Structural and Elemental Evidence for Edema in the Retina, Retinal Pigment Epithelium, and Choroid during Recovery from Experimentally Induced Myopia

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Purpose. The purpose of this study was to monitor temporal changes in the retina, retinal pigment epithelium (RPE), and choroid of chick eyes using biometric, ultrastructural, and elemental microanalysis techniques as a means of visualizing more detailed signs of the physiological processes underlying choroidal expansion and refractive normalization during recovery from form deprivation.

Methods. Axial dimensions and refractions were measured on form-deprived and fellow eyes of 117 experimental chickens reared with monocular translucent occlusion from days 1 to 15 and given different lengths of visual experience (T = 0–144 hours) before death. Tissue was analyzed ultrastructurally by electron microscopy and relative sodium (Na) and chloride (Cl) ion abundances, by using x-ray microanalysis to determine changes in the presence of these indicators of tissue hydration.

Results. Refractive error decreased from more than 20 D of myopia almost linearly over the first 144 hours after occlusion. Concurrent changes in thickness in the retina, RPE, and choroid were seen as a series of thickness increases and edema, which returned to normal thickness, first in the retina, and did not reach maximum until 3 days after occluder removal in the choroid. In freeze-dried tissue, Na and Cl ion concentrations were greatest in the RPE photoreceptor outer segments and extravascular choroid at T = 0, decreasing toward fellow eye levels by T = 48 in the RPE and choroid. Na and Cl ion abundances in the frozen lymph of choroidal lymphatics were nearly at control levels (T = 0) and increased later as the vessels became more distended after the extravascular edema became significant.

Conclusions. The results suggest that occluder removal induces edema across the retina and choroid and that this fluid may be the vector eliciting choroidal expansion during recovery from form deprivation possibly driven by the hyperosmolarity in the choroid, RPE, and photoreceptor outer segments that accompanies deprivation. (*Invest Ophthalmol Vis Sci.* 2004;45:2463–2474) DOI:10.1167/iovs.03-1009

Clinically significant refractive errors are the most common form of visual disorder, with myopia affecting approximately one half of the world's young adult population[1–4] and hyperopia affecting another 20%. In several Asian countries, the prevalence of myopia is said to be approaching epidemic proportions.[5] By comparison, the prevalence of myopia in primary school children in the United States is considerably lower,[6] and the prevalence in Australian children has recently been shown to be significantly lower still.[7] This suggests that not only do some populations show a genetic predisposition to axial myopia but also that differences in environmental lifestyle may affect the development of refractive errors in humans.

Similarly, studies of many vertebrate groups, including humans,[8,9] monkeys,[10] tree shrews,[11] and chicks[12] have demonstrated the presence of an environmental, visually mediated active emmetropization mechanism. This is achieved in the long term by changes in ocular growth. However, attempts to identify the neural signal driving such visually mediated refractive development and the vector by which this signal is translated into axial elongation have largely been unsuccessful, though it is usually accepted that the mechanisms responsible for such growth adaptations are predominantly local to the retina[13–15] (although see Wildsoet C, et al. *IOVS* 2001;42:ARVO Abstract 326).

In the chick, removal of the occluder after form deprivation (FD) initiates a normalization of the induced refractive error[16] with dramatic thickness changes in the retina and choroid within 30 minutes.[17] Initial biometric descriptions of these phenomena by Wallman et al.[16] reported a threefold increase in choroidal thickness in the first 4 to 8 days of recovery after 10 days of translucent occlusion and correlated this change with the refractive error. It was hypothesized that the dilated choroid was "pushing" the retina back into focus,[16] while acknowledging that this cannot be the only mechanism of re-emmetropization. The calculations of Wallman et al.[16] indicated that the choroidally driven displacement of the retina would contribute less than half of the refractive shift.

Currently, most recent theories seeking to explain the source of choroidal expansion in recovery from FD are still based in some way on increased choroidal vascular perfusion after occluder removal, though Wallman et al.[16] suggested that increased synthesis of extracellular matrix (i.e., proteoglycans) and/or modulation of the smooth nonvascular muscle spanning the choroid could be involved. Although both of these latter two hypotheses have since been supported by data, Pendrak et

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al.,[18] on the basis of biochemical studies, suggested that choriocapillaris leakage is the most likely primary source of the choroidal swelling that occurs after occlusion. Such a theory is consistent with our morphologic observations of an abnormality of the choriocapillaris vessels during the first 48 hours.[19] Shih et al.[20] have also shown that, although choroidal blood flow in the chick is significantly reduced in both eyes during periods of monocular occlusion, occluder removal leads to an immediate but relatively transient increase in blood flow[21,22] that peaks at least 10 hours before the same researchers reported significant choroidal expansion. Fitzgerald et al.[21] have hypothesized that the postocclusion increase in blood flow may trigger the subsequent onset of choroidal expansion, but offer no explanation as to why a bilateral increase in blood flow does not induce choroidal expansion in both eyes or why monocular occlusion that initially induces bilaterally reduced blood flow results in choroidal thinning only in the occluded eye. Thus, questions remain as to what other normal mechanism(s) commonly associated with retinal physiology might contribute to changes in ocular growth or refractive normalization and whether the observed increase in choroidal thickness during recovery is an active or passive component of the re-emmetropization process.

Normal Ocular Homeostasis

In general, fluid homeostasis in the various tissues throughout the body is under the control of epithelial cells. This is also the case in the eye, with anterior chamber volume regulated by ciliary epithelium and retinal edema regulated by the retinal pigment epithelium (RPE).[23] Circadian oscillation in choroidal thickness and the complementary but antiphase change in axial length and intraocular pressure, has been demonstrated in normal chicks,[24,25] FD chicks,[26] and marmosets,[27] with Nickla et al.[25] arguing that intraocular pressure rhythms play a role in the regulation of the growth of the eye. Mammals normally show a net physiological flow of fluid from the retina across the RPE to the choroid during the day and a net increase in fluid movement into the vitreous during the night (see detailed description in Ref. 28) which is consistent with the observation of Nickla et al.[25] of an increase in intraocular pressure and vitreous depth in the chick. In 1979, O'Leary and Millodot[29] also observed a myopic shift in refraction with eyelid closure in humans.

Morphologic Responses to FD

Two weeks of either opaque or translucent occlusion in the chick eye induces abnormal ocular elongation, associated with a significant reduction (30%) in retinal thickness and choroidal thickness around the hemisphere.[17,30–32] Our reports have also noted that the thinning of the choroid is primarily due to constriction, and even in some cases total collapse, of the lumen of the major blood vessels[30] and lymphatic vessels[33] and greatly reduced density of the minor vessels of the choriocapillaris.[17] These structural changes can all be considered representative of perturbations of tissue hydration—that is, fluid balance within the eye. The excessive vitreous elongation that accompanies FD and the simultaneous thinning of the retina and choroid are consistent with a net reduction in normal outflow of fluid into the choroid.[25]

One expects that such changes should be reversible after removal of the occluder. In fact, as our earlier report[17] mentioned, changes in the vascular and lymphatic spaces of the choroid are initiated within 30 minutes of occluder removal,[17] despite the fact that choroidal blood flow is reduced for at least 7 hours after occluder removal.[21] Much of the rapid increase in choroidal thickness appears to be due to the redilation of the lacunae of the suprachoroid[16,33,34] and edema in the extravascular space.[17,19] The lacunae have recently been shown to be true lymphatic sinusoids,[33,35,36] and exhibit ultrastructural correlates of active and passive fluid movements.[19] The frequency of open junctions between lymphatic endothelial cells (accepted as an anatomic indicator of passive fluid transfer) increased significantly over the first 72 hours after termination of FD, whereas the density of lymphatic fenestrations (an indicator of active fluid transfer[37]) increased significantly in the first 24 hours of recovery. However, there is still a need to explain the source of the extra fluid that results in dilation of the lymphatics.

Thus, we hypothesize that the temporal change in refraction after occluder removal is associated with anatomic evidence of increasing fluid retention in the retina-RPE-choroid complex. This edema should visible morphologically as a series of progressive changes in the thickness of the retina-RPE-choroid tissues associated with ultrastructural changes in the density and nature of RPE vesicular inclusions at the basal membrane. Also, given that extravascular edema of all bodily tissues is associated with Na and Cl ion shifts, increases in the relative concentrations of these ions are expected in all regions of the retina, photoreceptors, RPE, and choroid after removal of the occluder and the abrupt onset of normal illumination.

Hence, this article focuses on chicks recovering from FD and the temporal relationship between refractive changes and concomitant changes in thickness of the retina, RPE (approximated by outer limiting membrane to Bruch's membrane: OLM-BM) and choroid; ultrastructural evidence of changes in fluid movement in the basal membrane of the RPE; and ionic status of the ocular layers. The abundance of the key ion species related to extravascular tissue hydration in the choroid and retina was followed throughout the recovery period by scanning electron microscopy energy-dispersive x-ray microanalysis. Preliminary reports of some of these findings have been published in brief in conference proceedings (Liang H, et al. *IOVS* 1998;39:ARVO Abstract 2306).[17]

Materials and Methods

Hatchling meat chicks ($N = 117$) were raised in a controlled environment in a 12-hour light–dark cycle, a restricted temperature range of 29°C to 31°C, and unlimited availability of food and water. Monocular translucent occlusion was introduced on day 1 (the day after hatching) and maintained until day 15 in 96 animals. The occluders were heat-formed hemispheres molded from translucent white styrene sheets and attached to the periocular feathers of the chicks with cyanoacrylate glue. Occluders were removed on day 15 and the chicks given various lengths of unrestricted visual experience (T = 0, 2, 8, 24, 48, 72, 96, 120, and 144 hours) before death. Three normal chickens were included and killed on day 15 for qualitative comparison of ultrastructure. Fellow eyes to experimental eyes across the first 96 hours of recovery formed the comparison group. Not all animals used for biometric assessments were used for electron microscopy (described later). The T = 144 hour group ($n = 4$) were only assessed for refractive status and are not included in the anatomic studies reported herein.

Biometric Measurements

Biometric measurements (retinoscopy and ultrasonography) were collected from animals on day 1 before monocular occlusion, at occluder removal on day 15 immediately before death. The refractive status of the eyes was determined with retinoscopy for the following experimental groups: T = 0 hours ($n = 18$); T = 2 hours ($n = 5$); T = 8 hours ($n = 5$); T = 24 hours ($n = 12$); T = 48 hours ($n = 14$); T = 72 hours ($n = 14$); T = 96 hours ($n = 9$); T = 120 hours ($n = 6$); and T = 144 hours ($n = 4$). The refractive status of the fellow eyes was always measured and results reported as the difference between the two eyes.

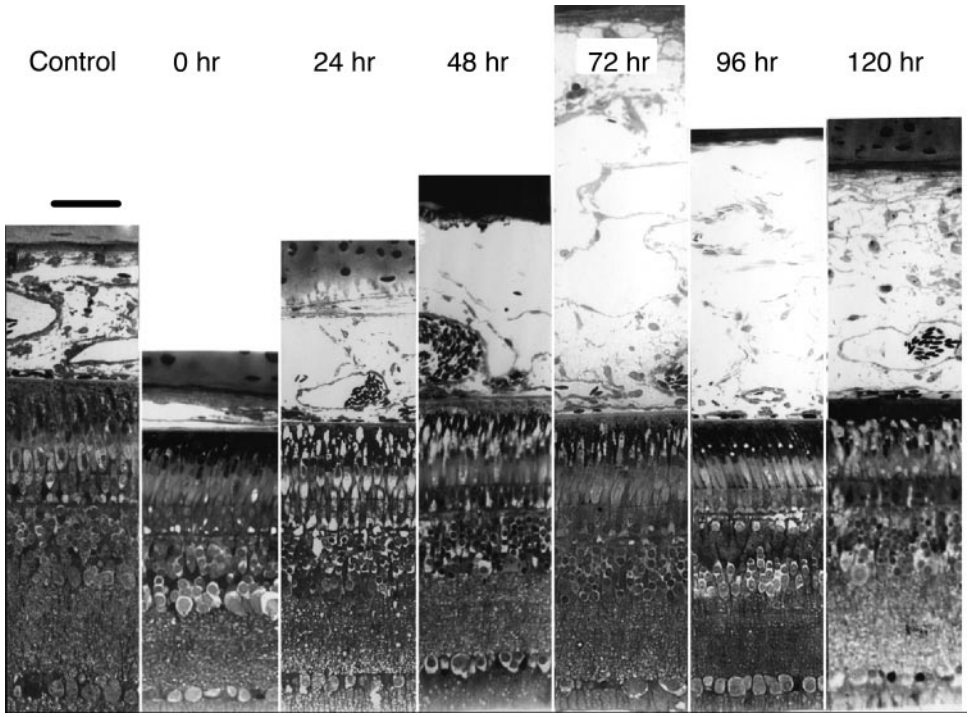

FIGURE 1. Light micrographs of typical transverse sections through the retina-RPE-choroid of chickens visually deprived by monocular translucent occlusion for 2 weeks, then given a variable number of hours of normal visual experience (from the left: control (fellow eye), after 0, 24, 48, 72, 96, and 120 hours of recovery from FD). Note the extremely rapid expansion of the choroid, especially the appearance of thin-walled lymphatic vessels away from the retina nearer the sclera and the lack of material in the edematous extravascular spaces. Red blood cells were seen only in the thicker-walled vessels closer to the choriocapillaris, which was well formed in the fellow eye or normal tissue but displayed an abnormal appearance in FD and early recovery. Seventy-two hours after occluder removal the mean choroid thickness had expanded to almost 300% of nondeprived thickness, with the most obvious features being the extreme distension of the lumina of the lymphatic sinusoids. Stained with toluidine blue. Scale bar, 50 μm.

For retinoscopy and ultrasonography, animals were lightly anesthetized with an intramuscular injection of a mixture of ketamine hydrochloride and xylazine (30 mg/kg and 3 mg/kg, respectively).

Transmission Electron Microscopy

Animals were killed by barbiturate overdose, and the eyes were immediately prepared for light and electron microscopic examination. Although perfusion fixation has been suggested as the method of choice in some situations, pilot studies showed that immersion fixation yielded extremely good tissue preservation and, more important, did not alter the physiological status or the dimensions of the lumina of the vascular tissue before investigation. Hence, eyeballs were immersion fixed in 2.5% glutaraldehyde and 2.0% paraformaldehyde in 0.1 M sodium cacodylate buffer. Tissue samples were obtained as 5-mm buttons, trephined 3.5 mm from the end of the pecten at the central posterior region of the eye cups to ensure consistent localization of morphologic analyses, then dissected to approximately 1 × 3 mm in size, postfixed in 2.0% osmium tetroxide, and dehydrated and embedded in Epon-araldite. Three tissue blocks were taken from each eye, and three sections were taken from each tissue block.

Semithin sections (0.5 μm) were stained with 1% toluidine blue for light microscopic examination. Ten measurements of each of the anatomic regions specified were taken from the midregion of each section by moving the microscope stage 20 μm for each subsequent measurement. *Choroidal thickness* was measured by light microscopy as the distance from the basement membrane of the choriocapillaris to the inner edge of the sclera in the following experimental groups: T = 0 hours (n = 8 animals, 21 sections); T = 2 hours (n = 5, 12 sections); T = 8 hours (n = 5, 8 sections); T = 24 hours (n = 6, 6 sections); T = 48 hours (n = 6, 6 sections); T = 72 hours (n = 6, 7 sections); T = 96 hours (n = 6, 11 sections); T = 120 hours (n = 6, 14 sections); and non-deprived fellow eyes from 16 animals taken evenly across the recovery period (37 sections; Fig. 1). *Retinal thickness* was measured from the inner limiting membrane to Bruch's membrane (thus including the RPE) and *RPE thickness* was measured as the region from the outer limiting membrane to the basal membrane of the RPE (OLM-BM) containing the subretinal space, in the following experimental groups: T = 0 hours (n = 8 animals, 8 sections); T = 24 hours (n = 6, 7 sections); T = 48 hours (n = 6, 7 sections); T = 72 hours (n = 6, 10 sections); T = 96 hours (n = 6, 10 sections); T = 120 hours (n = 6, 14 sections); and non-deprived fellow eyes (n = 16, 37 sections).

Ultrathin sections (60–90 nm thickness) were made on a microtome (Pharmacia LKB, Gaithersburg, MD) and stained with 4% uranyl acetate and 2% lead citrate before examination on a transmission electron microscope (model 7000; Hitachi Ltd., Tokyo, Japan). Thickness of Bruch's membrane was measured in the experimental groups T = 0 hours (n = 8 animals, 8 sections); T = 24 hours (n = 6, 7 sections); T = 48 hours (n = 6, 7 sections); T = 72 hours (n = 6, 10 sections); T = 96 hours (n = 6, 10 sections); and T = 120 hours (n = 6, 6 sections) and in the control eyes (n = 16, 37 sections).

All eyes were exposed to exactly the same procedures, including time of death.

Energy-Dispersive X-ray Microanalysis

Seventeen chicks were given 0 hours (n = 4), 24 hours (n = 3), 48 hours (n = 3), 72 hours (n = 4), and 96 hours (n = 3) of unrestricted visual experience after occlusion and killed for energy-dispersive x-ray spectral microanalysis to determine the abundance of particular ion species in the retina and choroid. Small sections of tissue dissected from the posterior pole in the same manner as described for transmission electron microscopy (TEM) were fixed immediately in liquid nitrogen slush and freeze dried for 12 hours. The dry tissue was mounted as for routine scanning electron microscopy (SEM) of transverse sections of the retina-choroid-sclera and coated with approximately 50 nm of carbon. For one eye at T = 48 hours, the remainder of the globe was embedded in Epon resin and sawed in half and the cut face highly polished and viewed by SEM[32] to visualize the choroidal architecture in three dimensions.

Specimens were examined with a scanning electron microscope (S-360; Leica/Cambridge Ltd., Cambridge, UK) fitted with an x-ray detector (Kevex, Foster City, CA). The electron beam was set to a cross-sectional area of 37 μm (parallel with the internal limiting membrane) × 26 μm (transverse across retina) and 10 kV. The resultant spectra were analyzed on computer (Iridium, Arlington, VA), using the auto background compensation auto elemental peak identification features. All raw elemental counts were standardized by defining a window in the background spectra that did not involve the elements under consideration, namely 5.0 to 7.0 KeV, and acquiring the spec-

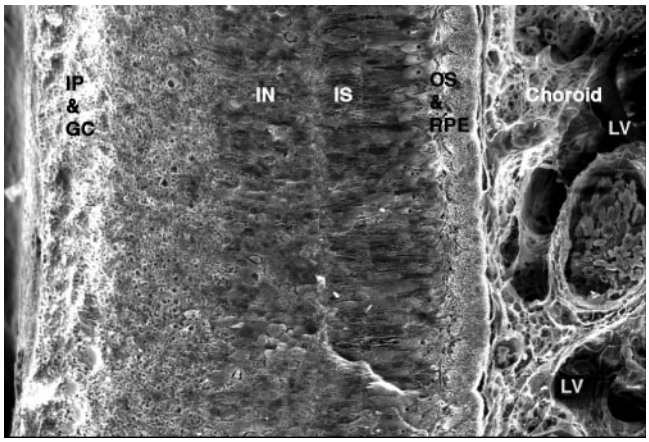

FIGURE 2. Fractured face of freeze-dried normal ocular tissue seen by SEM indicating each of the six locations examined: region of ganglion cell layer (GC) and inner plexiform layer (IP), inner nuclear layer (IN), region of inner segments (IS), region of outer segments (OS) and RPE, choroidal lymphatics (LV).

trum until 1000 counts had accumulated in the window. Data were collected from five regions, but only the Na and Cl data from the two outermost regions are reported herein (Fig. 2). Counts were repeated in adjacent locations within each region three to seven times (mean 4.0 ± 1.1), to achieve a reliable estimate.

Because freeze-dried tissue is an open mesh, an estimate of the volume of the tissue under analysis was calculated by running a Monte Carlo electron-scattering model for 10-kV electrons impacting solid carbon. This showed that 10-kV x-rays emanate from a depth of 500 nm in carbon, which represents the thickness of approximately 25 cell membranes.

Microanalysis of the contents of the lymphatic vessels cannot be ascertained from freeze-dried material, and so for this elemental analysis, a wet preparation (bulk-frozen tissue) was used.[38] A further 13 eyes from 13 chicks at various times of recovery (T = 0 hours, $n = 3$; T = 24 hours, $n = 1$; T = 48 hours, $n = 3$; T = 72 hours, $n = 2$; and control, $n = 4$) were plunged into liquid nitrogen immediately after enucleation. After freezing, the eyes were fractured under liquid nitrogen, and segments of the eye demonstrating a clear cross section of the vitreous, retina, and choroid were quickly mounted in a cryotrans system (Model E 7400; Polaron, Watford, UK) attached to the scanning electron microscope (S-360; Cambridge). X-ray analysis was performed at 265× on the surface of the lumen of the lymphatic vessels at approximately −150°C after sublimation of condensate from the surface of the specimen at −70°C. An 8.2-mM control solution with the elements Mg, S, Na, Cl, K, P, and Ca was prepared by adding equimolar amounts of $MgSO_4 \cdot 7H_2O$, NaCl, KH_2PO_4, $Ca(NO_3)_2 \cdot 4H_2O$ to enable quantitative analysis of ions in choroidal lymph.[38] Six replicates of readings from adjacent regions of choroidal lymph of each specimen were obtained and the results averaged.

The animals were raised and killed in accordance with the regulations of the National Health and Medical Research Council of Australia, and procedures conformed to the NIH Guiding Principles in the Care and Use of Animals (1996) and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Statistical Analysis

The influence of the duration of normal visual experience after occluder removal on the relative thickness of the retina, OLM-BM, and choroid were calculated with analysis of variance ANOVA (SPSS software; SPSS Science, Chicago, IL). Because the thickness of the regions was expected to be independent of one another, Bonferroni correction of the type I error rate was not applied across all analyses. The analyses for each location represent separate and therefore independent families of comparisons. Changes in the thickness of the various tissues measured at different times were analyzed using post hoc Student's unpaired *t*-tests.

The influence of the time from occluder removal on the abundance of ionic species was calculated using multivariate ANOVA (SPSS software) with three independent variables—namely, refractive category (FD or control eye), region of retina/choroid, and time.

RESULTS

Refractive Status

Refractive error was found to decrease with duration of unrestricted visual experience after the removal of the occluder (Table 1). The difference in refractive error between the two eyes in each group of chicks at any recovery stage was consistent, with standard errors typically less than 1 D. The rate of change in refraction was slower over the first 24 hours after occluder removal than in the period between 24 and 48 hours, but was basically linear over the first week (Fig. 3). Complete refractive recovery from myopia was achieved by the four animals examined at T = 144 hours (6 days) after occluder removal, with the eyes showing complete re-emmetropization and even a slight overcompensation resulting in a mild hyperopia.

Light Microscopy

Consistent with earlier reports,[16,17,19] the most marked anatomic change in the 120 hours after occluder removal was the massive choroidal expansion (Table 1) and the concomitant

TABLE 1. Comparison of Refractive Status and the Thickness of Choroid, Retina, OLM-BM, and Bruch's Membrane of Deprived and Nondeprived Control Eyes at various Times after Occluder Removal

Hours Visual Experience	Refractive Difference (D)	Choroid (μm)	Retina (μm)	OLM-BM (μm)	Bruch's Membrane (μm)
0	−22.15 ± 0.74	21.52 ± 1.41	194.50 ± 5.29	47.00 ± 0.66	0.34 ± 0.063
2	−20.20 ± 1.11	43.11 ± 3.35	—	—	—
8	−19.20 ± 1.02	88.75 ± 3.08	—	—	—
24	−19.33 ± 1.16	123.33 ± 7.70	223.43 ± 3.64	62.29 ± 1.45	0.57 ± 0.044
48	−11.64 ± 1.01	137.56 ± 4.27	231.43 ± 2.03	62.00 ± 0.76	0.57 ± 0.067
72	−8.04 ± 0.86	282.82 ± 18.64	226.40 ± 5.90	62.40 ± 0.27	0.53 ± 0.073
96	−3.00 ± 0.49	152.0 ± 2.60	200.80 ± 5.87	47.20 ± 0.61	0.98 ± 0.081
120	−1.17 ± 0.25	128.7 ± 3.22	192.80 ± 4.53	46.60 ± 1.04	0.95 ± 0.064
144	0.50 ± 0.21	—	—	—	—
Fellow eyes	—	92.23 ± 4.34	230.59 ± 6.23	58.57 ± 1.85	0.87 ± 0.12

Refractive data for the FD eyes is shown as difference between the two eyes. The mean ± SEM refraction of the group of fellow eyes was +1.14 ± 0.39 D. Mean ± SEM thickness comparison measurements for the fellow eye group are also shown.

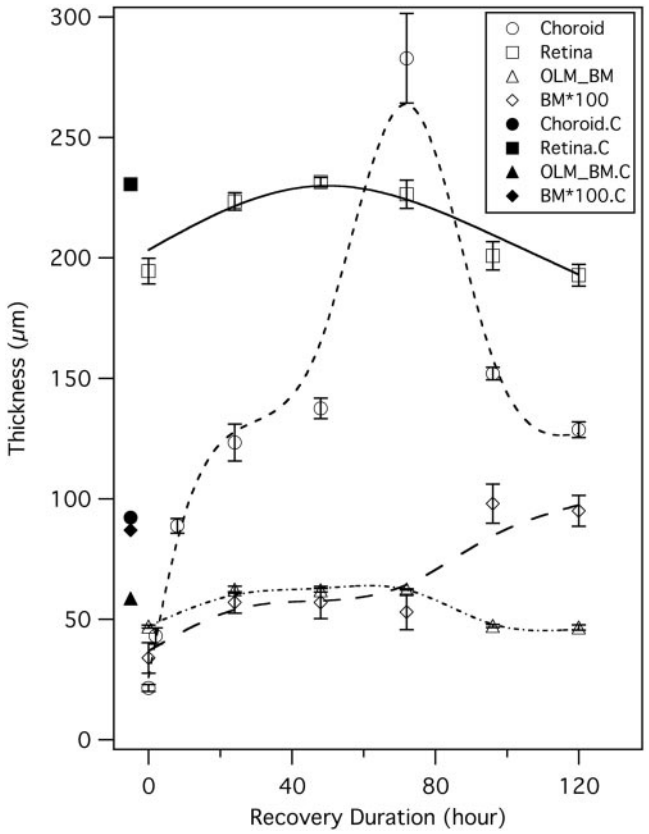

FIGURE 3. Temporal sequence of mean thickness measurements (in micrometers with standard errors indicated) in the retina (including RPE), RPE (OLM-BM), Bruch's membrane (shown at ×100 scale to allow representation on the same axes) ,and choroid of the form-deprived eyes (indicated by *unfilled symbols*) and fellow eyes (indicated by *filled symbols*, offset slightly to the left to aid visibility) during the first 120-hour period of normal visual experience after occluder removal. It is clear that the retinal thickness measurements peaked earlier than did the choroidal thickness. The thickness of Bruch's membrane showed some increase in the first 24 hours and then stabilized until after the choroid had passed its maximum thickness and the re-emmetropization was almost complete. The curves are smoothing spline fits,[40] weighted by standard deviations, with a smoothing factor of 1.0.

rapid and extensive dilation of the large, cell-lined suprachoroidal lymphatics (Fig. 1).

Compared with fellow nondeprived control eyes, the mean choroidal thickness in experimental eyes progressed from a 60% reduction in thickness at occluder removal, to a thickness similar to that of the control nondeprived eyes after 8 hours and then increased to approximately three times that of the control thickness after 72 hours of visual experience (Fig. 3). Choroidal thickness decreased significantly ($P < 0.0001$) in the period between 72 and 96 hours after occluder removal. Choroidal thickness continued to decrease more slowly until 120 hours after occlusion, by which time the choroid had not returned to the control thickness level (Table 1, Fig. 1). At all time points sampled after 8 hours after occluder removal, the mean difference in choroidal thickness between FD and control nondeprived eyes is significant ($F = 224.4$, $df = 7$; $P < 0.0001$).

At occluder removal ($T = 0$), the extravascular elements of the choroid of the deprived eye were extremely close packed and the lumina of the lymphatic sinusoids appeared to be compressed relative to the choroidal tissue of the nondeprived control eyes. However, over the next 72 hours, the extravascular tissue swelled significantly, giving an edematous appearance (characterized by a change in ultrastructural density and increasing sparseness of extravascular connective tissue elements between lymphatic vessels and the major blood vessels[37,39]; Fig. 1).

Retinal thickness in the formerly form-deprived eyes also showed a significant increase ($F = 11.4$, $df = 5$, $P < 0.0005$) during the recovery period (Table 1, Fig. 3). When compared with the retinas of the fellow eyes, the FD retinas were initially reduced in thickness as a result of the 2 weeks of deprivation, but expanded rapidly (~10%) in the first 48 hours and then thinned again after 72 hours. By 120 hours of recovery from FD, the thickness of the FD retinas had returned to that of the FD eyes at the time of occluder removal. This was approximately a 15% reduction in thickness compared with fellow nondeprived eyes. As can be seen in Fig. 1, the inner nuclear layer was approximately 12 to 13 cells thick in fellow eyes but was only 6 to 8 cells thick in the experimental eyes (Fig. 4).

The transverse thickness of the region between the outer limiting membrane and the RPE basal membrane (OLM-BM) was also measured by light and electron microscopy (Table 1, Figs. 3,4). This region contains the distal tips of the photoreceptor outer segments, the subretinal space, and the RPE cell layer (Fig. 5).

The results suggest that changes in the OLM-BM region contribute substantially to the changes in retinal thickness (whose measure includes the RPE) during the first 120 hours after occluder removal, though largely in proportion and with

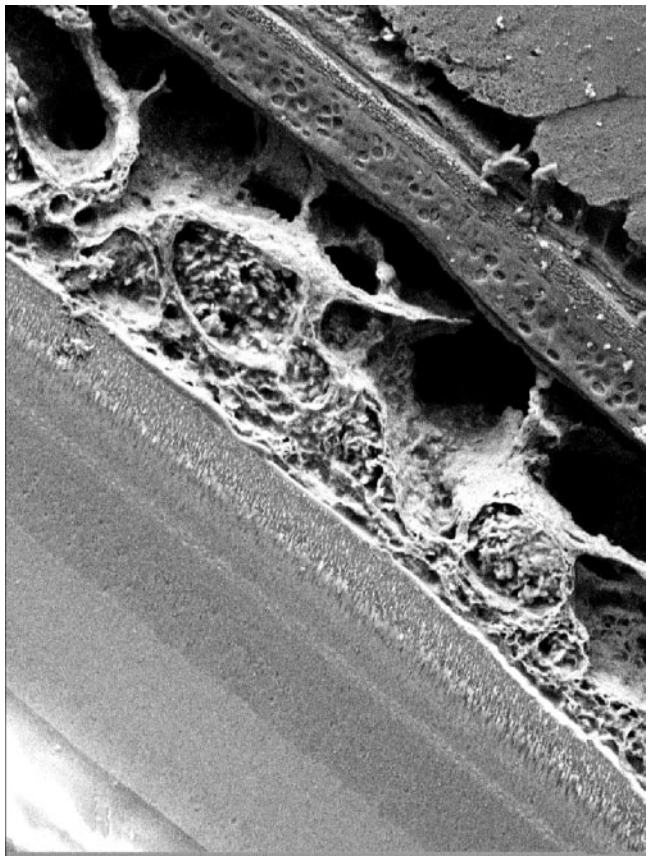

FIGURE 4. Scanning electron micrograph of the highly polished cut face of a resin-embedded chick eye 48 hours after recovery from 2 weeks FD. The architecture of the lymphatic sinusoids was enhanced by poor infiltration of the resin. Note the comparison between the thick-walled blood vessels filled with erythrocytes and the large, extremely thin-walled empty lymphatic sinusoids.

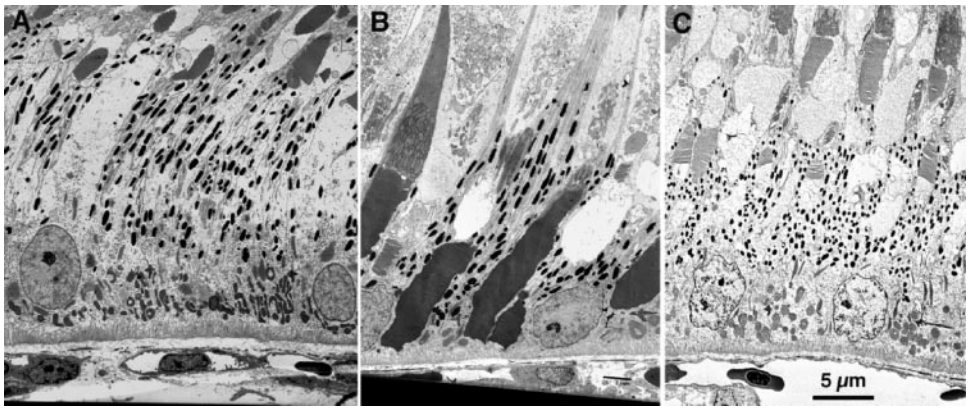

FIGURE 5. Transmission electron micrographs of outer retina, RPE and Bruch's membrane. (**A**) Nondeprived fellow eye. (**B**) Form-deprived eye immediately after removal of occluder, T = 0. Note the extended rod outer segments reaching almost to Bruch's membrane. (**C**) Form-deprived eye after 48 hours of normal visual experience. Note the loss of density of cellular inclusions and the changing morphology of the RPE nuclei. Scale bar, 5 μm.

the same time course as in the retina. The greatest rate of retinal thickness increase occurred in the first 24 hours after occluder removal. Once the initial swelling had occurred, the expansion was maintained for another 48 hours until T = 72 hours, after which time a decrease in transverse thickness to the level immediately after occluder removal was observed. Areas of detachment of the outer retina from the RPE appeared as opaque and edematous spaces around the subretinal space at 2 and 8 hours after occluder removal.

It is interesting to note that the RPE thickness (OLM-BM) attained after 24 hours of visual experience was comparable to the average thickness in the fellow nondeprived eyes, but that this expansion of the RPE was not maintained beyond 72 hours of recovery. This temporal sequence of thickness changes mimicked those observed in the transverse thickness of the whole retina during recovery (Fig. 3).

Temporal Dynamics of Thickness Changes between Tissues

A major purpose of this study was to investigate temporal changes in morphology that might suggest possible explanations for choroidal expansion during recovery from FD. As shown in Figure 3, at T = 0, all tissue components were thinner than the corresponding fellow eye components. The neural retina reached maximum thickness between 24 and 48 hours after the resumption of normal visual experience, considerably earlier than maximum choroidal thickness, reached at 72 hours. Choroidal thickness rapidly reduced on the fourth day, but less so on the fifth day. At this time, the choroid was still approximately one-third thicker than in fellow eyes, whereas the RPE had returned to fellow-eye thickness. Recovery from maximum swelling of the retina started at a time between 48 and 72 hours after removal. On the contrary, Bruch's membrane displayed an early increase in thickness as in the retina, but rather than achieving maximum thickness in the first day, this tissue remained at approximately 60% of final thickness for the same duration as the retinal-RPE thickness plateau, before rapidly regaining the thickness of fellow eyes by 96 hours.

Thus, this investigation of the temporal sequence of anatomic changes during recovery from FD indicates that, at a gross level, the retina and RPE reach maximum thickness before the choroid. Similarly, the recovery from maximum swelling is initiated in the retina before it occurs in the choroid.

Transmission Electron Microscopy

Outer Retina-RPE Complex. There are some very obvious differences in the ultrastructure of the outer retina-RPE complex of both the deprived and recovering eyes, compared with the fellow nondeprived eyes (Figs. 5, 6, 7). In particular, in the fellow eyes and the T = 0 experimental eyes, there was very little evidence of any degree of fluid retention or edema, and there was little evidence of extracellular space visible between the photoreceptor outer segments and the soma of the RPE cells. The lateral junctions between RPE cells were tightly apposed and regular, with few spaces between the apposing cell walls (Figs. 5, 6), and RPE nuclei were round and well displaced from the basal membrane. The mitochondria of the RPE cells of nondeprived fellow eyes were compact and plentiful, and in many cases were cigar shaped and generally oriented in a direction parallel to that of the infoldings of the basal membrane. The basal membrane was characterized by the presence of regular tightly packed infoldings that lay orthogonal to Bruch's membrane and that were generally approximately 1.25 μm in height (Fig. 8), though this array was often interspersed with much larger membrane-bound vesicles.

By comparison, the ultrastructure of the photoreceptors and RPE complex of the form-deprived eyes showed significant changes at all the times sampled after occlusion (Figs 5, 6, 7, 9). At T = 0, the distal tips of the rod photoreceptor outer segments were abnormally elongated and had densely packed photoreceptor discs (Fig. 5, 9). Within 2 hours of normal visual experience, the photoreceptors showed cleavage between outer segment discs that may be indicative of phagosome

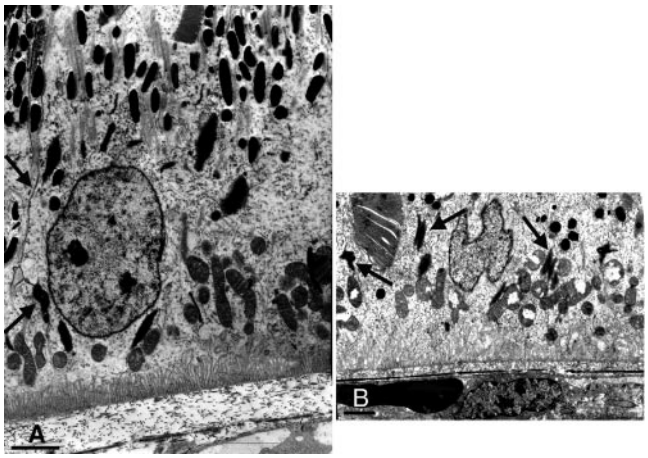

FIGURE 6. RPE and Bruch's membrane. (**A**) Nondeprived fellow eye. (**B**) Form-deprived eye, T = 0. Note the loss of regularity of basal infoldings of the RPE, polymorphism of RPE nuclei and mitochondria, and the compression of Bruch's membrane. *Arrows*: junctional complexes and the integrity of intercellular spacing in fellow and T = 0 eyes, which indicates that tissue fixation has led to minimal artifacts. Scale bar, 2 μm.

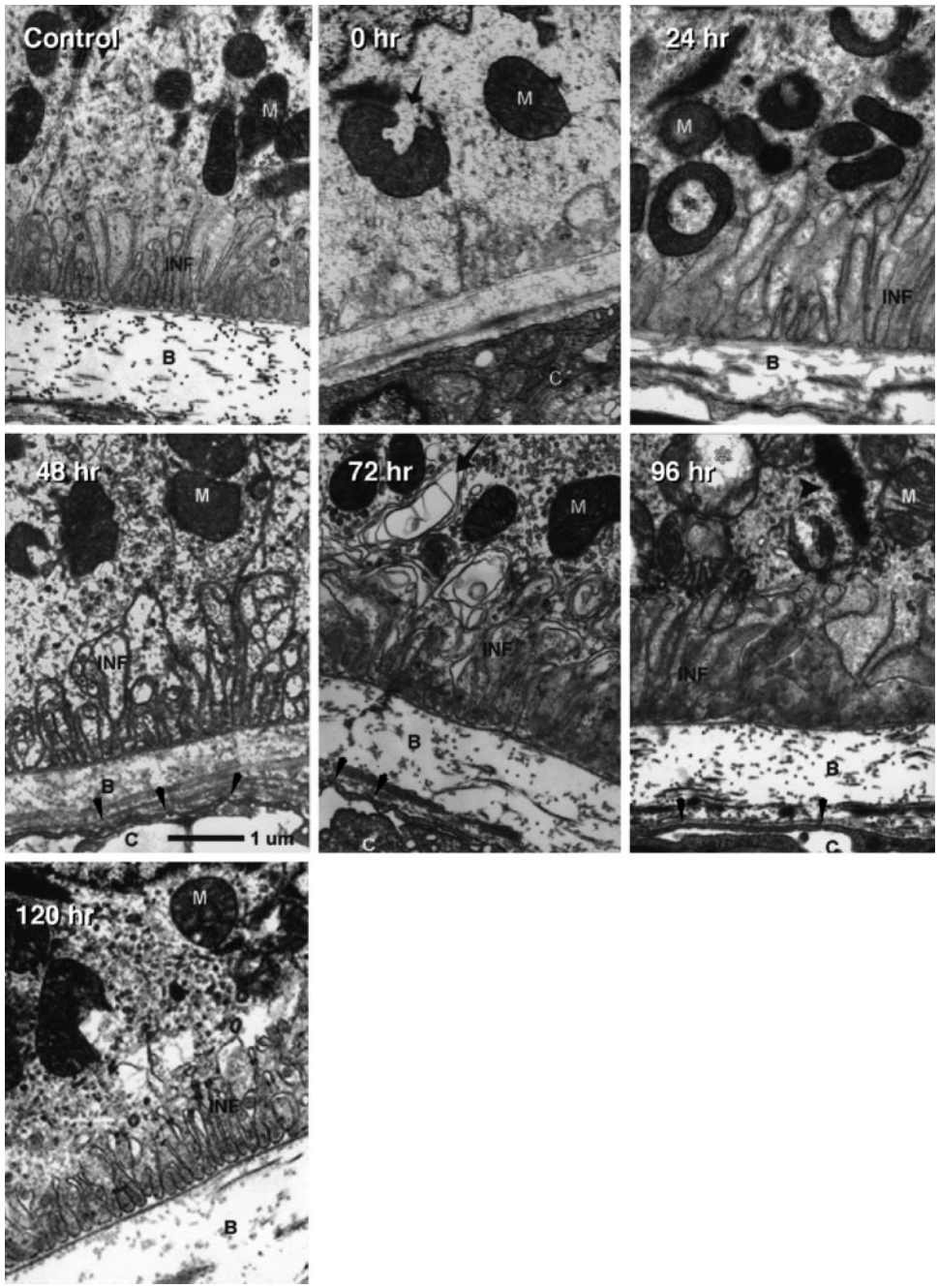

FIGURE 7. Electron micrographs comparing the morphology of a nondeprived fellow eye and recovering eyes (T = 0, 24, 48, 72, 96, and 120 hr) in the vicinity of the basal membrane of the RPE and Bruch's membrane. Note, the dramatic changes in the thickness of Bruch's membrane and the structure of the basal membrane of the RPE cells in the deprived eyes over the 120 hours. During the early stages of refractive recovery the inner and outer collagenous layers of Bruch's membrane, normally appearing as crisp dots (transverse section of collagen fibrils), were not readily apparent until the membrane had begun to increase in thickness at around 72 hours after occlusion. Likewise, the structure and regularity of the infoldings of the basal membrane of the RPE were dramatically compromised by form deprivation (compare Fig. 7, Control with T = 0 hr) but improved substantially over the recovery period, though there was still evidence of edema in the cytoplasm at 120 hours. In all examples of recovering eyes, the appearance of the basal membrane of the RPE of the deprived eyes was disordered with respect to the fellow eye. In particular at T = 0 hours there was greatly reduced density of infolding of the basal membrane and little evidence of bilaminar membranous inclusions, nor any well-formed vesicles expected in normal fluid movement across the RPE. *Short arrow,* 0-hr: a structurally abnormal mitochondrion that may infer impaired function. The micrograph at 72 hours showed evidence of a large amount of edema in the RPE cytoplasm with many large disorganized infoldings on the basal membrane and an increased frequency of membrane-bound, fluid-filled inclusions toward the inner region of the cell (*long arrow*). It is clear that the disorganization of infoldings is not a result of inadequate tissue preservation, as there is an example of the electron dense desmosome of a junctional complex between RPE cells clearly visible in the 96-hour micrograph (*arrowhead*). Fenestrations were evident on endothelial cells of the choriocapillaris in micrographs taken at 48, 72, and 96 hours (*arrowheads*). M, mitochondria; INF, infoldings of the basal membrane of an RPE cell; B, Bruch's membrane; C, the choriocapillaris. Scale bar, 1 μm.

formation as part of the shedding process[41] (Fig. 9). As noted in our earlier reports, the nuclei of the RPE cells from the deprived eye were generally smaller in profile, less compact, and more irregular in shape, frequently showing deep indentations and giving the appearance of recent direct contact with the photoreceptor outer segments (Fig. 9, layer C).[17,30] The mitochondria were also changed in shape (Figs 6, 7), often appearing rounder and with inclusions resulting in annular transverse profiles.[42] Although it is acknowledged that mitochondria of RPE cells in birds show circadian changes in morphology,[43,44] the changes observed in the present study are unlikely to be related to time of day, as the degree of change was greater than circadian changes reported, and all animals were killed at the same time of day (early afternoon). A gradual increase in edema was obvious in the temporal profile of changes in the electron density of the intracellular and extracellular spaces and the voids in the electron micrographs of eyes during recovery from FD (Figs 5, 6, 7, 9).

Basal Membrane of RPE. Notably, during recovery the height and the packing density of the convoluted infoldings of the basal membrane of the RPE were less in the myopic eye than in the nondeprived fellow eye (Fig. 7). This is indicative of lesser basal membrane area per unit length of Bruch's membrane. The low linear density of infoldings at T = 0 (infoldings per unit length along Bruch's membrane) began to increase in the first 24 hours after occluder removal (compare Fig. 7, 24

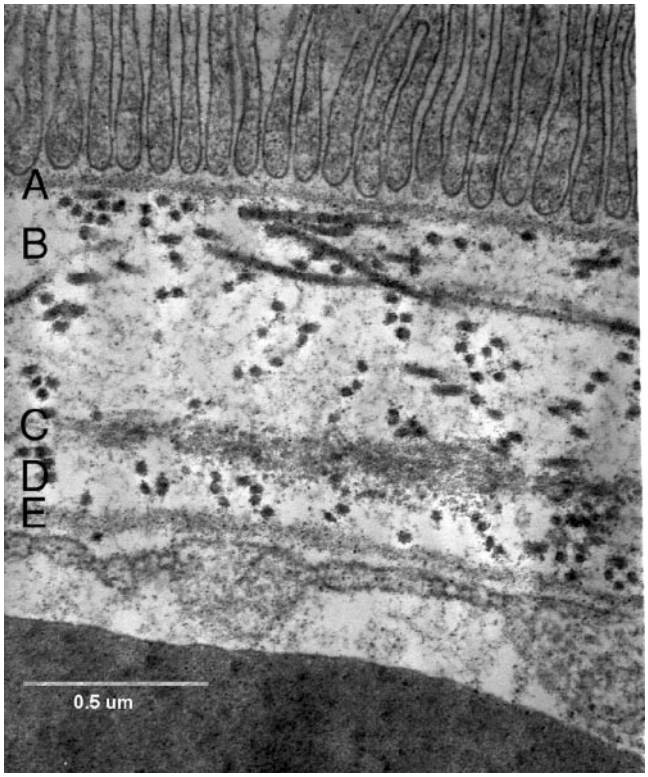

FIGURE 8. Electron micrograph of Bruch's membrane in a normal chick. (**A**) Basal membrane of RPE composed of fine filaments extending from basal surface of cells of RPE. (**B**) Inner collagenous zone composed of interwoven collagen filaments. (**C**) Noncontinuous elastic layer. (**D**) Outer collagenous zone. (**E**) Basement membrane of choriocapillaris. Note regularity of infoldings of the basal membrane of RPE cell.

hours with 0 hours). Further visual experience led to great irregularity of the infoldings on the basal membrane and a number of unusual membrane-bound, fluid-filled structures extending farther into the cytoplasm. This is usually interpreted as ultrastructural evidence of edema.[37] The linear density of infoldings and the number of fluid-filled vesicles continued to increase up to 96 hours. The most obviously swollen membranes were in the samples taken from animals given 72 hours of normal visual experience (Fig. 7). In the T = 96 hours group, fewer fluid vesicles were evident and by 120 hours after occluder removal, the basal membrane appeared to be regaining a more normal appearance.

Bruch's Membrane. Bruch's membrane is a five-layered structure that is contiguous with the basal membrane of the RPE on the retinal side and the basement membrane of the choriocapillaris on the choroidal side, with an inner collagenous zone, an (interwoven) elastic layer, and an outer collagenous zone between, as shown in Figure 8. There was an obvious difference ($F = 295.6$, $df = 6$, $P < 0.0001$) in relative thickness of Bruch's membrane between the deprived and the nondeprived fellow eyes (Table 1, Figs. 3, 6, 7). At the time of occluder removal, Bruch's membrane in FD eyes showed a greatly altered morphology, with very few discrete collagen fibrils in the inner and outer collagenous layers, plus compression of the elastic fibers. At T = 0 hours the average thickness of Bruch's membrane in the deprived eyes was less than 50% of the average thickness of that in the nondeprived fellow eyes (Figs. 6, 7). However, a rapid increase in thickness occurred during the first 24 hours of visual experience, which plateaued between 24 hours and 72 hours. At this time, Bruch's membrane showed a separation of the collagen filaments, as would be expected of edematous tissue. The architectural appearance of Bruch's membrane only began to approach normalcy at 96 hours. During this period of recovery the thickness changed significantly ($P < 0.001$). Thickness comparable to the fellow eye was not achieved until 120 hours.

An interesting feature of FD eyes, noted several times at 120 hours after occluder removal when the basal infoldings were returning to normal form and less frequently at other times, was the appearance of electron-dense material within membrane-bound vesicles between the RPE, Bruch's membrane, and the choriocapillaris (Fig. 10). Further studies of serial sections are needed to confirm whether the circular bodies are indeed sections of spherical bodies and therefore possibly indicative of the transfer of proteinaceous and/or lipid material, as their electron density suggests.

Edema

The most outstanding morphologic feature of tissue examined (by light and electron microscopy) from eyes recovering from FD was morphologic evidence of edema. The increasing edema was greatest in tissue taken 72 hours after occluder removal, where, in addition to the lessened clarity of electron-dense material, there were also areas of lowered intracellular organelle density. The edematous appearance was not related to tissue preservation, as many of the same sections also showed areas of high electron densities indicative of intact junctional complexes (Fig. 9C) and well-preserved tissue (e.g., Fig. 7, 96 hr). Other evidence for edema was provided by the looseness of the cytoplasm in the region of the apical membrane of the RPE (Figs. 5C, 9) with lowered transparency of interstitial spaces an ultrastructural sign of excess fluid. In addition, evidence was provided of a gradually increasing number of vesicles in the vicinity of the basal membrane of the RPE (Fig. 10).

Energy-Dispersive X-ray Microanalysis

Energy-dispersive x-ray microanalysis was performed on freeze-dried wholemount sections at five locations as indicated in Figure 2, at five times—T = 0, 24, 48, 72, and 96 hours—during recovery after occlusion. The microanalysis was also performed on large fragments of fully hydrated material to enable x-ray examination of the fluid within lymphatic vessels (T = 0, 24, 48, and 72 hours of recovery). In this method, the elements that are detectable in large quantities, apart from hydrogen, oxygen, and carbon, are Na, Cl, K, sulfur (S), and phosphorus (P), but only the relative concentrations of Na and Cl ions in the outer retina-RPE-choroid are reported herein. The relative x-ray counts of these elements sampled at all time points during recovery in the freeze-dried photoreceptor outer segments, RPE and choroid are illustrated in Figures 11 and 12 and are shown compared with the mean ionic concentration for the fellow eyes of the same animals. Figure 13 shows relative changes in Na and Cl ions in the fluid within lymphatic sinusoids of the choroid of bulk frozen eyes.

At occluder removal, T = 0 hours, it is readily apparent from Figures 11 and 12 that the ionic abundances of Na and Cl in choroid, RPE, and photoreceptor outer segments were significantly greater ($P < 0.006$) than those in the same areas in the fellow eyes. However, the change in Na and Cl ion concentrations was greater in the freeze-dried choroid than the RPE and outer segments, with the ionic abundances of the RPE and outer segments and choroid all returning toward normal range within 48 hours (Cl ion more rapidly than Na ion). Thus, the outer retina and RPE demonstrated signs of hyperosmolarity.

In contrast, the relative ionic concentrations of Na and Cl in the lymphatic vessels measured using frozen rather than freeze-

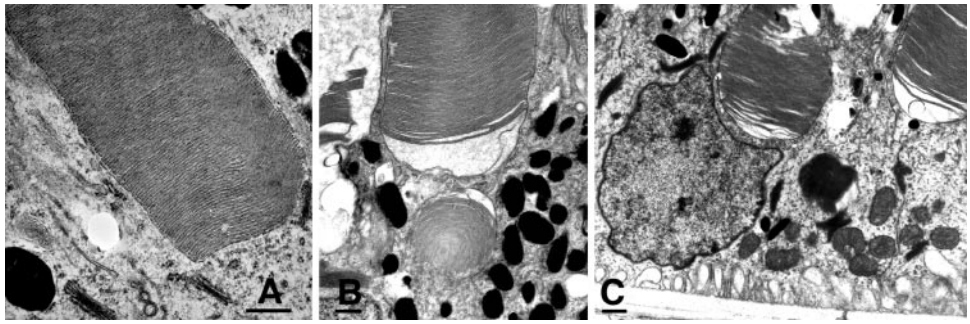

FIGURE 9. Electron micrographs of the distal tips of rod photoreceptors taken from a nondeprived fellow eye (layer A) and a form-deprived myopic chick eye after 48 hours of unrestricted visual experience (layers B, C). In the nondeprived eye the lamellae of the outer segments were tightly packed and integral with the abutting RPE cell. By contrast, the outer segment in the T = 48 hours chick showed evidence of edema—that is, less electron-dense organelles in the cytoplasm of the RPE cell and a large, fluid-filled gap at the base of the outer segment. In (layer B) the lamellae of the photoreceptor discs were not as densely packed as in layer A, and there was a phagosome nearby. In (layer C) there were indications of breaks in the discs that may indicate the beginning of the shedding process and the formation of a phagosome. Note the close apposition of the RPE nucleus and the rod PR. Scale bar, 0.5 μm.

dried samples (Fig. 13) was not significantly different (comparing FD and fellow eyes) over the recovery times from T = 0 to T = 48 hours, when it began to increase rapidly up to 72 hours. Data from times after 72 hours are not currently available.

DISCUSSION

The most important new finding of this study was the demonstration of significant amounts of extravascular edema and relative change in Na and Cl ion abundances related to duration of normal visual experience after occluder removal from form-deprived myopic chicks. The time of maximum tissue thickness was reached earliest in the retina and later in the RPE and choroid—in particular, in the lymphatic vessels. Ultrastructurally, the surface area of the basal membrane of the RPE increased dramatically, contemporaneously with the change in ion abundance and appearance of edema. We believe that such results have important implications for the understanding of the physiological mechanisms of ocular growth underlying active emmetropization.

Refractive normalization and choroidal thickening after FD have been described extensively in chickens[17,18,45] and to a lesser extent in other mammalian species (Hung L-F, et al. *IOVS* 1998;43:ARVO Abstract 2309),[46] but the temporal sequence of morphologic, ultrastructural, and ionic changes that accompany restoration of a normal visual environment after removal of the occluder has received little attention. In addition, this represents the first application of x-ray microanalysis to the study of the development of refractive error.

The use of energy-dispersive x-ray microanalysis to compare FD and fellow eyes provides further support for the existence of altered tissue hydration. There was a significant difference in the relative concentrations of Na and Cl ions in the outer retina, RPE, and choroid, between the FD myopic eyes at T = 0 and fellow nondeprived eyes. This difference in concentrations of Na and Cl ions progressively decreased over the first 48 hours in the freeze-dried samples in the outer segments and RPE and choroid, whereas the choroid expanded. This expansion of the choroid over the first 72 hours and the consequent lowering of spatial density of membranes, increasing the stopping distance of the electron beam in the freeze-dried preparation, has to be taken into account in interpreting the results in Figure 11. However, the similar progression of ionic abundances (Fig. 12) found in the RPE-outer segments (which does not show the 10-fold tissue expansion of the choroid) lends weight to the notion that there is an increased concentration of Na and Cl ions in the choroid immediately after FD and that the ion excesses dissipate around the time that the choriocapillaris fenestrations return to normal range.[30,47]

The bulk-frozen X-ray microanalysis method was used to give an accurate measurement of molar concentrations of ions directly in the lymphatic lumen under analysis. Because only the contents of the large choroidal lymphatics were sampled (through direct observation) and because the lumina of the vessels sampled were filled with frozen lymph, the electron beam stopping distance was constant across the recovery period samples, with electron scattering confined to a surface layer. Under these conditions, the changes in molar concentration of Na and Cl ions from the frozen lymphatics showed an opposite tendency to the freeze-dried choroidal preparation. Whereas in the T = 0 and T = 24 eyes, the ionic concentrations were only marginally different from the fellow eye values, a strong increase was observed at T = 48 and T = 72 hours with concentrations of both species a factor of 2 greater than in the fellow eyes. This supports our earlier work[19] detailing the increase in the number of passive endothelial junctions and active fenestrations in lymphatic vessels over the first 72 hours. The freeze-dried approach involved sampling from the inner part of the choroid and would have included blood vessels and extracellular matrix as well as some lymphatics. It appears that the lymphatics take up the Na and Cl ions from the extravascular space, increasing the luminal concentration in the lymphatics as the extravascular abundances normalize.

Thus, both morphologic and elemental aspects of this work suggest that occluder removal leads to edema across the retina, RPE, and choroid. The time sequences of thickness within

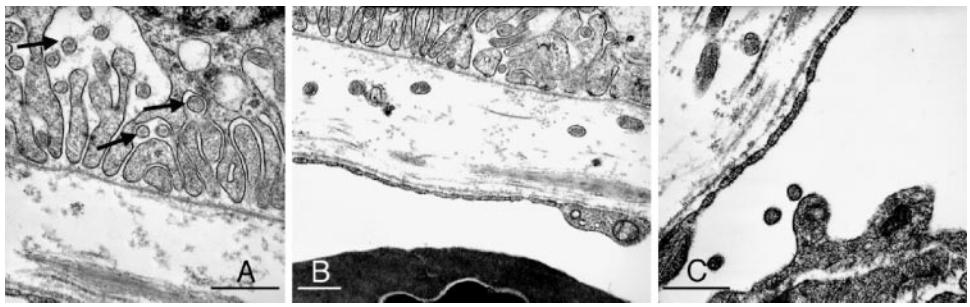

FIGURE 10. Electron micrographs of RPE, Bruch's membrane, and choriocapillaris 120 hours after removal of the occluder. (**A**) Round vesicular structures (*arrows*) were visible in the basal infoldings of the RPE. Note the apparent encapsulation of fluid in the proximal loops of the basal infoldings. (**B**) Similar vesicular structures within Bruch's membrane. (**C**) Round structures in the lumen of the choriocapillaris. Scale bars, 0.5 μm.

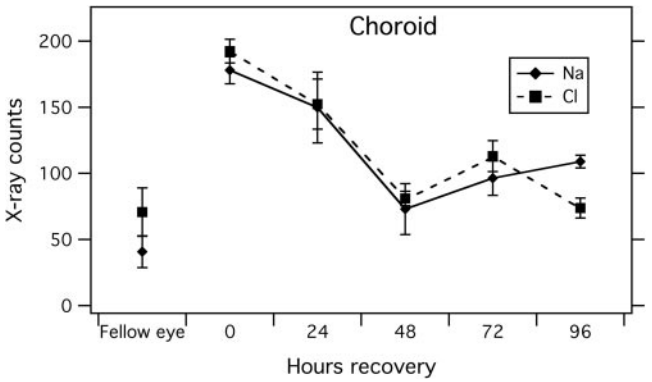

FIGURE 11. Relative abundances of Na and Cl ions measured in the inner choroid of freeze-dried tissue from fellow eyes compared with that measured in eyes recovering from FD-induced myopia. The abundance of both ion species was three to four times that of fellow eye choroid at eye-opening, but resolved to comparable levels by approximately 48 hours after occluder removal.

tissue components indicates that the return toward final thickness (from maximum) starts earlier in the retina than in the RPE and choroid (or Bruch's membrane) suggesting that fluid movement is a possible vector that could be used by the eye to transform rapidly the axial dimensions of the eye and its tunics.

Recovery from FD myopia in this breed of chickens, from more than 20 D of myopia to emmetropia, took the same time (120–144 hours of normal visual experience) as normalization of the occlusion-induced ultrastructural changes, thus further supporting the idea of physiological normalization and edema playing a central role in re-emmetropization.

The Implications of Biometrics and Ultrastructural Analyses for Understanding Refractive Recovery

In 1995, Wallman et al.[16] offered three possible sources of choroidal thickening: increased glycosaminoglycans in the extracellular matrix followed by passive water movement, increased drainage of aqueous humor into the choroidal compartment, and increased capillary permeability. Pendrak et al.[18] concluded that the most likely of these possibilities for choroidal thickening is increased passive choroidal vessel permeability due to a choriocapillaris abnormality[30,47] and a continuation of the deprivation-induced reduction in choroidal blood flow in the first couple of days of recovery.[20] However, a recent study found reduced vascular exchange at only 7 hours after occluder removal with a peak at 19 hours and a subsequent return to normal,[21] although the question of flow in the fellow eye is variable, with Jin and Stjernschantz[22] reporting an increase of flow in all tissues.

It is thus difficult to explain the development of edema in the choroid beyond this time (peaking at 72 hours) in terms of these transient flow changes. Pendrak et al.[18] also note that since the choroidal glycosaminoglycans are least when the choroid is thickest, the thickness increase cannot be simply explained by the tissue water's tracking the sulfated glycosaminoglycans.

The hypothesis of drainage of aqueous humor into the choroidal lymphatics as the cause of choroidal thickness changes is supported by reduced fluorescein dextran measured in the lymphatics when injected into the aqueous chamber of FD chick eyes and the observation of an increase during recovery.[22]

Another hypothesis involves intraocular pressure reduction during recovery from FD and the consequent lowering of resistance to blood flow, and such an increase in flow then contributes to choroidal expansion. Possibly related is the idea of van Alphen[48] that the choroid could be considered as a solid sheet of smooth muscle able to resist intraocular pressure in part and to regulate scleral stretch in the growing eye. Relaxation in this sheath would allow choroidal expansion to occur more readily.

Source of the Edema

Our morphologic observations of the reorganization of the RPE basal lamina infoldings during recovery from FD are consistent with the presence of hyperosmolarity at the time of occluder removal and the rapid establishment of edema across the retina and the RPE, gradually building in the choroid to peak 3 days after occluder removal. Two sources of fluid are possible: the choroidal blood flow and the retinochoroidal flow from the vitreous chamber across the RPE. We favor the vitreous chamber as the likely source, as it is capable of delivering the volume of fluid involved in the expansion of the choroid and the timing of transient changes in choroidal flow do not appear to correlate with the timing of choroidal swelling. Such a hypothesis of vitreous fluid excess under FD conditions is supported by studies showing an increased amount of sol (liquid) vitreous with normal hyaluronate in occluded chick eyes,[49,50] which would be consistent with a reduction in retinochoroidal flow during deprivation. In addition, alterations in electrolyte bal-

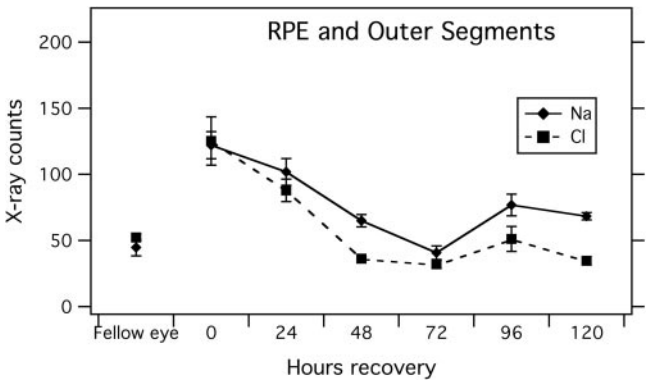

FIGURE 12. Relative abundances of Na and Cl ions in the outer segments and RPE of the freeze-dried tissue from normal fellow eyes compared with that measured in eyes recovering from FD-induced myopia. Cl ionic abundances seemed to recover to mean fellow eye values by 48 hours, though Na ion values remained higher than that in fellow eyes for considerably longer.

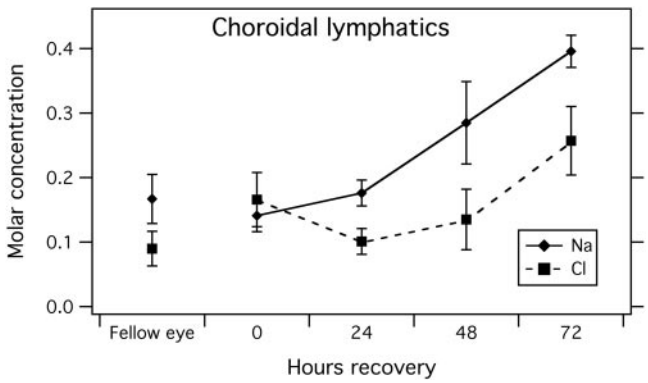

FIGURE 13. Molar concentration of Na and Cl ions in the lymph of the choroidal lymphatics of bulk-frozen tissue from fellow eyes and at various times after occluder removal of the eyes with FD-induced myopia, up to 72 hours after occluder removal.

ance have been observed in the vitreous of myopic eyes—namely, increased chloride, decreased potassium and phosphate, and similar sodium and calcium compared with the control.[51] The increase we observed in sodium and chloride ions through x-ray microanalysis in the RPE and choroid is consistent with the edema resulting from this hyperosmolarity, the decay in levels occurring over a time scale similar to that of the recovery of choroid.

Conclusion

Recovery from FD myopia may be interpreted as the reestablishment of normal physiological functions attenuated during occlusion. Removal of occlusion results in the gradual recovery of ultrastructural integrity and reestablishment of the normal appearance of the outer segments of the photoreceptors-RPE and the choroid. These refractive, ultrastructural, and ionic sequelae of occluder removal after FD have been demonstrated to occur concurrently with edema, which appears in the first day after occluder removal and recovers first in the retina and reaches a peak in the choroid only 3 days after occluder removal.

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References

1. Au Eong K, Taylor H, Lim M. Race, culture and myopia in 110,236 Singaporean males. *Singapore Med J*. 1993;34:29–32.
2. Fledelius H. Myopia profile in Copenhagen medical students 1996–98. Refractive stability over a century is suggested. *Acta Ophthalmol Scand*. 2000;78:501–505.
3. Mavracanas T, Mandalos A, Peios D, et al. Prevalence of myopia in a sample of Greek students. *Acta Ophthalmol Scand*. 2000;78:656–659.
4. Villareal M, Ohlsson J, Abrahamsson M, Sjostrom A, Sjostrand J. Myopisation: the refractive tendency in teenagers. Prevalence of myopia among young teenagers in Sweden. *Acta Ophthalmol Scand*. 2000;78:17–81.
5. Seet B, Wong T, Tan D, et al. Myopia in Singapore: taking a public health approach. *Br J Ophthalmol*. 2001;85:521–526.
6. Zadnik K. The Glenn A. Fry Award Lecture. (1995). Myopia development in childhood. *Optom Vision Sci*. 1997;74:603–8.
7. Junghans BM, Crewther SG. The prevalence of myopia among primary school children in eastern Sydney. *Clin Exp Optom.* 2003;86:339–345.
8. Nathan J, Kiely PM, Crewther SG, Crewther DP. Disease-associated visual image degradation and spherical refractive errors in children. *Am J Optom Physiol Opt.* 1985;62:680–688.
9. Meyer C, Mueller M, Duncker G, Meyer H. Experimental animal myopia models are applicable to human juvenile-onset myopia. *Surv Ophthalmol*. 1999;44:S93–S102.
10. Wiesel TN, Raviola E. Myopia and eye enlargement after neonatal lid fusion in monkeys. *Nature*. 1977;266:66–68.
11. Norton TT, Essinger JA, McBrien NA. Lid-suture myopia in tree shrews with retinal ganglion cell blockade. *Vis Neurosci.* 1994;11:143–153.
12. Wallman J, Turkel J, Trachtman J. Extreme myopia produced by modest change in early visual experience. *Science*. 1978;201:1249–1251.
13. Troilo D, Gottlieb MD, Wallman J. Visual deprivation causes myopia in chicks with optic nerve section. *Curr Eye Res*. 1987;6:993–999.
14. Wallman J, Gottlieb M, Rajaram V, Fugate-Wentzek L. Local retinal regions control local eye growth and myopia. *Science*. 1987;237:73–77.
15. Wildsoet CF, Pettigrew JD. Experimental myopia anomalous eye growth patterns unaffected by optic nerve section in chicks: evidence for local control eye growth. *Clin Vision Sci*. 1988;3:99–107.
16. Wallman J, Wildsoet C, Xu A, et al. Moving the retina: choroidal modulation of refractive state. *Vision Res*. 1995;35:37–50.
17. Liang H, Crewther SG, Crewther DP, Pirie B. Morphology of the recovery from form deprivation myopia in the chick. *Aust N Z J Ophthalmol*. 1996;24:41–44.
18. Pendrak K, Papastergiou GI, Lin T, Laties AM, Stone RA. Choroidal vascular permeability in visually regulated eye growth. *Exp Eye Res*. 2000;70:629–637.
19. Junghans B, Crewther S, Liang H, Crewther D. A role for choroidal lymphatics during recovery from form deprivation myopia? *Optom Vis Sci*. 1999;76:796–803.
20. Shih YF, Fitzgerald ME, Norton TT, Gamlin PD, Hodos W, Reiner A. Reduction in choroidal blood flow occurs in chicks wearing goggles that induce eye growth toward myopia. *Curr Eye Res*. 1993;12:219–227.
21. Fitzgerald ME, Wildsoet CF, Reiner A. Temporal relationship of choroidal blood flow and thickness changes during recovery from form deprivation myopia in chicks. *Exp Eye Res*. 2002;74:561–70.
22. Jin N, Stjernschantz J. Regional blood flow in the myopic chick eye during and after form deprivation: a study with radioactively-labelled microspheres. *Exp Eye Res*. 2000;71:233–238.
23. Hamann S. Molecular mechanisms of water transport in the eye. *Int Rev Cytol*. 2002;215:395–431.
24. Nickla DL, Wildsoet C, Wallman J. Visual influences on diurnal rhythms in ocular length and choroidal thickness in chick eyes. *Exp Eye Res*. 1998;66:163–181.
25. Nickla DL, Wildsoet CF, Troilo D. Endogenous rhythms in axial length and choroidal thickness in chicks: implications for ocular growth regulation. *Invest Ophthalmol Vis Sci*. 2001;42:584–588.
26. Nickla DL, Wildsoet C, Wallman J. The circadian rhythm in intraocular pressure and its relation to diurnal ocular growth changes in chicks. *Exp Eye Res*. 1998;66:183–193.
27. Nickla DL, Wildsoet CF, Troilo D. Diurnal rhythms in intraocular pressure, axial length, and choroidal thickness in a primate model of eye growth, the common marmoset. *Invest Ophthalmol Vis Sci*. 2002;43:2519–2528.
28. Marmor M, Wolfensberger T, eds. *The Retinal Pigment Epithelium*: *Function and Disease.* 2nd ed. Oxford, UK: Oxford University Press; 1998.
29. O'Leary DJ, Millodot M. Eyelid closure causes myopia in humans. *Experimentia*. 1979;35:1478–1479.
30. Liang H, Crewther DP, Crewther SG, Barila AM. A role for photoreceptor outer segments in deprivation myopia. *Vision Res*. 1995;35:1217–1225.
31. Beresford J, Crewther S, Crewther D. Anatomical correlates of experimentally induced myopia. *Aust N Z J Ophthalmol.* 1998 (suppl.);26:84–87.
32. Beresford JA, Crewther SG, Kiely PM, Crewther DP. Comparison of refractive state and circumferential morphology of retina, choroid, and sclera in chick models of experimentally induced ametropia. *Optom Vis Sci*. 2001;78:40–49.
33. Junghans BM, Crewther SG, Liang H, Crewther DP, Wareing L, Pirie B. Lymphatics in the chick choroid? *Aust N Z J Ophthalmol*. 1996;24:47–49.
34. Wildsoet C, Wallman J. Choroidal and scleral mechanisms of compensation for spectacle lenses in chicks. *Vision Res*. 1995;35:1175–1194.
35. Junghans BM, Crewther S, Pirie B, Crewther D. Are the lymphatics of the chick choroid an active storage reservoir? In: Rogers P, Cook B, Andrews F, Knight K, eds. *Progress in Microcirculation Research.* Melbourne, Australia: Monash University Press; 1997:84–86.
36. De Stefano M, Mugnaini E. The fine structure of the avian choroid: the lymphatic system. *Invest Ophthalmol Vis Sci*. 1997;38:1241–1260.
37. Földi M, Casley-Smith J. *Lymphangiology.* Stuttgart: FK Schattauer Verlag; 1983.

38. Wadley R, Junghans B, Liang H, Dickson M. A quantitative cryo-scanning x-ray microanalysis protocol for the examination of the eye. *Scanning*. 2002;24:34–38.
39. Casley-Smith J. The functioning and interrelationships of blood capillaries and lymphatics. *Experientia*. 1976;32:1–12.
40. Reinsch C. Smoothing by spline functions. *Numer Math*. 1967;10: 177–183.
41. Young RW. The daily rhythm of shedding and degradation of rod and cone outer segment membranes in the chick retina. *Invest Ophthalmol Vis Sci*. 1978;17:105–116.
42. Liang H, Crewther SG, Crewther DP. A model for the formation of ring mitochondria in retinal pigment epithelium. *Eye Science*. 1995;11:1–5.
43. Lauber JK. Retinal pigment epithelium: ring mitochondria and lesions induced by continuous light. *Curr Eye Res*. 1982;2:855–862.
44. Lauber JK. Diurnal mitochondrial changes in avian retinal pigment epithelium: a search for correlation with thyroid state. *Curr Eye Res*. 1982;2:863–868.
45. Westbrook AM, Crewther SG, Liang H, et al. Formoguanamine-induced inhibition of deprivation myopia in chick is accompanied by choroidal thinning while retinal function is retained. *Vision Res*. 1995;35:2075–2088.
46. Siegwart J, Norton T. The susceptible period for deprivation-induced myopia in tree shrew. *Vision Res*. 1998;38:3505–3515.
47. Hirata A, Negi A. Morphological changes of choriocapillaris in experimentally induced chick myopia. *Graefes Arch Clin Exp Ophthalmol*. 1998;236:132–137.
48. van Alphen GW. Emmetropization in the primate eye. *Ciba Found Symp.* 1990;155:115–120; discussion 120–125.
49. Pickett-Seltner RL, Sivak JG, Pasternak JJ. Experimentally induced myopia in chicks: morphometric and biochemical analysis during the first 14 days after hatching. *Vision Res*. 1988;28:323–328.
50. Lauber JK. Review: avian models for experimental myopia. *J Ocul Pharmacol*. 1991;7:259–276.
51. Seko Y, Shimokawa H, Pang J, Tokoro T. Disturbance of electrolyte balance in vitreous of chicks with form-deprivation myopia. *Jpn J Ophthalmol*. 2000;44:15–19.

E R R A T A

Erratum in: "Pharmacokinetic and Toxicity Study of an Intraocular Cyclosporine DDS in the Anterior Segment of Rabbit Eyes" by Theng et al. (*Invest Ophthalmol Vis Sci.* 2003;44:4895–4899).

The name of the second author was inadvertently misprinted. The correct form of the name is Seng-Ei Ti.

The online version of this article was corrected on June 17, 2004.

Erratum in: "Retinal Colocalization and In Vitro Interaction of the Glutamate Transporter EAAT3 and the Serum- and Glucocorticoid-Inducible Kinase SGK1" by Schniepp et al. (*Invest Ophthalmol Vis Sci.* 2004;45:1442–1449).

In the title of this article, the word *receptor* was inadvertently used in place of the correct word, *transporter.* The title should read as given above.