, a fibrous tissue providing structural integrity and resistance to the expansive forces of intraocular pressure.

A morphological examination was made, at the light and electron microscopic levels (Spurr-embedded, osmicated tissue stained with uranyl acetate and lead citrate), of the vascular choroid and sclera in normal developing tree shrew eyes. These were compared with eyes made myopic by 21 days of monocular form deprivation and eyes that recovered for 30 days from a deprivation-induced myopia.

During normal development, the thickness of the choroid remained relatively constant. The cross-sectional area of the choroid (a measure of its volume) increased with the size of the eye. The choroid in deprived eyes was significantly thinner than in recovering eyes. The thickness and cross-sectional area of the sclera increased with age. Compared to open control eyes, the sclera was significantly thinner in deprived eyes, but not in recovering

eyes. Measured from low-power EM montages, the fraction of the sclera comprised of lamellae of collagen fibrils increased with age. The fraction occupied by fibroblast processes gradually declined. The fraction occupied by "space" (possibly containing proteoglycans) remained relatively constant. In deprived eyes, the amount of the "space" component was significantly lower. In recovering eyes, the fibroblast and "space" components were significantly larger than in the deprived sclera. In both deprived and recovering eyes, the lamellae component did not change significantly. The number and thickness of lamellae, density of collagen fibrils within lamellae, mean collagen fibril diameter, ratio of collagen fibril area to total area and nearest neighbor distances did not change significantly with age, deprivation or recovery. These results suggest that the fibril-containing lamellae are important for overall structural integrity, but that control of the viscoelastic properties of the sclera, and axial length, may depend more on other extracellular matrix components, such as proteoglycans in the region between lamellae.

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INTRODUCTION

Emmetropia, Myopia and Hyperopia

The vertebrate eye (Figure 1) has remarkable anatomical and functional similarities across a wide range of species (Walls, 1942). A common feature is that these eyes focus images onto the retinal photoreceptor array. Through phototransduction, activation of retinal circuitry and its connections in central neural processing centers, the visual system is able to detect objects and produce visual perception.

Having an eye that provides clearly focused images on the retina has probably promoted survival throughout vertebrate evolution. As illustrated in Figure 2B, emmetropia is a condition where there is no refractive error; the axial length of the eye matches the focal length produced by the eye's optical components, so that parallel rays of light are focussed on the photoreceptor layer when the eye is in a relaxed (non-accommodating) state. Myopia (Figure 2C) is a condition where the axial length is longer than the focal length, so that parallel rays of light are focussed in front of the retina (e.g., toward the cornea). In hyperopia, the axial length is shorter than the focal length, so light rays would focus behind the retina (Figure 2A).

Given the utility of having clearly focused retinal images, it may not be surprising that emmetropia is very common in animals and in humans. In a

Figure 1. Schematic diagram of primate eye. The expanded box shows the layers in the neural retina and the choroid. Note the thinness of the choroid compared to the retina and the sclera. Modified from Krebs and Krebs (1991), Primate Retina and Choroid.

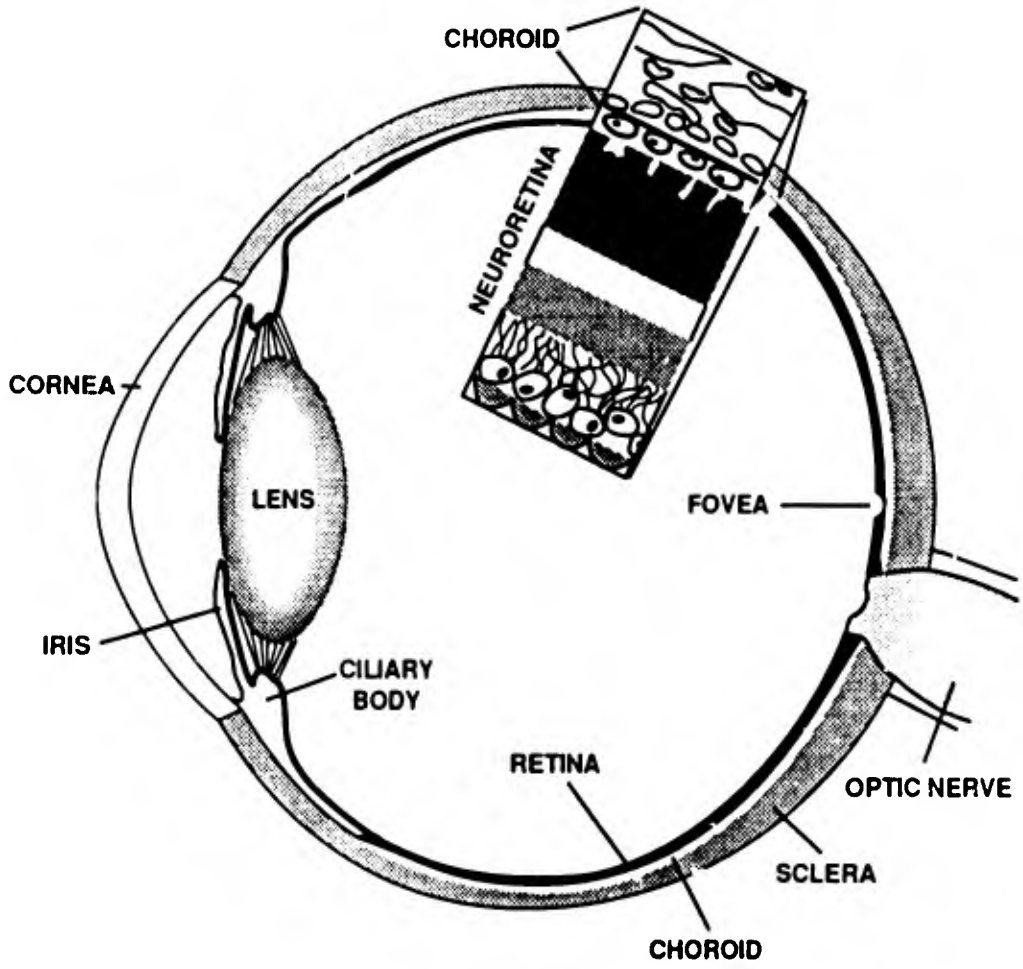
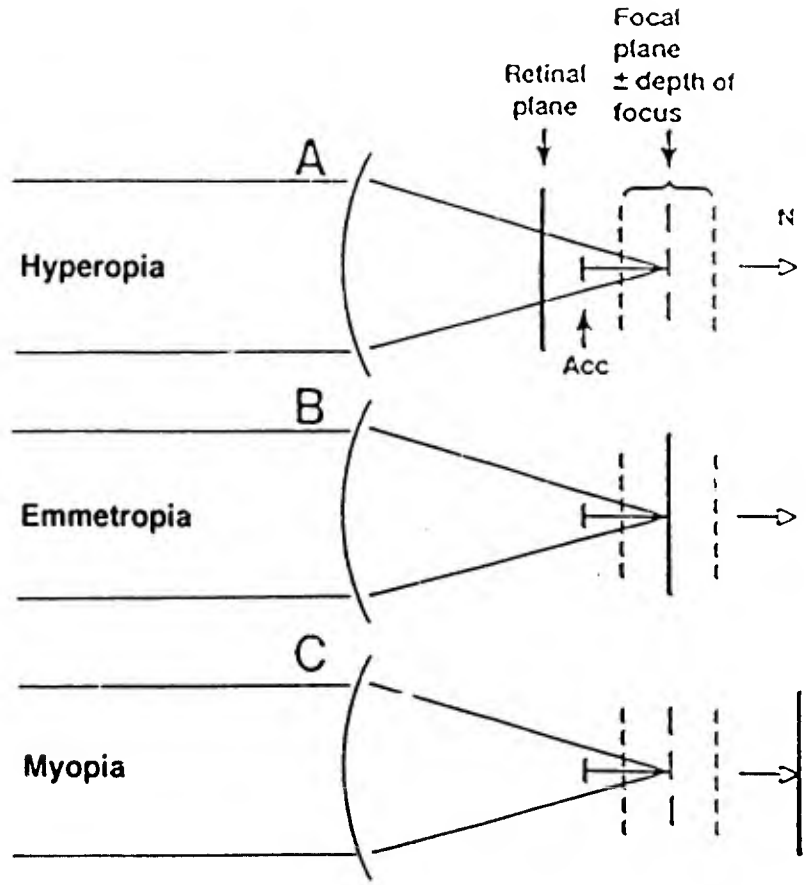

CHOROID
NEURORETINA
CORNEA
LENS
FOVEA
IRIS
CILIARY BODY
RETINA
OPTIC NERVE
SCLERA
CHOROID

Figure 2. Schematic diagrams of (A) hyperopia, (B) emmetropia, and (C) myopia. ACC, accommodative amplitude, the location of the focal plane during full accommodation. N, the direction the focal plane moves for near objects. In each example the focal length (focal plane) is the same. In hyperopia (A) the axial length of the eye is short, so that the retina (retinal plane) is in front of the focal plane. Accommodation moves the focal plane toward the retina. In emmetropia (B) the retinal plane is coincident with the focal plane. In myopia (C) the axial length is longer than the focal plane, so that images focus in front of the retina. However, the focal plane for near objects moves posteriorly (N) so that nearby objects can be in focus.


Retinal plane
Focal plane ± depth of focus
A
Hyperopia
Acc
N
B
Emmetropia
C
Myopia

sample of over 5,000 people, Stenstrom (1946) found that emmetropia occurred with far greater frequency than would be expected from a random combination of the ocular components of human eyes, each of which is normally distributed across the population (Tron, 1940). Further, Sorsby, Benjamin, Sheridan, Stone, and Leary (1961) found that the depth of the vitreous chamber was highly correlated with the refractive state of the eye, suggesting that coordination of vitreous chamber depth to match the focal length of the eye occurred to produce emmetropia. These, and other, data have lead to the suggestion that an active biological mechanism, an "emmetropization" mechanism, guides most eyes toward emmetropia during normal postnatal development by controlling the axial length (Norton, 1990; Wallman & Adams, 1987). One of the challenges that has faced ocular neurobiologists is to learn whether such a mechanism really exists and, if so, how it functions.

Despite the higher-than-random incidence of emmetropia in at least some human populations, refractive error, particularly myopia, is quite common. In the U.S., approximately 25% of the population is myopic, and in some Asian countries (Taiwan, Korea, Japan) the incidence is much higher, as great as 80% (Curtin, 1985; Sperduto, Seigel, Roberts & Rowland, 1983; Wilson & Woo, 1989). In human myopia, the posterior (vitreous) chamber is too long to match the focal length of the eye. Myopia is not just an inconvenience and an economic burden involving the wearing of corrective lenses. In some cases (pathological myopia) the axial length continues to increase beyond the ability of the retina to follow, resulting in retinal detachment. More than 10,000

people become legally blind in the U.S. each year from this progressive myopia and myopia has been ranked as the fifth most frequent cause of impaired vision (Curtin, 1985). Others, with large but less severe myopia are at increased risk for retinal detachment and glaucoma (Curtin, 1985). Thus, another challenge to ocular neurobiologists is to learn how the mechanism that produces emmetropia in most people can sometimes function improperly and result in varying amounts of myopia. The eventual goal is the prevention of myopia.

Environmental Effects on Ocular Development

For many years, epidemiological studies have suggested that environmental factors, such as literacy, education, and tasks involving extensive near work, may affect ocular development to produce myopia. However, demonstrating a causal relationship in humans has been extremely difficult. For example, there is no question that prevalence of myopia increases with the level of education (especially in Asia), but the question of cause and effect still remain unclear. Also, studies over the years have found contradictory results on the prevalence of myopia when, for example, comparing sexes, comparing urban and rural populations (review, Curtin, 1985).

The idea that the visual environment has an effect on ocular development received direct support when it was found that the eyes of monkeys (Wiesel & Raviola, 1977) and tree shrews (Sherman, Norton & Casagrande, 1977) become myopic if they are deprived of form vision during

postnatal development by means of eyelid closure. The same findings have been demonstrated in many other animals such as chicks (Wallman, Turkel & Trachtman, 1978), cats (Yinon & Koslowe, 1984), and marmosets (Troilo & Judge, 1993). In all of these species, if one eye is deprived of the opportunity for focused images to occur on the retina, by any of several methods, that eye elongates beyond the axial length of the fellow control eye and, consequently, becomes myopic. In human children, it is also known that such ocular conditions as ptosis (drooping eyelid) (O'Leary & Millodot, 1979) and cataracts (Curtin, 1985) which prevent image formation on the retina result in increased axial elongation and myopia.

The discovery that images need to be formed on the retina in order for normal emmetropia to develop has lead, through a series of experiments that will be described later in more detail, to the hypothesis that an active emmetropization mechanism occurs, certainly in animals and probably in humans, that depends upon retinal activity produced by visual images. This retinal signal apparently indicates that the optics and the axial length have become matched properly during development, thus "telling" the eye to stop elongating.

The Composition and Potential Role of the Choroid and Sclera

When a retinally-generated signal "tells" the eye to stop elongating, what "listens" to the signal, how does the signal reach it, and how does it implement the instructions? All these questions remain yet unresolved, but

two structures that are likely to play an important role are the vascular choroid and the fibrous sclera.

Choroid

The choroid (Figure 1) consists primarily of blood vessels and melanocytes, and functions to provide blood supply to the retinal pigment epithelium (RPE) and to the photoreceptor cells of the retina. It is the posterior part of the uvea, the middle tunic of the eye between the RPE and the outer fibrous sclera, and is continuous anteriorly with iris and ciliary body. The choroid is divided into three layers: the inner most layer is the Bruch's membrane which borders the RPE. The middle layer is the choriocapillaris, and the outer most layer is the vascular stroma consisting of large blood vessels and melanocytes. Embryologically, choroid develops from mesenchyme surrounding the eyecup which, in turn is an invagination formed at the surface of the embryonic forebrain at the end of first month in the development of human embryo (Ozanics, Rayborn & Sagun, 1978). The melanocytes originate from the neural crest.

Other than the obvious role of supplying the vascular need of the outer neural retina and, perhaps, in preventing thermal damage from focussed light at the outer retina by its high blood flow rate, the role of choroid is not clear. Van Alphen (1990) has suggested that the choroid, along with ciliary muscle in the ciliary body, forms a continuous sheet of smooth muscle to resist the expansive force of intraocular pressure (IOP).

In addition to a possible structural role, the highly vascularized choroid is located between the retina and the fibrous sclera of the eye. Thus, any retinally-generated signal must cross through the choroid which might serve as a barrier. However, the choroid might serve as an active relay of a retinally-generated signal, receiving, responding to, and then transferring a related signal to the sclera. For these reasons, it is possible that choroidal structure may affect the influence of retina on the development of the sclera.

Sclera

The sclera, which is the fibrous outer coating of the eyeball (Figure 1), probably plays a very important role in emmetropization. The sclera is continuous anteriorly with the cornea, and together they form a nearly spherical sheath covering the eye. The extraocular muscles attach to the sclera near the equator and in the posterior region, and the optic nerve penetrates the sclera near the posterior pole. The primary functions of the sclera are to provide a structural framework to the eye, providing protection to the internal neural, vascular, and optical tissues, and to resist the expansive force of IOP. The IOP is produced by 1) the formation of vitreous humor in the posterior chamber of the eye and 2) by the balance between production and outflow of aqueous humor in the anterior chamber (Sebag, 1992).

In mammals, the sclera consists of fibroblasts which produce an extracellular matrix of collagen fibrils and proteoglycans, and is organized in interwoven bundles of collagen fibrils called lamellae (Torczynski, 1982) (see Figure 17 for an example). Embryologically, sclera is thought to be induced

from mesodermal and ectodermal neural crest tissues by the neural retina and/or the RPE (Johnston, Noden & Hazelton, 1979). In human sclera, collagen content is approximately 50% by dry weight, consisting primarily of type I collagen (Keeley, Morin & Vesely, 1984), although small amounts of collagen types III, IV, V, and VI, as well as fibronectin has been found (Maza, Dutt & Foster, 1992). In tree shrew sclera, recent evidence suggests that collagen may be mostly type I (about 99%) (Norton, unpublished data). Associated with these collagens are proteoglycans with side chains of various types of glycosaminoglycans (GAGS) such as hyaluronic acid, heparan sulfate, dermatan sulfate, and chondroitin sulfate (Trier, Olsen & Ammitzboll, 1990).

During postnatal ocular development, the rate of growth of the sclera, including the rate of accumulation of collagen and proteoglycans, might reasonably be expected to be of great importance in regulating the axial length of the eye and in the emmetropization process. With respect to its role in regulating axial length, the viscoelastic property of the sclera and, hence, its resistance to intraocular pressure, must be critically important. Like other connective tissues, the biomechanical properties are derived from the collagen content, fibril size and orientation, and content and composition of other extracellular matrix such as proteoglycans with their GAG side chains (for review, see Parry & Craig, 1984). Collagen fibers provide tensile strength, whereas the proteoglycans cope with compressive forces and regulate hydration. These issues will be discussed in detail in a later section.

Despite its potential importance in ocular development and emmetropization, the sclera has been studied much less intensively than the cornea, with which it is contiguous but distinct both morphologically and biochemically. In particular, very little is known about how the sclera develops normally and how it is altered in deprivation-induced myopia. Even less is known about the choroid. As outlined in the following sections, a morphological examination of the sclera at the light and electron microscopic levels is a logical next step in understanding how choroid and sclera may participate in the control of axial length.

Emmetropization

The previous section introduced the idea that an emmetropization mechanism normally guides developing eyes to emmetropia. In this section, additional data on ocular development will be presented, along with a feedback model that summarizes a way that a retinally-generated signal could regulate axial length in developing eyes to produce emmetropia. With this model as a framework, more detailed information will be presented and examined in sections dealing with the generality of the model across species, the potential roles of the choroid and the sclera in normal ocular development, and the potential roles of the choroid and the sclera in induced and spontaneously occurring myopia.

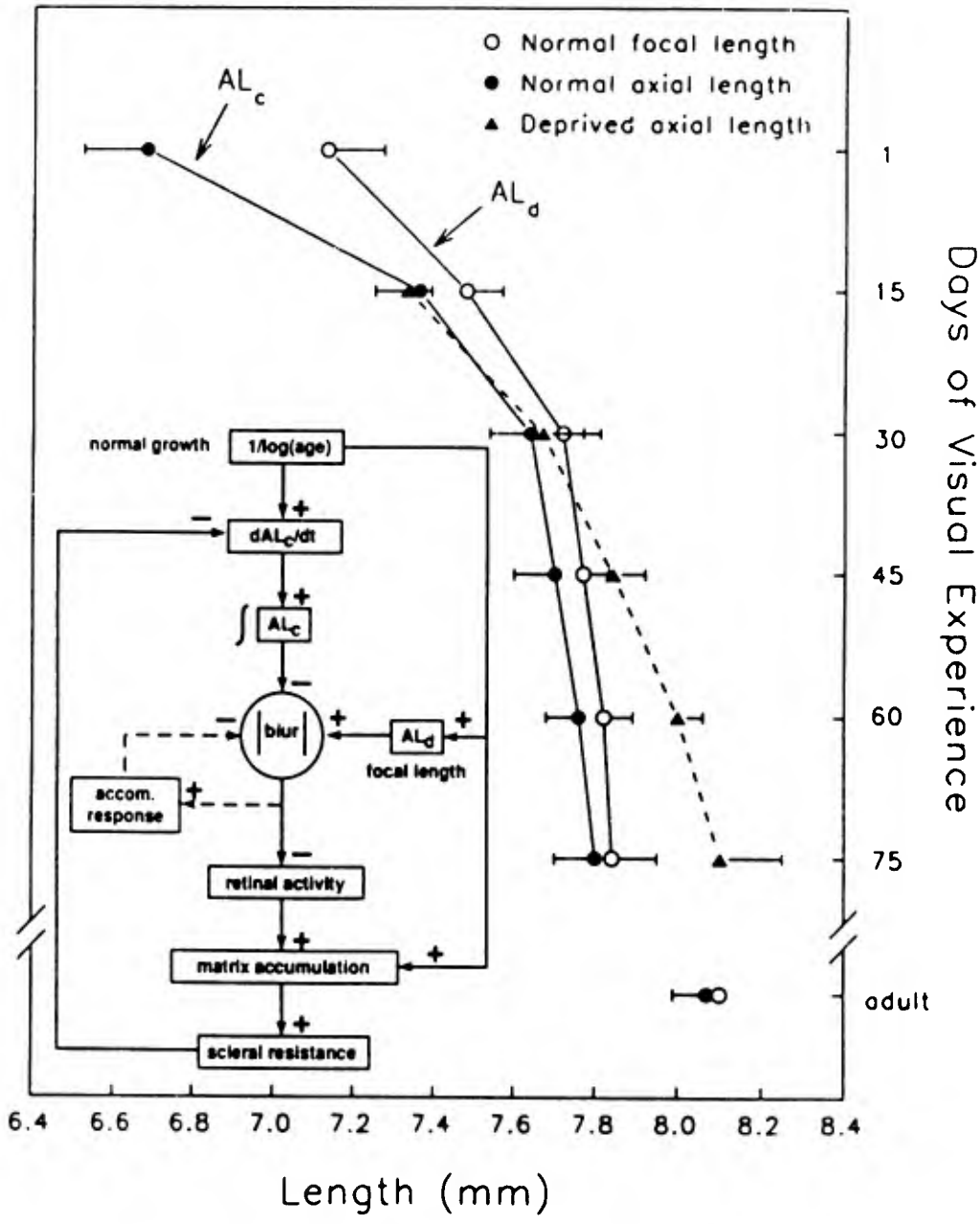
Ocular Development in Tree Shrews

Figure 3 summarizes data on ocular development in tree shrew (Norton & McBrien, 1992) and gives a schematic outline of the emmetropization model.

Figure 3. A feedback model of emmetropization. The model is based on retinal activity control of scleral matrix accumulation and resistance. The plot shows the relationship between the focal length and the axial length of tree shrews from eye opening (day 1) through late development. At eye opening, the axial length (AL_c) is much shorter than the focal length (AL_d), so the eye is hyperopic. Gradually, the axial length and the focal length become closely matched. In deprivation-induced myopic eyes, as the plot demonstrates, the axial length elongates beyond the focal length. The focal length is calculated from measures of refractive state using a schematic eye model of tree shrew eye (Norton & McBrien, 1992).

Focal and Axial Length Development

Model of Emmetropization



The tree shrew has been the subject of many studies of ocular development because it is a mammal, closely related to primates, and thus may provide information useful in understanding human ocular development and emmetropization. In addition, tree shrews develop rapidly, reaching sexual maturity in about 4 months. The open circles in Figure 3 denote the focal length of the eye as a function of the number of days after the eyes first are open ("Days of Visual Experience"). During the first several weeks of visual experience the focal length increases somewhat. This is due to a gradual flattening of the cornea and enlargement of the lens (Norton & McBrien, 1992). It appears that the increase in the focal length occurs as a consequence of normal growth, and without any apparent effect of the visual environment. Thus, it appears to be a genetically determined value.

The filled circles in Figure 3 show the axial length of the eye. At the time the eyes open, the focal length of the eye is much longer than the axial length. Parallel light rays entering the eye would be in focus a considerable distance behind the retina; the eye has approximately 20 diopters of hyperopia. The diopter (D) is a unit of power of lenses equal to the reciprocal of focal length in meters. In the first 2 weeks of visual experience (v.e.), the axial length increases rapidly (from about 82% of its adult value to about 91%). As a result, the initial hyperopia decreases to about 6 D. During the next several weeks, the rate of increase in axial length slows, and the eye enters a period of very gradually lessening hyperopia. Eventually, in adult animals, the axial length approximately matches the focal length and the eye is emmetropic.

The filled triangles in Figure 3 denote the axial length found in the eyes of tree shrews deprived of form vision for varying periods, starting at the day of eye opening and continuing for 15, 30, 45 etc. days of visual experience (McBrien & Norton, 1992). As may be seen, the axial length in animals deprived for the first 15 days after the eyes open is not significantly longer than normal. After 30 days or more of deprivation, the axial length increases beyond the normal value, and, in older animals, the axial length exceeds the focal length, producing myopia. It has also been shown that short periods of deprivation (5 or more days) can produce significant axial elongation and myopia if presented to animals that are older than 15 days of v.e. (McBrien & Norton, 1992; Norton, 1990; Norton & Siegwart, 1991).

The increased axial length in visually-deprived eyes shows clearly that the axial length in normal eyes could be much longer than it actually is (e.g., the eye normally is not elongating at a maximal rate during the period after 15 days of v.e.). This, in turn, suggests that something about normal visual experience, related to the presence of images on the retina, acts to restrain axial elongation and produces the very gradual decrease in hyperopia in the older animals. This sub-maximal increase in axial length, dependent upon a normal visual environment, suggests that the axial length is actively matched to the focal length during emmetropization via a visually-guided mechanism.

The absence of an effect of deprivation on axial length development in young animals (less than 15 days of v.e.) may be due to several factors. One is that the eye may be growing at a maximal rate during this period, so that

no further increase is possible. It may be that only in older animals, when the normal rate of elongation slows, the environment can have an effect. Another consideration is that the retina may be so immature before 15 days of v.e. that it may not respond differentially to the presence of normal visual input or visual deprivation. Evoked potential and behavioral studies in tree shrews (Casagrande & Condo, 1988; Norton & McBrien, 1992) support this suggestion; tree shrews do not appear to use visual cues to guide their behavior for 10-15 days after eye opening. A third factor may be that the mismatch between the focal length and the axial length is so great in very young animals that images are always quite blurred. Many studies have shown that retinal cells respond more strongly to clearly-focussed images than to blurred ones or to diffuse light (Kuffler, 1953). As the axial length approaches the focal length, the blur is decreased and, presumably, retinal responses increase. Thus, the susceptible period for deprivation-induced myopia may begin at a point when the retina begins to respond strongly to visual images.

A Feedback Model to Control Axial Length

The inset within Figure 3 is a model, based on the data in the figure and on other studies, that illustrates how a retinally-generated signal, dependent on the amount of blur on the retina, might interact with normal growth to guide the axial length so that it comes to match the focal length. The model utilizes the fact that, when the axial and focal lengths of the eye are not identical, the amount of blur of the retinal image will be greater than if the eye were emmetropic. If a retinal signal, inversely-proportional to the average

amount of blur, restrains the elongation rate of an initially hyperopic eye, then emmetropization will occur.

At each time-point during postnatal development, the eye has a "current axial length" (AL_c in Figure 3)) and a "desired axial length" (AL_d), which is, in fact, the focal length. In the first days after eye opening, the daily change in current axial length, dAL_c/dt is large. A primary factor producing this change is assumed to be normal growth, which typically is inversely related to age (on a log scale). If no other factors were involved, dAL_c/dt would gradually decline as the animal reached adulthood. The model assumes that the elongation rate, dAL_c/dt, provided by normal growth in lighted condition is greater than the growth-produced increase in the focal length, so that, if there were no restraining influence, the AL_c (the integral of the rate of change) would eventually exceed the focal length.

The necessary restraint is provided in the model by a blur-dependent retinal signal. At any given time, the difference between the AL_c and the focal length (AL_d) is the amount of blur (indicated as an absolute value because tree shrews appear to treat myopic blur as equivalent to hyperopic blur). As mentioned in a preceding paragraph, a strongly blurred image will produce relatively weak retinal responses.

The model assumes that the accumulation of extracellular matrix in the sclera is stimulated by retinal activity. In young animals, with apparently little retinal activity, the accumulation of matrix is driven only by normal growth mechanisms. However, as the retina matures and the blur decreases

in older animals, the retinally-generated signal increases, stimulating matrix accumulation beyond the level provided solely by growth mechanisms. The closer the axial length (AL_c) becomes to the focal length (AL_d), the less is the blur and the greater is the retinal signal and matrix accumulation. Finally, the model assumes that the retinally-stimulated increase in matrix, coupled with normal growth, increases the resistance of the sclera to intraocular pressure. This, in turn, reduces the rate of change in axial length (dAL_c/dt).

By means of this negative feedback mechanism, as the axial length (AL_c) approaches the focal length (AL_d), blur is reduced, retinal activity is increased, and matrix accumulation in the sclera is stimulated, further slowing the rate of elongation. If the elongation rate becomes too slow, blur increases, slowing matrix accumulation and allowing an increased rate of elongation. Similarly, if the elongation rate becomes too great, so that the eye is slightly too long, blur decreases, stimulating matrix accumulation, increased resistance and thus reducing the elongation rate. Experimental confirmation of this portion of the mechanism has come from the fact that tree shrews in which the axial length has been increased somewhat by visual deprivation will, when the deprivation is removed, slow the axial elongation rate and actually recover from the induced myopia (Norton, 1990; Norton & Siegwart, 1991). An additional prediction of this model is that if the focal length were artificially increased by having a developing tree shrew wear a minus-power spectacle lens, the axial length should increase until it matches the imposed focal length. A recent study has shown this to be the case (Siegwart & Norton, 1993).

The model readily explains the effect of visual deprivation: preventing images from forming on the retina with an occluder or eyelid closure is equivalent to blurring the image and should reduce matrix accumulation, slowing the development of scleral resistance and allowing a high elongation rate. Because deprivation opens the feedback loop, so that images never form, the high elongation rate continues until normal growth eventually stops.

Although this model offers no direct explanation for human myopia, if a similar mechanism normally operates in humans to produce emmetropia, then the mechanism must fail in some way in those people who develop myopia. Three obvious places where this could occur would be: 1) an absent or incorrect retinal signal, 2) a failure of transmission across the choroid to the sclera and/or 3) failure of the sclera to respond properly or fully to the signal.

Based on the available data in tree shrew and upon the predictions of the emmetropization model, the sclera, and possibly the choroid, are extremely important structures. After examining whether the emmetropization model seems to generalize to other species, the next sections will provide more detailed information about the normal development of the choroid and the sclera and the changes found in myopic eyes.

Generalization of the Model to the Other Species

Ocular Development and Emmetropization in Humans

In humans, the larger size of the eye reduces the size of refractive error (in diopters) which occurs from mismatching the axial length to the focal length in smaller eyes, such as tree shrews. Nonetheless, humans appear to

follow the same general developmental pattern as tree shrews, an early rapid growth phase followed by a more gradual phase lasting into early puberty. Although overall refraction is dependent on many elements in the eye, the refractive powers of the cornea and lens, and the axial length contribute the most. In humans, the cornea, which provides most of the optical power of the eye, develops "rapidly" (if human years equals tree shrew weeks in development). From birth, it increases its radius of curvature, thus decreasing its refractive power and increasing focal length, and reaches adult proportions by the age of two. After this early development, the cornea changes little in either refractive power or in size (Larsen, 1971a); the slight change is related to the increase in overall ocular size. The lens also develops rapidly after birth, becoming thinner anteroposteriorly with a reduction in refractive power (Curtin, 1985). The lens continues to get thinner till the ages of 8 to 10, gradually increasing the focal length. Afterwards, the lens changes slowly, but unlike the cornea, the lens continues to change throughout life, increasing in size and mass (Larsen, 1971b; Curtin, 1985). As a result of these corneal and lenticular changes, human focal length normally increases about 5.1 mm (approximately from 18.8 mm to 23.9 mm) during the first 3 years, and then increases more slowly by about 0.3 mm (approximately from 23.9 mm to 24.2 mm) between the ages 3 till 14.

As in tree shrews, this early change in the focal length of the human eye is accompanied by a slightly more rapid increase in the axial length. At birth, the axial length of the human eye is about 18 mm but increases rather rapidly

to about 22.9 mm by the age 3. After this rapid growth, the eye elongates rather slowly between the ages 3 till 13 or 14. By the age of 13 or 14, the axial length of the eyes have reached the adult size of 23.9 mm. The average increase in the axial length between the ages 3 and 14 is about 1 mm which is more than needed to compensate about 0.3 mm increase in the focal length (see above) during the same period, and as a result of these changes an initial hyperopia is gradually reduced and shifted toward emmetropia or slight myopia.

Given the slightly greater focal length compared to the axial length, at birth, humans are generally slightly hyperopic but become emmetropic during normal development. At birth the distribution of refractive errors is Gaussian in appearance centered slightly on hyperopic side with a mean of about 2.07 D with a standard deviation of about 2.75 D (Hirsch & Weymouth, 1991). By the start of school (5 to 6 years old), most of the extreme refractive errors have disappeared so that the refractive distribution is leptokurtotic and centered near emmetropia, but still on hyperopic side. About half of the children at the age of 6 have already reached the adult refraction values and their refraction changes very little during their school years. In adults, the distribution of refractive errors is shifted so that it is now centered near emmetropia or slightly on myopic side (0.5 D) and leptokurtotic in appearance (Stenstrom, 1946). These data suggest that an emmetropization mechanism is at work before the age 6.

The rate of ocular growth, which varies slightly among individuals, or the final size of the eye, appears to be independent of the final refractive state. It is true that the myopic eyes are often larger in size compared to the emmetropic eyes, but it is possible to have emmetropic eyes of different eye sizes (Curtin, 1985; Sorsby, Benjamin, Sheridan, Stone & Leary, 1961). What is important, then, appears to be not the final size, but the coordination of the axial length to the optics of the eyes as the eyes reach their adult size.

Ocular Development and Emmetropization in Other Species

Studies of normal ocular development in other animals are rare, but evidence in marmosets (a primate) and in chicks suggest that, as in tree shrews and humans, the ocular development can be characterized by early rapid changes in the focal and the axial length followed by slower changes as the animal reach maturity (Pickett-Seltner, Sivak & Pasternak, 1988; Sivak, Ryall, Weeheim & Campbell, 1989; Troilo & Judge, 1993; Wallman & Adams, 1987). In chicks, as in humans, the cornea provides the most of the optical power to the eye, and the corneal radius of curvature increases during early development, resulting in increase in the focal length during development. Unlike humans, however, the chick lens is found to increase in thickness with slight, or no changes in its optical power during development (Sivak et al., 1989; Wallman & Adams, 1987). These changes in the focal length are more than matched by the increase in vitreous chamber depth and, consequently, axial length so that an initial hyperopia with high individual variation in chick moves toward emmetropia with lowered individual variability.

In addition to the fact that the eyes normally develop to emmetropia, the evidence for the presence of active emmetropization process during ocular development in animals also comes from two types of experiments: 1) spectacle lenses with power to alter the focal length of the eye and 2) recovery from deprivation-induced myopia after form vision is restored.

Spectacle Lenses

The evidence in tree shrews, monkeys, and chickens suggest that when the focal length of the developing eye is experimentally elongated with either spectacle or contact lenses, the axial length of eye increases to match the new focal length as long as the lenses are not too strong (Crewther, Nathan, Kiely, Brennan & Crewther, 1988; Hung, Smith & Harwerth, 1992; Schaeffel, Glasser & Howland, 1988; Siegwart & Norton, 1993; Wildsoet & Wallman, 1992). The effects of plus lenses are variable across species. These lenses, which decrease the focal length, can make the eyes less hyperopic, myopic, or even emmetropic depending on the power of the lens and when it is imposed during development. Monkeys raised with the high plus lenses have become hyperopic, or myopic (Crewther et al., 1988; Hung et al., 1992). It may be that the imposed change in the focal length is too great. Perhaps beyond a certain physiological limit, too much blur is produced and emmetropization mechanism might fail in primates in such cases. Tree shrews respond appropriately to small plus lenses slowing down their elongation rate. Compared to tree shrews and monkeys, the results in chicks are more robust; chick eyes appear to be able to compensate for the greater amount of the focal length change resulting

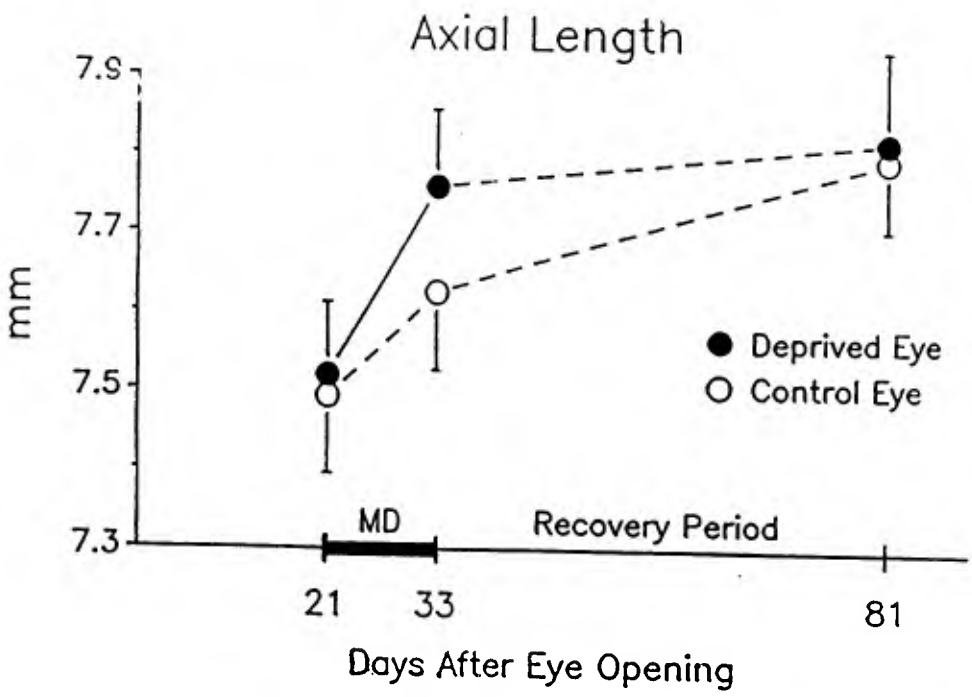
in good axial length compensation to the new focal length regardless of the sign of the lenses. The chick eyes raised with plus lenses consistently stop elongating sooner to match the shorter focal length.

Recovery

After a visual deprivation period has produced axial elongation and myopia, restoration of a normal visual environment results in "recovery" in the axial length and refraction so that the deprived eye is eventually not axially elongated or myopic compared to normal eyes. As Figure 4 demonstrates, in the eye recovering from an induced myopia, the axial elongation rate is slowed significantly in comparison with the control eye. The focal length however continues to elongate normally, reducing the myopia. Recovery from deprivation-induced myopia has been demonstrated in chicks (Troilo & Wallman, 1991; Wallman & Adams, 1987) and in tree shrews (Norton, 1990). In both tree shrews and chicks, it is the vitreous chamber depth that accounts for the recovery (Figure 4). In chicks, recovery can also occur from the hyperopic direction (Troilo & Wallman, 1991). In the case of chick eyes recovering from hyperopic direction elongation is increased, and since the contralateral control eye is continuing its normal elongation, recovery of axial length and refraction is achieved.

The recovery from deprivation-induced myopia is a visually guided phenomenon. In chicks, if the restoration of normal vision is prevented during recovery period by putting the animals in either dim illumination or in the dark, much less recovery occurs, suggesting that it is the visual cues that are

Figure 4. The axial length changes during recovery. A plot of axial length in control eyes and in eyes that are first deprived to induce myopia and then recover from the induced myopia in tree shrews (n=5). After 12 days of monocular deprivation, the axial length in the deprived eyes is longer compared to the control eyes, but after recovery the axial length is no longer different. The plot shows that the recovery is achieved by the recovering eye slowing its axial elongation while the control eye is continuing with normal elongation. Used with permission. (Unpublished data, Siegwart & Norton).


Axial Length
7.9
7.7
7.5
7.3
mm
Deprived Eye
Control Eye
MD
Recovery Period
21
33
81
Days After Eye Opening

important (Gottlieb, Marran, Xu, Nickla & Wallman, 1991; McBrien & Norton, 1987). The recovery is also a phenomenon independent of accommodation or visual cortex, since neither the lesion of the Edinger-Westphal nucleus nor cutting the optic nerve affect recovery (Troilo & Wallman, 1991). However, visual cortex might have a role in chicks in guiding the recovery process since the eyes with optic nerve sections overshot emmetropia, as if they did not know exactly when to stop (Troilo & Wallman, 1991).

The recovery from deprivation-induced myopia is also an age dependent phenomenon. The younger the animal is when normal vision is restored, the more likely for the recovery to occur (tree shrews, Norton, 1990; chicks, Wallman & Adams, 1987). It appears that, in these animals, the developing eyes are capable of recovery because the focal length is still increasing. Once the axial length has exceeded the adult focal length, the eyes cannot completely recover.

The Three Components of Emmetropization

The above evidence suggests that an emmetropization mechanism may occur in humans as well as animals and that it has three main components: 1) a retinal signal, 2) communication to the choroid and/or sclera and 3) some changes in choroid and/or sclera to control axial length.

Retinal Signal

The studies using various types of visual deprivation along with using plus and minus power lenses suggest that a retinal signal related to the

amount of blur is important. It seems that any manipulation that degrades the retinal image will induce myopia.

Lid suture in monkeys leaves a thin layer of translucent skin over the eye so that most of the light, although diffuse, gets through the lid, creating "form deprivation" where the retina is deprived of clear images of high contrast but not light *per se*. Monkeys raised with a translucent cornea, in effect producing similar visual condition as in lid suture but without the mechanical effects, also become axially elongated and myopic (Iuvone, Tigges, Stone, Lambert & Laties, 1991; Wiesel & Raviola, 1979). Also, when raised in complete darkness, with or without lid suture, myopia does not develop in monkeys (Raviola & Wiesel, 1978), in chicks (Gottlieb, Wentzek & Wallman, 1987), and in tree shrews (McKanna, Cassagrande, Norton & Marsh, 1983). These results suggest that it is only when light, with blur, is present during form deprivation that the eye elongates in an uncontrolled manner.

There is evidence in birds that the amount of retinal activity is a key feature in emmetropization. Gottlieb and Wallman (1987) found that when monocularly deprived chicks were raised with strobe light, to produce retinal activity without the presence of visual form, the amount of induced myopia was reduced. Little is known about the specific retinal neurons that encode the necessary visual signals. Lesion studies in chick suggest that deprivation myopia can be induced with as little as the photoreceptors and horizontal cells intact (Ehrlich, Sattayasai, Zappia & Barrington, 1990).

The retinal signal, as might be expected, is spatially local. More surprisingly, so is the control of axial length. Partial visual field deprivation causes "local" ocular elongation and myopia limited to the corresponding retinal region in tree shrews (Norton & Siegwart, 1991) and in chicks (Wallman, Gottlieb, Rajaram & Fugate-Wentzek, 1987). Non-deprived regions of the eyes have normal axial length and refraction. As in the case of recovery from deprivation-induced myopia, axial elongation and myopia appear independent of accommodation (tree shrews, Siegwart & Norton, 1993) and visual cortex since neither the sectioning of the optic nerve nor functional disconnection of the eye with tetrodotoxin affect the development of myopia (Norton, 1990; Troilo, Gottlieb & Wallman, 1987).

Local "myopia" has also been observed to occur naturally. In birds, it has been found that the dorsal part of the eye is myopic, and it has been suggested that the degree of this myopia corresponds to the distance to the ground when the animal is standing on the ground (Hodos, 1990). This "local myopia" or "near distance emmetropia" would serve to keep nearby ground in focus for feeding while keeping the distant horizon in focus for detection of predator at the same time. In addition, when raised in an environment with a low ceiling, chick eyes develop "local" myopia in the corresponding ventral part of the eye (Miles & Wallman, 1990).

The transmitter pathways that may be involved in the retinal signal for emmetropization are not yet clear. Dopamine has been implicated. It is found to be lower in the retina of the deprived eyes in chicks when compared to the

retina from the normal eyes (Stone, Lin, Laties & Iuvone, 1989), and application of the dopamine agonist, apomorphine, to form deprived eyes reduces the axial elongation and myopia in chicks (Stone, Lin, Iuvone & Laties, 1990) and in rhesus monkeys (Iuvone et al., 1991). However, haloperidol, a dopamine antagonist, has also been found to reduce the deprivation-induced elongation and myopia (Stone et al., 1990). In addition, 6-hydroxydopamine, which kills dopaminergic amacrine cells in the retina, has been reported to reduce deprivation-induced myopia (Li, Schaeffel, Kohler & Zrenner, 1992). Given what appears to be conflicting results, it is not clear how dopamine might affect ocular development.

In addition to dopamine, increased levels of vasoactive intestinal polypeptide (VIP) have been found in the retina of the lid sutured monkey eyes (Stone, Laties, Raviola & Wiesel, 1988). Perhaps more importantly, the level of VIP in the retina of deprived eyes is reported to be related to the amount of myopia in those eyes (Raviola, unpublished data). VIP has been implicated in chicks as well, but its role is not clear since the VIP as well as VIP antagonist have been found to reduce the deprivation-induced myopia (Pickett-Seltner, Tao, Rohrer & Stell, 1993).

There is evidence that other transmitter systems might also be involved in form deprivation myopia. McBrien, Moghaddams, and Reeder (1993) reported that, in chicks, vitreous injections of atropine (a muscarinic antagonist) blocked the effects of form deprivation. This effect was not related to accommodation, since when accommodation was measured, the atropine

treated eyes did not differ in accommodative response compared to the normal eyes. Atropine may have acted directly on muscarinic cholinergic receptors in the retina. In tree shrews, intravitreal injections of pirenzepine, a M_1 selective muscarinic antagonist, has also been found to reduce the deprivation-induced myopia (McBrien & Cottriall, 1993).

Communication of Retinal Signal

In order to affect the axial length of the eye, any retinally generated signal must be communicated at least to the choroid and, presumably, to the sclera. Possibly, retinal activity releases a transmitter which may directly cross to the sclera. As mentioned earlier, the choroid is a highly vascularized tissue located between the retina and the sclera. Thus, for any retinal signal to act directly on the sclera it must get across the choroid to affect the sclera in a spatially local manner. Given the high vascular flow in the choroid it seems unlikely that a simple diffusion would work. More likely is the possibility that the retinal signal affects the choroid which in turn affects the sclera in a cascading manner. Evidence of deprivation-induced changes in chick choroid support this possibility. In chick, proteoglycan synthesis in the choroid appears greater in the eyes with plus spectacle lenses than in those with minus lenses (preliminary report, Wallman, 1993). The choroid thins in deprived eyes and thickens in eyes that are recovering from myopia (Wallman, Xu, Wildsoet, Krebs, Gottlieb, Marray & Nickla, 1992). Also, co-culturing of the choroid from deprivation-induced myopic eyes or from eyes recovering from deprivation-induced myopia with the sclera from normal eyes lowers

proteoglycan synthesis in the sclera cultured with the recovery choroid compared to the sclera cultured with the myopic-eye choroid (Gottlieb, Nickla & Wallman, 1993).

It is also possible that alteration in choroidal blood flow might serve as a mediator of a signal from the retina to the sclera. In chicks with monocular visual deprivation, choroidal blood flow is significantly reduced in the deprived eyes (Reiner, Fitzgerald & Hodos, 1991; Shih, Fitzgerald & Reiner, 1992). This, in turn, might alter the scleral development. The cause and effect of this reduced choroidal blood flow is not clear, however, since choroidal blood flow is affected by other factors such as light and accommodation which are known to increase the flow (Fitzgerald & Reiner, 1990) and thus complicate the interpretation. Also, decreasing choroidal blood flow without form-deprivation does not lead to axial elongation (Fitzgerald, Shih & Reiner, 1992).

Effect of Retinal and/or Choroidal Signal on the Sclera

As in the case of the choroid, there is no direct evidence of what is the retinal and/or choroidal signal affecting the sclera or what the effects of the signal on the sclera might be. However, basic fibroblast growth factor (bFGF) has been found to prevent the deprivation-induced elongation in a dose-dependent manner in chicks (Rohrer, Negishi, Tao & Stell, 1993). Also, evidence from *in vitro* studies suggest that in chicks the proteoglycan synthesis by the scleral chondrocytes is higher in deprivation-induced myopic eyes compared to the normal sclera (Nickla, Gottlieb, Christensen, Pena, Teakle, Haspel & Wallman, 1992), and that the proteoglycan synthesis is visually

modulated (Rada, Cornuet, McFarland & Hassel, 1992). The effects on the sclera will be discussed in more detail later.

Are There Additional Mechanisms in Emmetropization?

It appears that direct retinal-choroidal-scleral communication is sufficient to mediate emmetropization, but there may be additional mechanisms. Even though the evidence shows that accommodation is not necessary for the emmetropization mechanism to function (Schaeffel, Troilo, Wallman & Howland, 1990; Siegwart & Norton, 1993), it is likely that accommodation has a role in the process. There is a large body of literature suggesting excessive accommodation as a significant cause of myopia in humans (review, Curtin, 1985). The nature of the role of accommodation in emmetropization is not known, but the model (Figure 3) shows how the input might work. The amount of blur on the retina is dependent not only on the coordination between the focal length and the axial length of the eye, but also on the accommodation system. Accommodation, which would reduce blur, is achieved by contraction of the ciliary muscle and, since the ciliary body (Figure 1) is continuous with the choroid, the tonus of the ciliary muscle might affect the tension of the choroid by pulling it forward during accommodation. The tension of the ciliary muscle/choroid might, in turn, place stress on the sclera.

A problem in trying to relate data from studies in animals to the development of myopia in humans is that in humans the accommodative system is dynamic and strong enough to compensate for small refractive errors quickly so that, in all probability, the retina may not experience significant

amount of blur most of the time. Despite this and without form deprivation, myopia still develops in humans. This might be because the emmetropization mechanism fails in one of the three components described above. It also might mean that the accommodative system might be especially important in the emmetropization mechanism of humans so that alterations in accommodation might influence axial length development more readily than in animals.

Normal Choroidal and Scleral Development

Development in Mammals

Any changes in choroid and/or sclera that are related to the control of axial length occur against a background of normal growth and development. Thus, in this discussion, as in the dissertation research, an examination of normal development must accompany an examination of myopia-related changes.

The development of the choroid has been little studied. The morphological characteristics of the vasculature in the choroid, the choriocapillaris and larger arteries and veins, are believed to be formed by the mid-gestation in monkeys and humans (monkeys, Ozanics et al., 1978; humans, Heimann, 1972). The choriocapillaris forms earlier than the larger vessels, developing from posterior to anterior direction and from the retinal pigment epithelium side to the scleral side. The development of choriocapillaris appears concurrent with the development of the retinal vessels. Vessel growth continues throughout the second half of gestation and continued

growth of capillaries have been observed after birth in monkeys (Ozanics et al., 1978).

The development of the sclera appears similar to the development of the choroid in that essential morphological characteristics of the sclera are formed early in gestation. The human sclera develops from anterior to posterior direction and from the choroidal side to outward and by the 13th week in gestation no differences in collagen morphology between the anterior and posterior regions as well as the inner and outer sclera are noted (Sellheyer & Spitznas, 1988). Fetal development of sclera in monkeys appears similar to the development of human sclera (Ozanics et al., 1976).

At birth, the human sclera is rather uniformly thin and somewhat transparent. This is why infant eyes have bluish appearance since the underlying uveal tissue is visible through the sclera. With increasing age, the sclera gradually gets thicker, accumulating extracellular matrix, and by the age of 10 years, regional differences in thickness are apparent. The sclera continues to get thicker and peaks in thickness around the fifth decade (Avetisov, Savitskaya, Vinetskaya & Iomdina, 1984).

Choroid and Sclera in Birds

The choroid and sclera in birds differ from the mammalian choroid and sclera. In birds, the retina is avascular, thus the retinal vascular needs are supplied by the choroid which is much thicker compared to the choroid in mammals. The avian sclera is also different in that it has both a cartilaginous layer and a fibrous layer. The inner cartilaginous layer is much thicker than

the fibrous layer and is composed of different types of collagens and proteoglycans than the outer fibrous layer (Mayne & Von Der Mark, 1982; Poole, Pidoux, Reiner, Coster & Hassel, 1982). Collagen in the cartilaginous layer is loosely organized, and its proteoglycans, primarily aggrecan with smaller amounts of decorin and biglycan, are considered to be structurally and functionally different from fibrous proteoglycans (Poole et al., 1982). The function of two layer sclera in birds remain unclear.

Development of Collagen Fibrils

Two primary components of the sclera are collagen and proteoglycans. It is believed that their content, organization and interactions are largely responsible for resistive properties of the sclera. As connective tissues in general, the scleral collagen fibril diameter distribution at birth is unimodal. Its shape is narrow for altricial animals and is broad for precocial animals (Parry & Craig, 1984). The sclera of humans at birth is not an exception (Schwarz, 1957). The fibrils, which are uniformly small in diameter size early in gestation and continue to grow in diameter throughout gestation, are still relatively small in diameter and have a narrow diameter distribution. With maturity, fibrils become much larger in diameter and more broadly distributed in size. In the mature sclera in monkeys and humans, an increase in the fibril size in diameter from the inner to the outer sclera in the posterior region has been reported (Funata & Tokoro, 1990; Spitznas, 1971). Since, as mentioned earlier, such regional differences are not seen in gestation, the inside-to-outside

gradient in the collagen fibril diameter across the sclera must take place later in development, presumably during visually guided emmetropization.

Collagen synthesis in normal developing tree shrews after eye opening through early adulthood (v.e. days 15 to 110) has been studied indirectly by measuring the expression of mRNA for type I and III collagen (Zorn, Hernandez, Norton, Yang & Ye, 1992). The results suggest that the type I mRNA expression was the highest in the 1 days v.e. animals (90% labeled fibroblasts), dropped to half that number by 45 days v.e., and to 0 by 75 days v.e. indicating that type I collagen, which is the primary collagen type in fibrous sclera, might be synthesized in greater amounts during the first few weeks of development after eye opening while the axial length is increasing rapidly. It is not clear, however, whether type I collagen synthesis or accumulation actually follows this time course, as mRNA expression may not directly relate to synthesis, accumulation and/or degradation. It also is not known whether type I collagen synthesis represents an increase in diameter of existing fibrils or synthesis of new fibrils resulting in the greater number of smaller fibrils. Type III collagen mRNA expression was low near eye opening and 0 by 45 days v.e.

In addition, an antero-posterior gradient in type I and III collagen mRNA expression was found in the 15 and 45 days v.e. animals with the highest expression seen in the posterior pole region. The antero-posterior gradient of mRNA for collagen types I and III agrees with the anterior to posterior direction of development of sclera in monkeys (Ozanics et al., 1976)

and humans (Sellheyer & Spitznas, 1988). This suggests that, as in monkeys and humans, the sclera in tree shrews might develop in anterior to posterior direction, and that this development might continue after eye opening, at least for a few weeks.

Proteoglycans and Glycosaminoglycans (GAGS)

Proteoglycans are macromolecules composed of a core protein to which side chains of glycosaminoglycans (GAGS) and oligosaccarides are attached. The glycosaminoglycan side chains of proteoglycans can number from one to over one hundred (Ruoslahti, 1988). An important functional feature of the GAGS is related to their capacity to occupy a large volume and their capacity to interact with other macromolecules through ionic interactions. As a result, proteoglycans are highly hydrophilic and have a tendency to expand. Proteoglycans are believed to play important roles in controlling the level of hydration in the sclera and in occupying space among collagen fibrils, perhaps keeping them apart and controlling lateral growth of fibrils.

Proteoglycans and glycosaminoglycans have been implicated in collagen fibril formation and growth (Borcherding, Blacik, Sitting, Bizzell, Breen & Weinstein, 1975; Poole, 1986; Ruggeri & Benazzo, 1984). In connective tissues in general, it has been found that certain GAGS are associated with collagen fibrils of certain sizes. For example, higher concentrations of hyaluronic acid have been associated with small diameter fibrils, whereas dermatan sulfate has been associated with larger fibrils. Another common GAG in human sclera, chondroitin sulfate, has been associated with the fibrils of intermediate

size in diameter. It is postulated that GAGS affect lateral growth of fibrils beyond certain diameter (Parry, Flint, Gillard & Craig, 1982).

Developmental studies of sclera in terms of spatial and temporal relationships between proteoglycans and collagen are not available. However, this relationship has been studied in rat tail tendon (Scott, Orford & Hughes, 1981), and results suggest that the relative concentrations of GAGS have an important role in collagen fibrillogenesis and that a temporal relationship between the two exists early in development.

Biomechanical Properties of Sclera

Strength of collagenous tissue in general is related to its thickness, collagen fibril size and organization, and its composition of proteoglycans and glycosaminoglycans (Parry & Craig, 1984). The sclera can be viewed as a tissue of proteoglycan gel with a tendency to expand that is held together under tension or compression by collagen fibril reinforcement. It is this relationship between collagen and proteoglycans that gives the sclera its viscoelastic property, being both able to resist the tensile and compressive forces. Tensile strength of any material is defined as the strength to resist the stress applied along the major axis. In practical terms applied to connective tissues, tensile strength means the ultimate strength before breakage occurs.

As in connective tissues in general, in the sclera the collagen fibrils are thought to provide the tensile strength and the proteoglycans to provide the compressive strength. The tensile strength of a collagenous tissue such as a tendon is related to the mass-average diameter of the component fibrils. It is

postulated that the density of stable covalent cross links occurring laterally between molecules within a microfibril increases with fibril diameter, and thus the larger diameter fibrils provide more tensile strength than smaller fibrils (Parry & Craig, 1984).

Creep refers to elongation under constant force. The smaller collagen fibrils are thought to provide greater creep-resistant properties than the larger diameter fibrils since higher proportion of smaller diameter fibrils would have greater surface area per unit volume of collagen allowing for greater interactions between fibrils and proteoglycans and hydrated GAGS (Parry & Craig, 1984). Thus, fibril diameter and distribution in the sclera might be important not only in dictating the tensile strength but also in providing creep-resistance.

Since the human scleral collagen fibrils become larger in diameter and broader in distribution from birth to maturity (see above, Schwarz, 1957), one would expect the human sclera to grow stronger with age. Indeed, Avetisov et al. (1984) have shown that human sclera elongates less given a fixed stress as a function of age. More importantly, they also have shown that the thinner myopic sclera elongates compared to emmetropic sclera. An important question, in both form-deprivation-induced myopia and human myopic eyes with excess elongation, is whether the scleral fibril morphology is related to the myopia.

The organization of the collagen fibrils is also important in determining the biomechanical properties and it is believed that collagen fibril distribution

pattern is related to the level of stress encountered by that tissue (Parry & Craig, 1984). In the sclera, collagen fibrils are organized in bundles (or lamellae) which vary in thickness and width, forming complex branching patterns (Komai & Ushiki, 1991). The lamellae appear to be organized to meet the biomechanical needs of the sclera, such that the orientation of the bundles varies so as to provide the sclera with the most mechanical strength around the high stress regions such as the limbus (cornea-sclera border), the extraocular muscle attachment sites, and the insertion region of the optic nerve. In other areas, the fibril bundles run primarily in anteroposterior direction.

Effects of Visual Deprivation on Choroid and Sclera

Effects of Visual Deprivation on Choroid

Besides the obvious vascular and possible mechanical roles mentioned earlier, the role of choroid in either mammals or birds in emmetropization mechanism is not clear because there have been no detailed histological or biochemical studies addressing this question. In chicks, as mentioned before, there is evidence that the choroid might be affected in form deprivation, and appears to have an important role in emmetropization mechanism.

Wallman et al. has suggested that choroid might function as an intermediate emmetropization mechanism, compared to the faster accommodative system and the slower axial elongation control. Contraction or expansion of the choroidal thickness could move the retina forward or backward, toward the focal plane of the optics of the eye. Indeed, as

mentioned earlier, the choroid is thinner in deprived myopic chicks, and thickens rapidly in recovery. Thus, the choroid may have a direct role, at least in chicks, in the emmetropization mechanism.

There is evidence that in adult humans the choroid in myopic eyes might be thinner compared to emmetropic or hyperopic eyes (Cheng, Singh, Kwong, Xiong, Woods & Brady, 1992), suggesting that the choroidal thickness change might be a constant feature of myopia in humans. In tree shrews, it is not known whether the choroid thickness is affected in deprivation-induced myopic eyes or in eyes recovering from induced myopia.

Effects of Visual Deprivation on Sclera

In mammals, the sclera in deprivation-induced myopic animal eyes or "spontaneously" myopic human eyes is thinner (Cheng et al., 1992; Curtin, Iwamoto & Renaldo, 1979; Curtin & Teng, 1958; Funata & Tokoro, 1990; Kang & Norton, 1993; Wiesel & Raviolar, 1977). Avetisov et al. (1984) found the human myopic sclera to be, in addition to being thinner, lower in collagen content and biomechanically weaker than the sclera from emmetropic eyes. Norton, Rada, and Hassel (1992) found that, in tree shrews, the glycosaminoglycan and collagen content in the sclera from deprivation-induced myopic eyes were reduced compared to normal sclera (collagen reduction was significant only in the posterior pole region). These findings are consistent with the hypothesis in the model of emmetropization (Fig. 4) that extracellular matrix accumulation might be reduced in form-deprived eyes, perhaps resulting in lower scleral resistance.

In chicks, the overall scleral thickness does not appear to change in the deprivation-induced myopic eyes (Gottlieb, Joshi & Nickla, 1990; Pickett-Seltner, Sivak & Pasternak, 1988). This is because the thinning in the fibrous layer is compensated by the thickening of the cartilaginous layer. In the sclera from deprivation-induced myopic eyes, the dry weight is greater, and the protein and DNA content is higher (Christensen & Wallman, 1991). Also, there is increased synthesis and accumulation of aggrecan, a cartilage proteoglycan (Rada, Thoft & Hassel, 1991). Thus, in chicks, the fibrous layer might be affected similarly as in mammals, but since the cartilaginous layer is thicker than the fibrous layer, axial elongation in deprivation-induced myopic eyes appears to be due to scleral growth primarily in cartilaginous layer (which does not exist in mammals).

An important question is whether, given what appears to be different scleral changes in mammals and birds, the emmetropization process might also be different. Based on the finding that cartilage proteoglycans and link proteins are found in bovine fibrous sclera (Poole et al., 1982), Wallman (1993) suggests that the differences between the two types of sclera are quantitative, the cartilage containing larger amount of proteoglycans compared to smaller amount in fibrous sclera.

Microscopic examination of the myopic sclera from humans shows not only marked thinning but also a reduction in collagen fibril size in diameter (Curtin et al., 1979; Curtin & Teng, 1958). In monkeys, in addition to the smaller fibril diameter size in the sclera from deprivation-induced myopic eyes,

fibrils are found to be uniformly small in diameter from inner to outer sclera, lacking the small to large fibril size gradient from inner to outer sclera found in the sclera from normal eyes (Funata & Tokoro, 1990). These findings suggest that changes in collagen fibrillogenesis as well as regional restructuring might occur in the sclera in myopic eyes.

In tree shrews there is indirect evidence that affecting normal collagen fibrillogenesis might have an effect on the ocular elongation and myopia development. Use of a lathyritic agent, beta-aminoproprionitrile, which prevents cross-linking of newly formed collagen, caused a marked increase in the axial elongation and scleral thinning in the deprivation-induced myopic eyes compared to the deprived eyes without lathyritic treatment (McBrien & Norton, 1988). Interestingly, the non-deprived eyes in lathyritic treated animals did not differ from normal control eyes, suggesting that visual deprivation somehow caused the sclera to be susceptible to the lathyritic agent, but that the drug itself did not affect normal ocular growth. Also, the same lathyritic agent treatment in chicks did not cause an increase in ocular elongation or scleral thinning in deprivation-induced myopic eyes. The reason for such selective effects of lathyritic agent is not clear at this time.

Summary

How does the eye regulate its refractive status? The above evidence suggests that the visual environment is important, that there is an emmetropization mechanism to coordinate the focal length and the axial length, that this mechanism is a direct and spatially local process within the

eye, and that the choroid and the sclera appear to have critical roles. There are three possible ways to examine the choroid and sclera: 1) morphological analysis for possible structural changes, 2) biomechanical analysis for what the effects of possible extracellular matrix changes might be in scleral resistance and 3) biochemical analysis to examine how changes in extracellular matrix synthesis and/or accumulation account for the morphological and biochemical changes. The purpose of the dissertation research was to examine the morphology and structure of the choroid and sclera in normal and deprivation-induced myopic tree shrew eyes. This provides basic data that can guide further biomechanical and biochemical studies. The choroid and sclera, and other ocular tissues, were examined morphologically at both the light and electron microscopic levels.

The specific aims of proposed dissertation research were: 1) To examine the structure of the choroid during normal development and in eyes with a deprivation-induced myopia or in eyes recovering from an induced myopia. In particular, how do choroidal thickness and cross sectional area develop and are they changed in deprived and in recovering eyes? 2) To examine the structure of the sclera during normal development. In particular, how does the scleral thickness and volume develop and, at the ultrastructural level, how does the fibrillar morphology and organization develop? The results provide an overview of the time course of extracellular matrix accumulation and collagen fibril development and how they might be related to scleral thickness and

volume changes. 3) To examine the scleral changes in eyes with a deprivation-induced myopia and in eyes recovering from an induced myopia. Is the sclera thinner in the deprived eyes and thicker in recovering eyes? Does the fibrillar size and organization change? Are there changes in other aspects of the sclera? These measures help us to understand how scleral extracellular matrix accumulation might be changed in the deprived, myopic eye and in recovering eyes, possibly affecting scleral resistance to IOP.

METHODS

Subjects

The subjects in this dissertation research were tree shrews (Tupaia belangeri). Tree shrews were raised on a 10/14 hour light/dark cycle in a colony maintained by the laboratory. Tree shrews are altricial and it is normally about 19 days after birth before the eyes open. The day of eye opening is designated as visual exposure (v.e.) day 1. The subjects were matched for days of v.e., and no more than one animal from the same litter were included in any single group. In deprived animals, only one eye was deprived, the other eye serving as within-animal control. Each group was balanced for whether the right or the left eye is deprived and for the sex of the animal.

Experimental Design

To address the specific aims listed at the end of Introduction, morphological measures were made at two levels, light and electron microscopic (EM). At the light microscopic level, choroidal structure in terms of its thickness and cross sectional area were examined. For the sclera, its structure in terms of its location (or position, an indicator of vitreous chamber depth or axial length), thickness, and cross sectional area (an estimate of volume) were examined. At the EM level, the sclera was examined for

lamellar organization, collagen fibril diameter, packing density and the ratio of fibril to non-fibrillar space as an indicator of collagen to proteoglycan ratio. EM evaluations were done at two scleral locations: the temporal equatorial region and near the posterior pole. Also, at each location the sclera was divided into inner, middle and outer third regions and evaluated separately. All of these measures were made on a series of normal animals, on a group of monocularly deprived animals, and on a group of animals recovering from monocular deprivation-induced myopia.

Animals

There were three groups of animals (Table 1): 1) normal animals at 5 different ages from eye opening through near puberty, 2) deprived animals age matched to the 45 days v.e. group of normal animals. 3) animals recovering from a deprivation-induced myopia, also age matched to the 75 days v.e. group of normal animals. There were 4 animals in each group. In normal animals, only one eye from each animal was examined. In deprived and in recovery group animals, both the treated and the control eyes were evaluated.

Normal Group

For the normal animals, the five age groups were v.e. days 1, 15, 45, 75, and 110 with four animals each. This range of v.e. days includes the period when the axial elongation rate is the highest (between v.e. days 1 and 15) and the period when the tree shrews' eyes are most susceptible to deprivation-induced myopia (between v.e. days 15 and 45). By v.e. day 110, the tree shrew eyes have achieved over 97% of their adult axial length and have slowed their

Table 1. Number of Animals and Eyes at the Light and EM Level Analysis.

Group		N(animal)	Number of Eyes	
			Light Level	EM Level
Normal	1	4	4	2
(age in v.e.)	15	4	4	2
	45	4	4	2
	75	4	4	2
	110	4	4	2

Deprived (24-45 day deprivation)		Treated Eyes	Control Eyes	Treated Eyes	Control Eyes
1) Whole Visual Field	4	4	4	4	4
2) Partial Visual Field	6	6	6	0	0
Recovery (24-45 day deprivation; 45-75 day recovery)	4	4	4	4	4

growth rate significantly and the animals are approaching sexual maturity. Previous studies have shown that in tree shrews there are no significant interocular differences in either axial length or refraction (indeed, the dimensions of the two eyes are highly correlated) (McBrien & Norton, 1992), and thus only one eye from each animal was used for morphological measures. All the eyes were evaluated at the light level, but for EM analysis, only two eyes per age group will be evaluated because of the labor intensiveness of EM analysis (total of 20 eyes for the light level and 10 eyes for the EM level analysis) (Table 1).

Deprivation-induced Myopic Groups

Whole Visual Field Deprived Group

There were four animals in this group that received deprivation with a translucent diffuser over one eye for 21 days from v.e. days 24 to 45. The 45 days of v.e. at the end of deprivation matches the 45 day group of normal developing animals. In tree shrews, 21 days of deprivation has been shown to produce reliable and significant axial elongation and myopia. Both the deprived and the control eyes were included for the light and the EM analysis (total of 8 eyes at the light level and 8 eyes at the EM level).

Partial Visual Field Deprived Group

This was a group of 6 animals, derived from another study in the laboratory (Norton & Siegwart, 1991) that were deprived with a translucent diffuser over one eye that covered only half of the visual field. This group was further divided into two groups: the nasal-field and the temporal-field deprived

groups with three animals each. The duration of deprivation was 21 days from v.e. 24 to 45 days. The 45 days of v.e. at the end of deprivation matches the 45 day group of normal developing animals. Both the deprived and the fellow control eyes will be evaluated at the light level, but EM analysis was not done on these animals (total of 12 eyes at the light level).

This group was included primarily to demonstrate that the fixation and histological preparation procedures of the eyes did not adversely affect the components of the eyes in terms of their structure or position. Could the local ocular axial elongation observed physiologically (A-scan ultrasound) in these animals be demonstrated morphologically in fixed and plastic embedded eyes?

Recovery From Deprivation-induced Myopia Group

There were four animals in this group that were monocularly deprived with a whole visual field translucent diffuser for 21 days from v.e. days 24 to 45 (same as the whole eye deprived group) and then allowed to "recover" from deprivation-induced axial elongation and myopia by removing the goggle and providing normal vision for 30 days from v.e. days 45 to 75. Both eyes were examined at both the light and the EM levels (total of 8 eyes for both the light and EM level analysis).

Procedure

Animal and Tissue Preparation

Immediately before all animals were sacrificed for histological preparation of the eyes, A-scan ultrasound measurement of the components of the eyes was made following the procedures of Marsh-Tootle and Norton (1989)

and Norton and McBrien (1992) (Table 2). This provided an *in vitro* measure of anterior segment depth, lens thickness, vitreous chamber depth and (summing these three) the axial length in each eye. During the A-scan measurement, the animals were anesthetized with Ketamine/Rompun mixture with Halothane supplement as needed and aligned with a bite bar so that ocular measurements could be made at the proper eye position. Ophthalmic atropine sulfate eye drops (administered >1 hour in advance) were used for cycloplegia, and excess mucus secretion was prevented with i.p. atropine sulfate. Animals were sacrificed under deep nembutal anesthesia, perfused with buffered glutaraldehyde, and the eyes enucleated for histological preparation.

The animals in the deprived and recovery groups also had an A-scan measurement before deprivation began to ensure their eyes were similar before treatment. In addition, the recovery group animals had a third measurement after the deprivation period, but before the start of the recovery period. The full pre-sacrifice ocular measurements for the deprived and the recovery groups included subjective refraction with a streak retinoscope and hand held trial lenses as well as ultrasound measures. Ophthalmoscopic examination of the fundus was also carried out to look for any possible ocular abnormalities. After the pre-treatment measurement at 24 days v.e., the animals in the deprived and recovery groups had dental-acrylic pedestal affixed to their skull following a procedure of Siegwart & Norton (submitted for publication). A goggle frame with a monocular translucent diffuser for deprivation was then

Table 2. Refractive and A-scan Measurement Schedule.

Group	A-scan Only	Refractive and A-scan
	(age in v.e. when measured)	
Normal		
(age in v.e.) 1	1	
15	15	
45	45	
75	75	
110	110	
Deprived		
(24-45 day deprivation)		
1) Whole Visual Field	24	45
2) Partial Visual Field	24	45
Recovery		
(24-45 day deprivation; 45-75 day recovery)		
	24	45 and 75

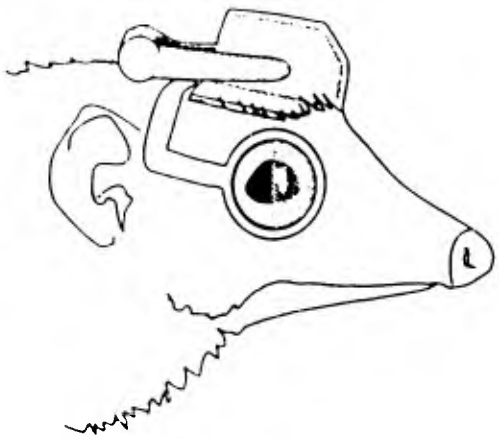
attached to the pedestal (Figure 5). This setup allowed for easy removal of the occluder for occasional cleaning. The use of the goggles did not cause any problems to the animal and produced reliable axial elongation and myopia in the deprived eyes.

Plastic Embedding and Sectioning

The fixed enucleated eyes were cleaned of extraocular tissues and the optic nerve trimmed so that only about 2 mm of it was left. A small square hole was made through the sclera and the retina on the ventral side of the eye for alignment purpose during plastic embedding later. This hole also allowed for better penetration of the internal structures by the plastic embedding media. The eyes were then washed of fixative with phosphate buffered solution, osmicated with 0.5% osmium tetroxide, dehydrated with a series of alcohol and propylene oxide. A standard solution of Spurr from Polysciences was used for embedding. Spurr is a common plastic embedding medium in electron microscopy (Spurr, 1969) and the process of tissue preparation for EM is also well established. During the process of washing, dehydrating, and embedding the eyes were put in a slowly rotating wheel. The eye was embedded in a plastic mold large enough to hold the whole eye, aligned, and then the Spurr hardened in the oven at 70 °C.

Once the Spurr has hardened, the dorsal third of the embedded eye was cut away with a jeweler's saw. This eliminated the need to section through the dorsal portion of the eye to get to the middle portion. The eye was then sectioned through the center in the horizontal plane in 2.5 μm thick sections

Figure 5. A diagram of a tree shrew wearing a goggle. The tree shrew is shown with a translucent diffuser covering only the nasal half of the visual field. The goggle is attached to the dental-acrylic pedestal affixed to the skull. The goggle frame made of aluminum is attached to a clip which can be quickly removed for cleaning or repair and replaced in the same position. A wide range of lenses including translucent diffusers and contact lenses with power can be used in the goggle. (From Siegwart and Norton, 1994, in press, reproduced with permission).



with Ralph glass knives (22 mm wide and 7.4 mm thick) on a LKB microtome. Each serial section was saved in individual wells in 96 well tissue culture plate (Spurr is hydrophobic and sections can be stored for later use), and every 10 to 20 sections a section was mounted on a glass slide. Each section was mounted flat on the slide and heated (60 °C) over night on a hot plate to prevent detachment of the section from the slide. Because the lens in the eye did not embed well and, if left in the block, the crystallin material ruined the glass knife easily, the lens nucleus was removed from the embedded eye with a surgical probe. Careful notes were taken during the cutting process to keep track of the location in the eye. The embedded eyes were cut past the level of the optic nerve and slightly past the horizontal equator. The remaining portion of the eye in the block was then prepared for electron microscopic examination.

Light Level Measurement Preparation

The slide mounted sections from the equatorial region of each eye was traced on paper at 25 X magnification using a microprojector (Baush and Lomb). The horizontal equator was located by using cornea/iris junctions as reference points for alignment, and by measuring sections serially for the section with the largest distance between the cornea/iris junctions. A section from near this equator was then selected for measurement. Sections were selected to avoid such irregularities as blood vessels and nerves in the sclera, and to find equivalent sections from both eyes in the same animal or across animals. Typically, equivalent sections were obtained within 50 microns in

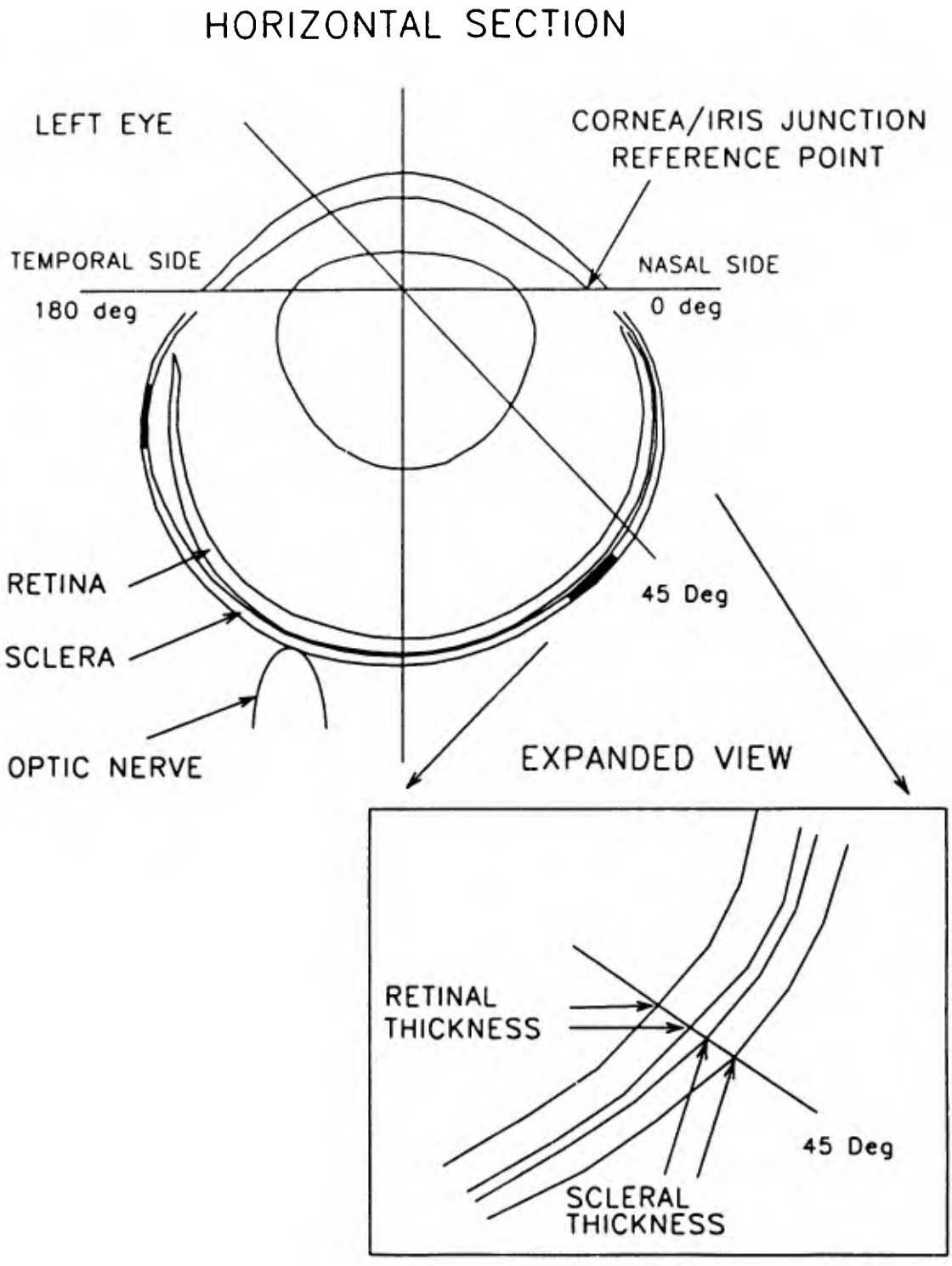
either direction from the equator. This system of alignment and selection of sections for measurements worked well, and has proven to be reliable. The other landmarks in the eye such as the optic nerve or the lens did not work as well. The optic nerve in tree shrews is located in the superior temporal quadrant above the posterior pole and thus its location changed unevenly with axial elongation. Also, the lens was sometimes found to be slightly displaced inferiorly in the eye after fixation and embedding procedures.

EM Level Measurement Preparation

Two locations in each eye were examined by electron microscopy. Preliminary results at the light level indicated that during normal development the scleral thickness changed the least in the equatorial region and changed the most near the extraocular muscle attachment site. Preliminary data also indicated that the changes in the scleral thickness in deprivation-induced myopic eyes may be greatest near the extraocular muscle attachment site compared to the control eyes. Thus, measuring from the nasal to the temporal side, a 10° wide sections from near the posterior pole (from 50° to 60°, just behind the extraocular muscle attachment site) and from equatorial region from temporal side (150° to 160°) (Figure 6) were cut out of the embedded eyes and prepared for EM procedures. The nasal posterior pole region was selected to avoid the optic nerve on the temporal side.

EM preparation involved trimming of the cutout sections, sectioning of tissue (about 120 nm in thickness) with a diamond knife, mounting the section on an EM grid, and staining with lead citrate and uranyl acetate. The EM

Figure 6. Plot of a digitized tracing of an eye. A tracing of an eye was aligned with cornea/iris junctions as reference points and the midpoint between the junctions was taken as the central reference point. From this central point, lines were drawn at 5° intervals around the globe, as the line at 45° demonstrates. The eye was digitized from nasal (0°) to temporal (180°) side at 5° intervals for the cornea, lens, retina, choroid, and sclera. The expanded box shows how the thickness measures for the retina and the sclera, for example, were made. The blackened areas in the sclera show the locations (10° windows) where sections for the EM analysis were taken from nasal posterior and temporal equatorial regions.


HORIZONTAL SECTION
LEFT EYE
CORNEA/IRIS JUNCTION
REFERENCE POINT
TEMPORAL SIDE
NASAL SIDE
180 deg
0 deg
RETINA
SCLERA
OPTIC NERVE
45 Deg
EXPANDED VIEW
RETINAL
THICKNESS
45 Deg
SCLERAL
THICKNESS

sections, like the Ralph glass knife sections, were in the horizontal plane. The location of the EM samples, relative to the light level tracing of the whole eye, was known to within about 100 μm. EM preparation was done mostly by the EM technician at the UAB Cancer Electron Microscope Center, but the operation of EM for selection of sites within the choroid and sclera for photographing was done personally (EM, Phillips model 301).

Once the section was in the EM, an area was selected subjectively for the uniformness of staining and for lack of holes, tears, or other abnormalities, i.e., the best area was selected. Then, at that location a serial photographs were then taken from inner choroid to outer sclera at 1900 X magnification. The 1900 X is the minimum magnification available on the Phillips EM and when printed at 3 X (5700 X final magnification, 10x12 inches in photomicrograph size) provided the largest field of study while providing enough details of choriocapillaris and collagen fibril bundle organization. A montage of choroid and sclera was then made from these serial photomicrographs and used to analyze the choroidal development and collagen fibril bundle organization during normal development and for any possible changes in the deprivation-induced eyes or in the eyes from animals recovering from deprivation-induced myopia.

Within the montage area, the sclera was divided into inner, middle, and outer third regions, and within each region an area of best cross section of collagen fibrils was selected and photographed at 25000 X magnification. These were printed at 75000 X (10x12 inches in size) for later analysis on a

digitizing tablet. Preliminary data indicated that the fibril diameter in tree shrew sclera range from about 20 to 300 nm, and 75000 X magnification provided enough resolution for the measurement of the smallest fibers while still providing enough field of study to have enough number of the largest fibers.

Measurement and Analysis Procedures

Light Level

Measurements were made on a digitizing tablet (Summagraphics Bitpad, resolution 500 lpi) using Sigma Scan (Jandel) software installed on a 386/25 Mhz computer. With this set up, distance and cross sectional measurements were made directly from the tracings of the sectioned eyes. Data in ASCII format were transferred to Lotus 123 spreadsheet software and analyzed.

The histological procedures outlined earlier for preparation of the eyes did not affect the ocular components significantly. The sclera, for example, did not collapse or deform so that even partial axial elongation in partial visual field deprived animal can be demonstrated morphologically at the light level (see below). However, on some eyes the cornea was occasionally found collapsed and/or the retina/choroid was found detached from the sclera. In such cases, location and thickness measures were not possible, but this was a rare problem and did not affect the data analysis significantly.

To ensure that each eye was measured at the same locations around the globe, the tracings of sectioned eyes were aligned using the cornea/iris junctions as reference points. After alignment, midpoint between cornea/iris

junctions was located as the central reference point and lines drawn at 5° intervals around the globe. All measurements were made from the nasal to temporal side of the eye by tracing all the around the each tissue. Figure 6 shows a plot of a such digitized tracing of an eye, and the expanded box demonstrates how the distances from the central reference point to front and back of the retina, choroid, and sclera were measured along one of the measurement lines.

Three primary measures at the light level were 1) the location of the sclera, 2) the choroidal and scleral thickness and 3) the choroidal and scleral cross sectional area.

Scleral Location

Location was defined as the distance from the reference point to the front of the sclera and was measured as an indicator of the vitreous chamber depth or the axial length to see if the excessive ocular elongation in deprived eyes morphologically. Scleral location was measured along the lines drawn at 5° intervals around the globe, as described above, from 10° (nasal side) to 170° (temporal side). The outer 10° regions were excluded because the sclera is continuous with the ciliary body and cornea anteriorly and no longer morphologically distinct beyond this point.

Choroidal Thickness and Choroidal Cross Sectional Area

Thickness was defined as the difference in distance between the front and back surfaces of the choroid. Choroid was often detached from the sclera in the periphery making precise measurements difficult. Thus, the thickness

measures were made only in the posterior region from nasal 45° to temporal 135°. Also, because of the location of central reference point, thickness measures, especially near the equator, were corrected for the obliqueness of the measurement lines. A mathematical correction was made so that thickness measurements were made normal to the surfaces as shown in Figure 7.

Cross sectional area, defined as the total area of the choroid from nasal 45° to temporal 135°, was measured by tracing all the way around the perimeter on the digitizing tablet. Cross sectional area gave an estimate of the volume of the choroid.

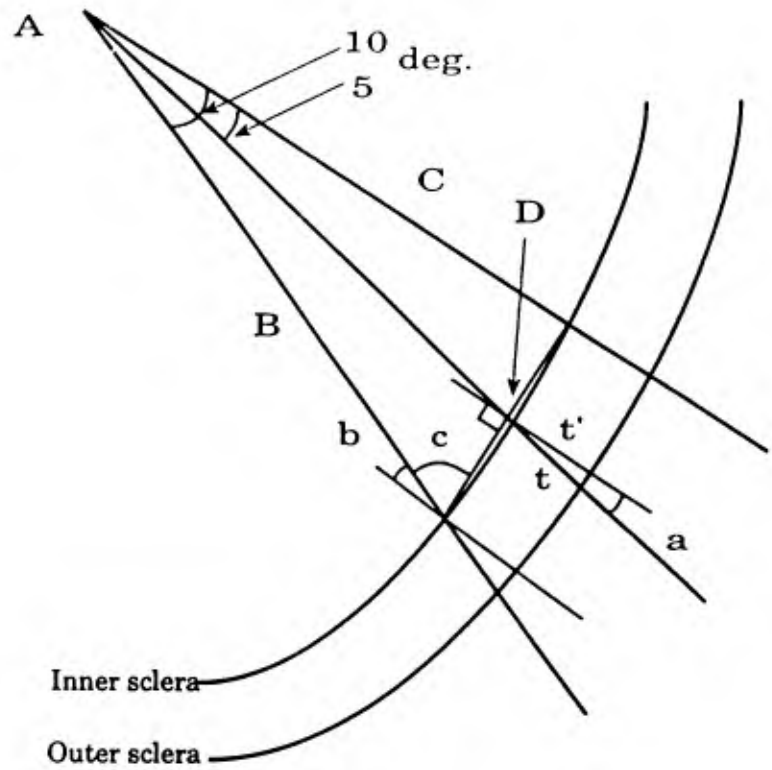
Scleral Thickness and Scleral Cross Sectional Area

Scleral thickness was defined as the difference in distance between the front and back surfaces of the sclera. The scleral thickness was measured from nasal 10° to temporal 160°. As in the choroidal thickness measures, mathematical correction for obliqueness of the measurement lines were also made for scleral thickness. Cross sectional area, defined as the total area of the sclera from nasal 10° to temporal 170°, was measured the same way as the choroidal cross sectional area by tracing all the way around the perimeter of sclera on the digitizing tablet. These measures gave an estimate of the volume of the sclera.

EM Level

At the EM level the following questions of the specific aims listed earlier were addressed: 1) How does the sclera develop normally? Collagen fibrils were measured in terms of a) lamellar organization, b) number of fibrils per

Figure 7. Schematic diagram illustrating the method of correction for oblique measurement lines. Because the measurement lines were not always normal to the front surface of the tissue being measured, a mathematical correction was made to obtain the thickness along the line normal to the front surface. Normal angle to the front surface of the sclera at each measurement line is obtained using two adjacent measurement lines, and since the angles between the measurement lines and the distances to the front surface are known, trigonometric equations provide the angle between the measurement line and the line normal to the front surface. For most of the posterior measurement lines, this correction does not make a significant difference.



A = central reference point
B, C= distances from A to inner sclera for two lines
D = distance between two lines at inner sclera
a, b, c = angles as noted

1) t = uncorrected scleral thickness
t' = t x cos a, corrected scleral thickness

2) to find angle a: a = b - 5
b = 90 - c

3) to find angle c: sin 10 / D = sin c / C
c = arcsin (sin 10 x (C / D))

4) to find distance D: D = (B x B) + (C x C) - 2 x B x C x cos10

given area of sclera, c) fibril diameter, d) the distance to the nearest neighbor as an indicator of spacing, and e) ratio of total cross sectional area of fibrils and non-fibril area as an indicator of collagen-proteoglycan relationship. 2) How is the sclera changed in the deprived or recovering eyes? The same measures for normal scleral development were made on these eyes also. In particular, if the sclera was indeed thinner and was reduced in extracellular matrix in the deprivation-induced myopic eyes compared to the control eyes, as preliminary data from light level analysis indicated, how are these changes reflected at the ultrastructural level?

For the analysis of collagen fibril morphology, a digitizing tablet with a cordless pen (CalComp Drawing Board II, resolution 1000 lpi), a 486/33 Mhz computer, and Bioquant System IV morphometric software was used. Using this system, collagen fibril diameter, number of fibrils, cross sectional areas of each fibril, and geometric centers of each fibrils were measured. During the measurement, the identity of photomicrographs was kept unknown. Lotus 123 spreadsheet software was used to analyze the data.

Lamellar Organization

The sclera is primarily composed of interwoven lamellae of collagen fibrils and fibroblasts which make the extracellular matrix in the sclera. As the sclera develops normally, it is important to assess how the lamellae across the thickness of the sclera changes in terms of its thickness and number. If, as suggested earlier, the sclera is formed adjacent to the choroid, it is possible that more lamellae will be formed as the animals age resulting in greater

number of lamellae. It is also possible, however, that the lamellae increases in thickness rather than in number as the fibrils inside the lamellae mature and the sclera becomes thicker with age. Thus, the number and thickness of lamellae and the fibroblasts which might indicate the level of extracellular matrix production level were measured across the thickness of the sclera in the EM montages in the normal eyes. The same measurement were also made in the treated and control eyes of the deprived and recovery groups to see if and how the lamellar organization might be affected in these treated eyes. These measurement were made in the montages from the near posterior region (50° to 60°) as the light level analysis indicated that the scleral thickness changes were primarily in the posterior region and not in the equatorial region.

To make the above measurements, perpendicular lines (mostly 3 but some 2) were drawn evenly spaced across the full thickness of the sclera from the choroid to the outer surface. Along these lines, the distances that were inside a lamellae, in fibroblast processes or cell bodies, or in "space" were measured. Because of the thickness of the sclera and the magnification used to generated the montages (1900 X, the lowest available on the scope), occasionally the wire mesh of the grid on which EM samples were mounted could not be totally avoided and in such cases blocked a portion of the sclera. In the few cases where this happened, the problem was minimized by making measurements on multiple measurement lines.

Number of Collagen Fibrils

The number of fibrils per given area of sclera was measured using the 75000 X photomicrographs. Within each photomicrograph, a 12x12 cm area was selected where collagen fibrils have been cut nearly in cross section, as judged from their appearance in the section, and measured. The fibrils are believed to be long spherical rods of even diameter (Chapman & Hulmes, 1984; Parry & Craig, 1984). The 12x12 cm area was selected because the collagen fibril lamellae in tree shrew sclera are thin, even at 75000 X, and, as a result, a region in which the fibrils have been cut in cross sections rarely fill entire 10x12 inch photomicrograph. This 12x12 cm area was small enough to find a measurable area of cross sections in most photomicrographs and yet large enough to contain about a hundred fibrils. However, in some eyes the fibril lamellae were so thin, especially in the inner third scleral region, that even this 12x12 measurement area was too large. Unfortunately, the fibrils were also small in diameter in the inner third region so that reducing magnification did not make things better. In these cases, a smaller area for measurement, the size of the area depending on the each photomicrograph, was used to maximize the number of fibril measured. However, even the smallest measurement area contained more than 50 fibrils and reliable measurements were thus possible.

The use of 12x12 cm measurement area introduces the problem known as the edge effect in stereology (Gundersen, 1977). To obtain an unbiased counting of fibrils, in addition to the fibrils inside the measurement area, only

the fibrils touching the top and the right borders were included. The fibrils touching the bottom or the left borders or the corners of left/top or bottom/right borders were not counted (Gundersen, 1977).

Collagen Fibril Diameter

Fibril diameter measurements were made in the same 12x12 cm measurement area on the same fibrils that were counted for the number of fibrils measurement by defining the minimum diameter subjectively on the digitizing tablet using the Bioquant software. Using the minimum diameter allowed measurement of fibrils even when they are not cut in perfect cross sections (not all fibrils run in same directions within a lamellae). Although only scleral regions that appear to be cross sections of fibrils were selected for photomicrographs, this selection was subjective. To correct for this variation, in addition to the minimum diameter, maximum diameter was also measured on each fibril and used to calculate the possible deviations from true cross section. The average of ratios between minimum and maximum diameters for each fibril indicates the degree of deviation from true cross section and correction can be easily (function of cosine) made to the measurement area. This was a minor problem as most photomicrographs contained fibrils appearing spherical. This correction was made for the nearest neighbor analysis. The minimum diameter and cross sectional area measurements determined as the ratio between fibrils and background were not affected by this problem.

Nearest Neighbor Analysis

To learn whether fibrils change their distance from other fibrils during development, a nearest neighbor analysis was used. As a by-product of the cross sectional area measures, geometric centers of each fibril were also obtained. These were then used to calculate the nearest neighbor distance for the fibrils. In the nearest neighbor analysis, the borders of measurement area created a different problem in that for the fibrils lying near the borders, analysis can only be made to the fibrils that are closer to the center of the measurement area and not to the fibrils that might be outside, creating perhaps a biased result. A smaller area (10x10 cm) was defined within the 12x12 cm measurement area so that only the nearest neighbor data from the fibrils inside this smaller area were used in analysis.

Cross Sectional Area of Fibrils Relative to Non-fibrillar Area

Cross sectional area measurements were made on the same 12x12 cm measurement area by tracing around each fibril on the digitizing tablet. For the fibrils lying on the borders of measurement area, only the portion of the fibril that is inside the measurement lines were traced. By this method, the ratio of total collagen cross sectional area to the measurement area were not affected by the selective inclusion or exclusion of fibrils lying on the borders. The total area of the 12x12 cm regions is, of course, also known and by dividing the sum of all collagen fibril areas into the total area gives the fraction of the section that is occupied by collagen fibrils. On the assumption that the region not occupied by the collagen fibrils is occupied by the

proteoglycans, this gives at least a rough estimate of collagen/proteoglycan ratio.

Data Analysis and Statistics

To examine whether changes occur in normal eyes as a function of age, an analysis of variance (ANOVA) was used with pair-wise comparisons to determine whether individual age groups differ from each other. To examine whether deprived eyes differ from control eyes and whether recovering eyes differ from their fellow control eyes, a dependent t-test was used. Assessment of whether deprived, control or recovering eyes differ from normal eyes was made with an independent t-test, with a Bonferroni correction for multiple comparisons where appropriate.

In the case of the EM data, the 2-way ANOVA was used across the age groups to examine whether regional differences in two scleral locations (posterior pole and temporal equatorial) or in three regions (inner, middle, and outer sclera) at each scleral location develop as a function of age. Deprived eye/control eye and recovering eye/control eye differences were assessed with a dependent t-test and independent t-test with a Bonferroni correction for multiple comparisons where appropriate.

RESULTS

Morphometric and Refractive Measurements

The data presented here are from the A-scan and retinoscopy measures, taken before the animals were sacrificed, in the 5 normal developing groups, the whole visual field deprived, and the recovery from induced myopia groups (Table 1, Methods). The data from the partial visual field deprived group will not be discussed here as these animal were obtained from a previous study (Norton & Siegwart, 1991) in the laboratory for the primary purpose of morphological analysis at the light level and thus will be discussed later.

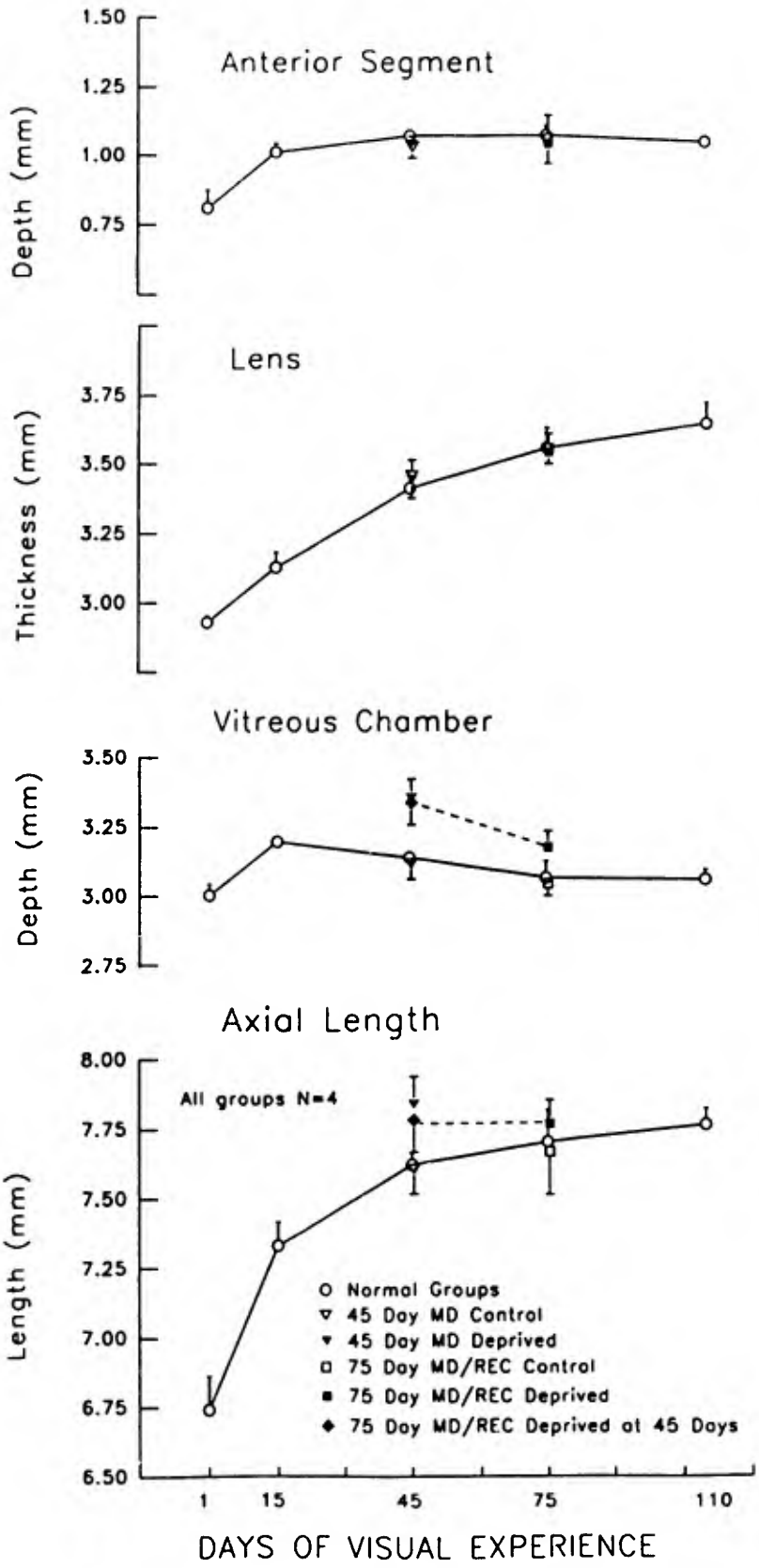
The results of A-scan ultrasound and refractive measurements for the normal groups are summarized in Table 3. The results of A-scan ultrasound measurements are summarized in Figure 8. Figure 8 shows the normal developing pattern of the anterior segment depth, lens thickness, vitreous chamber depth, and the axial length in the 5 normal groups. As expected (Norton & McBrien, 1992), the axial length was seen to increase rapidly between 1 and 45 days v.e. and to slow its rate of increase after 45 days v.e. The vitreous chamber depth also increased initially between 1 and 15 days v.e., but was seen to decrease gradually afterwards. This decrease was due to the continuing increase in the lens thickness with age. Overall, the pattern of ocular development of the 5 normal groups in this study agree with other

Table 3. Axial Dimensions of All Eyes in Normal Developing Animals.

		1 DAY (n=4)		15 DAY (n=4)		45 DAY (n=4)		75 DAY (n=4)		110 DAY (n=4)	
		RE	LE	RE	LE	RE	LE	RE	LE	RE	LE
Anterior Segment (mm)	MEAN	0.81	0.81	1.01	1.01	1.06	1.06	1.06	1.07	1.05	1.4
	S.D.	0.07	0.06	0.04	0.03	<0.01	0.01	0.03	0.05	0.04	0.02
Lens Thickness (mm)	MEAN	2.92	2.94	3.13	3.12	3.41	3.41	3.56	3.57	3.66	3.65
	S.D.	0.04	0.02	0.04	0.07	0.04	0.06	0.06	0.09	0.06	0.07
Vitreous Chamber Depth (mm)	MEAN	2.99	3.01	3.19	3.20	3.13	3.15	3.07	3.06	3.06	3.06
	S.D.	0.06	0.06	0.02	0.01	0.03	0.02	0.07	0.05	0.04	0.04
Axial Length (mm)	MEAN	6.72	6.76	7.33	7.33	7.60	7.63	7.71	7.69	7.77	7.75
	S.D.	0.15	0.12	0.09	0.09	0.04	0.05	0.12	0.11	0.03	0.10
Equatorial Dimension (mm)	MEAN	7.76	7.73	8.33	8.21	8.52	8.43	8.66	8.63	8.70	8.81
	S.D.	0.03	<0.01	0.13	0.06	0.02	0.03	0.17	0.21	0.01	0.03
Eye Weight * (g)	MEAN	0.21	0.21	0.26	0.26	0.29	0.30	0.30	0.31	0.31	0.32
	S.D.	<0.01	0.02	0.02	0.01	0.01	0.01	<0.01	0.02	<0.01	<0.01

* n=2, measurements from 1 eye per animal, 2 RE and 2 LE.

Figure 8. Summary of A-scan ultrasound measurement of ocular dimensions. Dimensions of the anterior segment, lens, vitreous chamber, and axial length for all the animals in the normal, deprived, and recovery groups are plotted as a function of age in days of visual experience (v.e.). In the normal animals, the axial length increased rapidly between the 1 and 15 days v.e. and slowed its rate after 45 days of v.e. The vitreous chamber depth also increased between 1 and 15 days v.e., but because of the increase in the lens thickness, it decreased gradually afterwards. In the deprived group (45 Day MD), after 21 days of monocular deprivation, there were no changes, in both the control and the deprived eyes, in the anterior segment or the lens thickness compared to the normal animals. In the deprived eyes, the vitreous chamber depth and the axial length were increased when compared to the control eyes. The control eyes in the deprived and the recovery groups were not increased in either the vitreous chamber depth or the axial length. In the recovery group (75 Day MD/Rec), after 30 days of normal vision, the differences in the vitreous chamber depth and the axial length between the control and the deprived eyes were reduced when compared to the differences in the deprived group. The axial length in the recovering eye changed very little during the recovery period. Error bars indicate standard deviations.


Anterior Segment
Depth (mm)
1.50
1.25
1.00
0.75
Lens
Thickness (mm)
3.75
3.50
3.25
3.00
Vitreous Chamber
Depth (mm)
3.50
3.25
3.00
2.75
Axial Length
Length (mm)
8.00
7.75
7.50
7.25
7.00
6.75
6.50
All groups N=4
Normal Groups
45 Day MD Control
45 Day MD Deprived
75 Day MD/REC Control
75 Day MD/REC Deprived
75 Day MD/REC Deprived at 45 Days
1
15
45
75
110
DAYS OF VISUAL EXPERIENCE

published results in tree shrews (Norton and McBrien, 1992) and will not be discussed further.

Figure 8 also shows the results from the whole eye deprived group (21 days of deprivation, 24-45 days). The results of the A-scan and refractive measures in the deprived and the recovery groups are summarized in Table 4. As expected, Figure 8 shows that the anterior segment depth and the lens thickness were not significantly affected by the deprivation. In the deprived eyes, the axial length was elongated, mean +/- sd, when compared to the fellow control eyes (0.23 +/- 0.02 mm, t test, $p < .05$). This increase in the axial length in the deprived eyes was primarily due to the increase in the vitreous chamber depth (0.23 +/- 0.04 mm, t test, $p < .05$), and caused the myopic refractive change in the deprived eyes.

In the recovery group, also shown in Figure 8, as in the deprived group, the recovery eyes were, after the same 21 days of deprivation, axially elongated (0.21 +/- 0.03 mm, t test, $p < .05$) when compared to the control eyes. After the recovery period (30 days of normal vision), the axial length in the recovery eyes was no longer significantly elongated (0.10 +/- 0.1 mm, t test, $p > .05$) when compared to the control eyes. As can be seen in Figure 8, as in the deprived group, these changes in the axial length were primarily due to the changes in the vitreous chamber depth. In the recovery eyes, during the 30 days of recovery, the axial length increased 0.01 +/- 0.05 mm, but in the control eyes the increases was 0.10 +/- 0.03 mm. These results suggested that the "recovery" in the axial length was primarily achieved by the recovery eyes

Table 4. Ocular Refraction and Axial Dimensions in Deprived and Recovery Animals.

		DEPRIVED GROUP (n=4)				RECOVERY GROUP (n=4)					
		PRE-DEPRIVATION		POST DEPRIVATION		PRE-DEPRIVATION		POST-DEPRIVATION		POST-RECOVERY	
		CONT	DEP	CONT	DEP	CONT	DEP	CONT	DEP	CONT	DEP
Retinoscopy (D)	MEAN	N/D*		9.4	-0.6	N/D*		9.8	0.1	9.1	5.9
	S.D.			1.7	4.6			2.8	2.7	0.5	2.0
Anterior Segment	MEAN	1.06	1.06	1.03	1.04	1.06	1.06	1.07	1.06	1.06	1.04
(mm)	S.D.	0.01	0.02	0.04	0.03	0.06	0.08	0.10	0.08	0.09	0.07
Lens Thickness (mm)	MEAN	3.23	3.28	3.46	3.44	3.19	3.19	3.41	3.38	3.56	3.55
	S.D.	0.05	0.07	0.06	0.07	0.07	0.07	0.06	0.07	0.06	0.04
Vitreous Chamber	MEAN	3.20	3.19	3.12	3.35	3.16	3.15	3.09	3.33	3.04	3.17
Depth (mm)	S.D.	0.04	0.03	0.06	0.07	0.04	0.03	0.04	0.08	0.04	0.06
Axial Length	MEAN	7.49	7.52	7.60	7.84	7.41	7.40	7.57	7.78	7.67	7.77
(mm)	S.D.	0.04	0.06	0.09	0.10	0.13	0.14	0.12	0.12	0.15	0.09
Equatorial Dimensions	MEAN	N/D		8.58	8.74	N/D		N/D		8.65	8.66
(mm)	S.D.			0.13	0.13					0.15	0.17
Eye Weight	MEAN	N/D		0.29	0.31	N/D		N/D		0.30	0.31
(g)	S.D.			0.01	0.01					0.01	0.01

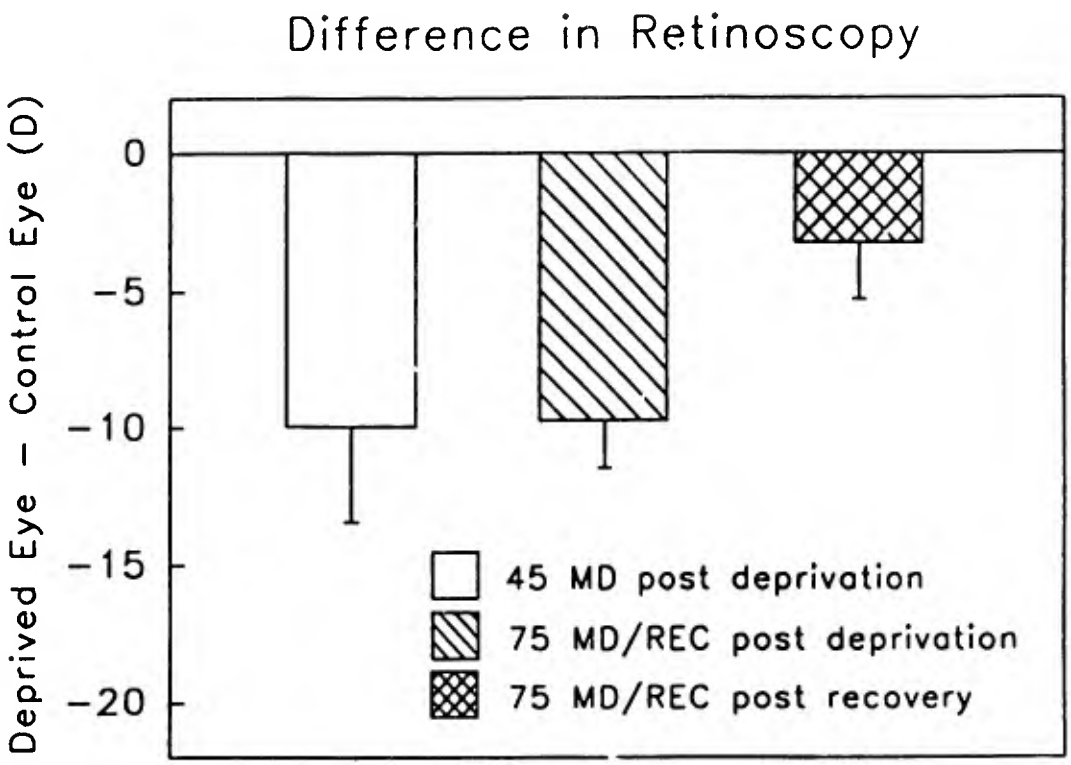
* N/D - not done

holding their axial length constant. The control eyes meanwhile continue elongating through their normal development. These findings of vitreous chamber depth and axial length changes in the deprivation-induced myopic eyes and in the eyes recovering from induced-myopia agree with other published results (McBrien & Norton, 1992).

Retinoscopy measurements, taken at the same time of the A-scan ultrasound (Table 2 in the Methods), are summarized in Figure 9. As can be seen, refractive changes matched the axial length changes as measured by the A-scan ultrasound. In the deprived group, the axially elongated deprived eyes were myopic relative to the control eyes (mean difference in refraction, -9.9 +/- 3.5 D). In the recovery group, the amount of myopia in the recovery eyes after deprivation was similar to the deprived group (as would be expected based on the similar axial elongation) (-9.7 +/- 1.7 D). After recovery, however, the recovery eyes were less myopic (-3.2 +/-2.0 D) when compared to the control eyes.

These physiological results suggested that the treatments applied to the treated eyes in the experimental groups achieved the intended result of axial length changes. The results also showed that the control eyes in both the deprived and the recovery groups were indeed normal in development pattern as measured by these methods. These results were compared to the morphological measurements.

Figure 9. Summary of refraction in the deprived and the recovery groups. Differences in refraction between the deprived and the control eyes for the deprived group and the recovery group are plotted. After 21 days of monocular deprivation, the deprived eyes in the deprived group were -9.9 D myopic compared to the control eyes. Similarly, after the same 21 days of monocular deprivation, and before 30 days of normal vision, the deprived eyes in the recovery group were -9.7 D myopic compared to the control eyes. After recovery, the differences were much reduced (-3.2 D).


Difference in Retinoscopy
Deprived Eye − Control Eye (D)
0
−5
−10
−15
−20
45 MD post deprivation
75 MD/REC post deprivation
75 MD/REC post recovery

Light Level Analysis

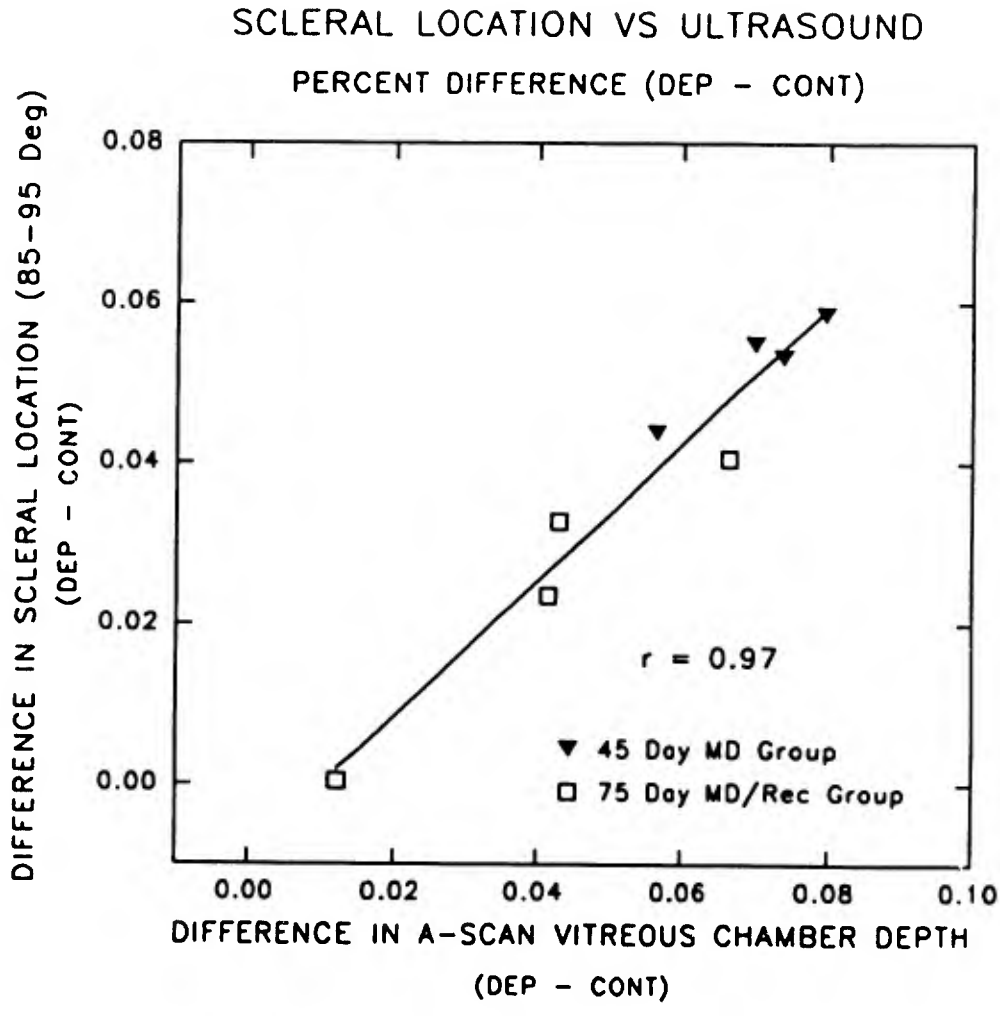
The reported data are derived from 48 eyes that were embedded in Spurr (Table 1 in the Methods). There were 20 eyes from 5 normal developing groups (1 eye per animal, 4 animals per group). There were also 20 eyes from deprived groups: 8 eyes from the whole visual field deprived group (both eyes, 4 animals) and 12 eyes from half visual field deprived animals (both eyes, 6 animals). In addition, there were 8 eyes from the recovery group (both eyes, 4 animals). After these eyes had been fixed, dehydrated, embedded, sectioned, as described in the Methods, measurements were made of scleral location, choroidal thickness, choroidal cross sectional area, scleral thickness, and scleral cross sectional area.

Scleral Location

As defined earlier, the scleral location was measured from the central reference point between the lateral and medial corneal/iris junctions to the front of the sclera. As explained earlier, it was not possible to measure the vitreous chamber depth directly in the Spurr embedded eyes. The scleral location measures were used as an indicator of vitreous chamber depth or the axial length.

Figure 10 shows the correlation between the scleral location and the A-scan vitreous chamber depth in percent difference between the deprived and the control eyes of the whole visual field deprived and the recovery groups. For this comparison, since the A-Scan was measured on-axis to the posterior pole, only the scleral location measures from the same posterior pole region

Figure 10. Correlation between the scleral location and the A-scan ultrasound measurements. Differences between the deprived and the control eyes, in percent, in the morphometric measurement of the scleral location are compared with the differences in the A-scan vitreous chamber depth in the same eyes for the deprived and the recovery groups. The results from the morphometric and ultrasound measurements were highly correlated, suggesting that the procedures used in eye preparation did not affect the structures of the eye significantly.


SCLERAL LOCATION VS ULTRASOUND
PERCENT DIFFERENCE (DEP - CONT)
DIFFERENCE IN SCLERAL LOCATION (85-95 Deg)
(DEP - CONT)
0.08
0.06
0.04
0.02
0.00
r = 0.97
45 Day MD Group
75 Day MD/Rec Group
0.00
0.02
0.04
0.06
0.08
0.10
DIFFERENCE IN A-SCAN VITREOUS CHAMBER DEPTH
(DEP - CONT)

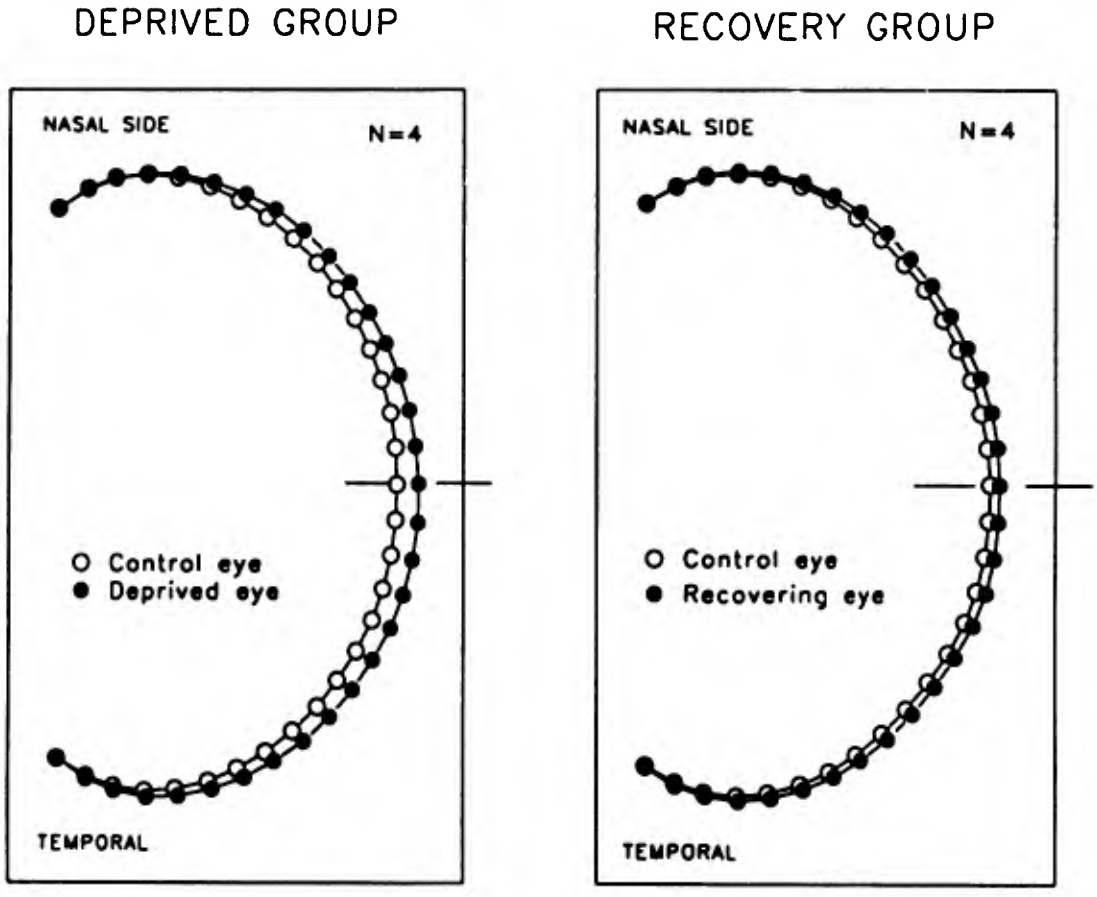
(85° to 95°) was used. As can be seen, these two measures were highly correlated ($r = 0.97$) suggesting that the scleral location as defined here was a good indicator of the changes in the vitreous chamber depth. Indeed, the high correlation suggests a minimum of changes induced by tissue processing.

In the deprived group, the scleral location in the deprived eyes was increased 5.3 +/- 0.6% when compared to the control eyes (t test, $p < .05$), suggesting that the sclera in these eyes were displaced posteriorly. Figure 11 shows the scleral location measurements in two of the animals in the deprived group. As can be seen, the scleral location measures clearly show the posterior displacement in the deprived eyes compared to the control eyes. In the recovery group, the scleral location in the recovering eyes was still increased but the differences were not significant (2.4 +/- 1.7%, t test, $p > .05$) when compared to the control eyes. These results provided morphological evidence that, in tree shrews, the sclera is displaced posteriorly in the deprivation-induced myopic eyes and that this posterior displacement is reduced in the recovering eyes allowing the recovery from induced myopia to occur.

In the partial visual field deprived group, the deprived eyes were deprived in only half of the visual field in the treated eyes. A-scan ultrasound and retinoscopy measurements were done off-axis into the deprived and the nondeprived half of the treated eyes and compared to the equivalent part in the fellow control eye. A significant myopia and axial elongation only in the deprived half in the treated eyes when compared to the equivalent half in the control eye was the result (Norton & Siegwart, 1991).

Figure 11. Effect of deprivation on the scleral location. The distances from the central reference point, mid-point between the cornea/iris junctions, to the front surfaces of the sclera were measured as an indicator of scleral position. After 21 days of deprivation, the sclera in the deprived eyes were displaced posteriorly compared to the control eyes. After 30 days of recovery, the differences between the recovering and the control eyes were reduced.

SCLERAL LOCATION



The scleral location measures from both the treated and the control eyes from the partial deprived animals were made. The scleral location was measured "off-axis" in both the nasal (65° to 75°) and temporal (105° to 115°) regions. The scleral location was found increased only in the deprived half of the treated eyes when compared to the equivalent half in the control eyes (2.5 +/- 0.5%, t test, $p < .05$). The non-deprived half in the treated eyes was not significantly different when compared to the equivalent half in the control eyes (-0.4 +/- 0.6%, t test, $p > .05$).

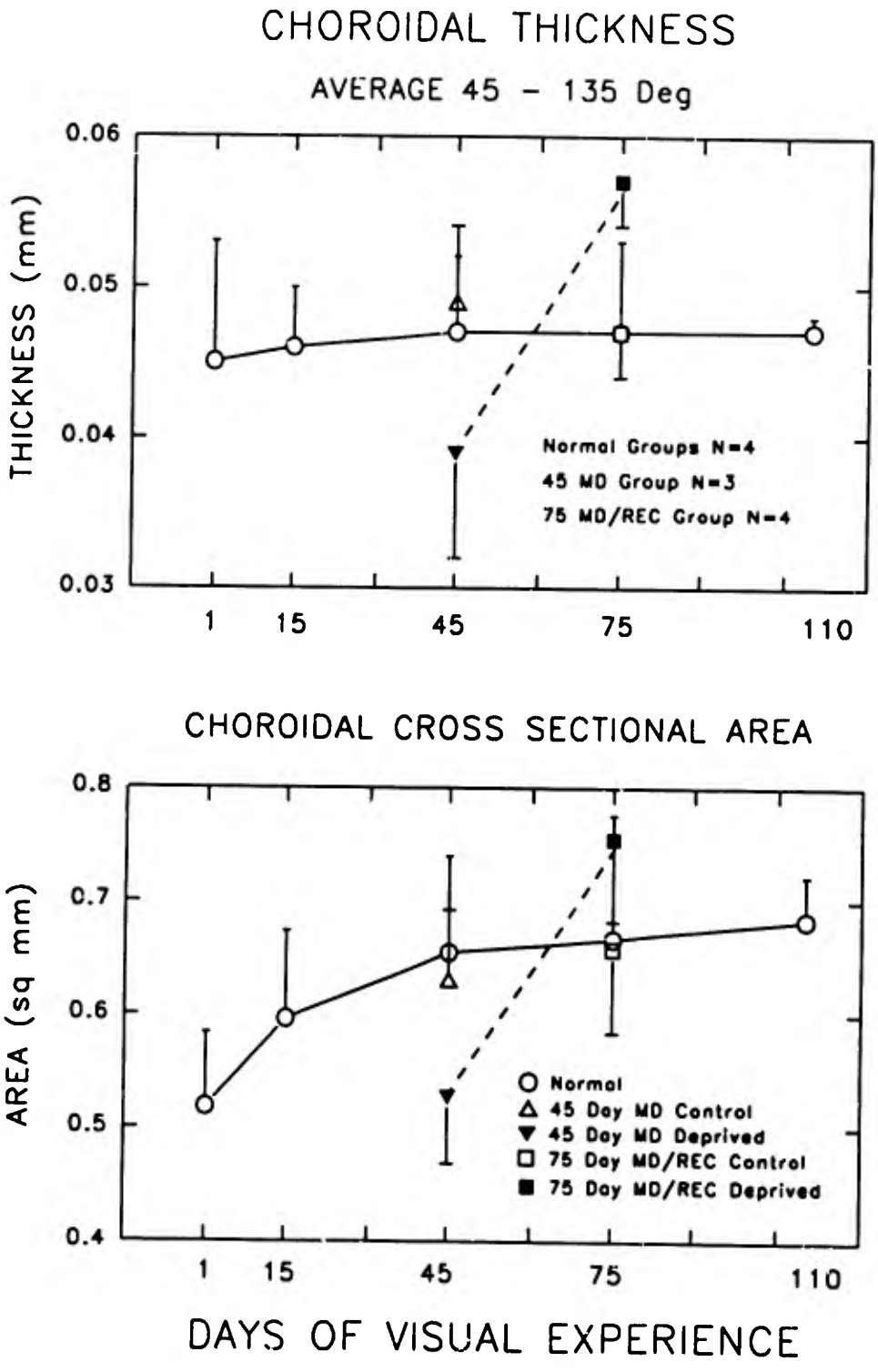
The above results clearly suggested that the process of fixation and embedding of the eye in Spurr did not significantly affect its structures so that even the local differences in the partial visual field deprived animals could be demonstrated morphologically.

Choroidal Thickness and Cross Sectional Area

Despite the difficulties in measuring a vascular tissue like the choroid in fixed and Spurr embedded eyes as discussed earlier, choroidal measurements of thickness and cross sectional area were possible on all the embedded eyes with the exception of two eyes from one animal in the whole visual field deprived group (retinal detachment during fixation).

The choroidal thickness and cross sectional results are summarized in Figure 12. In the normal developing animals, the choroidal thickness did not appeared to change much with age. In the deprived group ("45 day MD"), however, the choroid in the deprived eyes was thinner, although not significantly (-18.5 +/- 19.8%, t test, $p > .05$), when compared to the control

Figure 12. Summary of the development of choroidal thickness and choroidal cross sectional area. Average of the choroidal thickness measurements (top) from nasal 45° to temporal 135° is plotted as a function of age in the normal, deprived and recovery groups. The choroidal thickness changed very little in the normal animals with increasing age. In both the deprived and the recovery groups, the differences between the deprived and the control eyes were not significant (t test, $p > .05$). The choroid in the recovering eyes were, however, thicker (46.2%) when compared to the deprived eyes in the deprived group (dashed line) (independent t test, $p < .05$). The choroidal cross sectional area (bottom) data are plotted in the same way as the choroidal thickness. In the normal eyes, the choroidal cross sectional area (an estimate of volume) increased gradually. This is because, although the choroidal thickness remained constant, the eyes were increasing in size so there was more choroidal volume in the larger eyes of older animals. As would be expected, the changes in the deprived eyes compared to the control eyes were similar to the changes seen in choroidal thickness. Data suggest that the changes in the thickness were not due simply to the changes in the eye size (see text for explanation).


CHOROIDAL THICKNESS
AVERAGE 45 - 135 Deg
THICKNESS (mm)
0.06
0.05
0.04
0.03
1
15
45
75
110
Normal Groups N=4
45 MD Group N=3
75 MD/REC Group N=4
CHOROIDAL CROSS SECTIONAL AREA
AREA (sq mm)
0.8
0.7
0.6
0.5
0.4
1
15
45
75
110
Normal
45 Day MD Control
45 Day MD Deprived
75 Day MD/REC Control
75 Day MD/REC Deprived
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eyes. In the recovery group ("75 day MD/Rec"), the choroid in the recovering eyes was significantly thicker when compared to the control eyes (19.5 +/- 10.8%, t test, $p < .05$). In both the deprived and the recovery groups, the control eyes were similar to the age matched 45 and 75 day normal animals, respectively. The most obvious and dramatic change was the significant increase in the choroid thickness in the recovering eyes when compared to the deprived eyes in the 45 day MD group (46.2%, independent t test, $p < .05$), suggesting that the choroidal thickness increased a great deal during the recovery period.

Because the choroid is rather thin (about 45μm, Figure 12) in the embedded eyes and the variability in thickness is large, the A-scan ultrasound measures were reexamined to see if similar antecedent could be obtained. The wave forms generated at the back of the eye by the A-scan ultrasound is rather complex, but enough detail is present to differentiate the peaks into what might be the back of the retina peak and the front of the sclera peak. By measuring the distance between these two peaks, one can reasonably guess at the "choroidal" thickness from the A-scan ultrasound wave forms. In the deprived group, in the pre-deprivation measurement, the "choroid" in the deprived eyes was similar in thickness to the control eyes (0.5 +/- 0.9%, t test, $p > .05$). After deprivation, however, the "choroid" in the deprived was significantly thinner (-10.7 +/- 2.8%, t test, $p < .05$) when compared to the control eyes. In the recovery group, in the pre-deprivation measurement, the "choroid" in the recovery eyes was, as in the deprived group, similar to the

control eyes in "choroid" thickness (1.9 +/- 4.7%, t test, p > .05). After deprivation, however, the "choroid" was significantly thinner (-7.7 +/- 4.6%, t test, p < .05) when compared to the control eyes. After recovery, the "choroid" in the recovery eyes was thicker, but not significantly (3.6 +/- 4.0%, t test, p > .05) when compared to the control eyes. The "choroid" in the recovery eyes was, however, significantly thicker (13.9%, independent t test, p < .05) when compared to the deprived eyes in the deprived group.

Also shown in Figure 12 are the choroidal cross sectional area results. In the normal developing animals, choroidal cross sectional area increased with age. This suggested that the choroid expanded along with the increasing eye size with age, increasing in volume but maintaining a relatively constant thickness. In the deprived group, the cross sectional area was reduced in the deprived eyes, although not significantly (-16.2 +/- 21.7%, t test p > .05), when compared to the control eyes (Figure 12). In the recovery group, the cross sectional area in the recovering eyes was increased, also not significantly (16.7 +/- 21.9%, t test, p > .05) when compared to the control eyes. As in the thickness measures, the most dramatic change in choroidal cross sectional area was in the recovering eyes which showed a 43.3% increase (independent t test, p < .05) when compared to the deprived eyes in the deprived group. As with the choroidal thickness data, the cross sectional areas in the control eyes of both the deprived and recovery groups were not significantly different when compared to the age matched normal animals.

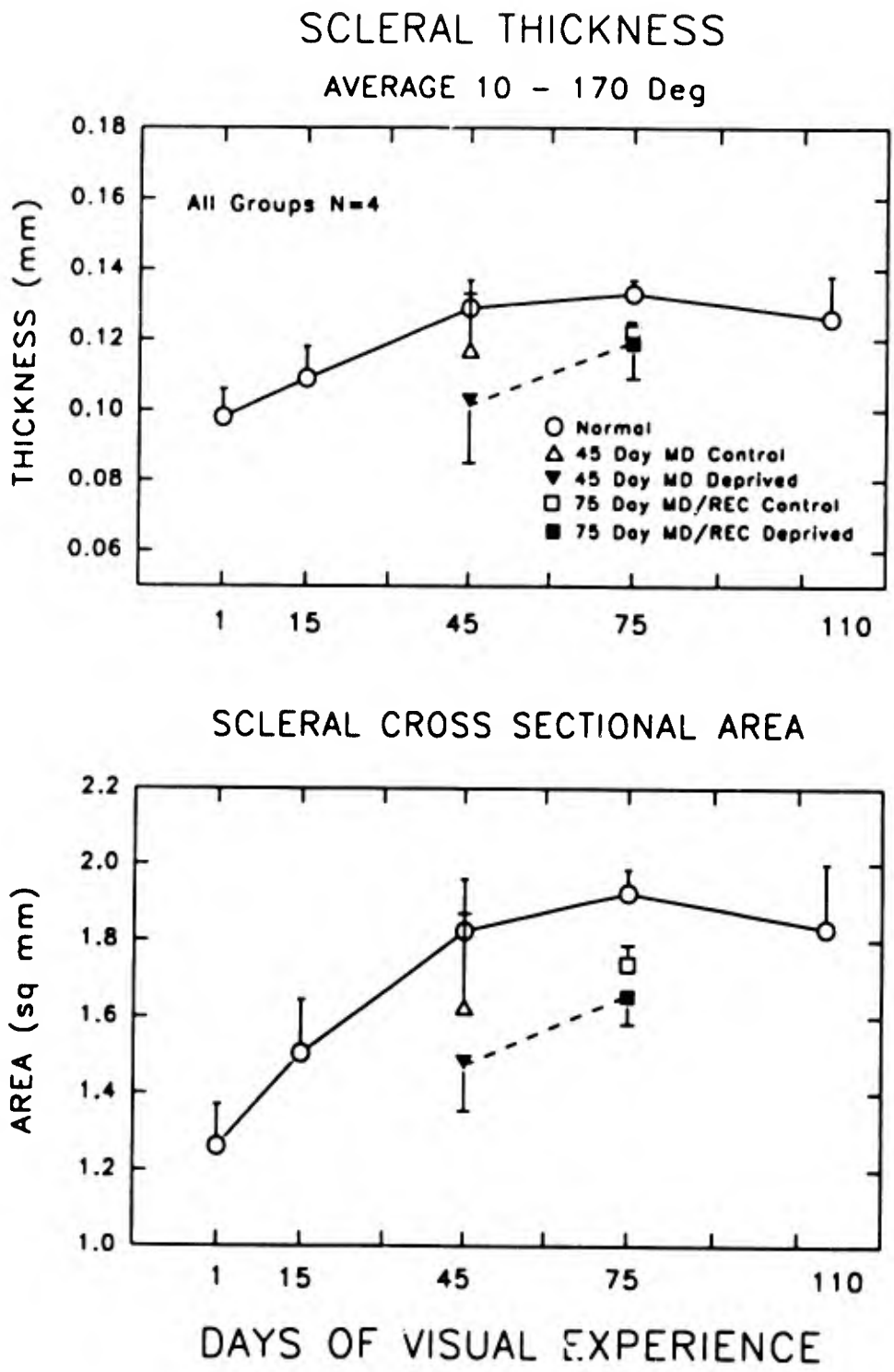
Because the deprived eyes in the whole visual field deprived group were larger (see scleral location) compared to the control eyes, the choroid in the deprived eyes might be thinner as a result of being stretched to cover the larger eye. The choroidal cross sectional area results indicated that such was not the case. The cross sectional area estimates volume and thus suggested that in the deprived eyes there was less choroidal "material" when compared to the control eyes. In addition, the fact that in the recovery group, the recovering eyes which were still larger than the control eyes had thicker choroid also suggested that eye size was not a factor.

As discussed earlier, changes in choroidal thickness has been reported in chick (Wallman et al., 1992). The above results provided the first evidence in tree shrews that changes in the choroid might be a feature of the deprivation induced myopia in a mammal as well. Perhaps more importantly, it is tempting to propose that the dramatic increase in the choroidal thickness seen in the recovering eyes might be pushing the retina forward compensating for the increased vitreous chamber depth. Indeed, recovery of refraction (about 65%) was greater than recovery of vitreous chamber depth (about 50%). Wallman has suggested that such an intermediate recovery system exists in chicks.

Scleral Thickness and Cross Sectional Area

Figure 13 summarizes the scleral thickness and cross sectional area results. Figure 13 shows that in the normal developing groups, the scleral thickness increased with development. It increased rapidly and steadily

Figure 13. Summary of the scleral thickness and scleral cross section area development. Average of the scleral thickness measurements (top) from nasal 10° to temporal 170° is plotted as a function of age in the normal, deprived and recovery groups. In the normal animals, the scleral thickness increased gradually between 1 and 45 days visual experience (v.e.) and changed very little afterwards. In the deprived group, the sclera in the deprived eyes was thinner when compared to the control eyes (t test, $p > .05$). In the recovery group, the sclera in the recovery group was not thinner compared to the control eyes, suggesting recovery in scleral thickness after 30 days of normal vision. The scleral cross sectional area (bottom) data are plotted the same way as in the scleral thickness. In the normal animals, the increase in the scleral cross sectional area is greater than observed in the scleral thickness (top) because the eyes are increasing in size with increasing age. As would be expected, the changes in the deprived and the recovering eyes were similar to the changes seen in the scleral thickness.


SCLERAL THICKNESS
AVERAGE 10 - 170 Deg
THICKNESS (mm)
0.18
0.16
0.14
0.12
0.10
0.08
0.06
All Groups N=4
Normal
45 Day MD Control
45 Day MD Deprived
75 Day MD/REC Control
75 Day MD/REC Deprived
1
15
45
75
110
SCLERAL CROSS SECTIONAL AREA
AREA (sq mm)
2.2
2.0
1.8
1.6
1.4
1.2
1.0
1
15
45
75
110
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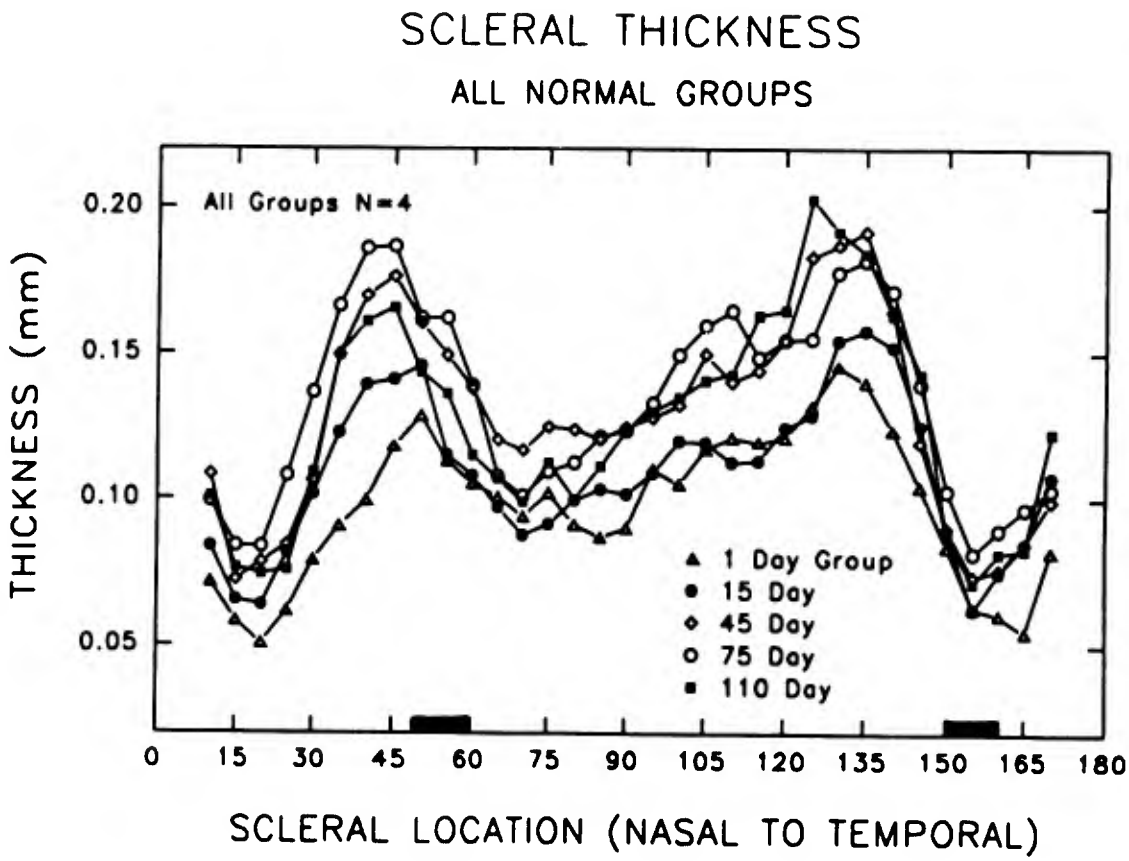
between the 1 and 45 days v.e. and more slowly between the 45 and 75 days v.e. and stayed about the same or perhaps even decreased a little in the 110 days v.e. group. Figure 14 plots the scleral thickness data at 5° intervals across the measurement region for the 5 normal developing groups to show the increase in thickness with increasing age in more detail. The most obvious change observed with increasing age was the increase in regional differences in thickness. The mid-peripheral regions (near nasal 45° and temporal 135°) where the extraocular muscles attach to the sclera increased in thickness more than the posterior region. As a result, the regional differences in thickness which were already observed in the 1 day v.e. group became more pronounced with increasing age. Figure 14 also shows that in the equatorial regions (near 20° nasal and 160° temporal) the scleral thickness changed relatively little compared to other regions.

The results of scleral thickness measures in the deprived and the recovery groups are also summarized in Figure 13. In the deprived group, the sclera was thinner in the deprived eyes when compared to the control eyes (-12.2 +/- 3.0%, t test, $p < .05$). In the recovery group, the scleral thickness in the recovering eyes was also thinner when compared to the control eyes, but the differences were not significant (-2.9 +/- 9.0%, t test, $p > .05$). Interestingly, the sclera in the control eyes in both the deprived and recovery groups were also thinner (-9.3% and -8.3%, respectively) when compared to the age matched 45 and 75 day v.e. normal animals. However, only the difference

Figure 14. Summary of the scleral thickness development in normal animals. The scleral location from nasal (0°) to temporal side (180°) is flattened on the X-Axis. The scleral thickness at each scleral location, at 5° intervals, is plotted for all 5 normal groups. In addition to the increase in overall scleral thickness, the regional differences develop with increasing age.


SCLERAL THICKNESS
ALL NORMAL GROUPS
All Groups N=4
0.20
0.15
0.10
0.05
THICKNESS (mm)
1 Day Group
15 Day
45 Day
75 Day
110 Day
0
15
30
45
60
75
90
105
120
135
150
165
180
SCLERAL LOCATION (NASAL TO TEMPORAL)

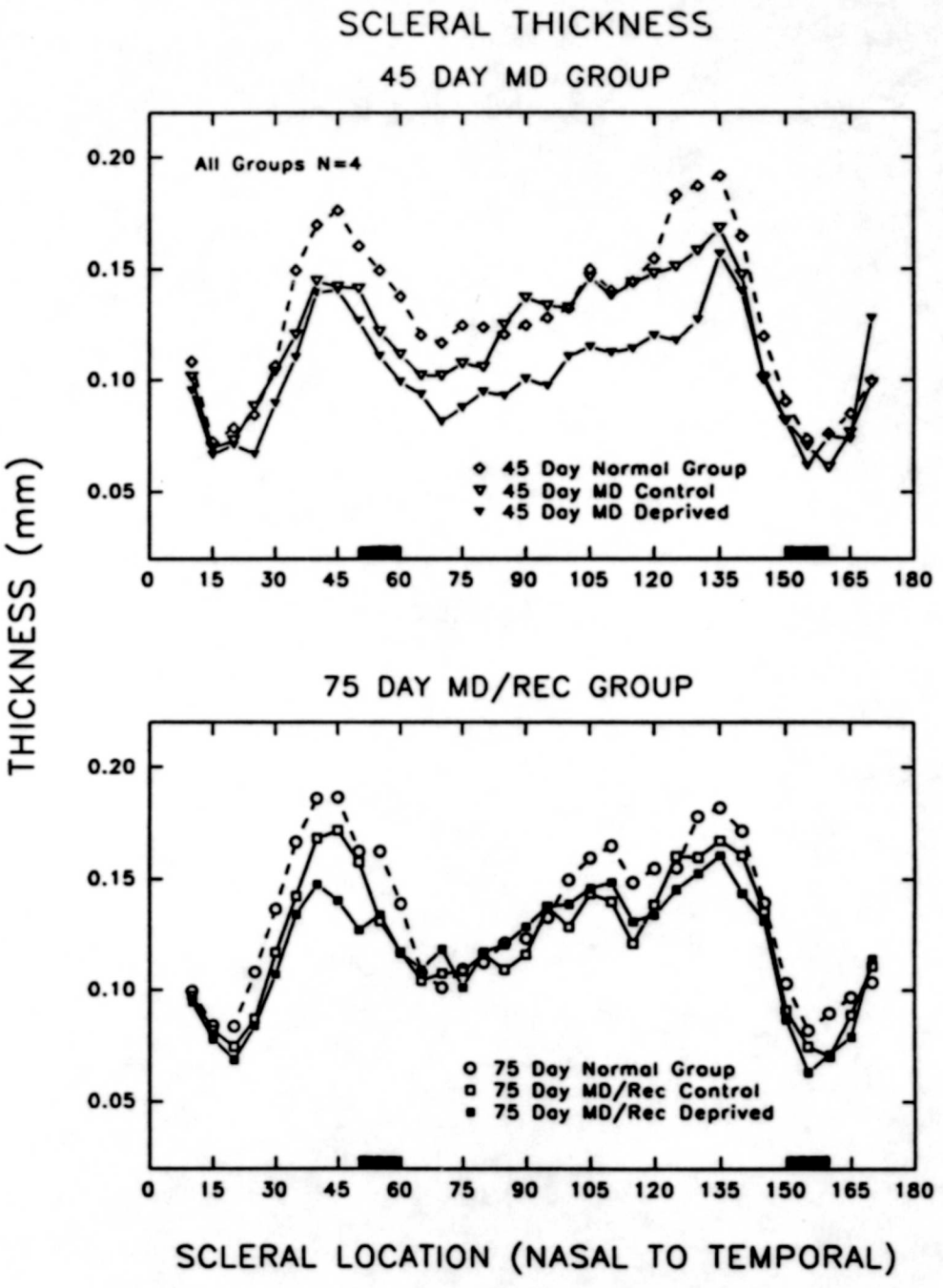
in the recovery control and the 75 day normals were significant (independent t test, $p < .05$).

The results of scleral cross sectional area measures in the deprived and the recovery groups are also summarized in Figure 13. The cross sectional area results were similar to the scleral thickness results. In the deprived group, the cross sectional area in the deprived eyes was reduced (-9.08 +/- 3.0%, t test, $p < .05$) when compared to the control eyes. In the recovery group, the cross sectional area in the recovery eyes was also reduced but the differences were no longer significant (-5.0 +/- 5.7%, t test, $p > .05$).

As pointed out earlier in discussing the choroid thickness, the combined results in the scleral thickness and cross sectional area measures suggested that eye size was not a factor. In the deprived eyes, the sclera was thinner and had less volume, suggesting that there was less scleral extracellular matrix in the deprived eyes compared to the control eyes. These results clearly support the model of emmetropization presented earlier. The model suggested that the sclera in the deprived might have reduced extracellular matrix and thus be biomechanically weaker and less able to resist the intraocular pressure (IOP), resulting in axial elongation and myopia.

Figure 15 summarizes the scleral thickness results in the two treated groups separately and also compares them to the age matched normal animals in more detail. Examined this way, it becomes clear that, in the deprived group, the sclera in the deprived eyes was thinner primarily in the posterior region compared to the control eyes. In comparing the control eyes in the

Figure 15. Summary of scleral thickness development in the deprived and the recovery groups. In the deprived group (top) both the deprived and control eyes are compared with the age matched 45 days visual experience (v.e.) normal eyes. The deprived eyes were thinner primarily in the posterior region when compared to the control eyes. The control eyes were thinner primarily in the two mid-periphery regions when compared to the normal eyes. In the recovery group (bottom), the recovery eyes are no longer thinner in the posterior region when compared either to the control or to the age matched normal eyes. The recovery eyes are, however, still thinner in the mid-periphery regions.


SCLERAL THICKNESS
45 DAY MD GROUP
All Groups N=4
45 Day Normal Group
45 Day MD Control
45 Day MD Deprived
75 DAY MD/REC GROUP
75 Day Normal Group
75 Day MD/Rec Control
75 Day MD/Rec Deprived
THICKNESS (mm)
0.20
0.15
0.10
0.05
0 15 30 45 60 75 90 105 120 135 150 165 180
SCLERAL LOCATION (NASAL TO TEMPORAL)

deprived group to the age matched normal eyes, the most obvious difference seemed to be the difference in thickness in mid-periphery regions. The deprived eyes were thinner in both the mid-periphery and the posterior region when compared to the normal eyes. As a result, it is interesting to note that the deprived eyes (45 days of v.e. at sacrifice) appeared rather similar to the 15 day v.e. normal eyes (see Figure 14, graphs have same scales and are same in size so they can be superimposed and compared) in terms of overall thickness and regional thickness differences. It is tempting to suggest that the scleral development might have been delayed during deprivation.

Also shown in Figure 15 is the scleral thickness results in the recovery group. Figure 15 shows that in the both the recovery and the control eyes appeared similar to the age matched 75 day normal eyes, although the recovery eyes were thinner in the mid-periphery regions compared to the other eyes. It is interesting to note that it appeared as if the control eyes of the recovery group, when compared to the control eyes in the deprived group, also underwent some "recovery" process so that it is thicker in the mid-periphery regions, although still thinner overall than the age matched normal eyes.

The observed changes in scleral thickness and cross sectional area in the control eyes of the deprived and recovery groups were puzzling, specially given the fact that the results from A-scan ultrasound and retinoscopy as well as the scleral location and choroidal thickness and cross sectional measures all failed to show any significant changes in these control eyes. However, this was the first morphological study of sclera measuring the scleral thickness and cross

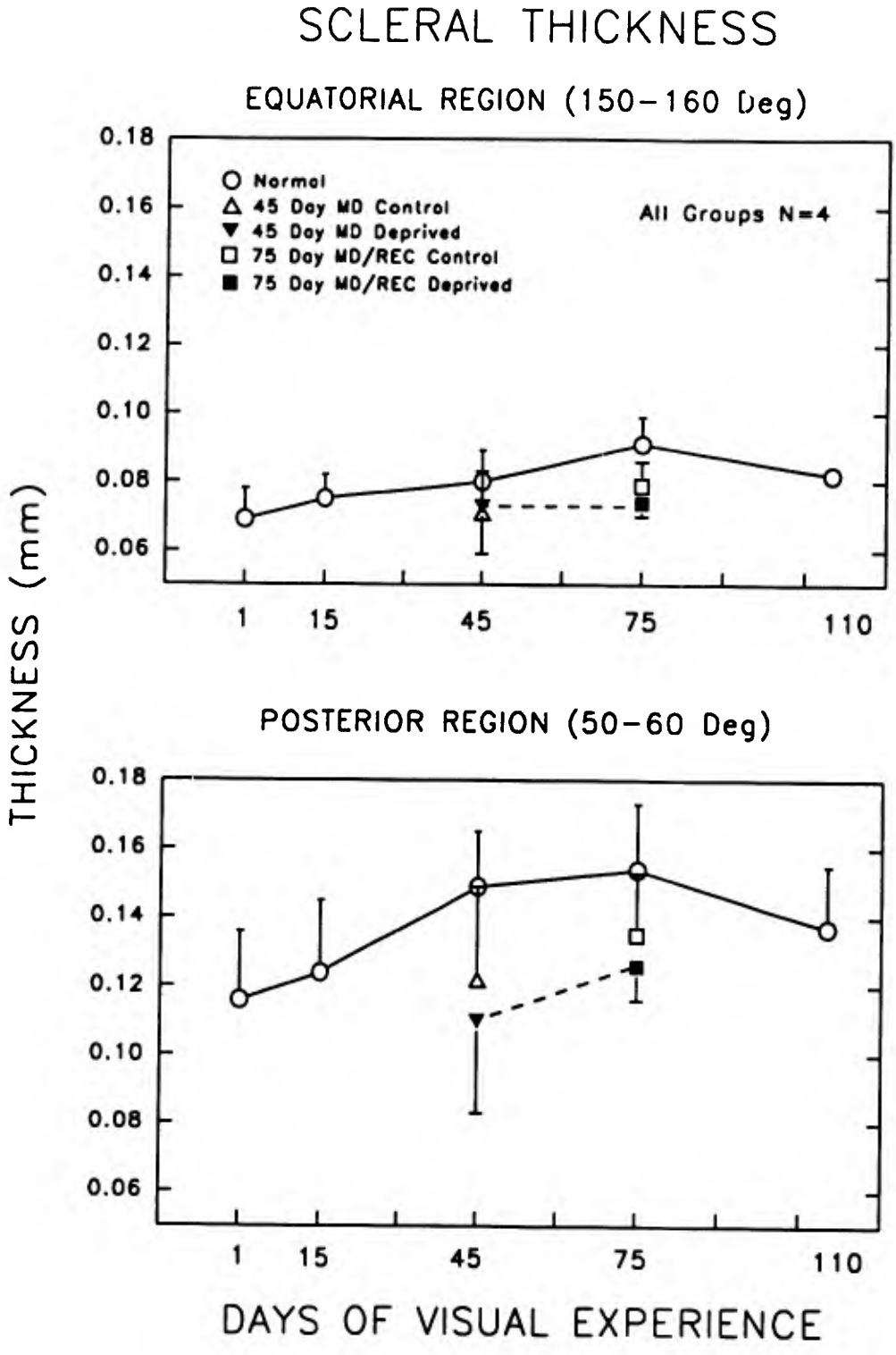
sectional area in a detailed and a systematic manner and it may be that monocular visual deprivation has effects on the control eyes as well but were not detectable before with physiological methods. This will be discussed further in the Discussion.

Since the scleral samples for the electron microscopic analysis were taken from the temporal equatorial region (150° to 160°) and from near the nasal posterior region (50° to 60°), scleral thickness measures at these two regions were examined separately and are summarized in Figure 16. In the normal animals, comparing the rate of change in thickness at the equatorial to the posterior region, the equatorial sclera changed much less. Also, the effects of deprivation and recovery in the equatorial scleral thickness was small when compared to the changes seen in the posterior region. This confirms the selection of this region as one with little change was indeed correct. In the posterior region, in the deprived group, the sclera in the deprived eyes was thinner (-10.4 +/- 1.8%, t test, $p < .05$) when compared to the control eyes. In the recovery group, the differences were not significant. Again, in the control eyes of the deprived and the recovery groups, the sclera appeared to be thinner, although in both cases the differences were not significant, when compared to the age matched normal eyes.

Electron Microscopic Level Analysis

Twenty-six eyes were evaluated at the EM level (Table 1 in the Methods). There were 10 eyes from 5 normal developing groups (2 eyes per group) and 16 eyes from the deprived and the recovery groups (both eyes, 4

Figure 16. Comparison of the two scleral locations used for electron microscopy. The scleral thickness in the two regions from which the electronmicroscopic samples were taken are plotted as the average at the 150° to 160° location in the equatorial region (top) and at 50° to 60° location in the posterior region (bottom). The data suggest that the differences between the deprived and the control eyes were greater in the posterior location.


SCLERAL THICKNESS
EQUATORIAL REGION (150–160 Deg)
Normal
45 Day MD Control
45 Day MD Deprived
75 Day MD/REC Control
75 Day MD/REC Deprived
All Groups N=4
0.18
0.16
0.14
0.12
0.10
0.08
0.06
1
15
45
75
110
POSTERIOR REGION (50–60 Deg)
THICKNESS (mm)
DAYS OF VISUAL EXPERIENCE

animals per group) in which the scleral lamellar organization and collagen fibrils were examined.

Overall, the results from the EM level analysis agreed with the results from the light level. The evaluation of the lamellar organization showed that, in the deprived and the recovery eyes, there were changes in the relative amounts of the components which make up the sclera when compared to the control eyes. However, the evaluation of the collagen fibrils suggested that the fibrils, in the deprived and the recovery eyes, were not significantly affected in density, in size, or in cross sectional area ratio to background when compared to the control eyes. Taken together, the results in the EM analysis suggest that the primary effect of deprivation (21 days) in the sclera of the deprived eyes might be a decrease in the activity of the fibroblasts, suggesting that the extracellular matrix synthesis and/or accumulation might be decreased also in these eyes, a finding which is consistent with the model of emmetropization discussed in the Introduction. The finding that the collagen fibrils were not significantly affected in the deprived eyes suggests that the 21 days of deprivation may not have been long enough in duration.

Lamellar Organization

Figure 17, Figure 18, and Figure 19 shows an example of the montage from the posterior region (from the inner, middle and outer third region, respectively) in a normal 45 days v.e. animal. As described in the Methods, measurements were made on the photomicrograph montages along a line across sclera from choroid to outer surfaces. The distances along that line that

Figure 17. Photomicrograph of the sclera showing the lamellar organization from the inner third region. Magnification is 3800 X. The photomicrograph is oriented with the choroid on the bottom left (as seen by the layer of pigment) and the outer sclera toward the top right corner. When put together with the photomicrographs in Figure 18 and Figure 19, a complete montage across the sclera is constructed. As shown, the lamellae (or collagen fibril bundles) in the sclera varies in thickness and orientation. A fibroblast cell body is seen toward the choroid with its cellular processes extending into space between the lamellae. The "space" between lamellae is seen as lighter areas, particularly visible on the top right corner. Scale bar = 100 μm.

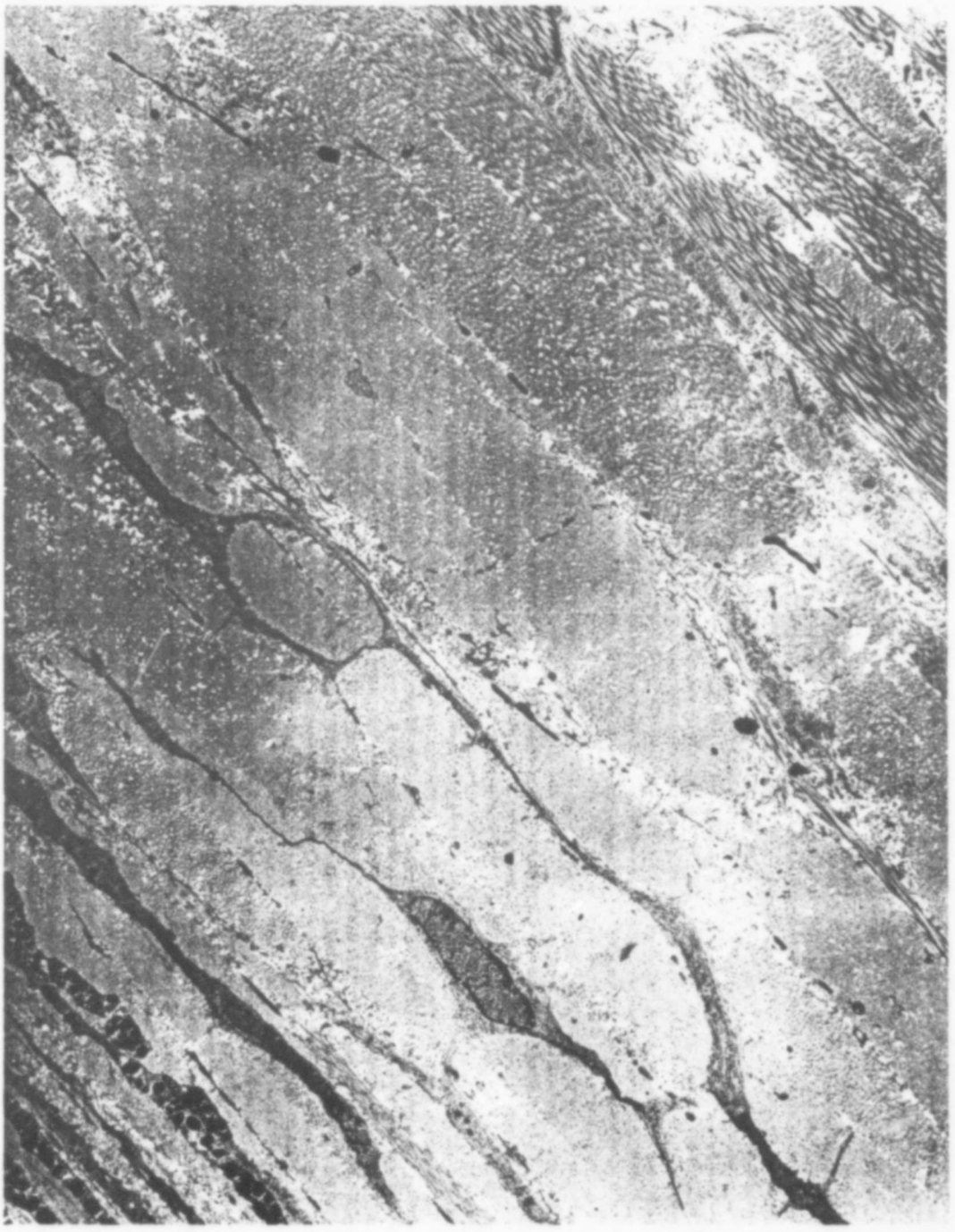


Figure 18. Photomicrograph of the sclera showing the lamellar organization from the middle third region. Magnification is 3800 X. The photomicrograph is orientated as in Figure 17 with the choroid toward the bottom left corner. Scale bar = 100 μm.

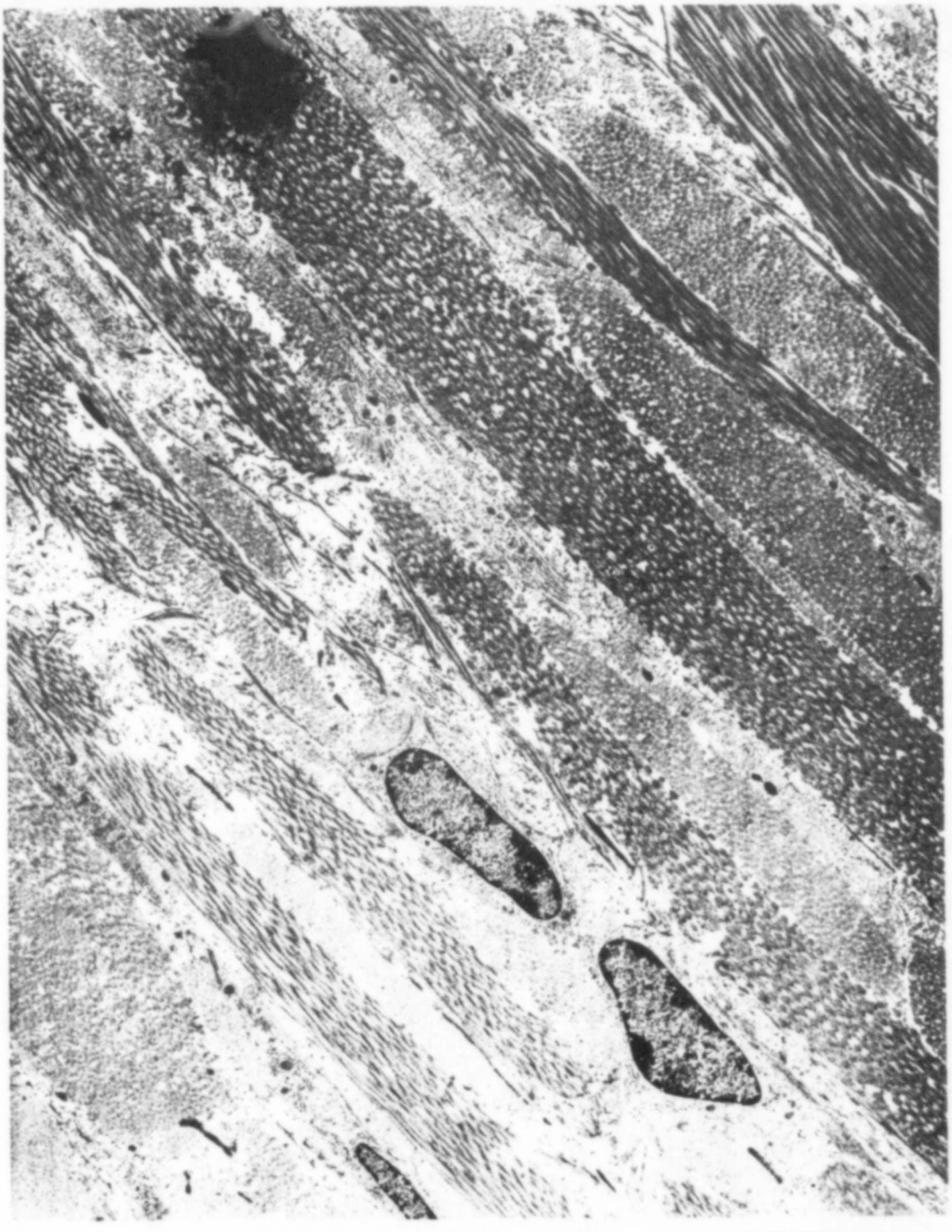
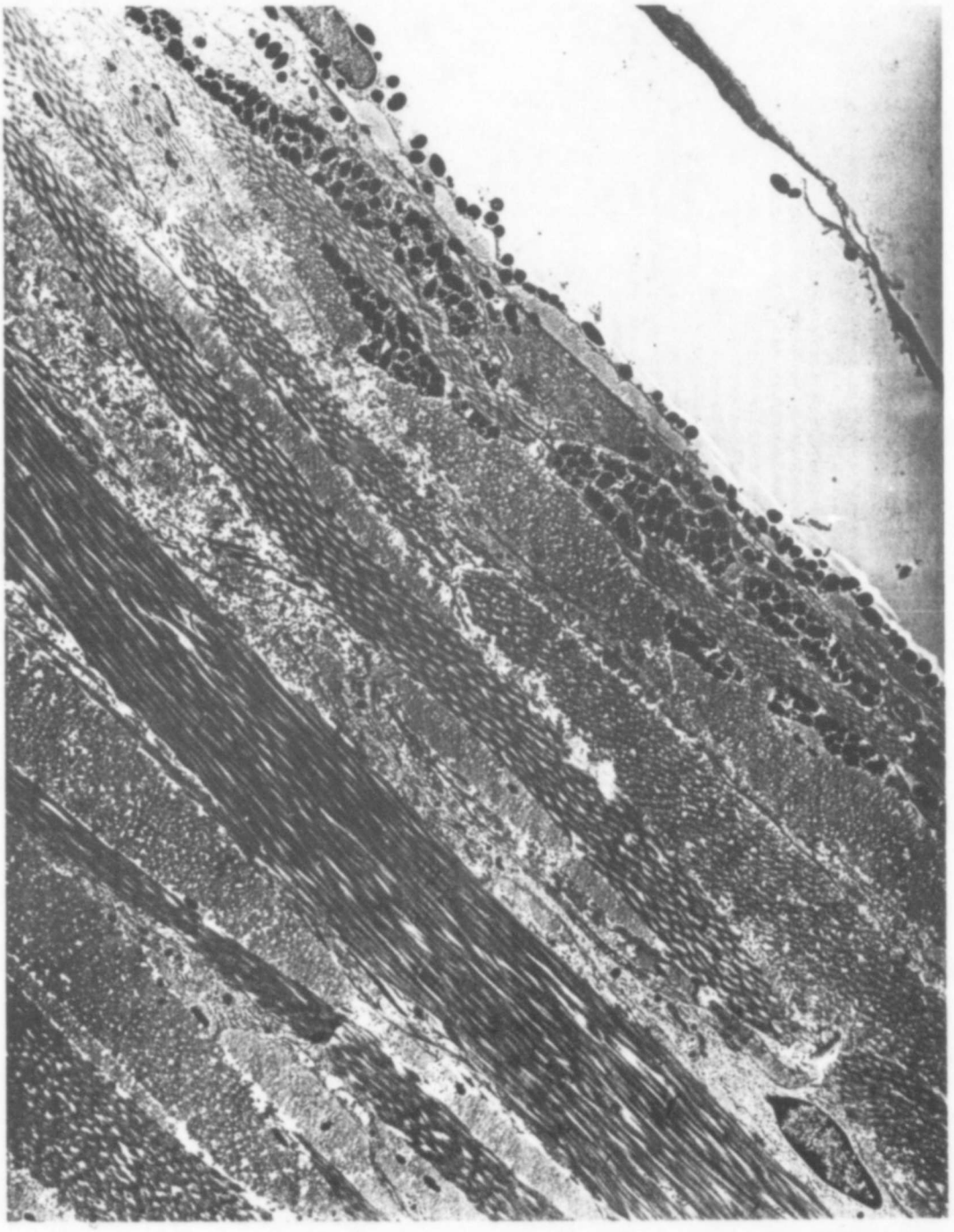


Figure 19. Photomicrograph of the sclera showing the lamellar organization from the outer third region. Magnification is 3800 X. The photomicrograph is oriented as in Figure 17 and Figure 18 with the choroid toward the bottom left corner. The outer scleral surface is visible toward the top right corner. Scale bar = 100 μm.

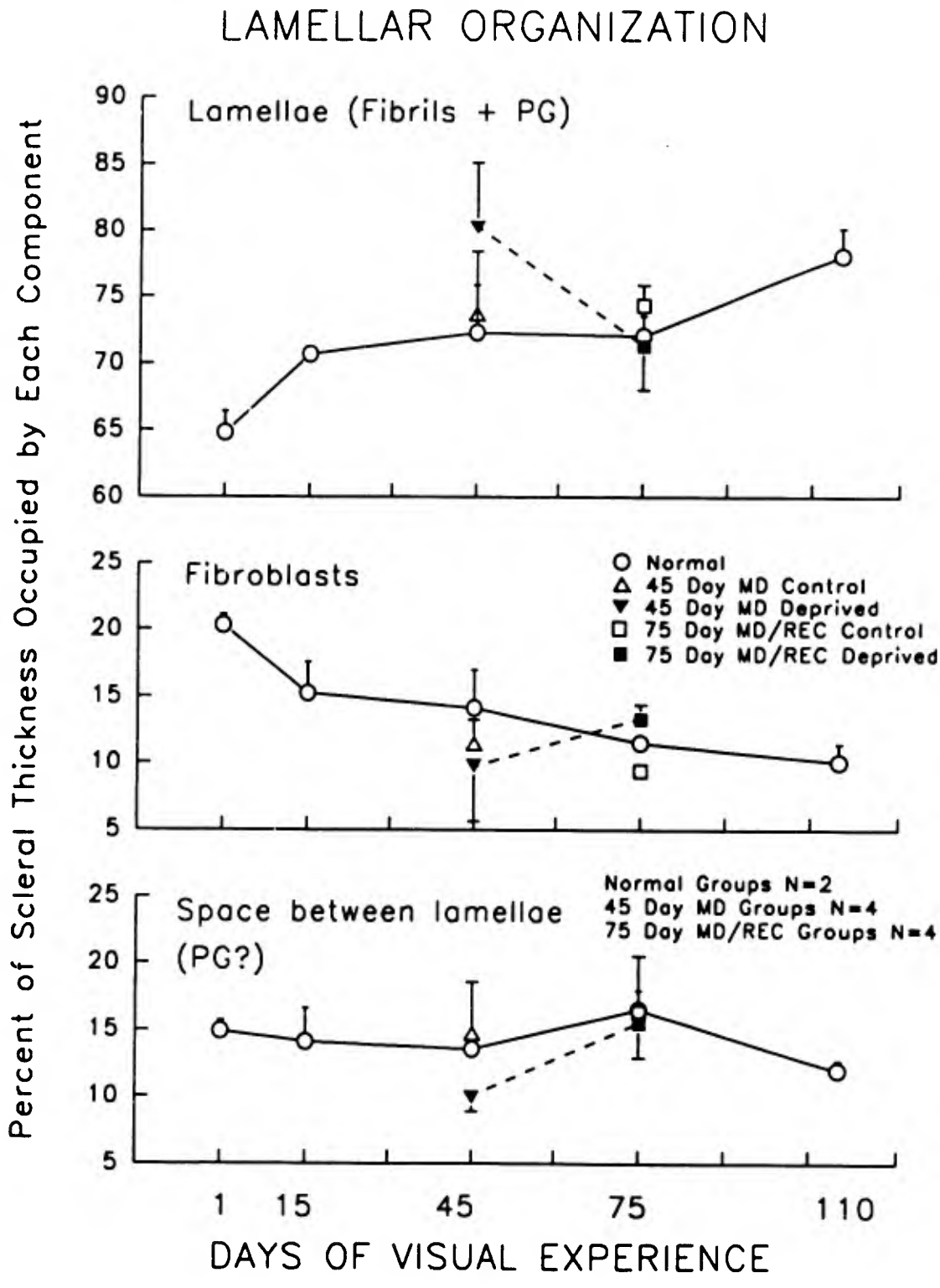


were inside a lamellae, in fibroblast processes or cell bodies, or in "space" was recorded. In the montages, there were areas between lamellae and also between lamellae and fibroblasts which might be best described as "space" which may be occupied by proteoglycans (PG) not associated with collagen fibrils. Including this "space," which was about 10 - 15% of the total thickness of the sclera, made the description of lamellar organization more complete.

The results of lamellar measurement are summarized in Figure 20. In the normal developing animals, the percent of the thickness of the sclera occupied by (or composed of) the lamellae increased with age while the percent occupied by the fibroblasts decreased. The "space" area was rather stable. These results suggest that, as the fibroblasts continue to make extracellular matrix and the sclera increases in thickness with age, the sclera probably becomes more fibrous in content, since the primary component of the lamellae is the collagen fibrils.

Another way to describe the development of the lamellar organization would be to measure the actual thickness of each of three components through development as a function of age. However, at the magnification of the photomicrographs (5700 X) from which the montages of the sclera were made, scleral thickness was rather variable as one moved across the montage. Also, as discussed earlier, in some montages, grid obscured some regions. Thus, to bypass this problem, the percent occupied by each component in Figure 20 were normalized, for each animal, to the scleral thickness results from the light level analysis at the same scleral locations (nasal 50° to 60°). For

Figure 20. Comparison of the three components of the sclera. For each of the components, total percent thickness across the scleral photomontage occupied by that component is plotted as a function of days of visual experience (v.e.). The data suggest that the lamellae component (top), which is made of mostly collagen and proteoglycans, comprised a gradually increasing fraction of the sclera with increasing age. The fibroblast component, which makes the extracellular matrix, appeared to decrease gradually with increasing age. The "space" component was defined as the space between the lamellae or between the lamellae and the fibroblasts and may be an index of the amount of proteoglycans outside the lamellae. The "space" component changed very little with increasing age. In the deprived and the recovery groups, the differences between the deprived and the control eyes were not significant. The fibroblast and "space" components were significantly increased, however, when compared to the deprived eyes in the deprived group.

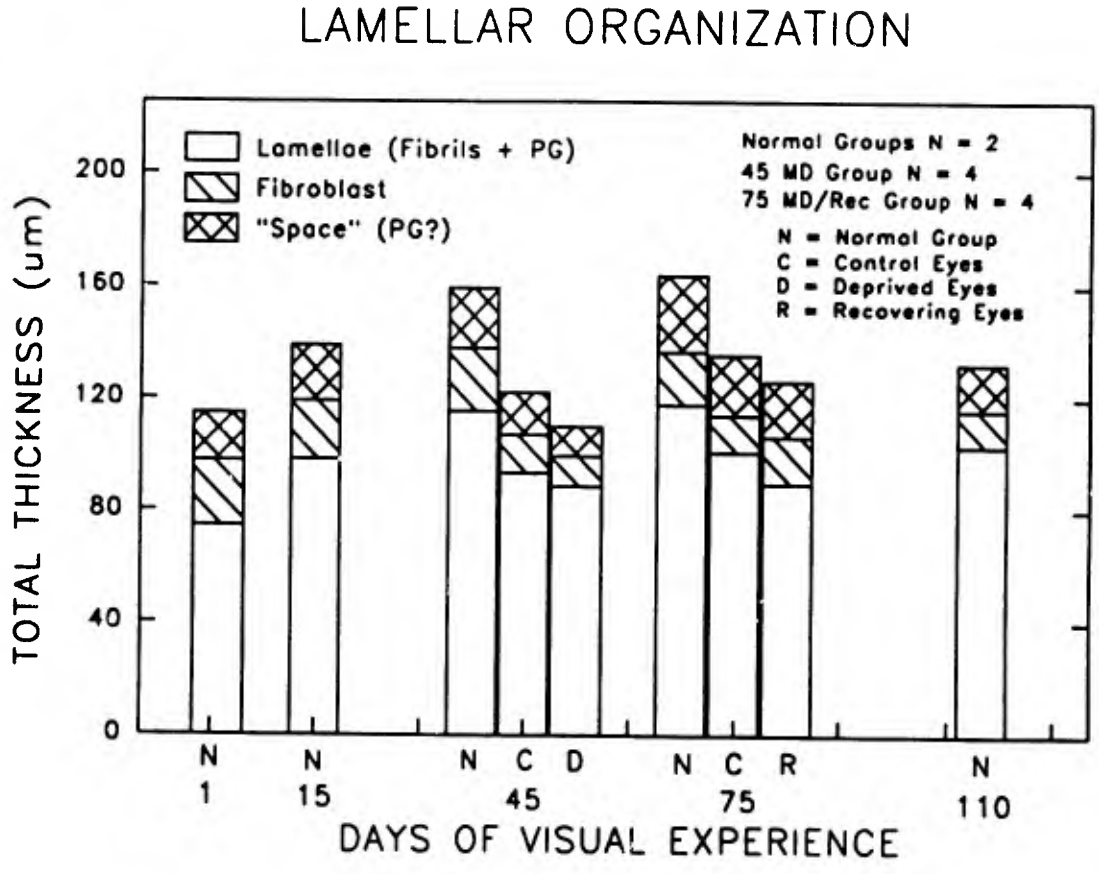

LAMELLAR ORGANIZATION
Percent of Scleral Thickness Occupied by Each Component
Lamellae (Fibrils + PG)
90
85
80
75
70
65
60
Fibroblasts
25
20
15
10
5
Space between lamellae
(PG?)
25
20
15
10
5
Normal
45 Day MD Control
45 Day MD Deprived
75 Day MD/REC Control
75 Day MD/REC Deprived
Normal Groups N=2
45 Day MD Groups N=4
75 Day MD/REC Groups N=4
1
15
45
75
110
DAYS OF VISUAL EXPERIENCE

instance, if the scleral thickness was 120 nm and the data from montages was 50% lamellae, then the lamellae would be 60 nm in thickness.

Figure 21 shows the result of such normalization. Described this way, it became clear that, in the normal animals, the total amount of lamellar tissue increased rapidly, between 1 and 45 days v.e. but remained rather stable from 45 to 110 days v.e. Interestingly, in the same normal animals, the total amount of fibroblast component was stable between the 1 and 45 days v.e. but decreased gradually between 45 and 110 days v.e. These results suggested that the decrease in normal animals of the percent occupied by the fibroblasts between 1 and 45 days v.e. (Figure 20) was due to the increase in the total thickness of the sclera and not to a decrease in the "amount" of fibroblasts. It might be suggested that during this early development period, the fibroblasts had enlarged processes because they were very actively producing extracellular matrix. After 45 days v.e., as the stable total thickness of lamellar tissue shows in Figure 21, accumulation collagen in the matrix slowed, perhaps as reflection of decreased activity by the fibroblast component Indeed, the percent occupied by the fibroblasts continued to decrease after 45 days v.e. (Figure 20) despite the fact that total scleral thickness decreased slightly in 110 days v.e. compared to 45 days v.e. (Figure 21). The "space" component did not appear to change in actual thickness.

In the deprived group, Figure 20 shows that there was an increase (not significant, 8.9 +/- 7.7%, $\underline{t}$ test, $\underline{p} > .05$) in the percent occupied by the lamellae when compared to the control eyes. The fibroblast component also was not

Figure 21. Comparison of the total thickness of each lamellar component. In each animal, the percent of the total scleral thickness, as measured on the photomontages, that each component occupied (see Figure 20) was normalized using the scleral thickness data measured at the light level, from the same sclera location (see text for details). The data suggest that the relative thickness of each of the three components were changing in a complicated manner throughout the development. The data also suggest that in both the deprived and the recovery groups, it was the fibroblast and the "space" components that were most prominently affected.


LAMELLAR ORGANIZATION
Lamellae (Fibrils + PG)
Fibroblast
"Space" (PG?)
Normal Groups N = 2
45 MD Group N = 4
75 MD/Rec Group N = 4
N = Normal Group
C = Control Eyes
D = Deprived Eyes
R = Recovering Eyes
TOTAL THICKNESS (um)
0
40
80
120
160
200
N 1
N 15
N C D 45
N C R 75
N 110
DAYS OF VISUAL EXPERIENCE

significantly different in the deprived when compared to the control eyes (- 8.5 +/- 44.4%, t test, p > .05). Both the control and the deprived eyes were slightly (but not significantly, independent t test, p > .05) decreased when compared to the age matched normal eyes. The "space" component in the deprived eyes was not different when compared to the control eyes (-22.1 +/- 10.6%, t test, p > .05).

In the recovery group, also shown in Figure 20, as in the deprived group, there were no significant differences noted in deprived eyes when compared to the control eyes. It was interesting to note, however, in contrast to the results in the deprived group, that the lamellae component in percent occupied in the recovery eyes appeared slightly decreased (-4.0 +/- 3.3%, t test, p > .05) when compared to the control eyes. Also, the percent occupied by the fibroblasts in the recovery eyes appeared to be increased (51.3 +/- 49.4, t test, p > .05) when compared to the control eyes. The "space" component was not different between the recovery and the control eyes.

When examined in normalized total thickness (Figure 21), in the deprived group, there were no significant difference noted in the lamellae and fibroblast components between the deprived and the control eyes. The "space" component was, however, decreased in the deprived eyes in total thickness (-30.1 +/- 9.4%, t test, p < .05) when compared to the control eyes. As discussed above in scleral thickness results, and as can be seen in Figure 21, both the control and the deprived eyes in the deprived group were thinner when compared to the age matched normal eyes. Analysis of the changes in

lamellae, fibroblast, and "space" in both percent occupied or in total thickness measures between the control eyes in the deprived group and the age matched normal eyes were, however, not significant.

In the recovery group (Figure 21), as in the deprived group, the apparent decrease in the lamellae thickness in the recovery eyes was not significant (-9.7 +/- 12.4%, t test, $p > .05$) when compared to the control eyes. The fibroblast and the "space" component changes were also not significant. The fibroblast component in the recovery eyes appeared to be increased, however, when compared to the control eyes (51.3 +/- 49.4%, t test, $p > .05$). When compared to the deprived eyes in the deprived group, the fibroblast and the "space" components in the recovery eyes were, however, significantly increased (independent t tests, $p < .05$). The total lamellae thickness was not different in the recovery eyes compared to the deprived eyes (5.7 +/- 22.7%, independent t test, $p > .05$). Indeed, the "recovery" of scleral thickness in the recovery eyes appeared to be from the increases in the total thickness of fibroblasts and "space" components as the total thickness of lamellae did not appear to have changed in the recovery eyes when compared to the deprived eyes in the deprived group.

The above results suggested that the effect of deprivation and subsequent restoration of normal vision in the recovery animals was to change the fibroblast and "space" components. It is tempting to suggest that, since the fibroblasts make the extracellular matrix, these changes in fibroblast in either percent occupied or in total thickness might reflect the level of synthesis and/or

accumulation of extracellular matrix material into the "space" between lamellae. In this regard, it is particularly interesting to note the dramatic increase in the total thickness of fibroblasts in the recovery eyes when compared to the deprived eyes in the deprived group.

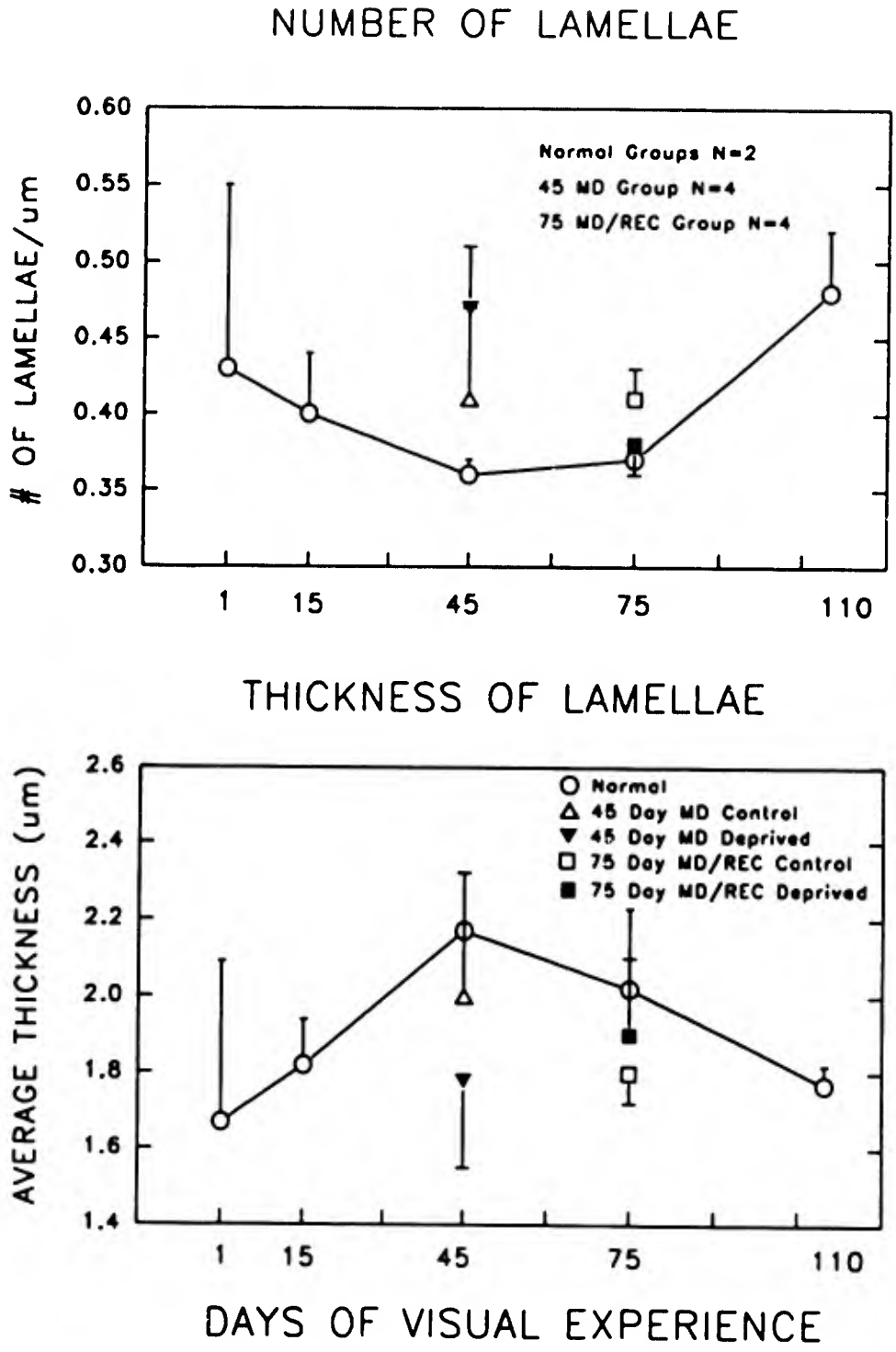
Number of Lamellae

Figure 22 summarizes the results of number and the average thickness of lamellae measurements across the montage. As would be expected, given the interweaving nature of lamellar organization in the sclera, the variability was high among animals within each group. In the normal animals, there was a slight decrease in the number of lamellae (expressed as # of observed lamellae per μm of sclera in the montages) from 1 to 45 days v.e. group and a slight increase in the 110 days v.e. group. The average thickness of lamellae, as would be expected, was nearly a mirror image of the number of lamellae in the same age groups.

In the deprived group, neither the number nor the average thickness of lamellae in the deprived eyes was significantly different from the control eyes (paired t test, $p > .05$). Figure 22 shows, however, in the deprived eyes, there appeared to be a slight increase in the number of lamellae (16.8 +/- 13.1%, not significant, t test, $p > .05$) and a decrease in the average thickness of lamellae (-5.5 +/- 10.9%, also not significant, t test, $p > .05$) when compared to the control eyes.

In the recovery group, there were also no significant changes (paired t test) in either the number or the average thickness of lamellae in the recovery

Figure 22. Summary of the number and thickness of lamellae. The total number of lamellae (top) counted along the measurement line across the sclera is plotted as number of lamellae per μm of sclera as a function of days of visual experience. The data suggest that the number of lamellae changed very little with increasing age. The differences between the deprived and the control eyes in both the deprived and the recovery groups are significant. The data also suggest that the average thickness (bottom) of lamellae counted across the sclera also changed very little with increasing age. The differences between the deprived and the control eyes in both the deprived and the recovery groups are not significant.


NUMBER OF LAMELLAE
OF LAMELLAE/um
0.60
0.55
0.50
0.45
0.40
0.35
0.30
Normal Groups N=2
45 MD Group N=4
75 MD/REC Group N=4
1
15
45
75
110
THICKNESS OF LAMELLAE
AVERAGE THICKNESS (um)
2.6
2.4
2.2
2.0
1.8
1.6
1.4
Normal
45 Day MD Control
45 Day MD Deprived
75 Day MD/REC Control
75 Day MD/REC Deprived
1
15
45
75
110
DAYS OF VISUAL EXPERIENCE

eyes when compared to the control eyes. As Figure 22 shows, in the recovery eyes the number of lamellae appeared to be decreased (-9.0 +/- 7.0%, not significant, t test, $p > .05$) when compared to the control eyes. When compared to the deprived eyes in the deprived group, however, the number of lamellae in the recovery eyes was significantly decreased (-20.9 +/- 5.1%, independent t test, $p < .05$).

Interestingly, the same comparison between the recovery and the deprived eyes for the average thickness measures were not different. This suggested that the decrease in the number of lamellae in the recovery eyes was the result of the "recovery" or the increase in the thickness of the recovery eyes compared to the deprived eyes. These results suggested that overall the number or the thickness of lamellae were not significantly affected by the deprivation or subsequent restoration of normal vision.

Figure 23 shows the number of lamellae results in more detail by examining the inner, middle, and outer third regions of the sclera separately. As can be seen, the significant decrease in the number of lamellae seen in the recovery eyes, compared to the deprived eyes (above), could be primarily attributed to the changes in the inner third region. Overall, the number of lamellae in the inner third region was higher than the middle and the outer third regions.

Figure 24 shows the average thickness of lamellae results in the inner, middle, and outer third regions. The thickness of lamellae in the inner region was thinner compared to the middle and the outer region. As can be seen, the

Figure 23. The number of lamellae in the inner, middle, and outer third sclera as a function of age. The number of lamellae counted along the measurement line across the sclera was analyzed in more detail by dividing the sclera into three regions; inner, middle, and outer third. The number of lamellae per μm of the sclera in each of the region is plotted throughout the development. The data suggest that the number of lamellae did not vary significantly as a function of the scleral region throughout development. In all three regions, the differences between the deprived and the control eyes in both the deprived and the recovery groups are not significant.

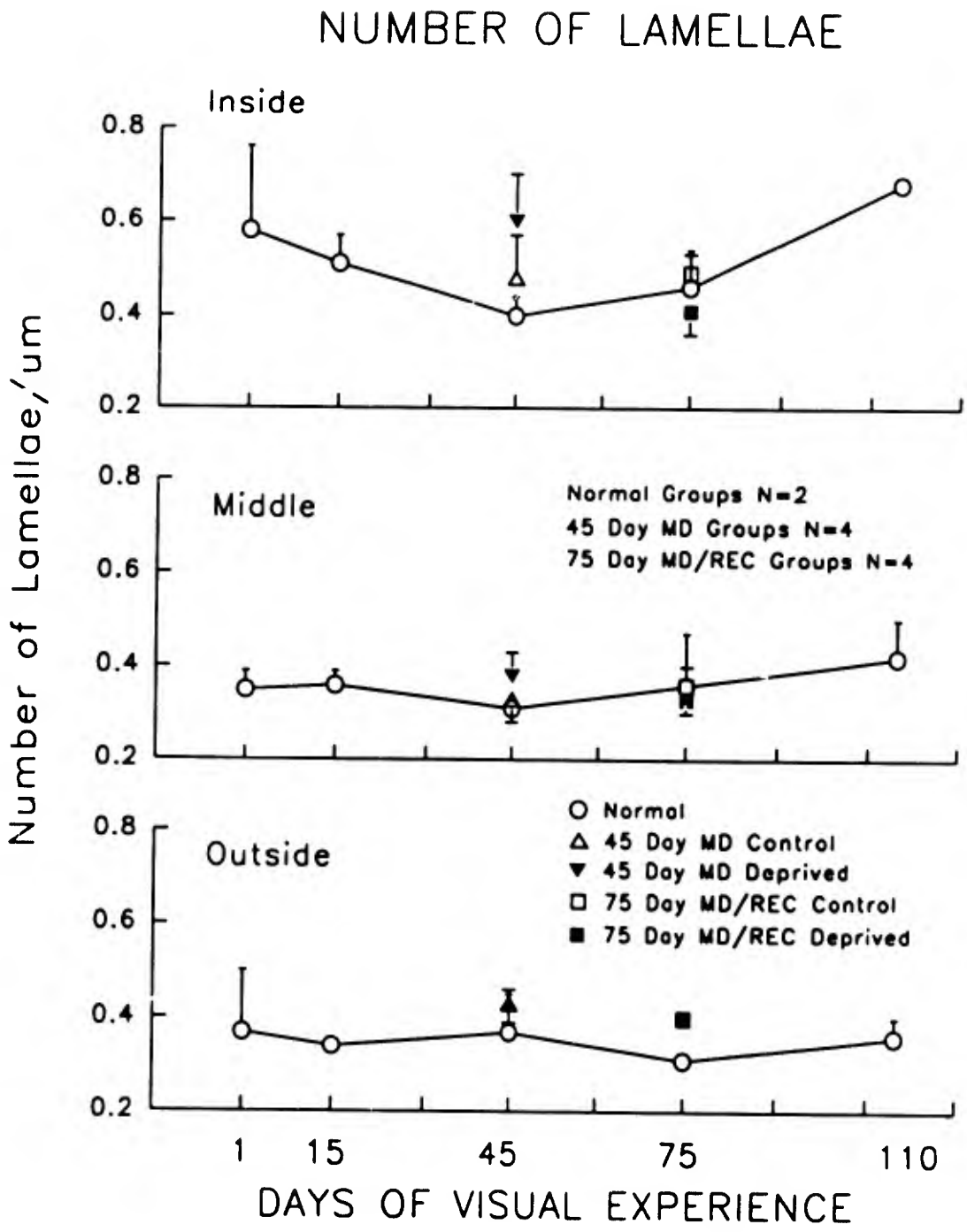
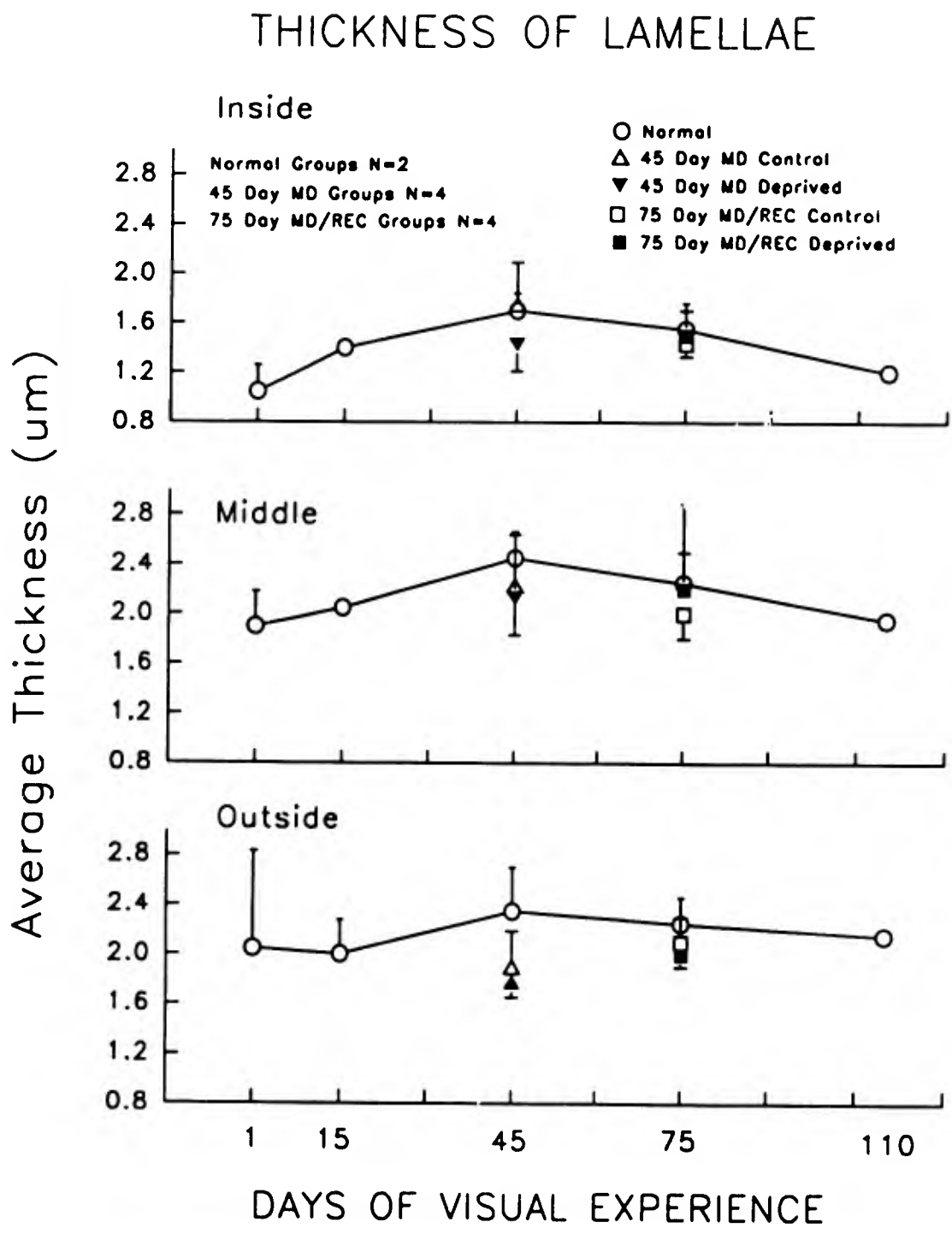

NUMBER OF LAMELLAE
Inside
Middle
Outside
0.8
0.6
0.4
0.2
Number of Lamellae/um
Normal Groups N=2
45 Day MD Groups N=4
75 Day MD/REC Groups N=4
Normal
45 Day MD Control
45 Day MD Deprived
75 Day MD/REC Control
75 Day MD/REC Deprived
1
15
45
75
110
DAYS OF VISUAL EXPERIENCE

Figure 24. The thickness of lamellae in the inner, middle, and outer third sclera. The average thickness of lamellae counted across the sclera was analyzed in more detail by dividing the sclera into three regions; inner, middle, and outer third. The data suggest that there were no significant effect of the scleral region on the thickness of lamellae throughout the development. In all three regions, the differences between the deprived and the control eyes in both the deprived and the recovery groups are not significant.


THICKNESS OF LAMELLAE
Inside
Normal Groups N=2
45 Day MD Groups N=4
75 Day MD/REC Groups N=4
Normal
45 Day MD Control
45 Day MD Deprived
75 Day MD/REC Control
75 Day MD/REC Deprived
Middle
Outside
Average Thickness (um)
2.8
2.4
2.0
1.6
1.2
0.8
1
15
45
75
110
DAYS OF VISUAL EXPERIENCE

changes in the normal developing animals or in the treated eyes in the deprived and the recovery groups were not significantly different compared to the control eyes in their group.

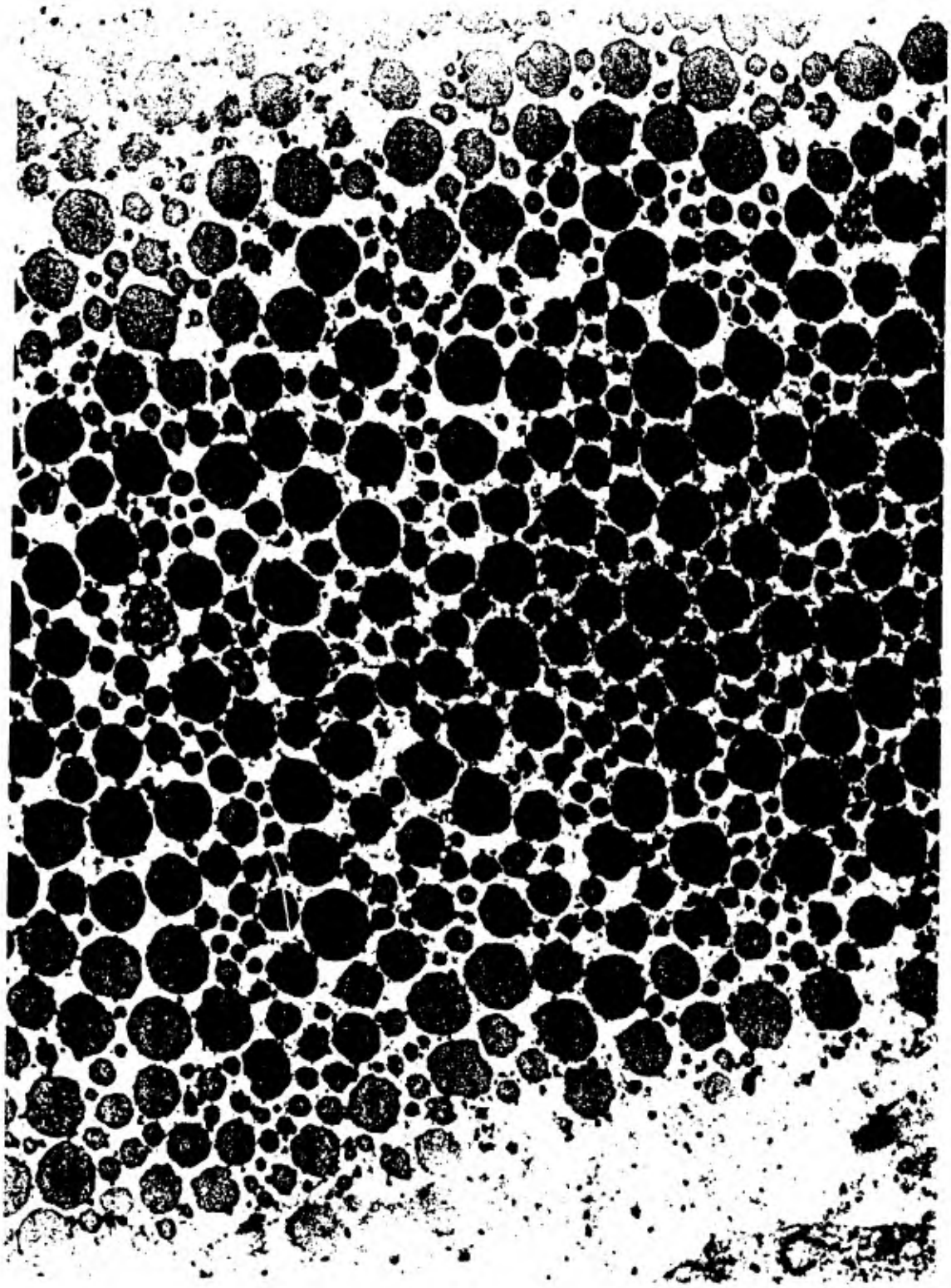
Collagen Fibrils

Examination of sclera was continued to high magnification EM level with an examination of collagen fibrils. Given the normal developmental changes in lamellar component, not surprisingly, the collagen fibrils changes as a function of age were not significant. Given the lesser changes in lamellar component in the deprived and recovery eyes, also not surprisingly, no significant changes related to the deprivation and recovery were found.

Collagen fibril measurements of the fibril density (per sq μm), fibril diameter, nearest neighbor distance, and cross sectional area ratio were made on the photomicrographs (75,000 X) of fibril cross sections. Figure 25 shows an example of photomicrograph of cross section of the collagen fibrils (in the posterior scleral location) in a 45 day normal animal. As described in the Methods, a measurement box was drawn on each of the photomicrographs and the fibrils inside the box were measured. Collagen fibrils were measured at both the equatorial and posterior scleral locations, and in the inner, middle, and outer third regions at each scleral location.

Initial analysis of the data from the inner, middle, and outer third regions at each scleral location made clear that there was a large variability in the collagen fibril measurements. Indeed, when more than one photomicrograph was taken within the same region, typically within a few

Figure 25. Photomicrograph of cross section of collagen fibrils at high magnification. Magnification is 50000 X. The sample was taken from the middle third region in the posterior scleral location from a 45 day normal animal. In this example, the entire thickness of a lamellae is shown as evidenced by the borders visible on the top left and the bottom right corners. A measurement box was drawn on each of the photomicrographs and the fibrils inside the box were measured for fibril density, diameter, nearest neighbor distance, and cross sectional area ratio of fibrils to background (see text for details). Scale bar = 1000 nm.



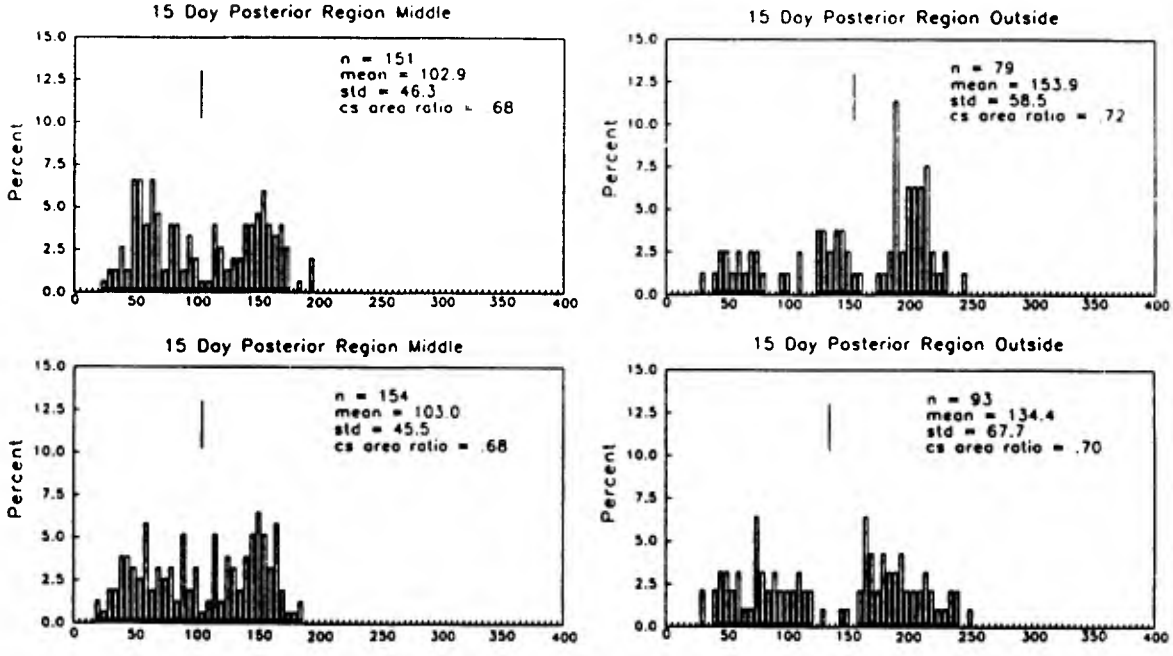
microns from each other, and the results of fibril measurements compared, the fibril distributions were found to be very similar in some cases, and very different in others. An example of two such comparisons is shown in Figure 26. In this 15 days v.e. normal animal, in the posterior scleral location, two photomicrographs from the middle and the outer third regions were measured. As can be seen, the two measurements at the middle region were very similar in terms of the number of fibrils measured, the mean diameter, and shape of the distributions, and cross sectional ratio to background. In the outer region, however, the number of fibrils and mean diameter were rather different in the two examples, and the fibril diameter distributions were also very different in appearance. Given these findings and because the number of animals examined at the EM level were small (N = 2 in the normal groups and N = 4 in both the deprived and recovery groups), the data from the inner, middle, and outer regions were pooled together at each location within each animal and analyzed. When significant results were found in these pooled data analysis, however, the inner, middle, and the outer third regions were examined also for a more detailed analysis.

In addition, as will be seen later, the analysis of the measurements of number of fibrils, diameter, nearest neighbor, and cross sectional area ratio to background, in the deprived group, failed to indicate any significant changes in the deprived eyes compared to the control eyes. Therefore, in the recovery group, only two animals were evaluated at 75,000 X level for the above measurements of fibrils. The results from these two animals indicated, as

Figure 26. A sample of variability observed in collagen fibril diameter distributions. In this 15 days of visual experience (v.e.) animal, at the posterior sclera, two photomicrographs were taken from very near each other within each of the middle and the outer third regions. The histograms show the distributions of the fibril diameter. The histograms show that, in the middle third region (left side) the two distributions were very similar when compared to each other, and that the fibril density, mean diameter, and cross sectional area (cs area) ratio of fibrils to background were also similar. In the outer third region (right side), however, the two distributions were rather different when compared to each other. The data also suggest that the fibril density and diameter were different.

FIBRIL DIAMETER DISTRIBUTION

15 Day Normal



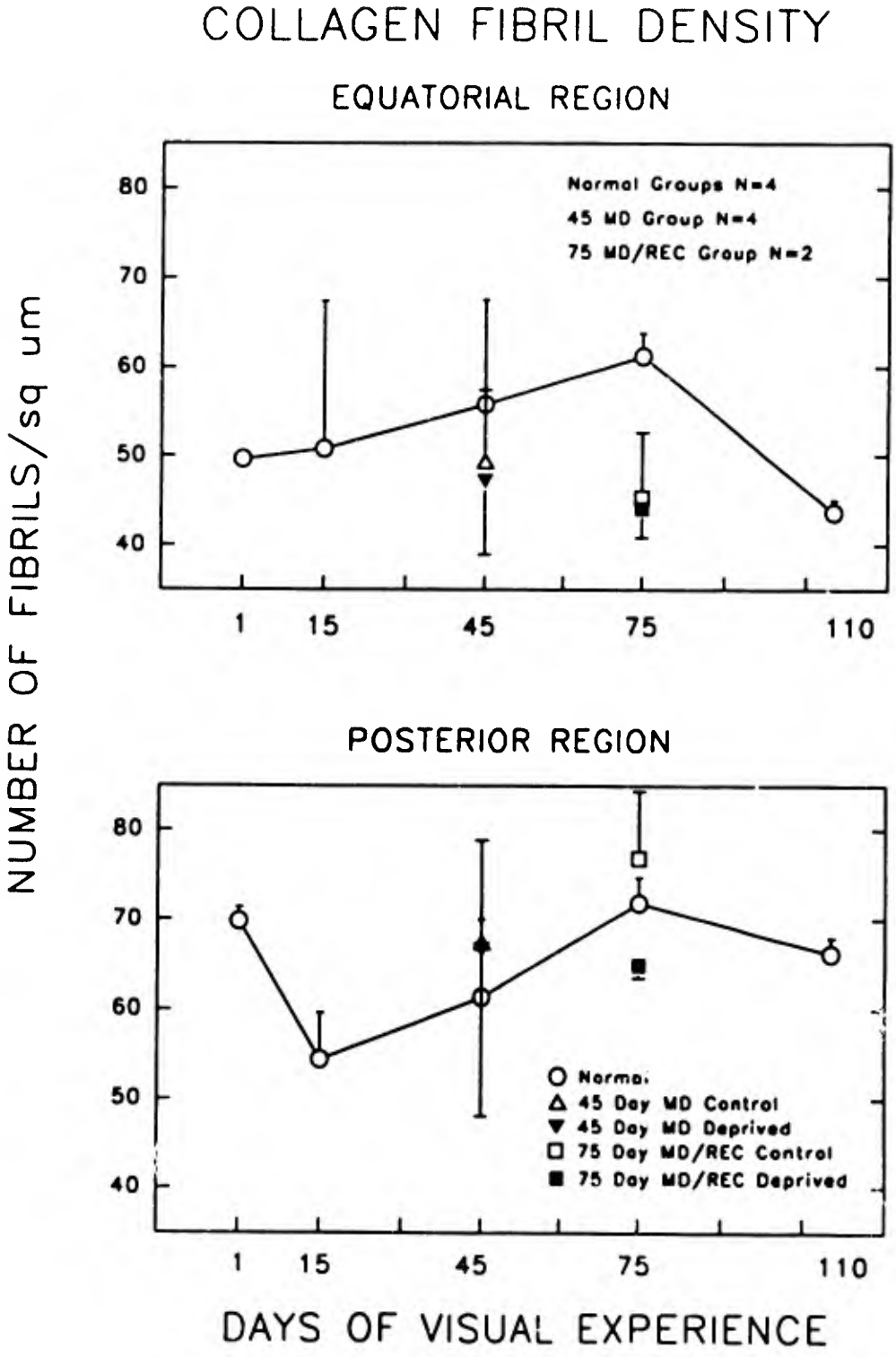
expected, that there was no "recovery" in the recovering eyes compared to the control eyes, since there appeared to be nothing to "recover from," based on the results from the deprived group.

Number of Collagen Fibrils

The results of the fibril density measurements are summarized in Figure 27. Because, on some photomicrographs, the areas measured were smaller than the standard 12x12 cm measurement area, the results are described as the number of fibrils counted per square μm. As can be seen in the Figure 27, in the normal developing animals, no clear trends were observed in either the equatorial or the posterior region. The 1-way ANOVA analysis confirmed that, in the normal developing animals, the fibril density was not significantly different as a function of age at either the equatorial region ($F(4,5) = 0.517$, $p = .73$) or the posterior region ($F(4,5) = 2.124$, $p = .215$). The 2-way ANOVA analysis indicated, however, that the equatorial and the posterior regions were different ($F(1,10) = 7.283$, $p = .02$) in fibril density.

In the deprived group, the deprived eyes were not significantly different in both the equatorial and the posterior regions when compared to the control eyes (t test). Also, in the recovery group, the recovering eyes were not significantly different when compared to the control eyes. Although Figure 27 shows that, in the equatorial region, both the control and the deprived eyes appeared to have decreased fibril density when compared to the age matched normal eyes, the differences between the control eyes and the 75 day normal

Figure 27. Summary of collagen fibril density. The number of fibrils per sq μm of scleral area is plotted for the equatorial (top) and the posterior (bottom) scleral locations throughout the development. The data suggest that the fibril density at each location changed very little throughout the development. The differences between the equatorial and the posterior regions were significant. The differences between the deprived and the control eyes in the deprived and the recovery groups were not significant in either the equatorial or the posterior region.


COLLAGEN FIBRIL DENSITY
EQUATORIAL REGION
Normal Groups N=4
45 MD Group N=4
75 MD/REC Group N=2
80
70
60
50
40
1
15
45
75
110
POSTERIOR REGION
80
70
60
50
40
1
15
45
75
110
Norma.
45 Day MD Control
45 Day MD Deprived
75 Day MD/REC Control
75 Day MD/REC Deprived
NUMBER OF FIBRILS/sq um
DAYS OF VISUAL EXPERIENCE

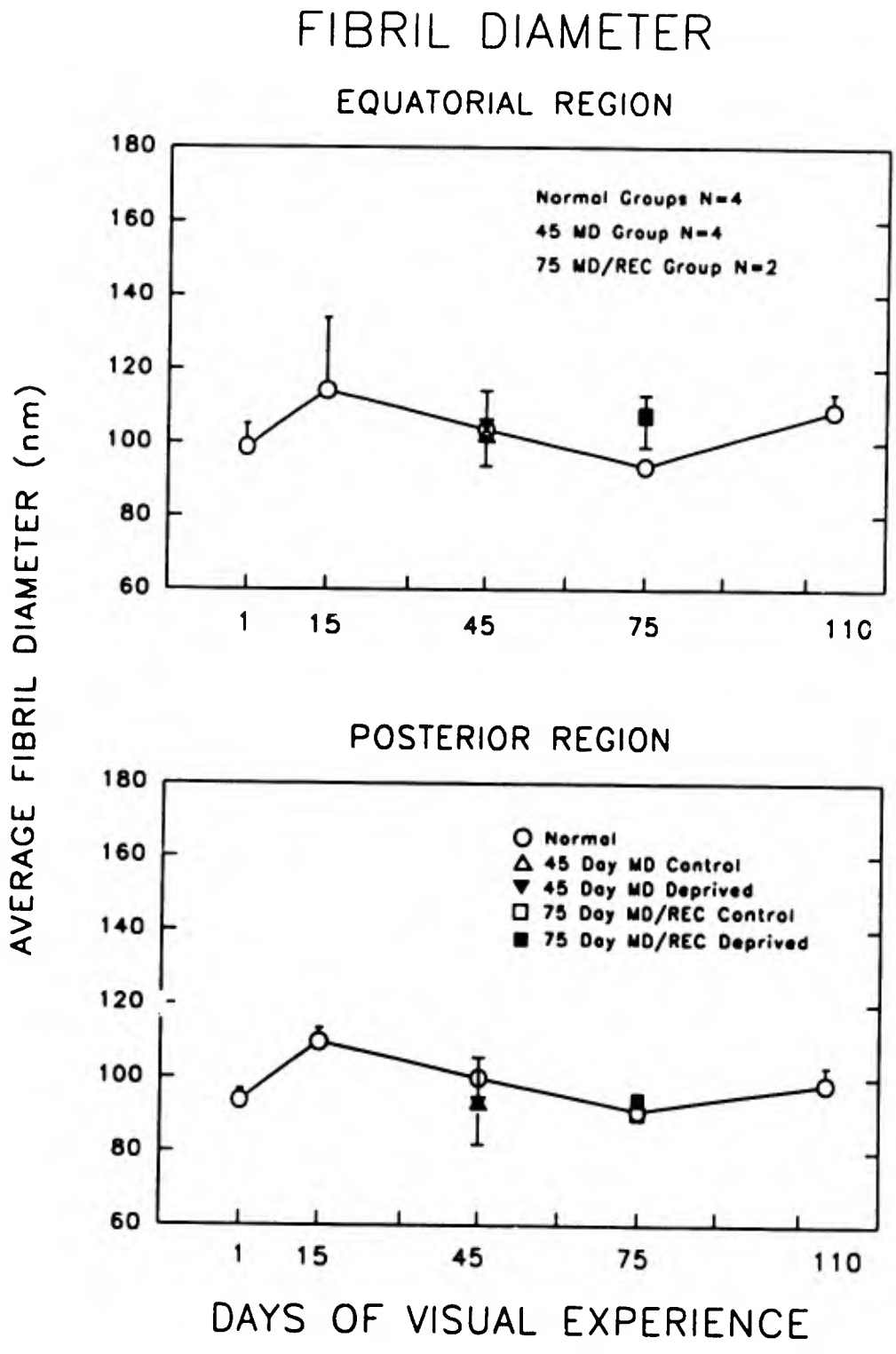
eyes were not significant (independent t test, control eyes compared to 75 day normal eyes, p > .05).

Collagen Fibril Diameter

The fibril diameter results are summarized in Figure 28. In the normal animals, in both the equatorial and the posterior regions, there was very little change in the mean diameter throughout development. The 1-way ANOVA analysis confirmed that there were no significant differences as a function of age in both the equatorial ($F(4,5) = 0.568$, $p = .687$) and in the posterior region ($F(4,5) = 4.921$, $p = .06$). The 2-way ANOVA also confirmed that the two scleral regions were not significantly different ($F(1,10) = 1.056$, $p = .328$). In the deprived group, there was not a significant difference between the deprived and the control eyes (t test, $p > .05$). Also, as can be seen, in the recovery group, the recovering eyes were not different when compared to the control eyes (t test, $p > .05$). In the recovery group, in the equatorial region, both the control and the recovering eyes appeared to have increased mean fibril diameter when compared to the age matched 75 day normal eyes, but the differences were not significant (independent t test, control eyes compared to 75 day normal eyes, $p > .05$).

As can be seen in Figure 28, the most obvious and surprising finding was the lack of increase in the fibril diameter as a function of age. Based on the studies in the past, it was thought that there would be an increase in the fibril size with increasing age. The fibril diameter results were further

Figure 28. Summary of collagen fibril diameter. The mean diameter of fibrils is plotted for the equatorial (top) and the posterior (bottom) scleral locations throughout the development. The data suggest that the mean fibril diameter at both the equatorial and the posterior locations changed very little throughout the development. The deprived and the control eyes in the deprived and the recovery groups were not significantly different when compared either to each other or to the age matched normal eyes.


FIBRIL DIAMETER
EQUATORIAL REGION
Normal Groups N=4
45 MD Group N=4
75 MD/REC Group N=2
180
160
140
120
100
80
60
1
15
45
75
110
POSTERIOR REGION
Normal
45 Day MD Control
45 Day MD Deprived
75 Day MD/REC Control
75 Day MD/REC Deprived
AVERAGE FIBRIL DIAMETER (nm)
DAYS OF VISUAL EXPERIENCE

analyzed to see if other than the mean diameter results would indicate significant changes.

To test the hypothesis that there is a gradient in the fibril mean diameter from the inner to the outer scleral region, the fibril diameter was evaluated in the inner, middle, and the outer regions separately at both the equatorial and the posterior regions. Figure 29 summarizes the results in the equatorial region. In the equatorial region, the mean diameter appeared to be increased from the inner to the middle and the outer regions. The 2-way ANOVA analysis confirmed that there was a regional effect in the mean diameter ($F(2,15) = 6.328$, $p = .01$). Bonferroni post hoc comparisons showed that the fibril mean diameter in the inner region was smaller compared to the middle region ($p = .014$), but not compared to the posterior region ($p = .043$). The middle and the outer regions were not significantly different ($p = .958$).

The results from the posterior sclera is shown in Figure 30. As in the equatorial region, the 2-way ANOVA analysis indicated that there was a regional effect in the fibril mean diameter from the inner to the middle and the outer regions ($F(2,15) = 8.797$, $p = .003$). Bonferroni post hoc comparisons showed that the fibril diameter was smaller in the inner region compared to the middle ($p = 0.017$) and to the outer region ($p = 0.004$). The middle and the outer regions were not significantly different ($p = 1.0$).

In Figure 29 and Figure 30, the results from the deprived and the recovery groups are also summarized. As can be observed, there were no significant differences in the treated eyes of both the deprived and the recovery

Figure 29. Summary of mean collagen fibril diameter in the equatorial sclera. The mean fibril diameter at the equatorial sclera is divided into the inner, middle, and outer third regions and plotted throughout the development. The fibril diameters changed very little with increasing age at all three regions. The fibril diameter was reduced in the inner third region when compared to the middle third region of the sclera. The middle and the outer third regions were not different. The differences between the deprived and the control eyes were not significant in both the deprived and the recovery groups.

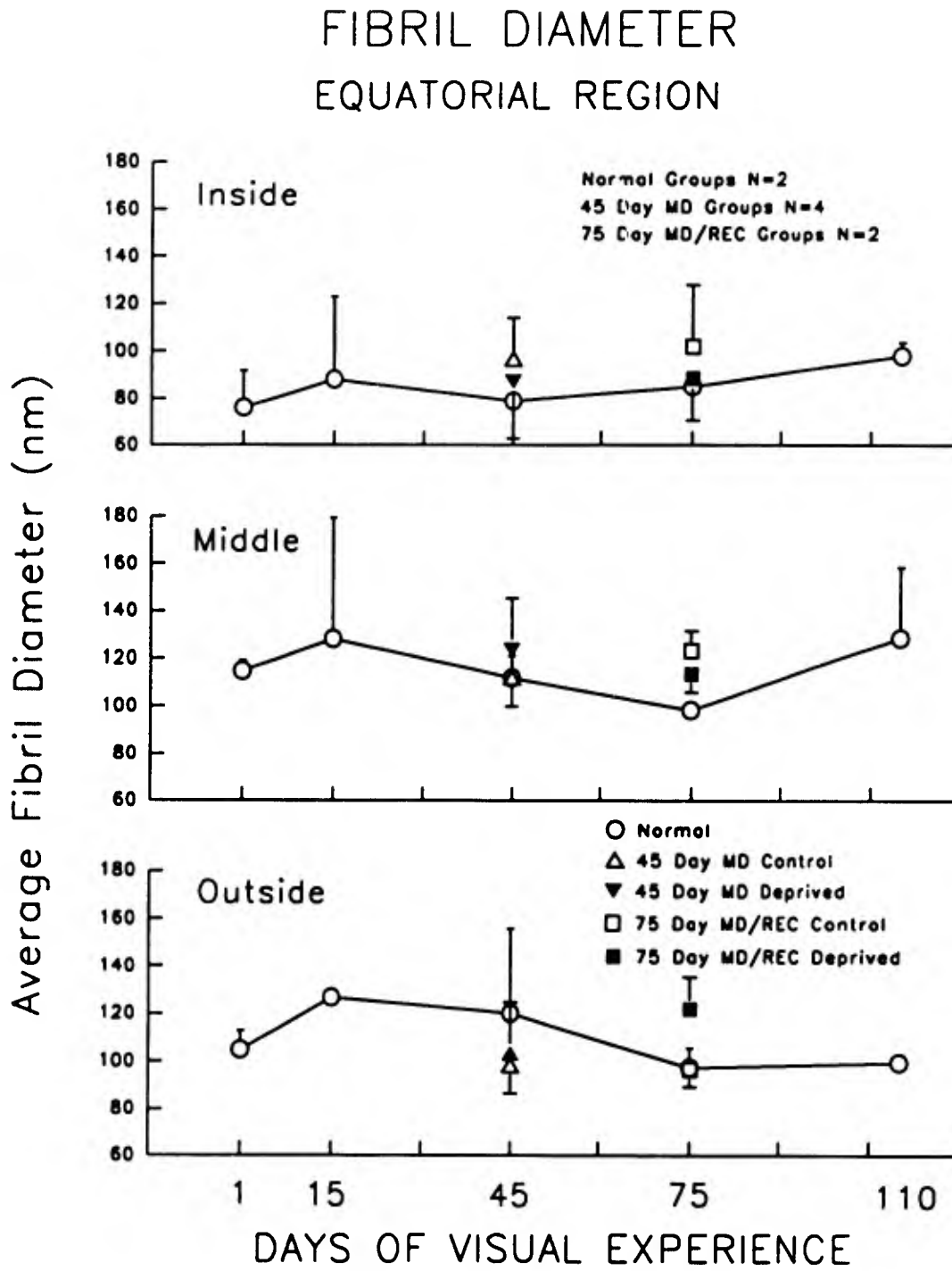
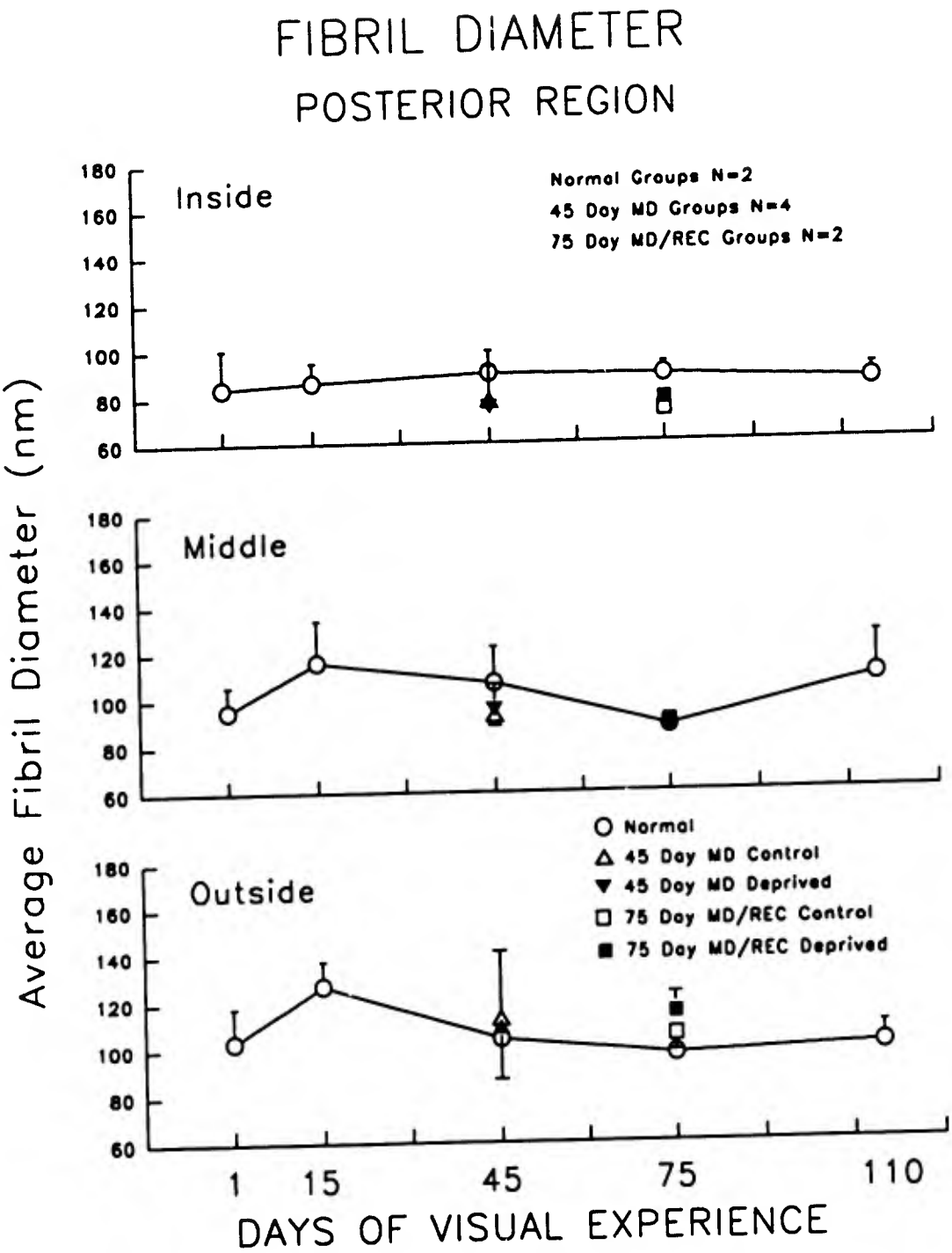

FIBRIL DIAMETER
EQUATORIAL REGION
Inside
Middle
Outside
Normal Groups N=2
45 Day MD Groups N=4
75 Day MD/REC Groups N=2
Normal
45 Day MD Control
45 Day MD Deprived
75 Day MD/REC Control
75 Day MD/REC Deprived
Average Fibril Diameter (nm)
180
160
140
120
100
80
60
1
15
45
75
110
DAYS OF VISUAL EXPERIENCE

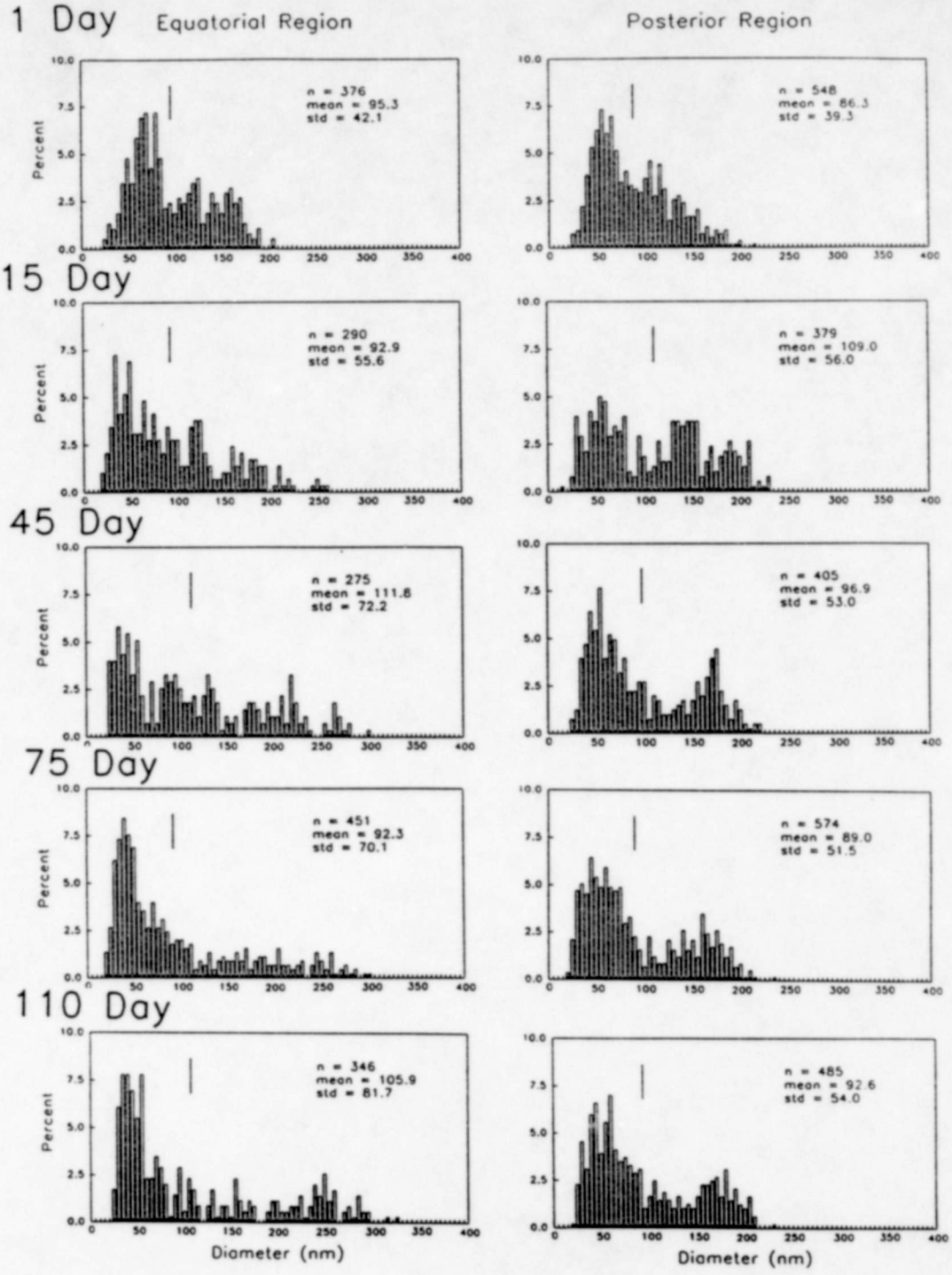
Figure 30. Summary of mean collagen fibril diameter in the posterior sclera. The mean fibril diameter at the posterior sclera is divided into the inner, middle, and outer third regions and plotted throughout the development. As in the equatorial sclera (Figure 29), the mean fibril diameter changed very little with increasing age at all three regions. The fibril diameter in the inner third region was reduced when compared to the middle or the outer third regions. There were no significant changes in the deprived or the recovery groups.


FIBRIL DIAMETER
POSTERIOR REGION
Inside
Middle
Outside
Normal Groups N=2
45 Day MD Groups N=4
75 Day MD/REC Groups N=2
Normal
45 Day MD Control
45 Day MD Deprived
75 Day MD/REC Control
75 Day MD/REC Deprived
Average Fibril Diameter (nm)
180
160
140
120
100
80
60
1
15
45
75
110
DAYS OF VISUAL EXPERIENCE

groups when compared to the control eyes in their group, or in the control eyes of these groups when compared to the age matched normal eyes (t tests).

Another aspect of the collagen fibril diameter results evaluated was the fibril diameter distributions. An example which shows the development pattern in fibril diameter distribution in 5 normal animals in the normal groups is shown in Figure 31. Because of the variability problem discussed above (Figure 26), statistical tests (Kolmogorov-Smirnov 2-sample test of cumulative distributions) yielded mixed results which made interpretations difficult. However, as can be seen in Figure 31, in both the equatorial and the posterior regions, it appeared that there was an increase in the dispersion of the distributions (the extremes of diameter are more widely separated) and an increase in the "bimodality" of the distributions. In the equatorial region, there appeared to be an increase in the number of both small and large fibrils with increasing age which made the distributions to appear more dispersed with increasing age. The fibril diameter distribution in the equatorial sclera was already bimodal in appearance at 1 days v.e. In contrast, in the posterior sclera, the bimodal appearance was not observed until later in the 15 or 45 days v.e. animals. Also, the fibril diameter distributions in the posterior region appeared to be less dispersed compared to the equatorial regions in all age groups. In particular, in the 75 and the 110 day v.e. animals, there appeared to be a fewer number of large fibrils in the posterior region compared to the equatorial region.

Figure 31. Development of the collagen fibril diameter distributions in normal animals, shown for one animal from each normal group. Histograms of fibril diameter distributions in the equatorial (left side) and in the posterior (right side) sclera are plotted. In both the equatorial and the posterior regions the fibril diameter distributions become more broadly distributed and bimodal in appearance with increasing age. The two scleral locations differ from the day that tree shrews open their eyes (1 day group). The differences between the scleral locations were significant in all age groups (Kolmogorov-Smirnov two sample test, $p < .05$).


1 Day
Equatorial Region
Posterior Region
n = 376
mean = 95.3
std = 42.1
n = 548
mean = 86.3
std = 39.3
15 Day
n = 290
mean = 92.9
std = 55.6
n = 379
mean = 109.0
std = 56.0
45 Day
n = 275
mean = 111.8
std = 72.2
n = 405
mean = 96.9
std = 53.0
75 Day
n = 451
mean = 92.3
std = 70.1
n = 574
mean = 89.0
std = 51.5
110 Day
n = 346
mean = 105.9
std = 81.7
n = 485
mean = 92.6
std = 54.0
Percent
Diameter (nm)

In the deprived group, fibril diameter distribution analysis also yielded mixed results, suggesting no consistent, large effect. For example, Figure 32 shows an example of fibril diameter distribution comparisons made between the deprived and the control eyes of the two deprived group animals. In the first animal shown in Figure 32 (top), the fibril diameter distributions at both the equatorial and the posterior region were not significantly different (Kolmogrov-Smirnov two sample test; $p > .05$). In the second animal, however, the distributions in the deprived and the control eyes were significantly different in both the equatorial region and the posterior region ($p < 0.05$). These results suggested that the possible differences between the deprived and the control eyes in the deprived groups were perhaps smaller than the large variability observed between the animals. Thus, given the small number of animals in this study, the possible differences may have been beyond the resolution available.

The results of fibril diameter distribution analysis in the recovery group, as in the case of the deprived group, yielded similar mixed results. An example of comparison of distributions in two recovery animals is shown in Figure 33.

Taken together, the results of fibril diameter distributions indicated that the fibrils were changing as a function of age, but these changes were rather complicated so that the analysis of the mean diameter alone was perhaps not sufficient to show the full extent of these changes.

Figure 32. Comparison of the collagen fibril distributions in the deprived animals. As an example, in two of the deprived animals, histograms of the fibril diameter distributions at both the equatorial and the posterior sclera are plotted. In the first animal (top), the histograms are rather similar at both the equatorial and the posterior sclera (Kolmogorov-Smirnov two sample test, $p > .05$). In the second animal (bottom), the histograms are different at both the equatorial and the posterior sclera. There was a lot of variability among the animals in the deprived group, suggesting no consistent, large effect.

FIBRIL DIAMETER DISTRIBUTION

45 MD

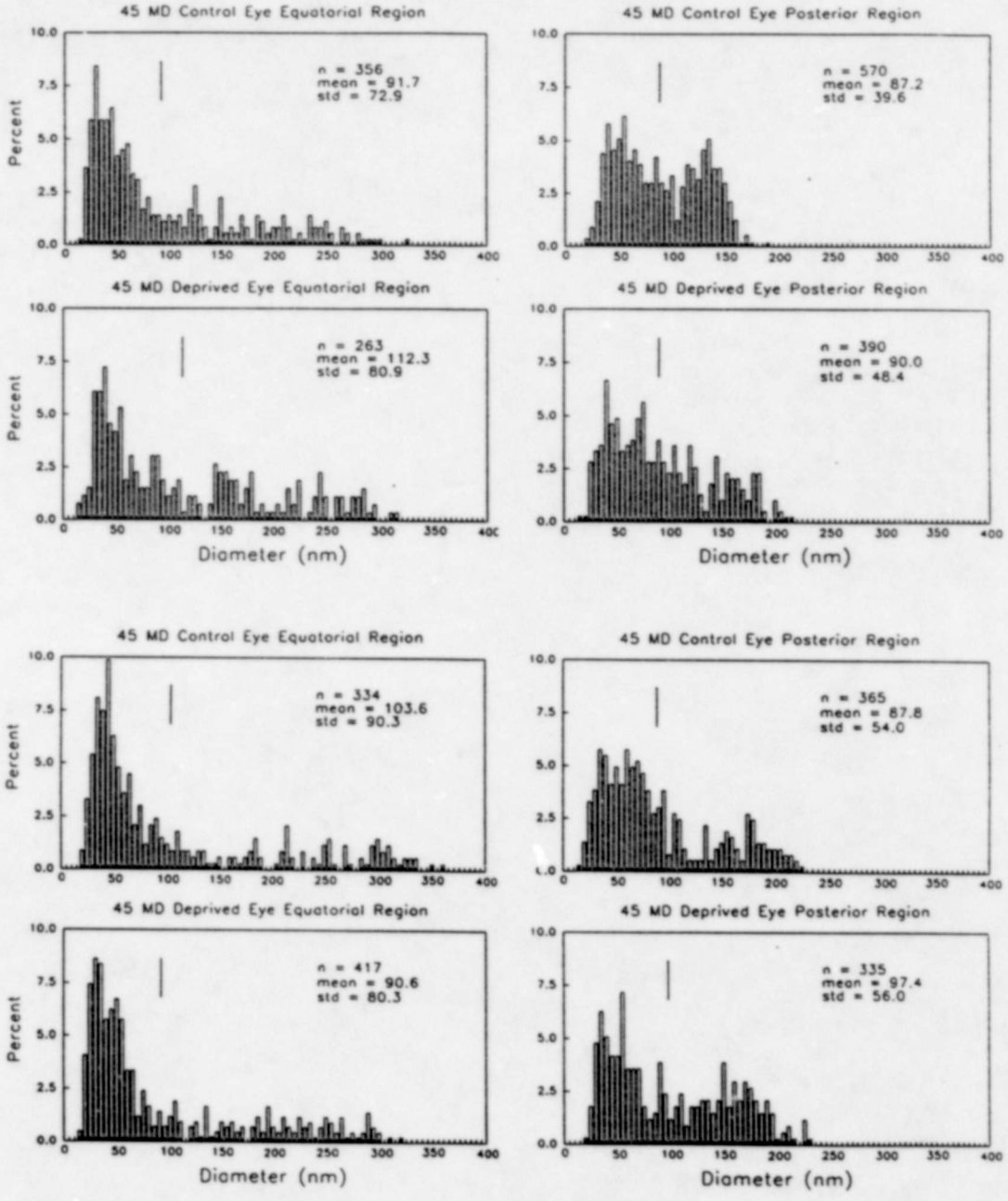
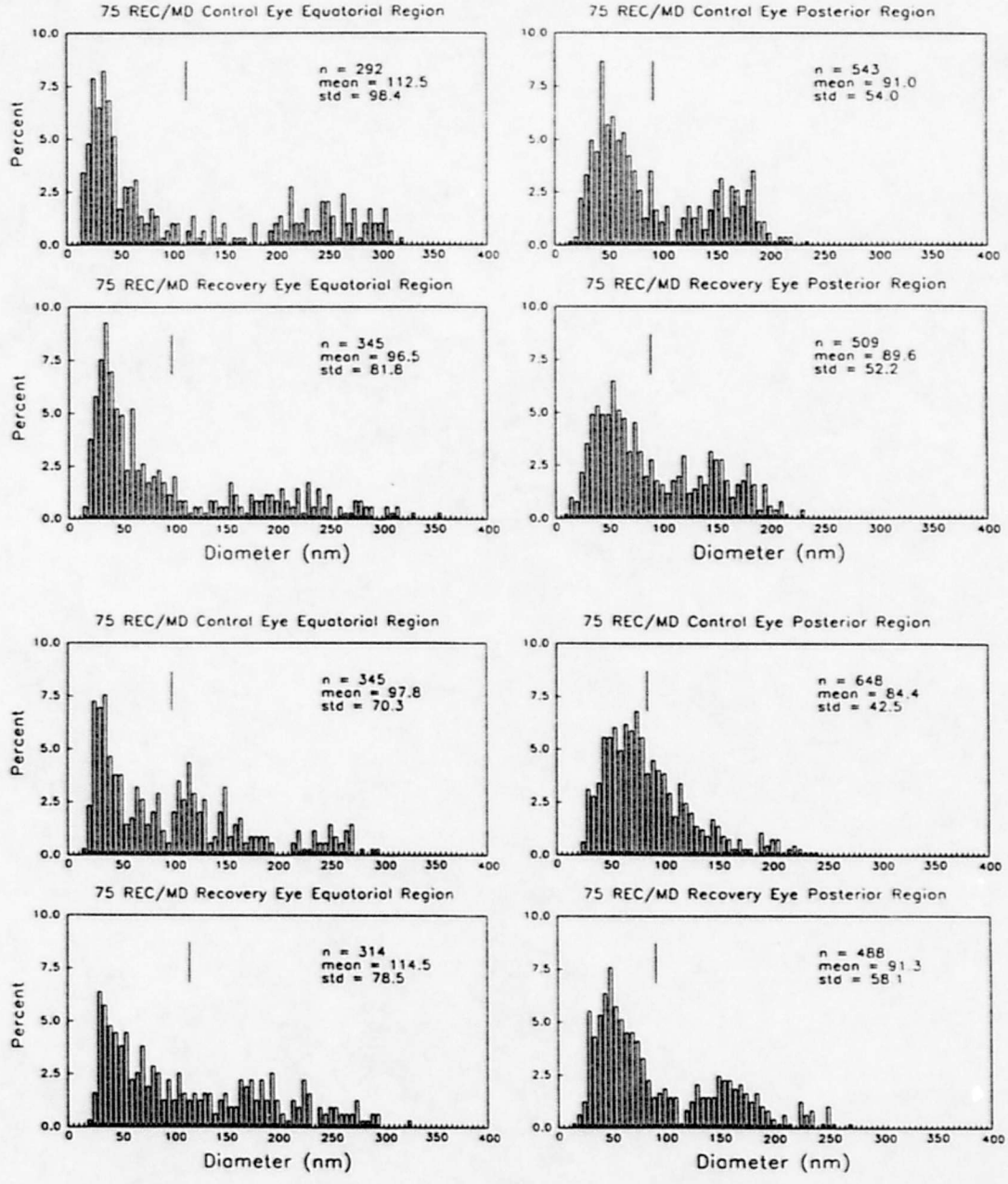


Figure 33. Comparison of the collagen fibril distributions in the recovery group. As an example, in two of the recovery animals, histograms of the fibril diameter distributions at both the equatorial and the posterior sclera are plotted. As in the case of the deprived group (Figure 32), there were no large, consistent effects in the recovery group.

FIBRIL DIAMETER DISTRIBUTION

75 REC



Nearest Neighbor Analysis

The results of the nearest neighbor analysis are summarized in Figure 34. The 1-way ANOVA analysis indicated that, in the equatorial region, there were no significant changes during development ($F(4,5) = 0.767$, $p = .59$). In the posterior region, however, the nearest neighbor distance was significantly different in the normal developing animals ($F(4,5) = 5.684$, $p = .042$). Tukey's post hoc comparisons indicated that the 15 days v.e. animals was different compared to the 75 days v.e. group ($p = 0.013$). In the deprived group, as can be observed, there was not a significant difference between the deprived and the control eyes. In the recovery group, it appeared that, in the equatorial region, both the recovering and the control eyes might be different when compared to the age matched normal eyes. However, these differences were not significant (independent t test, $p < .05$).

Overall, the nearest neighbor results appeared to be closely related to the fibril diameter results (Figure 28), suggesting that the distance from one fibril to its nearest neighbor was related to its size. Smaller fibrils appeared to be more closely distributed compared to larger fibrils.

Cross Sectional Area of Fibrils Relative to Non-fibrillar Area

The results from cross sectional area measurements are summarized in Figure 35. The cross sectional area of collagen fibrils to background ratio appeared to be increasing during development. The 1-way ANOVA analysis confirmed that the cross sectional ratio of fibrils were changing with increasing age ($F(4,5) = 23.51$, $p = .002$). Tukey's post hoc comparisons indicated that the

Figure 34. Summary of the collagen fibril nearest neighbor distance. The mean nearest neighbor distance is plotted for both the equatorial (top) and the posterior (bottom) regions of the sclera throughout the development. At both scleral regions, the distance between fibrils changed little with increasing age. There were no significant changes in the deprived and the recovery groups.

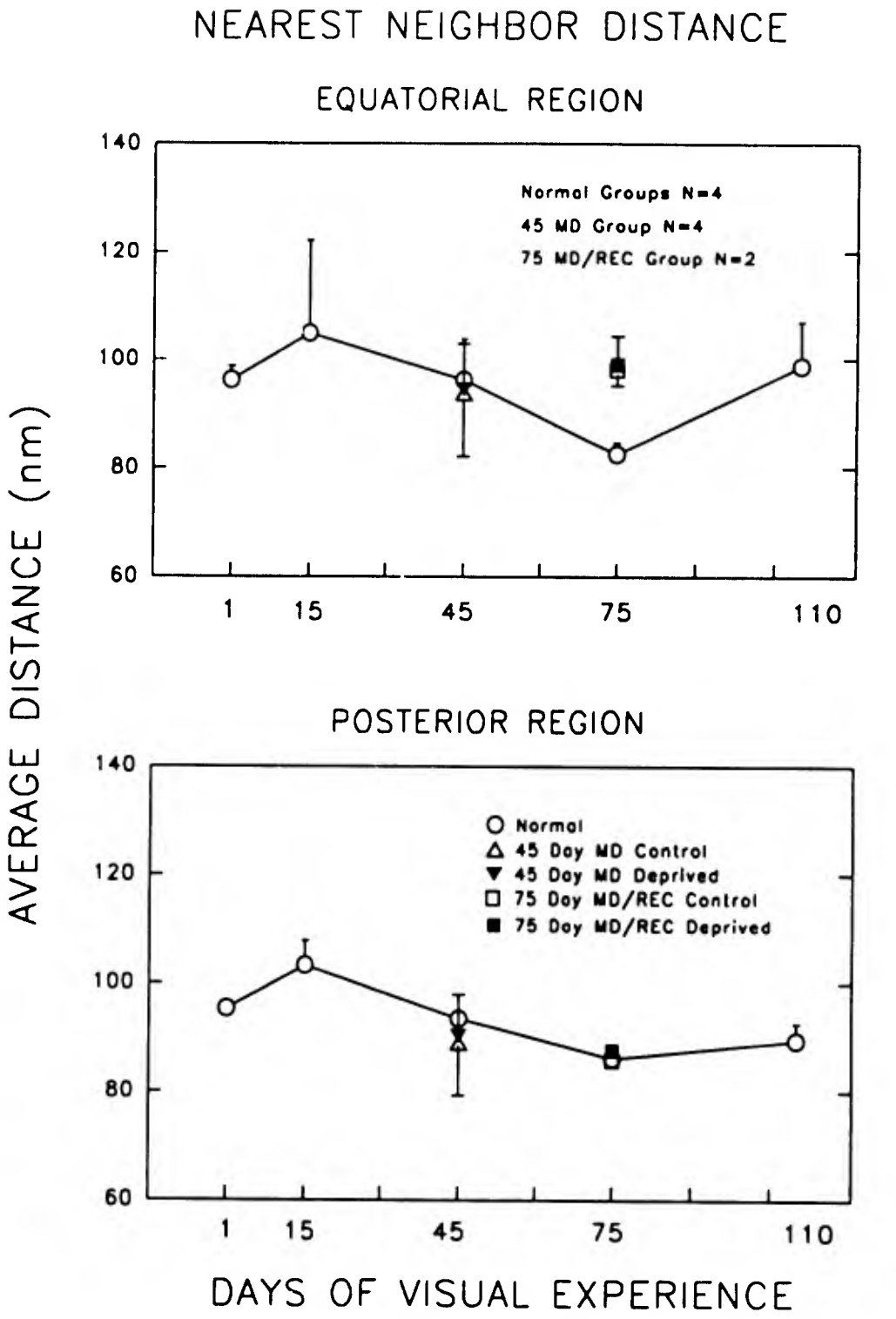
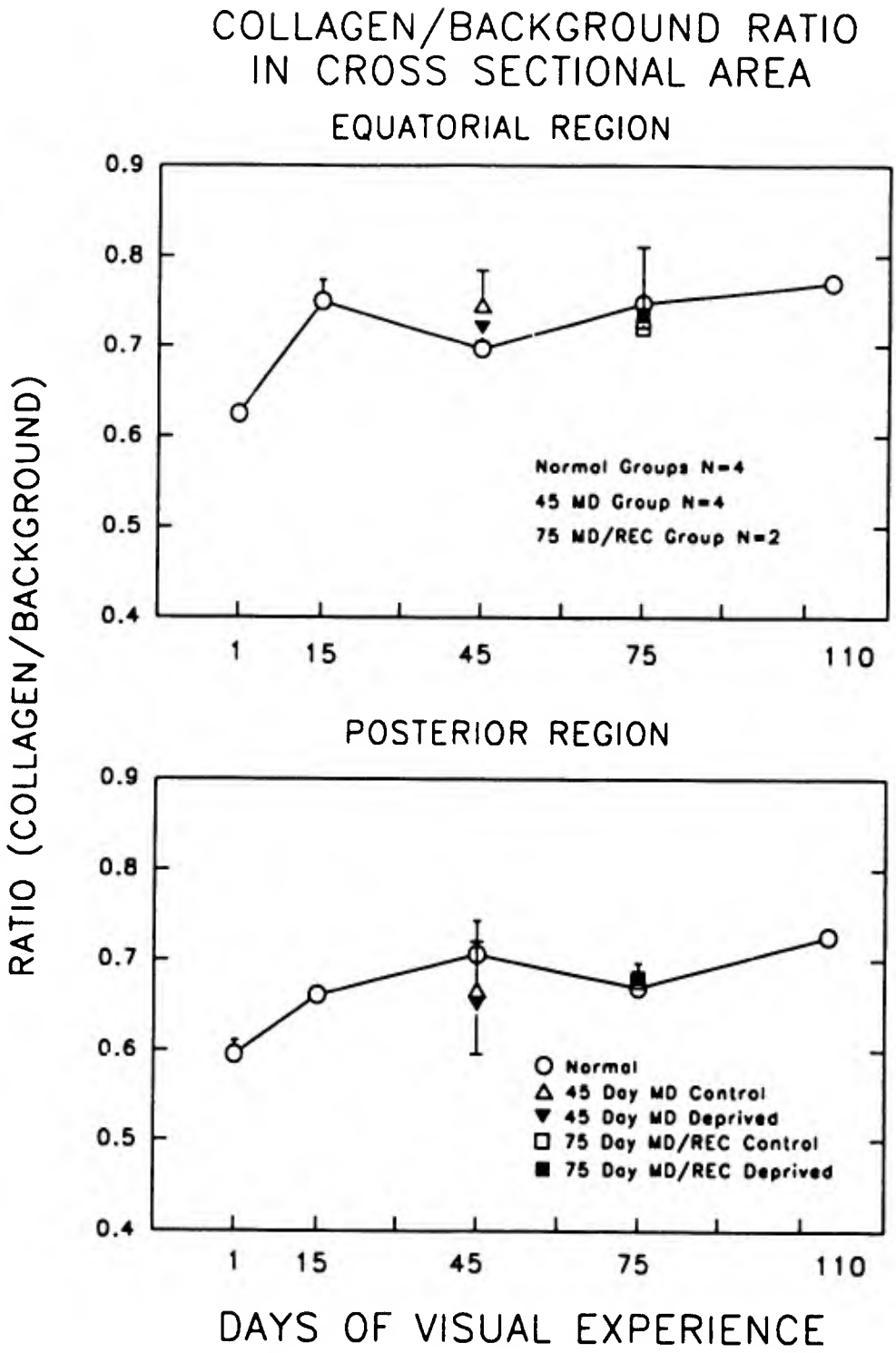

NEAREST NEIGHBOR DISTANCE
EQUATORIAL REGION
Normal Groups N=4
45 MD Group N=4
75 MD/REC Group N=2
140
120
100
80
60
1
15
45
75
110
POSTERIOR REGION
Normal
45 Day MD Control
45 Day MD Deprived
75 Day MD/REC Control
75 Day MD/REC Deprived
AVERAGE DISTANCE (nm)
DAYS OF VISUAL EXPERIENCE

Figure 35. Summary of the collagen to background ratio in cross sectional area. The ratio of collagen to background (possibly a rough indicator of collagen to proteoglycan ratio within a lamellae) is plotted for both the equatorial (top) and the posterior (bottom) regions of the sclera throughout the development. At the equatorial region, the observed increase between the 1 and 15 days v.e. groups was significant. In the posterior region, the collagen to background ratio changed very little with increasing age. The data suggest that in both the deprived and the recovery groups, there were no large, consistent changes in this measure.


COLLAGEN/BACKGROUND RATIO
IN CROSS SECTIONAL AREA
EQUATORIAL REGION
0.9
0.8
0.7
0.6
0.5
0.4
Normal Groups N=4
45 MD Group N=4
75 MD/REC Group N=2
1
15
45
75
110
POSTERIOR REGION
0.9
0.8
0.7
0.6
0.5
0.4
Normal
45 Day MD Control
45 Day MD Deprived
75 Day MD/REC Control
75 Day MD/REC Deprived
1
15
45
75
110
RATIO (COLLAGEN/BACKGROUND)
DAYS OF VISUAL EXPERIENCE

1 day v.e. group was different compared to all the other normal groups ($p < 0.005$). In the posterior region, however, the changes were not significant ($F(4,5) = 4.947$, $p = .06$). Comparing the two scleral locations, the 2-way ANOVA analysis indicated that the equatorial and the posterior locations were significantly different ($F(1,10) = 16.352$, $p = .002$). As can be observed, the treated eyes in both the deprived and the recovery groups were not significantly different when compared to the control eyes in their group (t tests).

Overall, the results from the high magnification analysis of collagen fibrils suggest that the fibril morphology or the pattern of distribution in the lamellae are not significantly affected by the visual deprivation. This is significant because it suggests that, in a manner which is consistent with the results from the lamellar organization analysis, the collagen fibril need not be affected to result in the sclera which is perhaps biomechanically weaker. In tree shrews, it has been reported recently (Siegwart & Norton, 1994) that, even after as little as 4 days of deprivation, the creep (elongation of tissue under constant stress) in the sclera of deprived eyes was increased compared to the control eyes.

DISCUSSION

In the Introduction, the idea was introduced that there is an active, vision dependent emmetropization mechanism guiding the axial length elongation to match the focal length of the eye during normal development. A model of emmetropization was also introduced suggesting that the choroid and/or the sclera might have important roles in the emmetropization mechanism. In particular, it was suggested that the resistance to the intraocular pressure (IOP) provided by the sclera might be related to the rate of extracellular matrix synthesis and/or accumulation in the sclera. In this regard, the morphometric evidence obtained in this study is consistent with the idea of emmetropization during normal development and supports the model of emmetropization.

When a retinally-generated signal "tells" the eye to stop elongating, what "listens" to the signal? The results in this study suggest that both the choroid and the sclera might be listening. The finding that the choroidal thickness might be decreased in the deprivation-induced myopic eyes and increased in the eyes recovering from induced myopia suggests that the choroid might have a more active role in emmetropization mechanism, rather than just being a "messenger." The finding that, in the sclera, the fibroblast may be the component of the lamellae which is affected the most by the deprivation, rather

than the collagen fibrils in the lamellae, at least after 21 days of deprivation, suggests that the metabolic rate of fibroblasts, particularly in terms of what they are making, in the sclera might be an important factor in emmetropization mechanism as well as in deprivation-induced myopia.

Morphometric and Refractive Measurements

The results from the A-scan ultrasound and retinoscopy measurements were important, and served as the foundation to which the results from the morphometric measurements could be compared. With such a comparison, it was shown that the procedures used in this study did not affect the structures of the eye significantly, and that the morphometric measurements were indeed accurate and reliable.

In addition, the A-scan ultrasound and retinoscopy results demonstrated that the animals in the normal groups were normal in their ocular development and that their eyes were indeed emmtropizing. In the deprived group, as expected, the deprived eyes were found to be axially elongated with increased vitreous chamber depth when compared to the fellow control eyes. In the recovery group, the results showed that the recovering eyes did "recover" in axial length and refraction. The control eyes in these treated groups were also found to be normal when compared to the age matched normal eyes.

Light Level Analysis

There are three main findings from the light level analysis. One is that the results showed morphologically that the sclera in the deprived eyes was displaced posteriorly compared to the control eyes. This finding confirms the

A-scan ultrasound measurements. The other important finding is that the choroid in tree shrews was found to be affected in both the deprived and the recovering eyes, suggesting that the choroid in mammals, as in chicks, might also be an important part of the emmetropization mechanism. Evaluation of the wave forms from the A-scan ultrasound supported the morphological finding of the choroidal thickness changes. The third and probably the most important finding was that the sclera was found to affected in the deprived and the recovery eyes in a way that suggested that the extracellular matrix synthesis and/or accumulation in the sclera might be an important part of the emmetropization mechanism. The sclera is thinner and reduced in volume in the axially elongated and myopic deprived eyes.

Scleral Location

The most obvious finding in the scleral location measurements is that the sclera in the deprived eyes was indeed displaced posteriorly compared to the control or to the age matched normal eyes. In addition, the results in the recovery group showed that, during the recovery, the scleral position in the recovery eyes was held in place, while the control eyes continued elongating.

Taken together, the above findings suggest that the sclera might be actively regulated during normal development and that this regulation might be important in controlling the axial elongation. The finding that the deprived eyes were elongated when compared to the control eyes or to the age matched normal eyes clearly indicates that the sclera in tree shrews is capable of elongating faster than seen in the normal developing eyes. This suggests that

during normal development, axial elongation may be actively restrained so that it could be gradually matched to the focal length of the eye. The other important finding from the above results is that the sclera in tree shrews is not only capable of faster elongation, but is also capable of slowing its elongation, to an almost stop, as seen in the recovering eyes. Thus, the emmetropization mechanism in tree shrews is a robust phenomenon able to control the axial length elongation actively and precisely.

Choroidal Thickness and Cross Sectional Area

As discussed in the Introduction, the emmetropization mechanism appears to be a vision dependent mechanism. Therefore, any retinal signal intended for the sclera must cross the choroid. In this regard, the results in the choroidal thickness and choroidal cross sectional area measurements are important. As shown in Figure 12, the choroid was affected in both the deprived and the recovery eyes compared to the control eyes. The choroid in the recovery eyes in the recovery group was significantly thicker (46.2%) and increased in cross sectional area (43.3%) when compared to the deprived eyes in the deprived group. These changes suggest that the choroid in tree shrews could be regulated in its thickness and "volume," as indicated by the cross sectional area, in response to the changes in the visual environment. In addition, the above results suggest that the choroidal thickness changes might be a part of emmetropization mechanism. The increase in the choroidal thickness could serve, during the recovery, to push the retinal focal plane

forward, partially compensating for the increase in the axial length, at least until the "recovery" in the scleral position is completed.

The increase in the choroid thickness in the recovery eyes compared to the deprived eyes, however, could be a by-product of increased blood flow rate. Althougn it is not known if the choroidal blood flow rate in tree shrews is affected in either the deprivation-induced myopic eyes or in the eyes recovering from induced myopia, in chicks, the choroidal blood flow rate is reduced in the deprived eyes (Reiner et al., 1991; Shih et al., 1992). Also, there is evidence that the metabolism in the sclera of deprived eyes might be reduced compared to the control eyes (Reeder & McBrien, 1994). Therefore, although the differences were not significant, the slight increases in scleral thickness (15.2%) and in cross sectional area (11.6%) in the recovering eyes compared to the deprived eyes suggest that, at the least, the metabolic rate in the sclera of the recovering eyes might be higher when compared to the deprived eyes. The higher metabolic rate in the recovering eyes might require higher choroidal blood flow rate.

Whether the choroid is actively regulated or passively changed, the results from this study have provided the evidence that in tree shrews, as in chicks, the choroid might have an important and more active than previously believed role in emmetropization process. The results also re-emphasizes the need for additional studies of the choroid in experimental myopia research.

Scleral Thickness and Cross Sectional Area

There are three important findings in the scleral thickness and scleral cross sectional area results. One is that, in the normal animals, the scleral thickness was found to be increasing with age (Figure 13), in a pattern which was similar to rate of axial elongation (Figure 8). The other finding is that, in the deprived eyes, the sclera was thinner when compared to either the control eyes or the age matched normal eyes. The cross sectional area results indicated that the scleral thinning in the deprived was not a result of the sclera being stretched to cover a larger eye. There was less scleral "stuff" in the deprived eyes. The third important finding is that, in the recovering eyes, the sclera was no longer thinner compared to the control eyes, suggesting "recovery" in the scleral thickness. These results suggest that there is an active remodelling of the extracellular matrix in the deprived and the recovering eyes as reflected in their scleral thickness changes. Also, these results match the results in the lamellar organization analysis at the EM level which suggested that the fibroblast component in the sclera might be decreased in the deprived eyes and increased in the recovering eyes.

The results in the normal developing animals are significant in two aspects. As expected, an increase in the average scleral thickness with increasing age (Figure 13) was found. The results showed that the rate of scleral thickness increase with increasing age was not constant. In fact, the scleral thickness increased rapidly between 1 and 45 days v.e., and did not change much after 45 days v.e. to 110 days v.e. group. As suggested in the

Introduction, if the gradual increase in the axial length after the initial rapid elongation represents the process of gradually matching the axial length to the focal length by regulating the axial elongation rate, the similar pattern of scleral thickness development suggests that, as the model of emmetropization suggests, the scleral extracellular matrix synthesis and/or accumulation may be related to the control of axial length elongation.

Another important aspect of the results in the normal animals is the development of regional differences in scleral thickness observed with increasing age. As shown in Figure 14, the regional differences appeared to increase with increasing age. The results indicated that the increase in the scleral thickness in the mid-peripheral regions was greater than the increase in the posterior region. It is not clear, however, what the purpose of such large regional differences might be, other than to suggest that these are the locations where the extraocular muscles attach and, therefore, needing the extra thickness to withstand the stresses exerted by these muscles.

The results in the deprived groups are significant for three reasons. One is that the results provided the morphological data confirming that, in tree shrews, the sclera in the deprived eyes are indeed thinner and reduced in volume compared to the control eyes. In chicks, as described earlier, the sclera in the deprived eyes grow with increased DNA, protein, and proteoglycan synthesis (Christenson & Wallman, 1991; Rada et al., 1991). Thus, unlike chicks, in tree shrews the sclera in the deprived eyes might be remodelled with reduced synthesis and/or accumulation of extracellular matrix. Does the

reduced thickness in the sclera, a fibrous tissue, indicate that it might be biomechanically weaker? Indeed, Siegwart and Norton (1994) found greatly increased creep in the sclera of deprived eyes compared to the control eyes.

The results indicate that the regional differences in scleral thickness might be important in axial elongation. The changes seen in the regional scleral thickness in the control and the deprived eyes in the deprived groups were different when compared to each other (Figure 15). Both eyes were also different when compared to the age matched normal eyes. The scleral thickness differences between the control and the normal eyes were primarily in the mid-periphery regions, whereas the differences between the deprived eyes and the control eyes were in the posterior region. It might be suggested that the differences in the scleral thickness in the posterior region are more important than the differences in the mid-periphery. Norton et al. (1992) has found that in the deprived tree shrew eyes, collagen content in the sclera was reduced only in the posterior region and not in the other regions when compared to the same regions in the control eyes. These results suggest that the development of regional thickness might be important in deprivation-induced myopia development. In this regard, as indicated earlier, it is interesting to note that the scleral thickness and regional thickness differences seen in the deprived eyes of the deprived group resembled the 15 days v.e. normal animals.

An interesting question which might be raised here is why the control eyes in both the deprived and the recovery groups which showed a slight

decrease in the scleral thickness and cross sectional area compared to the age matched normal eyes, were not axially elongated. The fact that these control eyes were not elongated compared to the age matched normal eyes suggests, again, that it may the scleral thickness in the posterior region which is important, rather than the overall scleral thickness. Indeed, the results indicated that, in the posterior pole, the scleral thickness in the control eyes of both the deprived and the recovery groups were similar when compared to the age matched normal eyes.

The results in the recovery group are important in that they suggest the scleral thickness, like the axial length, might also "recover" from the thinning observed in the deprived eyes. This finding indicates active regulation of the sclera. In the recovering eyes, the differences in the scleral thickness were no longer significant when compared to the control eyes. Siegwart and Norton (1994) found that, in tree shrews, the creep also recovers in the recovering eyes so that it was no longer increased compared to the control eyes.

Electron Microscopic Level Analysis

One of the main questions addressed in the EM level analysis was how the sclera, in terms of its lamellar organization and collagen fibril morphology, developed during normal development. The other question addressed was, given the scleral thinning observed at the light level analysis, how this scleral thinning might be accounted for by the lamellar organization and in the collagen fibril morphology.

Results in the normal animals suggest that the sclera becomes more fibrous as it increases in thickness with increasing age. The results indicate that the relative portion of the sclera occupied by the lamellae component increased with age, partly from the accumulation of material but also from the decrease in the fibroblast and "space" components in older animals. The results also indicate that the collagen fibrils were changing, in a rather complicated way, so that the distribution of fibrils becomes more broad and bimodal in appearance with increasing age.

Results in the deprived and the recovery groups suggest that it was the decreases in the fibroblast and the "space" components in the sclera rather than the lamellae component which was primarily responsible for the scleral thinning observed at the light level. This is a significant finding because it suggests a more active role for the fibroblasts than previously thought in the remodelling of the sclera in the deprived eyes. In addition, the results also suggest that short term changes in the collagen fibrils within the lamellae may not be as important as previously believed in the development of axial elongation or scleral thinning in the deprived eyes. The 21 days of deprivation might not have been sufficient to produce significant changes in the collagen fibril morphology. Therefore, and perhaps not surprisingly, the results from the measurements of collagen fibrils indicated that the fibrils were not significantly changed in the deprived eyes when compared to the control eyes.

Lamellar Organization

The results indicated that the fibroblast component is relatively high in the 1 day v.e. animals and remained similar through 45 days v.e. group, suggesting that the fibroblast activity, and, thus, the synthesis and/or accumulation of extracellular matrix might be greater during this early development. During this period, the axial length as well as the scleral thickness and volume also increased rapidly. In this regard, it is significant that the expression of mRNA for type I collagen in the normal developing tree shrews was the highest in 1 day v.e. animals, reduced by half in the 45 days v.e. animals, and reduced to 0% by the 75 days v.e. animals (Zorn et al., 1992) Overall, these results suggest that affecting the metabolic rate of fibroblasts during early development might have a propound effects on the development of the sclera.

In the deprived group, as would be expected, the most significant finding in the lamellar organization results was the changes observed in the fibroblast and "space" components in the sclera of the deprived eyes compared to the control eyes. This is significant in that, as mentioned above, it suggests that metabolic rate of the fibroblasts might be reduced in the deprived eyes and, thus, the proteoglycans which may be occupying the "space" between the lamellae might be reduced in amount. It needs to be pointed out, however, that the "fibroblast" thickness measured in this study may not be related to the number or the activity level of the fibroblasts. In the sclera, the fibroblasts appear to have rather complex 3-dimensional shape with many cellular

processes that branch out to fill the spaces between the lamellae. However, the data in this study are consistent with the reported findings that, in the deprived tree shrew eyes, the metabolic rate is decreased (Reeder & McBrien, 1994) and the level of proteoglycans is decreased (Norton et al., 1992).

The "space" as measured in this study represents the spaces between the lamellae. As suggested above, if these spaces are occupied by proteoglycans, the results suggest that the regulation of the proteoglycans in the sclera might be important, perhaps more so than the collagen fibrils, in the deprivation-induced myopia. In the sclera of deprived tree shrew eyes, even after as short as 4 days of deprivation, the creep rate has been found to be increased when compared to the control eyes (Siegwart & Norton, 1994). Therefore, it is tempting to suggest that the increased creep found in the sclera of the deprived eyes may be largely due to the decrease in the proteoglycans in the "space" component. The lamellae in such a sclera may be, when stressed, more likely to slide past each other, resulting in the increased creep.

The results found in the recovery group are significant in that they are in agreement with the findings in the deprived group. Based on the above argument, one might expect that the fibroblasts and "space" components would be increased in the recovering eyes when compared to deprived eyes. The results summarized in Figure 21 has shown that, indeed, such was the case.

Taken together, the results in lamellar organization indicate the need to identify the types of proteoglycans that might be present in the mammalian

sclera, where specific types of proteoglycans might be located within the sclera, and how they might be affected in the deprivation-induced myopic eyes.

Number of Collagen Fibrils

There may be several reasons why, overall, the results from the measurements of the fibril density, diameter, nearest neighbor distance, and cross sectional area ratio to background failed to show any significant changes in the normal developing animals or in the treated eyes of the deprived and the recovery groups. One reason might be, as discussed earlier, that the collagen fibrils are not involved in deprivation-induce myopia development. More likely reason might be, however, that the duration of deprivation in this study was not sufficient to result in the morphological changes in collagen fibrils. In monkeys where collagen fibril morphological changes (fibrils were reduced in diameter) associated with deprivation-induced myopia have been reported, the duration of deprivation was between 1 to 2 years (Funata & Tokoro, 1990). In tree shrews, also, this association between the reduction in fibril diameter and deprivation-induced myopia has been found but only in the animals which were deprived for long term (up to 11 months) (Cornell & McBrien, 1994).

Another reason for the results in the fibril measurements might be that the method of selection used in choosing the area of the sclera for fibril cross sections for photomicrographs was biased. To find a large enough area of fibril cross sections so that a meaningful number of fibrils could be measured, it was necessary to limit the search to the middle of the larger lamellae. It may be

that the larger lamellae are the "older" ones which were formed perhaps before the deprivation was applied and, thus, less likely to be affected by or to show the effects of deprivation than the smaller lamellae might have.

The results in the number of collagen fibril measurements are important in two aspects. The one important finding in the results was that during the normal development, the fibril density was found to be rather stable at both of the scleral locations. Taken together with the thickness and number of lamellae results, this finding suggests that the fibrils inside the lamellae might not be changing greatly during the development. The results also demonstrated that during development, the fibril density was higher in the posterior region than in the equatorial region. This is significant because it suggests that, in tree shrews, the equatorial and the posterior sclera might be different in their collagen fibril development.

The data in Figure 27 suggested that in both the deprived and the recovery groups, as would be expected, the fibril density was not affected significantly in the treated eyes when compared to the control eyes. As discussed earlier, this finding is in agreement with the data in the lamellar organization measurements. If the number of fibrils did not change significantly, then, how did the size of fibrils change?

Collagen Fibril Diameter

The data in Figure 28 suggest that the fibril size described as mean diameter did not change significantly during the normal development. Taken together with the fibril density data discussed above, it suggests that the

fibrils do not change greatly in their size or packing density within the lamellae. However, the data summarized in the histograms showing the distributions of diameter during the normal development (Figure 31) suggests that, indeed, the fibrils were changing in their sizes as shown by the changes in the distributions. The distribution data suggest that the relationship between the number and the size of fibrils during the development may be a complicated one. The one possible explanation of the data could be that the fibrils were changing in size relatively, so that some fibrils were increasing in size while others were decreasing in size.

Another important aspect of the fibril diameter results is the differences observed in the distribution of fibril diameter between the equatorial and the posterior sclera. Data in Figure 31 suggest that the fibrils in the equatorial sclera, as suggested in the Introduction, might be maturing earlier compared to the posterior sclera, as shown by the histograms which were more broadly distributed and appeared bimodal earlier in younger animals. For example, the fibril distribution of 1 day v.e. animal at the equatorial region already shows evidence of bimodal distribution and, when compared to the posterior fibril distribution, has more larger diameter fibrils. In contrast, in the same animal, the fibrils in the posterior region were more uniform in size, narrowly distributed, and lacked the bimodal appearance. This finding is in agreement with the finding that, in normal tree shrews, in 15 days and 45 days v.e. animals the expression of mRNA for type I and III collagen was higher in the posterior region when compared to the equatorial region.

In addition to the equatorial to posterior scleral location differences, the results indicate that there may be regional differences within each scleral location. In both scleral locations, the fibril size increased from the inner third region to the middle or the outer third region. This finding in tree shrews is in agreement with reported findings in monkeys (Funata & Tokoro, 1990) and in humans (Spitznas, 1971). These results in monkeys and in humans were, however, derived from the posterior sclera. The evidence of similar gradient found in both scleral locations suggest that, in this regard, the two scleral locations in tree shrews may be similar.

Overall, the data in the normal animals are consistent with the results in the lamellar organization. As would be expected, there were no significant changes in the deprived and the recovering eyes.

Nearest Neighbor Analysis

The results in the nearest neighbor analysis suggest that the nearest neighbor distance of the fibrils was related to the size of the fibrils. Evidence of this can be seen by comparing the data from the fibril diameter (Figure 28) and the data from the nearest neighbor distance (Figure 34) measurements. As discussed in the Methods, the nearest neighbor distance was measured from the center of one fibril to the center of other fibrils. This finding suggests that as some fibrils were changing in size their spatial relationship to the other fibrils also changed, so that distances between the outer surfaces did not change much. Proteoglycans are believed to play important roles not only in collagen fibril formation and growth (Borcherding et al., 1975; Poole, 1986) but

controlling the level of hydration and occupying space among fibrils and perhaps keeping them apart. As would be expected, based on the evidence discussed above, there were no significant changes found in either the deprived or the recovering eyes compared to the control eyes.

Cross Sectional Area of Fibrils Relative to Non-Fibrillar Area

Given the assumption that the space among fibrils was occupied by the proteoglycans, the ratio of collagen to background might at least provide a rough estimate of collagen/proteoglycan ratio. The results suggest that the collagen/proteoglycan relationship remained rather stable during development. Also, there were no significant changes found in either the deprived or the recovering eyes compared to the control eyes. The data is consistent with the evidence discussed above. The results suggest that the decrease in the proteoglycans in the sclera of the deprived tree shrew eyes (Norton et al., 1992) may reflect the changes in the proteoglycans outside the lamellae, most likely in the "space" between the lamellae.

CONCLUSIONS

The data in this study indicate that the choroid and the sclera are involved in the emmetropization mechanism. In the Introduction, a feedback model of emmetropization was introduced suggesting how the retinal activity dependent emmetropization mechanism could regulate axial length during normal development. The model suggested that by controlling the synthesis and/or accumulation of extracellular matrix in the sclera and, thus, affecting the resistive property of the sclera to the intraocular pressure the axial length could be regulated. The data in this study support the model. The data clearly indicate that the scleral thickness and the scleral volume are changed in both the deprivation-induced myopic eyes and in the eyes recovering from induced myopia in a manner which is consistent with remodelling of the sclera as suggested by the model. In addition, the data in this study indicate that the choroid might also be an integral part of the emmetropization process.

The Role of Choroid in Emmetropization

The data in this study indicate that the choroid has an active role in the emmetropization process. The role of the choroid may be more than to receive and transmit the retinally-generated signal to the sclera. The choroid itself may be affected by the signal. One way that the choroid might be affected and, hence, involved in emmetropization, may be through the changes in blood flow

rate, by affecting the metabolic rate of the fibroblasts in the sclera. Given that one of the roles of the choroid is to supply the vascular needs of the outer retina and the largely avascular sclera, it may be that any change in the choroid may reflect the changes in the metabolic rate of these tissues. Whether the choroidal thickness changes reflect the rate of metabolism in the sclera or may even be responsible for it, the effect on the sclera might be significant, particularly during the early development when the synthesis and accumulation of extracellular matrix is great. In chick, the choroidal blood flow is decreased in the deprived eyes (Reiner et al., 1991; Shih et al., 1992) compared to the control eyes. In humans, it has been reported that, even in adults, the choroid in the myopic eyes is thinner compared to the emmetropic eyes, suggesting that perhaps choroidal change might be a permanent feature in myopic eyes (Cheng et al., 1992).

Another way by which the choroid might participate in the emmetropization mechanism is by acting as an intermediate "accommodative" system (Wallman, 1993). By adjusting its thickness, the choroid may be able to partially compensate for the mismatch between the focal length and the axial length of the eye, at least until the recovery in the axial length is completed.

Based on the morphological data in this study, it is not possible to suggest how the choroid participate in the emmetropization process in tree shrews. The data indicate, however, that the choroidal changes are related to the scleral changes seen in the deprived eyes.

The Role of Sclera in Emmetropization

The data in this study indicate that the sclera has a role in regulating axial length during development. In particular, the data indicate that biomechanical property of the sclera and, hence, its resistance to the intraocular pressure may be critical in normal ocular development and in deprivation-induced myopia. In this regard, the data support the model of emmetropization which suggest that the axial length of the eye is gradually matched to the focal length of the eye during normal development by controlling the rate of axial elongation by actively controlling the extracellular matrix synthesis and/or accumulation in the sclera.

The biomechanical property of the sclera is primarily derived from the collagen fibrils and proteoglycans in terms of their content, organization, and interaction. Thus, the development of the extracellular matrix in the sclera must be critical in determining the "viscoelastic property" of the sclera. The data in this study suggest that the organization of fibril bundles or lamellae may also be important in determining the "viscoelastic property" of the sclera. It is not known how the development of lamellar organization is controlled in normal development but the data in this study indicate that it may be related to the metabolic rate of the fibroblasts in the sclera. In particular, the data suggest that, in the deprivation-induced myopic eyes, the proteoglycans in the "space" between the lamellae might be critical in determining the creep component of biomechanical property of the sclera. In the deprived eyes, these "space" proteoglycans may be more decreased than the proteoglycans inside the

lamellae and such a change might result in a sclera with lamellae which are not held tightly to each other and becoming more "creepy".

The morphometric data from collagen fibrils indicate that the development of fibrils in the sclera may not be critical in the development of the short term deprivation-induced myopia. At the least, the data suggest that the 21 days of deprivation in tree shrews was not sufficiently long enough in duration to affect the collagen fibril morphology. This finding is consistent with the finding that the sclera in the deprived tree shrews is increased in creep after only 4 days of deprivation (Siegwart & Norton, 1994). It may be argued that the 4 days of deprivation was not long enough to cause any significant changes in the fibrils inside the lamellae but may have been long enough to cause a change in the amount or the composition of proteoglycans in the "space" between the lamellae. Evidence for this may also be found in the finding that the lathyritic agent which blocks cross linkage formation between newly formed collagen fibrils did not cause axial elongation or myopia in the normal eyes.

In conclusion, this study has provided basic morphological data which has improved our understanding of the processes that normally guide the ocular development in tree shrews. In addition, the data have shown how the choroid and the sclera participate importantly in normal development and in deprivation-induced myopia. The data from electron microscopic examination of lamellar organization and collagen fibril morphology may be particularly

important in guiding future studies into biochemical and biomechanical properties of these tissues.

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