THE DEVELOPMENT OF THE CILIARY EPITHELIUM IN THE EMBRYONIC CHICKEN EYE

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REICHMAN

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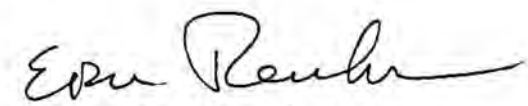

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ABSTRACT

Title of Dissertation: The Development of the Ciliary Epithelium of the Embryonic Chicken Eye

Eric F. Reichman, Doctor of Philosophy, 1989

Dissertation directed by: David C. Beebe, Ph.D.
Professor and Chairman
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The embryonic optic cup consists of two layers. The inner layer consists of the presumptive neural retina and nonpigmented layer of the ciliary epithelium. The outer layer comprises the presumptive retinal pigmented epithelium and the pigmented layer of the ciliary epithelium.

The pigmented and nonpigmented layers of the ciliary epithelium begin to differentiate ultrastructurally from the adjacent retinal pigmented epithelium and neural retina, respectively, by day four of development. By the sixth day of development, the pigmented and nonpigmented layers of the ciliary epithelium have acquired the morphological characteristics of fluid and electrolyte transporting cells. It is possible that the cells of the ciliary epithelium are secreting embryonic aqueous humor and are responsible, at least in part, for intraocular pressure in the chicken embryo.

The present work provides the initial utilization of acetylcholinesterase, the enzyme which hydrolyzes acetylcholine, as a marker for ciliary epithelial development

in the chicken embryo. Specific histochemical staining for acetylcholinesterase was initially detected in the presumptive ciliary epithelium on day four of embryonic development whereas it did not appear in the retina until day six. On this basis, it was possible to differentiate the presumptive ciliary epithelium from the adjacent presumptive retina early in development. The function of acetylcholinesterase in the developing ciliary epithelium is unknown, but from the data currently available it seems unlikely to be involved in the degradation of the neurotransmitter, acetylcholine.

I have examined changes in cellular dynamics during the early development of the ciliary epithelium of the embryonic chicken eye. Striking changes were observed in the labelling indices, cell packing density, cell height and cell volume of the pigmented and nonpigmented layers of the ciliary epithelium. Reduction of intraocular pressure, by intubation of the embryonic eye with a hollow tube, affected all these parameters. The data raise the possibility that active transport by the cells of the ciliary epithelium cause an increase in cell volume which precedes the formation of the ciliary folds. If this interpretation is correct, the epithelial layers of the ciliary epithelium may indirectly regulate their own mitotic activity and contribute to their own morphogenesis through the generation of intraocular pressure in the embryo.

The Development of the Ciliary Epithelium in the Embryonic Chicken Eye

by

Eric F. Reichman

Dissertation submitted to the Faculty of the Department of Anatomy Graduate Program of the Uniformed Services University of the Health Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy 1989

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

The size of the vertebrate eye at any stage in its growth and development is the result of the interaction of a large number of factors which change throughout development (Coulombre, 1965; Beebe,1986). These factors include contact between the optic cup and overlying head ectoderm (McKeehan, 1954), genetics (Harrison, 1925; Twitty, 1931), the effect of the lens on the development of the cornea, vitreous body, and ciliary epithelium (Mangold, 1931; Amprino, 1949; Neifach, 1952a,b; Lopashov and Stroeva, 1961, 1963; Lopashov, 1963; Coulombre & Coulombre, 1964; Coulombre, 1965), the effect of the presumptive neural retina on the development of the lens (Spemann, 1901; Lewis, 1904; McKeehan, 1951; Woerdeman, 1962), the invagination of the optic vesicle and the lens placode (McKeehan, 1958; Schook, 1978), the temperature during the time of development (Fielder, Levene, Russell-Eggitt and Weale), and intraocular pressure (Coulombre, 1956, 1957; Coulombre & Coulombre, 1957, 1958).

Researchers have been studying the development of the embryonic chicken eye for many years. The eye is one of the first organs to form in the developing chicken embryo. The early appearance and large size of the optic primordia in the embryo, the ex utero development, the easy accessability of the eye through a small hole in the shell, the low maintenance and embryo cost makes the chicken embryo an ideal system in

which to study eye development. In addition, the eye develops in a similar manner in all vertebrates (Duke-Elder and Cook, 1963; Coulombre, 1965; Pei and Rhodin, 1970; Aguirre, Rubin and Bistner, 1972; Bistner, Rubin and Aguirre, 1973; O'Rahilly, 1975). The only major differences are in the timing of the events. Most of these studies have focussed on the development of the optic vesicle, optic cup, lens, and retina (O'Rahilly and Meyer, 1959; Hunt, 1961; Weiss and Jackson, 1961; Domenech-Ratto, 1974; Camatini and Ranza, 1976; Hilfer, Brady and Yang, 1982; Hilfer, 1983). Except for the Coulombres (1955-1965) and Bard and Ross (1982a,b), researchers have ignored the development of the ciliary epithelium.

In the adult eye, the ciliary epithelium produces the aqueous humor, which nourishes the avascular tissues in the anterior segment and generates intraocular pressure (IOP). IOP is the pressure contained within the eye that is above atmospheric pressure. Normal IOP in adult humans ranges from 10-20 mm Hg. Given the importance of the ciliary epithelium in ocular physiology and pathology (Hoar, 1982; Beebe, 1986), it is surprising that so little is known of its development.

The Light Microscopic Development of the Ciliary Epithelium in the Embryonic Chicken

The chicken eye begins to develop as an outgrowth of the primitive diencephalic portion of the forebrain (prosencephalon) at stage 9 (O'Rahilly and Meyer, 1959; and Hilfer, 1983). At this stage, the outgrowth is called the optic vesicle (figure 1a). Its formation appears to be due to localized cell proliferation (Schook, 1978). The optic vesicle begins to adhere to the overlying surface ectoderm, the lens placode (figure 1b). The optic vesicle and overlying lens placode begin to invaginate at late stage 13 (figure 1c) to become the bilayered optic cup. The remnant of the optic vesicle (the optocoel) is gradually eliminated and the two layers of the optic cup become apposed over their entire surface between stages 15 and 17 (O'Rahilly and Meyer, 1959; Hilfer, 1983; figure 1d). The ciliary epithelium is formed from the two layers of cells which form the edge, or rim, of the optic cup (Beebe, 1986). The outer, pigmented layer of the ciliary epithelium is continuous with the retinal pigmented epithelium (figure 2b). The inner, nonpigmented layer of the ciliary epithelium is continuous with the neural retina (figure 2b). The folding of the optic vesicle does not occur uniformly in the eye (Hilfer, 1983). A groove, the choroid fissure, is formed in the ventral margin of the optic cup and extends into the optic stalk. The choroid fissure is

the area where blood vessels enter the eye. The iris is not present in the early embryo, but eventually develops as an outgrowth from the edge of the optic cup (Ferrari and Koch, 1984a,b).

The ciliary body consists of the ciliary epithelium, ciliary muscle and stromal tissue and is derived from the embryonic ectoderm (figure 58). The inner and outer layers of the ciliary epithelium develop from the optic cup and thus originate from neural plate ectoderm (Coulombre, 1965; Beebe, 1986). The ciliary muscle and stroma develop from head mesenchyme, whose origin is the cephalic neural crest (Johnston, et.al., 1979).

The development of the ciliary epithelium of the chicken eye occurs over a five- to six-day period (Coulombre, 1957, 1965). On E4, the edge of the optic cup is in contact with the lens equator and no ciliary zone or iris is grossly visible. The future ciliary zone is visible on day four due to greater accumulation of melanin in this region. This was confirmed by Harrison (1951), who showed tyrosinase activity appeared on day four of development. Tyrosinase is the enzyme which catalyzes the formation of melanin. As the eye continues to grow and develop, tyrosinase activity increases. At this early stage, the ciliary epithelium is attached anteriorly to the lens and the ciliary folds are not yet seen. From five to seven days of development, this zone increases in area and diameter while remaining symmetric about the lens

axis.

The appearance of the pericorneal (scleral) cartilage ring occurs during the eighth day (Weiss & Amprino, 1940), as does the beginning of the increase in corneal curvature (Coulombre, 1957; Coulombre & Coulombre, 1958a). The growth of the anterior portion of the eye slows due to the limits placed on it by the developing cartilage ring. The eye continues to expand not as a sphere; but as two spheres with a common base (Coulombre & Coulombre, 1958a). This is indicated by the corneal bulge anterior to the cartilage ring and the scleral bulge posteriorly; as well as by the asymmetric shape of the ciliary processes. They are narrowest anteriorly and widest posteriorly. This difference persists throughout life.

The iris first appears at the end of the ninth day or the beginning of the tenth day as a growth from the free edge of the ciliary epithelial zone. The iris rapidly increases in width until the seventeenth day of development. After day seventeen, the iris slows its growth to a constant rate. This rate, which is the same for the eye as a whole, allows the eye to grow uniformly.

The Role of the Lens in the Development of the Ciliary Epithelium

Many investigators who removed or transplanted the lens, did not examine the ciliary epithelium. The Coulombres (1964) found that removal of the embryonic chicken lens prevented eye growth. Replacement of the lens restored growth, suggesting that the surgical procedure was not the cause of the decrease in eye growth. Boiling of the lens to kill the cells before reimplantation prevented the eye from growing. In these studies, the effect of lens removal on the ciliary epithelium was not examined. It is possible that these eyes failed to grow because, in the absence of the lens, the ciliary epithelium did not form, resulting in reduced intraocular pressure and microphthalmia. Woerdeman (1963) extracted embryonic rat lenses *in utero* to determine if a lens could regenerate in mammals. He reported the effects on the iris, lens and retina. There were no comments on the state of the ciliary epithelium or if the eye was microphthalmic. Hilfer (1983) showed that the optic cup, in the chicken, formed without the presence of the lens placode and the eye remained microphthalmic. Genis-Galvez (1966) removed lens primordia from two or three-and-a-half day old chicken embryos and implanted them in the anterior region of the optic cup of a host embryo of the same age. The embryo thus possessed two lens primordia, its own and an implanted one. The embryos

were allowed to survive for periods ranging from a few hours to four days after surgery.. Upon examination, the host had two optic axes; one through its own lens and one through the implanted lens. The implanted lens induced the formation of a new cornea, anterior chamber, and pupillary margin. The survival times for these embryos was not long enough to determine if a second iris and ciliary epithelium developed.

Cultivation of embryonic rat eyes, in the anterior chamber of adult rat eyes, after removal of the lens, resulted in the absence of the ciliary epithelium. It appeared that the tissue that would have formed the ciliary epithelium was replaced by retina (Stroeva, 1967). Lens removal also resulted in microphthalmia (Stroeva and Panova, 1983). A similar experiment was performed by McKeehan (1961) who removed the lens from three- to five-day-old chicken embryos to determine if the "iris" could regenerate a new lens in vertebrates. He showed that the cells of the anterior rim of the optic cup or "iris underwent metaplasia in the direction of the neural retina from which they [the iridial cells] were microscopically indistinguishable". Even though the formation of the ciliary epithelium was not mentioned, the area referred to as "iris" was in fact the ciliary epithelium. The iris does not exist at these early developmental stages (Ferrari and Koch, 1984a,b).

In man, a similar morphologic condition exists. The overgrowth of the retinal-like tissue, without the formation of the normal architecture of the ciliary epithelium, is known as a glioneuroma (Zimmerman, 1970; Yanoff and Fine, 1982; Shields, 1983; DeJong, Barrsma and Polak, 1985). This benign, congenital tumor occurs on the edge of the nonpigmented layer of the optic cup and may cause compression and subluxation of the lens.

It is clear from the above experiments that the lens plays an important role in the growth and development of the eye. Specifically, it affects the differentiation of the cornea, anterior chamber, pupillary margin, iris and ciliary epithelium.

The Role of Intraocular Pressure in the Development of the Eye and the Ciliary Epithelium

Experiments on the role of intraocular pressure in eye development were performed in the late 1950's and early 1960's (Coulombre, 1956, 1957; Coulombre & Coulombre, 1957, 1958, 1964). They performed a series of operations on chicken embryos *in ovo* and observed the effects several days later. In the egg, the chick embryo lies on its left side. Therefore, all experiments were performed on the right eye and the left eye served as the contralateral control eye.

The first experiment involved intubating the eye with

a hollow capillary tube, which allowed the vitreous humor to leak out, thus presumably, decreasing the intraocular pressure. Upon examining these eyes, the Coulombres observed that: 1) the ciliary folds did not develop, 2) the ciliary zone progressively thickened, 3) the neural retina was thrown into large, asymmetric folds which adhered tightly to the lens, 4) eye growth slowed so that the eye was one-sixth the diameter of the control eye, 5) the corneal bulge did not appear, 6) the cornea was thicker due to proliferation of the stroma and Bowman's membrane, and 7) the folded neural retina was normal only where it was in close proximity to the retinal pigmented epithelium.

In the development of the human eye, intraocular pressure may act in a similar manner (Seefelder, 1923). In human embryos 10-15mm long, before closure of the choroid fissure, folds appeared in the presumptive neural retina, whereas the retinal pigmented epithelium was unfolded and adherent to the eye wall. After closure of the choroid fissure, when the vitreous could not escape, the folds disappeared and the presumptive neural retina was closely opposed to the retinal pigmented epithelium. This finding lends credence to a theory that the hydration of the vitreous is responsible, at least early in eye development, for increased intraocular pressure and for causing tension in the eye wall (Coulombre, 1956, 1957; Coulombre & Coulombre, 1957, 1958, 1964). It does not, however, rule out another source

of the intraocular pressure. Could the embryonic ciliary epithelium, neural retina, or retinal pigmented epithelium be pumping fluid into the eye causing, at least in part, the intraocular pressure?

These results resemble a pathologic, congenital condition in man. The cessation of eye growth brought about in chicken embryos by the presence of the hollow tube is similar to microphthalmia with associated cyst (Mann, 1937; Yanoff & Fine, 1982). In this developmental anomaly, the vitreous humour passes through a fistula and drains into a cystic space.

The cause of microphthalmia in mice appears to be the lack of intraocular pressure. In the ZRDCT-AN strain of microphthalmic mouse, the eyes do not have a developed ciliary epithelium (Silver, 1984), the tissue responsible for the generation of intraocular pressure. In the colombomatous microphthalmic mouse, the lack of complete closure of the choroid fissure allows the vitreous and aqueous humors to escape (Packer, 1967; Scholtz and Chan, 1987). The authors suggested that the lack of retention of the vitreous was the cause of the microphthalmia. They did not consider the reduction of intraocular pressure as a possible cause. Recently, it has been suggested that, in mice, the failure of the choroid fissure to close prevents the establishment of normal intraocular pressure and results in microphthalmia (Jackson, 1981; Kobayashi and Otani, 1981).

The Coulombres' second operation, which was similar to the first, involved removal of the tube after twenty-four hours. The operated eyes were smaller than the contralateral control eyes but normally proportioned. The eyes showed a decrease in the number of ciliary folds and the folds were not very large. This decreased eye size was thought to be due to resistance to growth caused by the continued differentiation of the pericorneal cartilage and a generalized thickening of the sclera during the period the eye was not growing. When the eye resumed growth, its size was limited by the already-differentiated surrounding tissues. There were also several low folds of the neural retina.

The eyes were intubated with a solid glass rod in the third operational group. These eyes served as operational controls. The eyes showed no significant changes from the unoperated eye in terms of eye growth and ciliary epithelial development. These operational controls prove that the trauma and other non-specific factors associated with the operation were not responsible for the failure of the intubated eyes to grow and develop.

The fourth experiment was to test the role of extraocular tissues in eye growth. The periocular mesoderm was stripped away from a small area on one surface of the eye. These eyes grew normally and appeared normal except for herniation of the eye wall through the area where the surrounding tissue had been stripped away. This indicated

that resistance to eye growth was provided by the extraocular tissues.

These four operations performed by the Coulombres provided indirect evidence that the intraocular pressure as well as the extraocular tissues contributed to the growth of the eye and ciliary epithelium.

The Coulombres concluded that the formation of ciliary folds was due to two factors. First, toughening of the pericorneal sclera, which is cartilage in the chicken embryo prevented the underlying area from expanding outward. If cell division continued in the ciliary epithelium, the epithelium would have to bulge inward. Secondly, radial tension from the intraocular pressure (vitreous humour ?) would cause folds to form radially between the lines of tension. They rationalized that the intraocular pressure was due to the production of the vitreal mucopolysaccharide, hyaluronic acid. The hydration of hyaluronic acid caused vitreal swelling which increased the pressure within the developing eye. It may be true that this causes, in part, the intraocular pressure (IOP) in the embryonic eye. It still does not rule out the ciliary epithelium, neural retina, or the retinal pigmented epithelium as contributing to the intraocular pressure. Throughout all of these experiments, intraocular pressure was never actually measured.

In subsequent experiments, Bard and Ross (1982b) showed that ciliary folds formed precociously when embryonic

chicken eyes had enlarged when placed in dilute ethanol solutions. These investigators also showed that, in chicken embryos, the rate of increase in the diameter of the eye slowed between stages 28-29 (embryonic day 5.5-6, E5.5-6), increased sharply between stages 29-30 (E6-6.5), then returned to the original rate (Bard and Ross, 1982a). These changes in growth rate occurred just prior to the formation of the ciliary folds. One interpretation of these data is that IOP may change significantly at about the time of fold formation.

Zauberman and co-workers (1978) tried several treatments to affect eye growth and intraocular pressure, including cryothermy and physical prevention of eye expansion. The only treatment which had any effect was cryothermy of the ciliary body. It caused reduced eye growth in in young rabbits for 10-14 days post-operative, at which point normal growth resumed. The experimental eye was always microphthalmic when compared to the control eye. They showed that intraocular pressure decreased during the period of growth retardation. The cells of both layers of the ciliary epithelium were severely damaged by the treatment and remained so during the period of reduced intraocular pressure and decreased eye growth. When the ciliary epithelial cells regenerated, intraocular pressure increased and eye growth resumed.

Carbonic Anhydrase and Embryonic Intraocular Pressure

In adult animals, including humans, carbonic anhydrase activity plays an important role in aqueous humor secretion and the generation of IOP. Carbonic anhydrase immunoreactivity first appears in the prospective ciliary epithelium at day two-and-a-half of embryonic development (E2.5, Linser and Cohen, 1989). The enzyme reaches maximal activity in the eye at E5. When carbonic anhydrase activity was inhibited, eye growth was decreased 25-50% in four- to seven-day-old chicken embryos (Linser and Plunkett, 1989). In addition, the offspring of pregnant mice treated with carbonic anhydrase inhibitors had eyes which were either missing or small (Scott, Lane, Randell, and Schreiner, 1984). These observations suggest that carbonic anhydrase activity in the presumptive ciliary epithelium may be involved in the generation of IOP and play an important role in ocular morphogenesis (Linser, Sorrentino, and Moscona, 1984; Linser and Cohen, 1989).

The Pecten Oculi and Intraocular pressure

The pecten oculi is a membranous, pigmented, vascular structure protruding from the optic nerve head into the vitreous body of birds (O'Rahilly and Meyer, 1961; Semba, 1962). The pecten develops about day eleven in the chicken embryo. It is known to have carbonic anhydrase and Na^+/K^+-ATPase activity; two of the major enzymes involved in aqueous humor secretion (Eichhorn and Flugel, 1988). Numerous

hypotheses have been postulated for the function of the pecten (Abelsdorf and Weesely, 1909; Semba, 1962; Wingstrand and Munk, 1965). It appears that the pecten may act in conjunction with the ciliary epithelium to secrete intraocular fluid and maintain intraocular pressure late in development and after birth, although this has never been proved.

The Onset of Secretion of Aqueous Humor

Although the timing of the onset of embryonic aqueous humor secretion is not accurately known for any species, circumstantial evidence is available. Generally, cells which are active in the process of fluid and electrolyte transport have elaborate, infolded and interdigitated cell membranes and numerous mitochondria (Pease, 1956; Tormey, 1963). This includes cells of the ciliary epithelium (Pappas and Smelser, 1958; Holmberg, 1959), the choroid plexus (Maxwell and Pease, 1956) and the nephron (Rhodin, 1954; Pease, 1955).

At birth, cells of the rabbit ciliary epithelium show no basal membrane infoldings or lateral intercellular interdigitations (Smelser and Brandt, 1959). Additionally, the volume of aqueous humor at birth was not measurable due to its extremely small quantity (Kinsey, Jackson and Terry, 1945). Aqueous humor formation in the rabbit is dependent on carbonic anhydrase activity which begins to appear in the nonpigmented layer of the ciliary epithelium two days postnatal (Bhattacherjee, 1972). At this time the cells of the ciliary epithelium first acquire the membrane

specializations associated with the secretion of aqueous humor (Weinquist, 1970). At six days postnatal, the cells of the nonpigmented layer of the ciliary epithelium contain the enzymes required for aqueous humor secretion (Friedenwald, 1949). At seven days postnatal, the cells resemble, morphologically, those seen in the adult ciliary epithelium (Pappas, Smelser and Brandt, 1959) and ascorbate is being actively transported (Kinsey, Jackson and Terry, 1945). From these data, one can infer that secretion of aqueous humor in rabbits begins soon after birth and is correlated with the acquisition of a characteristic cellular morphology.

In primates, similar observations have been made. By day 65 of embryonic development (E65) in monkeys, the blood-ocular barrier (Cunha-Vaz, 1979) is present and resembles that of the adult (Townes-Anderson and Raviola, 1981). Takei (1978) suggests that aqueous humor formation may begin about E100 in monkeys; the time at which membrane specializations and intracellular organelles required for secretion are evident. The ciliary epithelium in the human fetus begins developing the ultrastructural characteristics of a secretory epithelium at three months (Wulle, 1966). Folds begin to form in the ciliary epithelium near the end of the third month and are completed by the end of the fourth month (Wulle, 1966, 1962). It has been suggested that the ciliary epithelium in the human fetus begins secreting aqueous humor between the fourth and sixth month of development (Wulle, 1966, 1967,

1972).

Further evidence for the association of specific morphological characteristics required for the secretion of aqueous humor involved the alteration of aqueous humor secretion by the use of an inhibitor of carbonic anhydrase, known as diamox or acetazolamide. The loss of basal membrane infoldings and lateral intercellular interdigitations in adult rabbits treated with diamox were considered a morphological indicator of the disruption of normal aqueous humor secretion (Pappas and Smelser, 1958, 1961). Diamox also caused the accumulation of vesicles in the cytoplasm of the cells of the ciliary epithelium (Holmberg, 1959). Holmberg considered these to be secretory vesicles since they accumulated following inhibition of aqueous humor secretion. This finding may be an artifact of tissue processing since it could not be repeated (Tormey, 1963).

Intraventricular Pressure and its Relation to Intraocular Pressure

Intraventricular pressure has been shown to be necessary for the normal growth and morphogenesis of the central nervous system, as has IOP for the eye. The first demonstration of the existence of pressure, above atmospheric pressure, within the ventricular system of the developing central nervous system in the chick embryo was provided by Jelinek and Pexieder (1968). This pressure was required for

expansion of the walls of the brain vesicles (Pexieder and Jelinek, 1970). When intraventricular pressure was artificially decreased by fluid removal, the pressure was restored within a few hours (Jelinek and Pexieder, 1970; Pexieder and Jelinek, 1970). The source of the fluid responsible for the expansion of the walls of the central nervous system is unknown. The choroid plexus, the source of cerebrospinal fluid in the adult, is not developed in the early chicken embryo (El-Gammal, 1981).

There are several mechanical factors which are similar in both eye and brain development (Coulombre and Coulombre, 1957; Coulombre and Coulombre, 1958b). These include an internal expanding force (intraocular pressure or intraventricular pressure) and the resistance of the overlying tissue to limit growth. Intubation of the developing chick central nervous system with a hollow tube or an incision in the midbrain area caused the brain and ventricular system to be as little as one-fifth the normal size (Coulombre and Coulombre, 1958b; Domenech-Ratto, 1974; Desmond and Jacobson, 1977; Desmond, 1985). In addition, when pressure was reduced, the epithelium of the neural tube continued to grow and folded into the lumen, as the neural retina does in the eye. In some cases, incisions or intubations with a hollow tube to release intraventricular pressure in the early embryo caused folding in the central nervous system and the neural retina (Domenech-Ratto, 1974; Desmond and Jacobson, 1977). Early

investigators, using a variety of experimental operations, have named this phenomenon "neural overgrowth" (Patten, 1952; Sjodin, 1957; Burda, 1968).

The present work represents the beginnings of an attempt to understand the development of the ciliary epithelium and the events which occur during its morphogenesis. This work examines three separate, yet interrelated, studies on the development of the ciliary epithelium. The first study examines the morphological development of the ciliary epithelium and its differentiation from the adjacent neural retina and retinal pigmented epithelium. The second study examines acetylcholinesterase as a tissue specific marker for the development of the ciliary epithelium. The final section examines the cellular dynamics of the developing ciliary epithelium and the effects of mechanically reducing IOP.

THE LIGHT AND ELECTRON MICROSCOPIC DEVELOPMENT AND DIFFERENTIATION OF THE CILIARY EPITHELIUM

Introduction

The morphology of the ciliary epithelium at E4, E6, E10, E14, hatching and in adult chickens was examined by Porte, Stoeckel, Brini and Metais (1968). They showed the cells of the presumptive ciliary epithelium had some of the characteristics of ion transporting epithelia as early as the fifth day of embryonic development. They were the first and, until recently (Beebe, 1986), the only researchers to suggest that the cells of the ciliary epithelium might generate intraocular pressure early in development. This study examines the morphology of the ciliary epithelium throughout dveelopment and its differentiation from the adjacent neural retina and retinal pigmented epithelium.

Materials and Methods

Quantitation of ciliary fold formation: Embryos were removed from the shell, staged, enucleated, and the eyes bisected into anterior and posterior halves. Looking in from the cut surface of the anterior half of the eye, it was determined if the eye contained folds and the extent of the folding around the circumference of the ciliary body.

Transmission electron microscopy: Embryos were removed from the egg, immediately staged and enucleated. The eye was bisected into anterior and posterior halves and immersion

fixed in 1.0% paraformaldehyde, 1.25% glutaraldehyde, 0.1M sodium cacodylate buffer, and 0.0125% anhydrous $CaCl_2$, pH 7.2 for 4-6 hours at 4^0C. The tissue was then transferred to a more concentrated fixation solution (4.0% paraformaldehyde, 5% glutaraldehyde, 0.1M sodium cacodylate buffer, and 0.05% anhydrous $CaCl_2$, pH 7.2) overnight at 4^0C. Fixed tissues were rinsed 3 times for 10 minutes each in cold 0.1M sodium cacodylate buffer and post-fixed in 2% osmium tetroxide at 4^0C for 2 hours. The tissue was rinsed 3 times for 10 minutes each in 0.1M sodium cacodylate buffer, once for 10 minutes in 0.05M sodium cacodylate buffer, and treated with 0.05M sodium cacodylate buffer containing 1.0% tannic acid for 30 minutes. The tissue was rinsed twice for 4 minutes each in 0.05M sodium cacodylate buffer containing 1.0% sodium sulfate, dehydrated in a graded series of ethanol and propylene oxide to 100% propylene oxide and infiltrated and embedded in an epon-araldite mixture (Electron Microscopy Sciences, Fort Washington, Pa.). Thick (1um) and thin (60-80nm) sections were cut on an LKB ultratome. Thick sections were stained with toluidine blue and examined by light microscopy. Thin sections were stained with lead citrate and examined at 80KV in either a Zeiss 10A or JEOL 100cx transmission electron microscope. Tissue sections were photographed at a magnification of 10,000x and printed at a final magnification of 25,000x.

Preliminary electron microscopic examination was

performed on the eyes of 4,5,6,7,9,13, and 19-day-old chicken embryos. Electron microscopic examination of the ciliary epithelium was then focussed on the following embryonic ages: stages 24-26 (day 4), 24 hours after the optic cup is formed; stage 29 (day 6), just prior to ciliary fold formation; stage 32 (day 7), the time when the ciliary folds occupy half of the circumference of the ciliary body; and stage 45 (day 19), after the ciliary epithelium matures and just prior to hatching. The retina just posterior to the ora serrata was examined at these same developmental stages. Montages were made through the complete thickness of the ciliary epithelium (pigmented layer [PE] and nonpigmented layer [NPE]) and retina (neural retina and retinal pigmented epithelium [RPE]).

Results and Discussion

Light microscopy

On day seven, the first ciliary folds appear at stage 31 (figure 3) in the anterior-inferior rim of the optic cup, adjacent to the closed choroid fissure. This is the same area where the scleral cartilage will first form on day eight (Weiss and Amprino, 1940; Coulombre, 1956b). Folds continue to form, one at a time, in an orderly progression around the circumference of the optic cup. The processes increase in length and number until the end of the eighth day (stage 34) when they number approximately seventy primary folds (figure 3). On days nine and ten, about twenty secondary folds appear between the primary folds to bring the total to about ninety

radially-oriented folds. The number of folds does not increase beyond this, but they do increase in depth as the eye grows (stage 39, figure 3). In birds, the tips of these processes adhere to the lens capsule. Spaces appear between them which allow the future anterior and vitreous chambers to communicate with each other.

Electron microscopy

The electron microscopic study of the development of the ciliary epithelium was performed qualitatively. The purpose was not a quantitative study of the morphology; but rather a general morphological description of the development of the ciliary epithelium and its differentiation from the adjacent neural retina and retinal pigmented epithelium. A summary of the major changes in the ultrastructural development of these tissues can be found in table 1.

Pigmented ciliary epithelium-day 4

The cells of the PE were simple cuboidal in shape with the basal surfaces facing the vascular connective tissue core of the prospective ciliary body (figure 4). Numerous polysomes, free ribosomes, smooth vesicles and coated vesicles were dispersed within the cytoplasm. Smooth endoplasmic reticulum (SER) and stacks of Golgi apparatus were found scattered in the apical portion of the cell. An occasional basal body or cilium was present on the apical surface facing the apex of the NPE cells. Large lipid droplets were occasionally found in the apical portion of the cell.

Throughout the cytoplasm are occasional cisternae of rough endoplasmic reticulum (RER); but no lamellar pattern was seen. The basal cell membrane had a few infoldings with sparse mitochondria in the basal cytoplasm. The apical surfaces of the PE cells were connected to each other by densely stained junctional complexes, which included gap junctions and tight junctions below which were lateral intercellular spaces of variable width.

Retinal pigmented epithelium-day 4

The cells formed a simple columnar epithelium. They had numerous polysomes and free ribosomes scattered throughout their cytoplasm (figures 5 & 6). The apical cytoplasm contained sparse RER and stacks of Golgi apparatus with a few smooth vesicles and an occasional lipid droplet (figure 5). The amounts of smooth vesicles, lipid, and pigment appeared to be less than that seen in the pigmented layer of the ciliary epithelium. The basal cytoplasm contained sparse RER and mitochondria and membrane specializations were not detected (figure 6).

Nonpigmented ciliary epithelium-day 4

The cells were pseudostratified with their basal surfaces facing the posterior chamber and their apices facing the apices of the PE cells. The apical cytoplasm contained occasional RER with large cisternae, small stacks of Golgi apparatus, and mitochondria (figures 7 & 8). A few smooth and numerous coated vesicles were found in the apical cytoplasm

(figure 8). Junctional complexes, including what appeared to be gap junctions and tight junctions, connected the apicolateral membranes of the NPE cells and the apices of the NPE cells to the PE cells (figure 7). Bundles of microfilaments spanned the apical cytoplasm between the apicolateral junctional complexes. An occasional basal body or cilium were seen at the apical surface and projecting toward the PE (figure 7). Scattered throughout the cytoplasm were numerous polysomes, free ribosomes and long mitochondria (figures 7-10). Large spaces were seen between the two layers of the nuclear membrane suggesting that the nuclear membrane may be involved in protein synthesis until the RER becomes more developed. Occasional coated vesicles, smooth vesicles, RER and mitochondria were seen in the basal cytoplasm but the principle constituents were polysomes and free ribosomes (figure 10). The lateral cell surfaces contained numerous omega profiles facing the intercellular spaces. The majority of these omega profiles were smooth, thus it is unknown if they were in the process of pinocytosis or exocytosis. An occasional coated omega profile was seen, presumably indicating endocytosis (Anderson, Brown and Goldstein, 1977).

Neural retina-day 4

These cells appeared to be very different from those in the nonpigmented layer of the ciliary epithelium. The cells were stratified in numerous layers. The cytoplasm contained large amounts of free ribosomes and polysomes

(figures 11-13). Occasional smooth and coated vesicles were scattered throughout the cytoplasm and around the few mitochondria and sparse RER. The cells lacked junctional complexes or obvious membrane specializations.

Pigmented ciliary epithelium-day 6

The cells developed RER and stacks of Golgi apparatus apically and increased pigmentation between E4 and E6 (figure 14 and 15). Numerous smooth vesicles were present throughout the cytoplasm with a few scattered coated vesicles. The basal cell membrane had infoldings and lateral intercellular interdigitations formed between adjacent cells (figure 15). Basolateral gap junctions were visible joining adjacent pigment cells (figure 15). Coated and smooth omega profiles were found on both the basal and lateral surfaces of the cell membrane. At this time the cells of the PE showed the morphological characteristics of cells which are involved in fluid and electrolyte transport: basolateral cell membrane infoldings surrounded by mitochondria and lateral cell membrane interdigitations (Pease, 1956; Pappas and Smelser, 1959; Tormey, 1963).

Retinal pigmented epithelium-day 6

The cells appeared similar to those of E4 (figure 16). The cells contained less pigment than that seen in the pigmented cells of the ciliary epithelium. The cells of the RPE could be differentiated from those of the pigmented layer of the ciliary epithelium because the RPE cells lacked

extensive basolateral and lateral cell membrane elaborations and cytoplasmic vesicles that are seen in the pigmented layer of the ciliary epithelium (figures 16 & 17).

Nonpigmented ciliary epithelium-day 6

The cells were well differentiated from those of the neural retina. The amount of RER had increased apically and the cisternae were dilated (figure 18). Mitochondria have increased in the apical cytoplasm. Junctional complexes, including gap junctions and tight junctions, connect the apicolateral surfaces of the NPE cells and the apices of the PE and NPE. The lateral intercellular interdigitations were extensive (figure 19). The size of the lateral intercellular spaces had increased. The basal cytoplasm now contained coated vesicles and RER (figure 20). The basal and lateral cell membranes demonstrated numerous omega profiles of both the smooth and coated type. Basolateral cell adhesions are present between adjacent cells. By day 6, the cells had the morphological characteristics of cells which are involved in aqueous humor transport: basolateral cell membrane infoldings surrounded by mitochondria and lateral cell membrane interdigitations (Pappas and Smelser, 1959).

Neural retina-day 6

These cells (figures 21-23) have changed little relative to E4 and were quite distinct from those of the nonpigmented layer of the ciliary epithelium. The principle structures in the cytoplasm are still the numerous free

ribosomes and polysomes but little or no RER. Large Golgi apparatuses were apparent in the cytoplasm of some cells near the RPE (figure 23). Near the vitreal surface occasional coated and smooth vesicles and mitochondria were found (figure 21) while near the RPE, mitochondria and RER were the chief cytoplasmic organelles (figure 23).

Pigmented ciliary epithelium-day 7

The cells resembled those of day 6 with only a few exceptions. The basolateral cell membrane infoldings appeared to have increased in size and number (figure 24). Interdigitations were present over most of the lateral cell surface. The apical cytoplasm contained more SER and RER (figure 25). The number of apical smooth and coated vesicles appears to have also increased. The ultrastructure of cells in the folded and soon-to-be-folded regions of the PE appeared similar.

Retinal pigmented epithelium-day 7

The cells did not appear different from those at E6.

Nonpigmented ciliary epithelium-day 7

The cells appeared similar to those of day 6. There was a further distension of the RER cisternae (figure 26). An occasional cilium or basal body was seen at the cell apex. The cells had developed basal mitochondria. The ultrastructure of the cells in the folded and soon-to-be-folded regions of the NPE appeared similar.

Neural retina-day 7

The cells did not appear different from those at E6.

Pigmented ciliary epithelium-day 19

The cells had increased pigmentation and the basolateral infoldings are striking (figure 27). The lateral intercellular spaces appeared larger than at E7. Otherwise, the cellular ultrastructure was similar to that at E7.

Retinal pigmented epithelium-day 19

The amount of cytoplasmic pigment granules appeared to be increased. They have developed basal membrane infoldings and cytoplasmic vesicles similar to a secretory epithelium were detected, but not to the same extent as seen in the ciliary epithelium.

Nonpigmented ciliary epithelium-day 19

Infoldings and intercellular interdigitations of the cell membrane were more elaborate. The basal cytoplasm contained numerous coated and smooth vesicles, and omega profiles (figure 28). An extensive network of RER was distributed throughout the cytoplasm (figures 27 & 28). The cisternae of the RER were not as dilated as those seen at E7. The remainder of the ultrastructural characteristics were similar to that seen at E7.

Neural retina-day 19

The distinct layers of the mature retina were seen. The cells of the neural retina are clearly distinguishable from those of the nonpigmented layer of the ciliary

epithelium.

Comments on the presence of cilia

Occasionally, basal bodies and cilia were situated in the apical portion of the PE and NPE cells (figures 4,7 and 19). The existence of cilia in widely varied locations, including the central nervous system, has been a frequently reported finding at the ultrastructural level (Perez del Cerro and Snider, 1967). Cilia have been previously observed in the cornea (Hogan, Alvarado and Weddell, 1971), iris (Hogan, Alvarado and Weddell, 1971), trabecular meshwork (Vrabec, 1971), retina (Allen, 1965), retinal pigmented epithelium (Amemiya, 1975) and ciliary epithelium (Ohnishi and Tanaka, 1980; Tenkova and Chaldakov, 1988). Cilia have been found in the following cells which, like the ciliary epithelium, are derived from the central nervous system: astroglia, hippocampal neurons, developing hypoglossal motoneurons, cerebellar neurons, leptomeningeal fibroblasts, retinal ganglion cells and retinal bipolar cells (Dahl, 1963; Allen, 1965; Perez del Cerro and Snider, 1967; Borke, 1963). The function of cilia in the central nervous system is unknown; although it has been suggested at different times that they are vestigal remnants from development, chemoreceptors, or osmoreceptors.

A well developed Golgi apparatus is visible near of the base of the cilia in the apices of the PE and NPE. The association between the base of the cilium and the Golgi

apparatus has been previously described (Poole, Flint and Beaumont, 1985; Tenkova and Chaldakov, 1988). It has been suggested that this cilium-Golgi complex may be a way in which the cells of the ciliary epithelium monitor the content and production of aqueous humor and extracellular fluid (Tenkova and Chaldakov, 1988) or the contents of the extracellular fluid in a variety of epithelia (Webber and Lee, 1975; Poole, Flint and Beaumont, 1985). This would allow local responses to altered extracellular fluid composition. Cyclic adenosine monophosphate (cAMP) and cAMP-dependent protein kinases have been localized to the Golgi apparatus and the immediate surrounding area (Farquhar and Palade, 1981; Nigg, Schafer, Hilz and Eppenberger, 1985). It appears that cAMP is processed in the Golgi apparatus and secreted in vesicles that fuse with the plasma membrane (Farquhar and Palade, 1981). It has been suggested that kinase activation of cAMP may occur near the Golgi apparatus or that phosphorylation of the Golgi constituents may contribute to membrane traffic and secretion (Nigg, Schafer, Hilz and Eppenberger, 1985).

Cilia which are thought to be sensory in nature do not have the typical 9+2 microtubule pattern seen in motile cilia (Dahl, 1963; Webber and Lee, 1975; Ohnishi and Tanaka, 1980). The microtubules in these "sensory" cilia may provide a "gated" access to the plasma membrane for vesicles from the Golgi apparatus to the plasma membrane surrounding the cilium (Poole, Flint and Beaumont, 1985). This may maintain a unique

plasma membrane composition in the region of the cilium.

The blood-aqueous barrier and its relation to aqueous humor secretion

The NPE is a typical example of a cell specialized in fluid and electrolyte transport (Pease, 1986; Tormey, 1963). This includes large numbers of mitochondria, cell membrane specializations and tight junctions (zonula occludens) in the apicolateral cell membrane. It is the system of apicolateral cell membrane tight junctions which form the blood-aqueous barrier (Cunha-Vaz, 1979). The blood aqueous barrier is formed by these tight junctions in all species examined. This includes chickens (Smith and Raviola, 1983), frogs (Dabagian, Ganieva and Eshkind, 1979), humans and monkeys (Vegge, 1971; Shabo and Maxwell, 1972; Raviola, 1977; Raviola and Raviola, 1978), rabbits (Raviola and Raviola, 1978; Hirsch, Montcourrier, Arguillere and Keller, 1985) and rats (Hirsch, Montcourrier, Arguillere and Keller, 1985; Arguillere, Patey and Hirsch, 1986). The tight junctions at the apical surfaces of the nonpigmented epithelial cells resist flow and diffusion of substances directly into the aqueous humor. Most substances entering the aqueous humor are actively transported into the eye by the cells of the ciliary epithelium (Reddy, Rosenberg and Kinsey, 1961; Cunha-Vaz, 1979).

The ciliary body has been shown to contain high concentrations of the enzymes Na^+/K^+-ATPase (Bonting, Simon and Hawkins, 1961) and carbonic anhydrase (Wistrand and Garg,

1979). These enzymes are known to be involved in the secretion of aqueous humor. Inhibitors of these enzymes reduce aqueous humor production and intraocular pressure (Bonting and Becker, 1964; Cole, 1966; Becker, 1980).

Na^+/K^+-ATPase has been found to be localized on the basolateral cell membrane infoldings and lateral interdigitations of the cell membrane of the NPE cells (Flugel and Lutjen-Drecoll, 1988; Usukura, Fain and Bok, 1988). Carbonic anhydrase has been found to be localized on the basolateral cell membrane infoldings, lateral interdigitations of the cell membrane and the apical cell membrane of the NPE cells (Lutjen-Drecoll and Lonnerholm, 1981; Lutjen-Drecoll, Lonnerholm and Eichhorn, 1983; Lutjen-Drecoll, Eichhorn and Barany, 1985).

The cells in the pigmented and nonpigmented layers of the ciliary epithelium begin to differentiate from the adjacent retinal pigmented epithelium and neural retina, respectively, by E4. The ciliary epithelial cells develop the ultrastructural characteristics of fluid and electrolyte secreting cells by E6. Therefore these cells, may secrete embryonic aqueous humor and generate intraocular pressure in the embryo. For this reason, it will be important to study a) the electron microscopic localization of Na^+/K^+-ATPase and carbonic anhydrase and b) when they appear in the nonpigmented layer of the embryonic chicken ciliary epithelium. This information should be correlated with the time of formation

of the blood-aqueous barrier, which appears, from morphological criteria, to be present at E4. These studies would aid in the determination of the onset of secretion of embryonic aqueous humor begins in the chicken embryo.

ACETYLCHOLINESTERASE AS A MARKER FOR THE DEVELOPMENT OF THE CILIARY EPITHELIUM

Introduction

The current investigations involved studying the development of the ciliary epithelium and the identification of mechanisms which cause it to differentiate along a pathway that is distinct from that taken by the adjacent retina. These experiments required an early, tissue-specific marker to distinguish the ciliary epithelium from the adjacent retina. Several potential markers were examined. These markers were monoclonal antibodies found by others to specifically label the ciliary epithelium in mammals and monoclonal antibodies against enzymes known to be localized there (Hsi, Fredj-Reygrobellet, Yeh, Elena, Moulin and Lapalus, 1986; Fredj-Reygrobellet, Hsi, Yeh and Lapalus, 1987; Tsuji, Takagi and Fujisawa, 1989). None of these markers appeared early enough or was sufficiently specific to identify the ciliary epithelium in the early chicken embryo. However, I discovered that a histochemical stain for acetylcholinesterase (AChE) labelled the prospective ciliary epithelium early in eye development and not the adjacent retina.

Acetylcholinesterase Histochemistry

Cholinesterase (ChE) is an enzyme which catalyzes the hydrolysis of short-chain carboxylic esters. In mammals, two types of ChE exist (Butcher, 1983). Type I ChE shows a substrate preference for acetic esters such as acetylcholine. Type II ChE shows a substrate preference for other kinds of esters. These include butyrylcholine and propionylcholine.

Acetylcholinesterase (AChE), like most other enzymes, is not absolutely specific for the hydrolysis of acetylcholine (ACh), the usual physiological substrate. AChE and related enzymes have the ability to catabolize both naturally-occurring and synthetic, structurally-related substrate analogs (Butcher, 1983). For this reason, specific inhibitors must be used to identify unambiguously the enzyme responsible for AChE activity.

The incubation/reaction medium used in the current work utilized acetylthiocholine iodide as the substrate, glycine as a chelating agent, copper II ions as a capturing agent. Promethazine (phenergan) or tetraisopropyl pyrophosphoramide (iso-OMPA) was used to inhibit staining by nonspecific cholinesterases. Acetylthiocholine is hydrolyzed by AChE at a rate greater than that for ACh. It is believed that the sulfur linkage in acetylthiocholine is weaker than the oxygen bond of ACh, thus allowing a more rapid hydrolysis of acetylthiocholine (Lewis, 1977; Butcher, 1983). The thiocholine liberated by acetylcholinesterase combines with

the glycine-chelated copper II ions to form a white, chalky copper-thiocholine complex. When reacted with sodium sulfite, the copper-thiocholine complex may either be replaced by a copper-sulfite complex or covered over to form a copper-thiocholine-sulfite complex. This new complex, when reacted with silver nitrate, forms a brown to black precipitate, which can be visualized by light or electron microscopy.

This study uses AChE to study the development of the ciliary epithelium and its differentiation from the adjacent retina in the chicken embryo. Electron microscopy was performed to localize the AChE reaction product. Embryonic and postnatal rats were examined to determine if AChE may be used as a marker for the development of the ciliary epithelium in in a mammal or was this marker limited to chickens.

Materials and Methods

Acetylcholinesterase histochemistry: Chicken embryos were removed from the egg, staged (Hamburger and Hamilton, 1951), decapitated, embedded in O.C.T. (Miles Scientific, Naperville, Il.) and frozen at -20^{0}C in a cryostat (Hacker-Bright, Fairfield, N.J.). Twenty to twenty-five chicken embryos were used for each stage examined from E4-E9, E19 and hatchlings. Timed-pregnant, pigmented, Long Evans rats (Charles River, Boston, Ma.) weighing 150-300gms were killed by cervical dislocation, the embryos were dissected out of the uterus, decapitated and frozen as above. The rats used in this study were embryonic days (E) 15,16,18, & 20, and

postnatal days (P) 1 and 15. Twelve to twenty rats were used at each embryonic age and eight to ten rats at each of the postnatal ages. Sections were cut at 10 microns, collected on chrome-alum slides, and allowed to air dry for 1-3 hours. Tissue sections were stained for AChE by a modification of the thiocholine methods of Hardy, et.al. and Koelle and Friedenwald (Koelle and Friedenwald, 1949; Hardy, Heimer, Switzer, and Watkins, 1976). The sections were hydrated in two 15 minute changes of buffer (0.05M sodium cacodylate, 0.02M cacodylic acid, 0.002M calcium acetate, and 2.57% sodium sulfate; Lewis and Schute, 1969), then preincubated for 30 minutes at 37^0C in the same buffer containing 0.006% promethazine or 10^{-4}M tetraisopropyl pyrophosphoramide (iso-OMPA; Butcher, 1983) to inhibit non-specific esterases, especially butyrylcholinesterase (BuChE). Results were indistinguishable when either inhibitor was used. The sections were placed in the incubating solution (10mM glycine, 2mM $CuSO_4$, 0.05M sodium acetate buffer (pH 5.0), 0.006% promethazine or 10^{-4}M iso-OMPA, and 0.12% acetylthiocholine iodide) overnight in a 37^0C oven, rinsed 6 times for 30 seconds each in buffer, then incubated in 1.25% sodium sulfite for 1 minute. Sections were rinsed 4 times for 30 seconds each in buffer, once for 4 seconds in distilled H_2O, then incubated in 1.0% silver nitrate for 5 minutes. After staining they were rinsed for 5 seconds in distilled H_2O, 6 times for 30 seconds each in buffer, dehydrated in 3 changes

of 5 minutes each of 95% ethanol, 2 changes of 10 minutes each absolute ethanol, cleared in xylene and coverslipped using DPX mounting media (Gallard-Schlesinger, Carle Place, N.Y.). Alternate sections, after being stained for AChE, were stained with either toluidine blue or hematoxylin and eosin. All rinses and incubations, after the overnight incubation, were carried out at room temperature.

Transmission Electron Microscopy: Eight chicken embryos at E4 and E6 were removed from the egg, immediately staged and enucleated. The eye was bisected into anterior and posterior halves. These halves were stained for AChE activity as described above and immersion fixed in 1.0% paraformaldehyde, 1.25% glutaraldehyde, 0.1M sodium cacodylate buffer, and 0.0125% anhydrous $CaCl_2$, pH 7.2 for 4-6 hours at 4^0C. The tissue was then processed as previously detailed for ultrastructural examination.

Results

Light Microscopy-chicken embryo

Through the beginning of E4 (stage 24), there was no AChE positive staining detectable in any ocular structure. By stage 25 (the middle of E4), AChE positive staining was visible at the rim of the optic cup, adjacent to the lens (figure 29). This is the presumptive nonpigmented layer of the ciliary epithelium. Staining was more intense in the nasal half of the eye than the temporal half. Due to the

accumulation of melanin in the pigmented layer of the optic cup, it was not possible to determine, at the light microscopic level, whether this region was stained. On late E4 (stage 26), the staining became more intense in the presumptive ciliary epithelium. Staining initially appeared in the lens at stage 25 and this staining persisted throughout development. No staining was detected in the neural retina during E4 (stages 24-26). If acetylthiocholine iodide was omitted from the incubation media, no reaction product was observed.

The AChE staining pattern at E5 (stages 27-28) was similar to that of E4, although the intensity of staining in the ciliary epithelium increased (figure 30). The nasal ciliary epithelium continued to stain more intensely than the temporal half. The neural retina remained unstained.

At E6 (stages 29-30), the staining intensity again increased throughout the NPE (figure 31). Staining was seen in the developing ganglion cell layer in the posterior region of the neural retina (stage 29). The AChE staining in the ganglion cell layer increased on late E6 (stage 30) and began to appear in other layers of the posterior neural retina.

At E7 and E8 (stages 31-34), the staining of the NPE was intense and differences in the staining intensity between the nasal and temporal halves could no longer be observed (figures 32 & 33). The staining of the ganglion cell layer and other layers of the neural retina increased and proceeded

anteriorly as retinal differentiation continued, approaching the ora serrata, the transition zone between the ciliary epithelium and the retina, by stage 34.

At E9, staining of the neural retina had reached the ora serrata (figure 34). The boundary between the ciliary epithelium and retina could still be distinguished, because staining was more intense in the ciliary epithelium than the adjacent retina (figure 35). This pattern of AChE staining remained throughout the rest of development.

Electron Microscopy

The NPE contained AChE reaction product primarily in the cisternae of the RER and the cisternal space between the two layers of the nuclear membrane at the stages examined (E4 and E6; figures 36 & 37). A small amount of the reaction product was contained in intracellular vesicles and in the lateral intercellular spaces. No AChE reaction product was found free in the cytoplasm or associated with any other organelle. Relative to the NPE, the PE contained a small amount of the reaction product in the cisternae of the RER and the cisternal space between the two layers of the nuclear membrane (figure 36). This pattern remained throughout development.

Light Microscopy-rat

In the rat, AChE staining was first visible in the ganglion cell layer of the neural retina in the posterior aspect of the eye beginning on E16 (figure 38). The E16 rat

is developmentally equivalent to the E6 chick (Hebel and Stromberg, 1986; Butler and Juurlink, 1987). There was no staining detected in the presumptive ciliary epithelium.

At E17, staining was visible in the entire neural retina, ending at the ora serrata. No AChE staining was evident in the ciliary epithelium. This staining pattern persisted until birth. After birth, the only change seen was the appearance of light, punctate staining in the nonpigmented layer of the ciliary epithelium and over lens fiber cells at P15 (figure 39).

Discussion

Acetylcholinesterase appears to be useful as a marker for the early development of the ciliary epithelium in the chick embryo. The prospective ciliary epithelium was AChE positive from E4 through hatching. From E4 through E7, an abrupt transition was present between stained and unstained tissue at the ora serrata, the boundary between the ciliary epithelium and the retina. The neural retina did not stain for AChE until E6, when the newly-formed ganglion cells at the posterior of the retina showed detectable staining. AChE activity is the earliest known feature that distinguishes the ciliary epithelium from the retinal tissues at the light microscopic level. However, additional studies have shown that the ciliary epithelium acquires additional ultrastructural and functional features which distinguish it from the retina between E4 and E6 (see previous EM studies).

It is known that the neural retina differentiates from posterior to anterior as well as from the ganglion cell layer towards the photoreceptors (Coulombre, 1955; Layer, 1983; Layer, Alber, and Sporns, 1987; Spira, Millar, Ishimoto, Epstein, Johnson, et.al., 1987). The retinal differentiation, by morphology above, corresponds to the pattern of AChE and choline acetyltransferase (ChAT) staining in the chicken neural retina (Spira, et.al., 1987). In the present studies, AChE staining was found in the posterior ganglion cell layer at early E6 (stage 29) and proceeded anteriorly with time. On late E6 (stage 30), enzyme activity appeared at the posterior pole of the eye in the other layers of the neural retina, also proceeding anteriorly with development. The current results indicate that the prospective ciliary epithelium followed a distinctly different developmental sequence with respect to AChE staining. In addition, the ciliary epithelium exhibited earlier and more intense staining in the nasal than in the temporal regions, revealing what appears to be an underlying spatial difference in the early development of these tissues.

For many years researchers have been studying the development of the derivatives of the optic cup. In some cases, investigators have clearly discriminated between the developing ciliary epithelium and retina (Bard and Ross, 1982a,b; Stroeva and Mitashov, 1983; Young, 1985a,b). In other instances, authors have either ignored the existence of

the ciliary epithelium, or referred to it as the "anterior retina" or "iris" (Mckeehan, 1961; Sidman, 1961; Bodenstein and Sidman, 1987; Halfter and Fua, 1987; Fitch, Mentzer, Mayne, and Linsenmayer, 1988; Linser and Cohen, 1989). These studies included measurements of mitotic activity, labelling index and lens regeneration, as well as immunohistochemical staining for laminin, type IX collagen and carbonic anhydrase. In these previous studies, the data shown verify that the region referred to as "anterior retina" or "iris" corresponds to the prospective ciliary epithelium, as detected by AChE staining. In light of this, some previous studies of retinal development have misinterpreted events occurring in the prospective ciliary epithelium as those taking place in the retina.

Previous studies have described AChE staining in the chicken eye during early development (Layer, 1983; Layer, Rommel, Bulthoff, Hengstenberg, 1988). Staining of the ciliary epithelium was evident in these studies. This included the presence of a naso-temporal gradient which was not remarked upon by the authors. In other recent work by Halfter and Fua (1987) and Fitch, et.al. (1988) a similar gradient appears to be present in chicken embryo eyes immuno-stained for laminin and type IX collagen.

Layer (1983) ascribed positive cholinesterase staining in the anterior of the optic cup to BuChE. This conclusion was based on the presence of staining when either acetyl- or

butyrylthiocholine was used as the substrate. Butcher (1983) has pointed out that substrate specificity is not sufficient to distinguish between these enzymes, because both substrates are hydrolyzed by either of these enzymes, although at different rates. In order to assure that only AChE activity was detected, the inhibitors iso-OMPA or promethazine were used at concentrations which prevent the detection of BuChE activity when acetylthiocholine is used as a substrate (Butcher, 1983).

My interpretation of the results presented by Layer (1983) is that both acetyl- and butyrylcholinesterase are present in the anterior region of the optic cup. However, BuChE activity seems to be more widely distributed, appearing at the apical ends of the retinal cells, as well as in the ciliary epithelium. My results indicate that, between stages 25 and 29, AChE activity is restricted to the prospective ciliary epithelium, where staining for it reveals a sharp boundary between this tissue and the adjacent retina.

In neurons and other cells of neural origin that produce AChE, staining is consistently restricted to the cisternae of the rough endoplasmic reticulum, the cisternal space between the two layers of the nuclear membrane and extracellular spaces (Lewis and Shute, 1966; Flumerfelt and Lewis, 1975; Miki and Mizoguti, 1982; Mastrolia, Bichi, Arizzi, and Manelli, 1986). This AChE staining pattern is consistent with that seen in the ciliary epithelium, an ocular

structure which develops from a primordium of the embryonic nervous system. Since no reaction product was detected in mitochondria or lysosomes in the present studies, it is unlikely that the thiocholine esters were hydrolyzed by non-specific esterases.

Electron microscopic examination of the developing ciliary epithelium shows numerous coated pits and coated vesicles, particularly in the nonpigmented layer (see previous EM studies). Some of these contain the AChE reaction product. It has been suggested that, in rat and chick myotubes, coated pits and vesicles contain AChE for exocytosis (Bursztajn, Nudleman, and Berman, 1987; Benson, Porter-Jordan, Buoniconti, and Fine, 1985; Porter-Jordan, Benson, Buoniconti, and Fine, 1986).

Layer (1983) suggested that AChE may provide spatial clues for development. Others have speculated that the transient increase in AChE levels during brain development in the mouse and rat may aid in morphogenesis of the brain (Hohmann and Ebner, 1985; Robertson, 1987; Robertson, Hanes, and Yu, 1988) by effecting cell movement, cell recognition, and cell adhesion (Mastrolia et.al., 1986) as well as axon guidance (Robertson, 1987). Still others have shown AChE to have peptidase activity, which may influence other aspects of development (Chubb, Hodgson, and White, 1980; Millar and Chubb, 1984).

The developmental significance of early ChAT and AChE

activity in the eye is not presently known. This is particularly true because synaptogenesis in the chick neural retina does not begin until E10, at least four days after the appearance of ChAT or AChE activity in the retina (Coulombre, 1955; Spira, et.al., 1987). It has been suggested that ChAT and/or acetylcholine (ACh) may have an ontogenic role regulating other aspects of development, or that ACh is used as a signal to control the outgrowth and spread of neurites. Whatever the function of ChAT and AChE, the fact that enzymes involved in ACh metabolism appear early in the development of a large number of vertebrate species suggests that these enzymes are crucial for normal neural development (Puro, 1985; Spira, et.al., 1987).

According to Spira (Spira, et.al., 1987), AChE activity in chickens appears on E4 in the ganglion cells of the posterior retina. This is two days before these authors first demonstrated ChAT activity in this region and one to one-and-a-half days before AChE activity was detected in the current study and in several previous reports (Shen, Greenfield, and Boell, 1956; Layer, et.al., 1987). This discrepancy in timing may be due to the sensitivity or the specificity of the techniques used. If so, AChE activity may appear in the ciliary epithelium even earlier than indicated in the present results.

The role of AChE activity in the development or function of the ciliary epithelium is unknown. Previous

research using immunocytochemical assays has identified nerves containing norepinephrine, acetylcholine, calcitonin gene-related peptide and serotonin in the ciliary body. These axons innervated the blood vessels in the stroma of the ciliary body and the ciliary muscle of chickens, dogs, humans, monkeys, pigs, rabbits, and rats (Laties and Jacobowitz, 1964; Ehinger, 1966; Lucas and Cech, 1966; Staflova, 1969; Tobin, Unger, and Osborne, 1988). In these studies, and in my own electron microscopic studies (see previous section), there were no axons which crossed the basement membrane and innervated either layer of the ciliary epithelium. Recently, there have been rare, free nerve endings found which cross the basement membrane and innervate only the pigmented layer of the iridial processes of the ciliary body in the rabbit eye (Yamada, 1988). These nerves did not cross the basement membrane and innervate the ciliary epithelium proper.

The effects of agonists and antagonists of alpha- and beta-adrenergics, cholinergics, and many other compounds affecting the formation of aqueous humor and regulating intraocular pressure have been studied for many years (Miichi and Nagataki, 1983; Kaufman, Wiedman, and Robinson, 1984; Lotti, LeDouarec, and Stone, 1984; Sears, 1984a,b; Lapalus and Elena, 1988). These substances appear to work by binding to specific cell surface receptors. The ciliary epithelium has been found to have alpha- and beta-adrenergic receptors (Mittag and Tormay, 1985; Polansky, Zlock, Brasier, and Bloom,

1985; Elena, Kosina-Biox, Moulin, and Lapalus, 1987; Wax and Molinoff, 1987), and muscarinic cholinergic receptors (Polansky, et.al., 1985; Konno and Takayanagi, 1986; Mallorga, Babilon, Buisson, and Sugrue, 1989; Helbig, Korbmacher, Wohlfarth, Coroneo, Lindschau, et.al., 1989). Cholinergics are thought to alter the concentration of inorganic ions in aqueous humor and influence the secretion of the ciliary epithelial cells (Bito, Davson, and Snider, 1965; Walinder, 1966; Walinder and Bill, 1969). It is not presently known if nicotinic and/or muscarinic acetylcholine receptors exist on the embryonic chick ciliary epithelium. If they do, the AChE there may be hydrolyzing exogenous ACh to clear the receptor. If not, AChE may have some other developmental role in the ciliary epithelium.

AChE appears to be synthesized, processed, and secreted in the chick ciliary epithelium in a manner similar to that seen in neurons. Although the function of the AChE in the ciliary epithelium is presently unknown, it serves as an excellent marker to study ciliary epithelial differentiation and development. This finding will be of significance in studying the effects of experimental manipulations on the developing ciliary epithelium, including lens removal and the effects of reducing IOP.

CELLULAR DYNAMICS OF THE DEVELOPING CILIARY EPITHELIUM AND THE EFFECTS OF REDUCING INTRAOCULAR PRESSURE

Introduction

In the only previous study of cellular dynamics in the ciliary epithelium, Bard and Ross (1982b) labelled stage 29 chicken embryos with ^{3}H-thymidine for six hours, a relatively long labelling period. They found a difference in the labelling indices of the PE and NPE. According to these authors, "nearly all" of the NPE cells, but only about half of the PE cells, had detectable ^{3}H-thymidine grains. However, the long labelling period and the use of only a single time point provided little information about how the proliferative behavior of these cells might change during the development of the ciliary epithelium and the formation of ciliary folds.

I have pulsed-labelled embryos with BrdU from E5 to E8, a period that spans the formation of the ciliary folds, in order to examine the cellular dynamics of the cells of the ciliary epithelium during development. The effects of an apparent reduction in IOP, by intubation of eyes with a hollow tube, was examined. Labelling patterns differed in the PE and the NPE. Some of these changes appeared to be dependent on IOP. In the course of the studies, large changes were detected in cell packing density, cell height, and cell volume, most of which occurred just before or during fold

formation. These changes, too, appeared to be affected by reductions in IOP.

Bromodeoxyuridine Immunohistochemistry

Within the last few years, a non-autoradiographic method for distinguishing DNA-synthesizing (S-phase) cells has been developed. This immunocytochemical technique uses monoclonal antibodies, developed by Gratzner in 1982, to measure the incorporation of 5-Bromo-2'-deoxyuridine (BrdU) into DNA. BrdU, a thymidine analog (figure 40), becomes incorporated into cells synthesizing DNA. The monoclonal antibodies are specific for bromodeoxyuridine and iododeoxyuridine and do not cross react with DNA, thymidine, or uridine (Gratzner, 1982; Miller, Heyneman, Walker, and Ulrich, 1986). BrdU can be incorporated into cells in vivo (Schutte, Reynders, Bosman and Blijham, 1987a; Greenberg, Chandler, Diaz, Ensminger, Junck, et.al., 1988) or in vitro (Raza and Preisler, 1985). The tissue may then be processed for frozen sections or embedded in paraffin with equally good results (Schutte, Reynders, Bosman and Blijham, 1987a,b).

The use of BrdU instead of ^{3}H-Thymidine has many advantages (Schutte, et.al., 1987a,b; Raza, et.al., 1985). Detection of BrdU incorporation is more sensitive than detection of ^{3}H-thymidine incorporation due to the use of monoclonal antibodies specific for BrdU which can detect individual molecules. The radioactive background characteristic of autoradiography is also eliminated and thus

less pulse-time is required to label the DNA. Experiments with BrdU are less time consuming than autoradiography. BrdU studies may be completed in one day; whereas autoradiography takes at least one week to complete. It is also more economical to use BrdU instead of ^{3}H-thymidine. There is no need for expensive radioactive chemicals, radioactive permits, and costly radioactive material disposal methods. The use of BrdU, instead of ^{3}H-thymidine, eliminates the possibility of radioactive exposure.

A major prerequisite for BrdU immunocytochemistry is the accessibility of the antibody binding site (BrdU). Since DNA is usually double stranded, denaturation is necessary to unmask the binding sites and increase sensitivity. Treatment with mild acid for denaturation works well and preserves tissue integrity and antigenicity (Schutte, Reynders, Bosman and Blijham, 1987a,b). Alternative methods for DNA denaturation include heat (Dolbeare, Beisker, Pallavicini, Vanderlaan and Gray, 1985) and nuclease digestion (Gonchoroff, 1985). However, these latter methods have proved to be rather harsh, causing the tissue sections to dissolve and/or float off the slide. The sensitivity of the method can be improved by increasing the number of binding sites available for the antibody (Beisker, Dolbeare and Gray, 1987) by enhancing denaturation, with longer incubation times in stronger acid. This treatment increases sensitivity by increasing the amount of single stranded DNA; but the tissue morphology is very

poor. This method appears adequate for cultured cells which are analyzed using a flow cytometer in which morphology is not a prime goal. These methods are not suited for studies of embryonic tissue when morphology is an important factor.

The embryonic chicken eye has a large degree of background autofluorescence. Another method to reduce autofluorescence is to incubate the tissue in sodium borohydride or sodium azide (Bock, Hilchenbach, Schauenstein and Wick, 1985; Beisker, Dolbeare and Gray, 1987). These methods have proved to be inadequate in reducing autofluorescence in embryonic chicken tissue. For the present studies, F_{ab} fragments of the primary antibody rather than whole immunoglobulin G (IgG; Brandon, 1985) which contains F_{ab} and F_c fragments were used to reduce background autofluorescence and increase sensitivity. The F_c fragments tend to bind non-specifically and thus increase background fluorescence (Farr and Nakane, 1981). The use of a secondary F_{ab} antibody against the F_{ab} portion of the primary antibody also increases sensitivity over using whole IgG molecules.

The Effect of Mechanical Pressure on Cell Division

In the following results, the high rates of cell division in the ciliary epithelium may be due, in part, to the tension placed on the cells by intraocular pressure. Mechanical stretch or tension is known to control cell growth in other biological systems. The tension in the vitelline membrane is associated with cell proliferation and expansion

of the chick epiblast (Trinkaus, 1984). Relaxation of this tension severely slows, and can prevent, early development. Mechanical stretching of the skin in vivo is known to increase the mitotic activity and the labelling index of the epidermis (Lorber and Milobsky, 1968; Francis and Marks, 1977; Squier, 1980). Force on, or exercise of human skeletal muscle also stimulates cell division (Basset, 1971). Similar stresses applied to growing and/or repairing bone in vivo also stimulate cell proliferation (Frost, 1987).

Stretching or mechanical tension of cells in culture has also been examined. Increased tension on cultured: arterial smooth muscle cells (Leung, Glagov, and Mathews, 1976), bone cells (Hasegawa, Sato, Saito, Suzuki, and Brunette, 1985), epithelial cells (Brunette, 1984), fibroblasts (Curtis and Seehar, 1978), osteoblasts (Buckley, Banes, Levin, Sumpio, Sato, et.al., 1988) and skeletal muscle cells (Vandenburgh, Hatfaludy, Karlisch, and Shansky, 1989) caused an increase in the amount of DNA synthesis, an increase in the number of cells entering the cell cycle, and an increase in the labelling index. Growing cells in hypergravity, which causes tension on the monolayer, also increased proliferation in many transformed and non-transformed cell lines from a variety of species (Tschopp and Cogoli, 1983).

Most cells grown in culture are maintained in relatively static conditions. The thin layer of medium

immediately above the cells has been thought to form a "diffusion boundary layer". This layer may become depleted of nutrients and may accumulate cellular waste products, thus preventing cells from dividing. When cells grown in static cultures were transferred to shaking cultures, their mitotic and labelling indices significantly increased (Stoker, 1973; Stoker and Piggott, 1974). This finding was taken as proof of the existence of the diffusion boundary layer. It was hypothesized that the shaking of cultures prevented a diffusion boundary layer from forming by exposing the cells to fresh nutrients and removing waste products. It is now believed that the flow of media over the surface of the cultured cells, itself, caused a fluid shear stress which stimulated DNA synthesis and cell proliferation (Whittenberger and Glaser, 1978; Eskin, Ives, McIntire and Navarro, 1984; Davies, Remuzzi, Gordon, Dewey and Gimbrone, 1986; Ando, Nomura and Kamiya, 1987; Sumpio, Banes, Levin and Johnson, 1987). The shear stress translates into a pressure equal to the product of one-half the density of the media and the square of the velocity of the media (Weast and Astle, 1980).

Materials and Methods

BrdU Immunocytochemistry: A small hole was made in the blunt end of the shell of fertilized White Leghorn chicken eggs (Truslow Farms, Chestertown, Md.) from E4-E9 using a blunt probe. Through the hole, 0.96 ml of a solution containing 9.32×10^{-3}M BrdU (Sigma Chemical Co., St. Louis,

Mo.) in 0.01M phosphate buffered saline (PBS), was deposited on the vitelline membrane. This dose was approximately 50mg/kg or 2.75mg/egg (Schutte, Reynders, Bosman and Blijham, 1987a,b). The hole was sealed with tape and the egg reincubated at 37^{0}C in a humidified chamber for 1 hour. The embryo was removed from the shell, placed in 10% neutral buffered formalin (NBF; Schutte, et.al., 1987b) for 2 hours, staged (15-30 chicken embryos were used for each of the stages examined), decapitated, and the head immersion fixed in 10% NBF for an additional 10 hours. Alternatively, the embryo was removed from the shell, staged, and frozen at -20^{0}C. The head was subsequently positioned to allow transverse sections of both eyes, embedded in O.C.T. (Miles Scientific, Naperville, Il.) and sectioned at 5um in a cryostat. Sections were collected on chrome-alum slides and allowed to air dry overnight.

The fixed embryonic chicken heads were processed overnight in a Tissue Tek II tissue processor (Fisher Scientific, Silver Spring, Md.). The next morning, heads were oriented, embedded in paraffin (Schutte, et.al., 1987a,b), sectioned at 5um and floated onto clean glass slides. The slides were allowed to air dry and placed in a 37^{0}C oven overnight. The slides were deparaffinized, dehydrated in ethanols, and hydrated in running tap water for 10 minutes. Paraffin and frozen sections were rinsed in PBS for 5 minutes and placed in 2M HCl for 30 minutes in a 37^{0}C oven to denature

the DNA (Beisker, Dolbeare and Gray, 1987; Schutte, et.al., 1987a,b). After HCl treatment, slides were dipped for 3 minutes in 0.1M sodium tetraborate pH 8.5, then in 0.5% Tween-20 (polyoxyethylenesorbitan monolaurate in PBS; abbreviated hereafter as TBS). The slides were drained of excess TBS and 41ul of a primary antibody mixture consisting of a 2:5 dilution of unconjugated mouse IgG Anti-BrdU, $F(ab')_2$ (Becton Dickinson, Mountain View, Ca.) to TBS was added to each slide. The slides were incubated at 37^0C in a humidified chamber for 30 minutes, rinsed in TBS and placed in fresh TBS for 10 minutes. A secondary antibody mixture consisting of a 1:50 dilution of FITC-conjugated goat anti-mouse IgG(H+L),$F(ab')_2$ (Tago Inc., Burlingame, Ca.; Brandon, 1985) in TBS was allowed to warm to ambient temperature, slides were drained of excess TBS and 41ul of the secondary antibody mixture was added to each slide. The slides were incubated at ambient temperature for 30 minutes, rinsed in TBS to remove the excess secondary antibody and placed in fresh TBS for 10 minutes. The slides were counter stained for exactly 1.25 minutes in 0.04ug/ml propidium iodide and immediately placed in running water to prevent overstaining. After washing for approximately 10 minutes, the slides were coverslipped using Permafluor (Lipshaw Immunon, Detroit, Mi.), an aqueous, permanent mounting media, and were allowed to air dry for 24 hours in a light-tight slide box.

Three sets of control slides were processed as above with the following modifications: control #1 was not treated with the primary antibody; control #2 was not treated with the secondary antibody; and control #3 was not treated with either the primary or secondary antibody (Childs, 1983; Petrusz, 1983).

A Zeiss Photomicroscope III (Zeiss, Oberkochen, FRG) equipped with epifluorescence optics and filters for rhodamine (for propidium iodide) and fluorescein was used. Total cells (cell packing density), labelled with propidium iodide, and fluorescein-labelled cells that had incorporated BrdU into DNA were counted along a 49 micron horizontal length (in the plane parallel to the optical axis) of the ciliary epithelial region of the developing eyes, adjacent to the equator of the lens (figure 2A). The labelling indices were calculated from the number of labelled cells and the cell packing density.

Calculation of average cell volume: We have assumed that the cells of the ciliary epithelium have similar widths in the horizontal plane and the plane perpendicular to the optical axis. With this assumption, and a measure of cell height, one can estimate the volume of these cells. If there are X cells in a 49um length of the ciliary epithelium, then there are also X cells in a 49um depth of the ciliary epithelium. Therefore, average cell volume was calculated as follows:

Average Cell Area (um^2)= $(49/X)^2$= $2401/X^2$

Average Cell Volume (um^3)= Average Cell Area x Average Cell Height

Because cell packing density was measured by counting nuclei in tissue sections, there is a potential sampling error. Cells at the cut surface of a section may be counted as being within the section, because their nucleus is included within the section. This will result in an over-estimation of the number of cells in a section and, consequently, systematic under-estimation of cell volume. In addition, if cell volume increases but nuclear volume does not increase to the same degree, the volume of the larger cells will be under-estimated to a lesser degree.

Quantitation of ciliary fold formation: Embryos were removed from the shell, staged, enucleated, and the eyes bisected into anterior and posterior halves. Looking-in from the cut surface of the anterior half of the eye, the number of ciliary folds in each eye were counted and the percentage of the surface of the ciliary body covered with folds was determined.

Shell-less culture: Embryos were grown in shell-less culture, as described by Auerbach, Kubai, Knighton, and Folkman (1974). Growing chicken embryos in shell-less culture allowed them to be staged at varying times prior to and during

the time of intubation. This could not be accomplished if the embryos were allowed to remain in ovo with a portion of the shell removed to make a window. After incubation for three days, fertile eggs were placed in the horizontal position for one hour in preparation for shell-less culture (Auerbach, et. al., 1974). The eggs were swabbed with 70% ethanol and placed in a sterile, forced-air hood to dry. The eggs remained in the horizontal position in the hood to allow the embryos to rise to the surface. The underside of an egg was cracked against a sterile glass beaker and the contents carefully placed in an ultraviolet light-sterilized 20x100mm Falcon petri dish. If there appeared to be hemorrhages along the yolk vessels or if the yolk was scrambled, the embryo was discarded. The uncovered petri dish was placed in a sterile 25x150mm Falcon petri dish and placed in a clean, humid, 37°C incubator. Autoclaved, sterile H_2O (approx. 50-60ml) was added to the larger dish to maintain a sterile and humid environment immediately surrounding the embryo and to minimize the embryo's heat loss upon opening the incubator. A cover was placed on the large dish and the embryo incubated until required for intubation of the eye.

Ocular intubations: Micropipettes were pulled from Pasteur pipettes by hand. The tips were broken off and filled with an aqueous toluidine blue solution to assure that they were patent. The glass tubes were washed, ground down to about 2mm in length, and some of them were heat sealed on one

end (solid tubes). Tubes were sterilized with ultraviolet light and placed in sterile H_2O. Since the chicken embryo floats upon the yolk on its left side, all operations were performed on the right eye. Using sterile technique, the embryo was removed from the incubator and staged under a dissecting microscope. Embryos were intubated early on day five of development, stages 26 and 27. Being careful not to break any extraembryonic blood vessels, a tear was made in both the amnion and chorion above the head to expose the eye. A sterile hollow tube or solid tube (open end inward) was placed in the eye through the dorso-lateral aspect of the retina and into the vitreous body. The embryos were allowed to develop to stages 28-32, exposed to BrdU for one hour and processed as described above.

To determine if the hollow tubes were patent, one or more days after intubation of a set of control embryos, 2ul of an aqueous toluidine blue solution was injected into the intubated eyes opposite the entry point of the hollow tube. The embryos were observed periodically to determine whether the dye was leaking out the tube and thus, was patent. In 8 cases out of 8, the tubes were patent.

Transmission Electron Microscopy: Embryos were removed from the egg and processed as previously described. Tissue sections were photographed at a magnification of 10,000x and printed at a final magnification of 25,000x.

A transparent grid containing dots every 5mm was placed over the prints of the electron micrographs. The percent extracellular volume was determined by dividing the number of dots over intercellular spaces by the total number of dots over both intercellular spaces and tissue. Determination of extracellular space was made for the PE and NPE at days 4-7 of development.

Statistics: The data from the individual chicken embryos in each experimental group were compared to each other and then pooled. Student's T-test was used to determine the significance of the difference between the controls and the experimentals at the individual stages. A p-value ≤ 0.05 was considered significant. Analysis of variance and multiple range testing were performed to determine the significance of the difference between successive stages within the control or experimental groups. Error bars in the graphs represent the standard error of the mean.

Results

Labelling Indices

Significant changes occurred in the labelling index of the pigmented and nonpigmented layers of the ciliary epithelium during development. These changes were distinctly different in the two layers (figure 41). The labelling index of the PE declined at a constant rate from 28% at stage 26 (E4.5-5) to 8% at stage 34 (E8). The NPE had a labelling index of approximately 26% from stage 26 (E4.5-5) through 30

(E6.5). The labelling index then rapidly increased to 37% at stage 31 (E7), declining to 31% by stage 34 (E8).

The ciliary folds first began to form in the anterior-inferior rim of the optic cup below the closed choroid fissure at stage 31 (E7; figure 3 and 41). Folds continued to form, one at a time, around the circumference of the ciliary body in a counter clockwise direction. By stage 35 (E8-9), the ciliary folds were present around the entire circumference of the ciliary body.

As described above, when the ciliary folds first began to form (stage 31), the labelling index of the NPE increased from 26% to 37% (figure 41). The labelling indices of the NPE at stages 31-33 (E7-8) were derived from the folded and the soon-to-be-folded regions of the ciliary epithelium. Table 2 shows the labelling index for the separate layers of the ciliary epithelium over the stages when folds did not completely cover the circumference of the ciliary body. There was no statistically significant difference between these two regions in either the PE or NPE. These data suggest that the increase in the labelling index of the NPE may be required, but is not immediately or solely responsible for the formation of folds.

There were several morphological differences between eyes intubated with hollow tubes and eyes in the three control groups (unoperated right eyes, contralateral eyes in operated embryos and eyes intubated with solid tubes). Intubation with

hollow tubes resulted in eyes that resembled those described by Coulombre (1956b). Eyes intubated with hollow tubes were one-third to one-half the size of the control eyes (figure 42) and the neural retina formed large folds which filled the vitreous cavity (figure 43). The presumptive ciliary epithelium was also much thicker than in control eyes and it failed to form ciliary folds. Normal eye growth and morphogenesis occurred in all control groups.

Intubation had a differential effect on the labelling index of the PE and NPE (figures 44-46). In the PE, there was no significant difference between eyes intubated with either solid or hollow tubes, or eyes intubated with solid tubes and unoperated eyes (figures 44 and 46). In contrast, the reduction in IOP caused by intubation with a hollow tube resulted in a decrease in the labelling index of the NPE, both before and after the time of onset of fold formation (figure 45). These data suggested that IOP may maintain the high labelling index of the NPE and that the increased labelling index in the NPE at stage 31 may be caused by an increase in IOP.

The neural retina and retinal pigmented epithelium (RPE), which are derived from the same epithelial layers that form the two layers of the ciliary epithelium, had quite different labelling indices from those observed in the ciliary epithelium. The labelling index of the RPE was constant at approximately 6% over stages 29-32, while that of the neural

retina remained near 18% (E6-7.5; figure 47). Reduction of IOP by intubation with a hollow tube had no effect on the labelling index of the neural retina or RPE. These data indicate that, at these early stages of development, the epithelia of the ciliary epithelium differ from those of the adjacent retina in two ways; their labelling indices are higher and only the labelling index of the nonpigmented ciliary epithelium is affected by IOP.

Cell Packing Density

The cell density of the PE and NPE of the ciliary epithelium was measured before and during the formation of the ciliary folds (figure 48). The cell density of the PE declined from 14 cells in a standard 49um length at stage 28 (E5.5) to 8.5 cells at stage 30 (E6.5). Packing density increased slightly at stage 31 (E7), then declined slowly to 8 cells per unit length at stage 34 (E8). The average cell density of the NPE remained relatively constant at about 25 cells/49um through stage 30 (E6.5), rapidly declined to about 14 cells at stage 31 (E7), and continued to decline slowly, reaching 10 cells/49um at stage 34 (E8).

The cell density of both layers of the ciliary epithelium was affected by insertion of a hollow tube. In the PE, eyes intubated with hollow tubes had cell densities that were not significantly different from controls at stages 29 and 30 (E6 & E6.5; figures 49 & 51). While the cell density of the controls increased from stages 30-31 (E6.5-7), the cell

density of the eyes intubated with hollow tubes continued to decline. The differences were statistically significant (figure 51). In the NPE, intubation with a hollow tube prevented the normal rapid decline in cell density at stages 30-31 (figure 50). This difference was also statistically significant (figure 51). Eyes intubated with a solid tube followed the course of unoperated control eyes with no significant difference in cell density in both the PE or NPE (figures 49 & 50).

Cell Height

The height of the PE and NPE cells varied greatly between stages 23 and 33 (figure 52). The cell height of the PE was approximately 24um through stage 25 (E4.5), then rapidly and significantly declined to 11um at stage 26 (E4.5-5). The cell height remained at 11um through stage 28 (E5.5), increased to 17um at stage 29 (E6) where it remained through stage 33 (E7.5-8). The cell height of the NPE varied between 43um and 58um from stages 23-33 (E3.5-8). The only statistically significant changes in cell height were the decrease from stages 25-26 (E4.5-5) and the increase from stages 30-31 (E6.5-7).

In the stages preceeding (29 & 30) and during (31 & 32) fold formation, cell height in eyes intubated with a solid tube was not significantly different from that of the unoperated, control eyes (figures 53 & 54). In eyes intubated

with hollow tubes, the cell height in the PE was three times greater than in control eyes (figure 53). Interestingly, Seaman and Himelfarb (1963) showed that cell height in the PE increased two-fold in adult chicken eyes treated with drugs to reduce IOP. During this same period, cell height in the NPE was approximately two times greater than in control eyes (figure 54). Unlike the eyes of untreated embryos, there was no increase in cell height in the NPE between stages 30 and 31.

Average Cell Volume

Average cell volume in the two layers of the ciliary epithelium (computed from measurements of cell height and cell packing density as described in the Materials and Methods) showed large changes during development (figure 55). The calculated average volume of cells in the PE remained about 120um^3 through stage 28 (E5.5) then increased four-fold over stages 28-30 (E5.5-6.5). There was a slight decrease in cell volume at stages 30-31 (E6.5-7), followed by an increase at stages 32 and 33 (E7.5-8). The average cell volume of the NPE remained about 200um^3 through stage 30 (E6.5), then rapidly increased over three-fold between stages 30-31 (E6.5-7). Cell volume continued to increase at a slower rate from stages 31-33 (E7-8).

These values for average cell volume are actually over-estimates because they do not take into account the extracellular space. The percent of tissue volume represented

by extracellular space was measured in electron micrographs by stereology (table 3). The changes in extracellular volume were small relative to the changes in cell volume. It is conceivable that they might account for minor changes in total tissue volume between adjacent stages, but they could not account for the large changes in cell volume seen in the PE and NPE between stages 28 and 31.

The presumed decrease in IOP that results from intubation with a hollow tube affected both the cell volume and the changes in cell volume of the PE and the NPE (figures 56 and 57). The average cell volume of the PE was three- to six-fold greater in the hollow intubated eyes than in the control eyes (figure 56). The cell volume of the NPE was two-fold greater than control eyes at stages 29 and 30 (figure 57), but increased only slightly at stages 31 and 32. Because there was a rapid increase in cell volume in control eyes between stages 30 and 31, the cell volume of eyes intubated with hollow tubes was lower than in the controls at these stages.

Discussion

Although the role of IOP in the development of ciliary folds has been studied previously (Coulombre, 1956; Coulombre, 1957; Coulombre and Coulombre, 1957; Bard and Ross, 1982a,b), the cellular events associated with this process are less well known. The present studies describe the changes in cellular dynamics that precede and accompany fold formation. Several

changes were observed in cell behavior before and during fold formation. Some of these changes were affected by experimental alteration of IOP.

Control of morphogenesis and cell division by hydrostatic pressure

In these experiments, the results of intubation with hollow tubes were similar to results reported previously (Coulombre, 1956; Coulombre, 1957; Coulombre and Coulombre, 1957). The only difference observed was that, in the earlier studies, the size differential between intubated and control eyes was more pronounced. This discrepancy was probably due to the fact that eyes were intubated for four days in the Coulombre's experiments and for only one to two days in the present work. The morphology of the neural retina and RPE was similar to that described by the Coulombres and to malformations found in certain aborted human fetuses (Patten, 1952) and in chicken embryos whose brains were intubated to reduce intraventricular pressure (Sjoden, 1957; Desmond and Jacobson, 1977).

Although the cells of the PE and NPE abut at their apices, are joined by gap junctions and tight junctions (see previous EM studies) and are not separated by a basement membrane, they behaved as distinct epithelia in these studies. Changes in labelling index, cell density, cell height and cell volume occurred in each layer independently of changes in the other layer.

The labelling indices of the PE and NPE were quite different. An increase in the labelling index of the NPE correlated with the onset of ciliary fold formation. Reduction of IOP, by intubation with a hollow tube, reduced the labelling index and prevented fold formation in this tissue, both before and during the formation of ciliary folds in controls. This observation suggests that the labelling index of the NPE is controlled by IOP. The increased mitotic activity of the NPE apparently did not, by itself, immediately cause fold formation, because a similar increase in labelling index was found in folded and soon-to-be-folded regions of the ciliary epithelium. However, the increase in labelling index may be a necessary prerequisite for folds to form.

The RPE and retina did not change their labelling indices during the time of ciliary fold formation and their labelling indices were unaffected by a reduction in IOP. The labelling index data for the RPE confirm the results of Stroeva and Mitashov (1983), who showed the labelling index of the RPE to vary between 5-8% from E5 through E8. It is apparent that the NPE and the retina have differentiated from each other by this stage with respect to the mechanisms controlling cell division.

The mechanical force generated by IOP on the epithelial layers of the eye may be responsible for the high labelling index in the NPE (26-27%) at stages 26-30. Similarly, the sharp increase in the labelling index of the

NPE between stages 30-31 may be due to an increase in IOP. Mechanical stretch or tension is known to control cell growth in other biological systems (see Introduction).

While measuring the labelling indices in the ciliary epithelium in the present studies, large changes were noted in cell density and cell height. From these measurements, the average cell volume could be calculated for the PE and NPE. There was a decrease in cell density and an increase in cell height in the PE just prior to ciliary fold formation. Because a decrease in cell packing density translates to an increase in the cross-sectional area of the average cell, these changes resulted in an apparent four-fold increase in average cell volume over a twenty-four hour period (stages 28-30). At the onset of formation of the ciliary folds (stages 30-31), the labelling index increased, the cell density decreased, and the cell height increased in the NPE. This resulted in an apparent three-fold increase in average cell volume over a twelve hour period.

Coulombre (1956a) studied extensively the RPE of the chicken embryo. Beginning on E5, the number of cells in a unit area of the RPE decreased, meaning that the individual cell area increased. While cell area increased, cell height remained constant. Thus, the volume of the cells must have increased. This is the same time period when the cells of the pigmented layer of the ciliary epithelium increase their

volume. A common factor may be responsible for the concurrent increases in cell volume of the pigmented cells in the retina and the ciliary epithelium.

The cause of these rapid increases in cell volume in the ocular epithelial cells are, as yet, unknown. It is conceivable that they may be passive adjustments to compensate for the stretching of the epithelia caused by expansion of the globe. Several observations argue against this interpretation. First, the increase in cell volume in each tissue is accompanied by an increase in cell height. If stretching were occurring, cell height should decrease or remain constant. Second, the increase in average cell volume began in the PE two stages (24 hours) before any change was seen in the average volume of the cells of the NPE. If simple expansion of the eye were responsible for stretching these epithelia, they should respond at the same time. Finally, when IOP (and the associated stretching force) was reduced by intubation, the average cell volume of the PE and NPE cells increased. This is the opposite of what would be expected if increased cell volume was compensating for stretching of the epithelium.

All of these considerations, taken together, suggest another explanation: active transport by the cells of the ciliary epithelium results in the increase in cell volume that precedes the formation of the ciliary folds. This could have several implications. If cell volume is a result of active

transport across the ciliary epithelium, such transport could be associated with the onset of aqueous humor production by the ciliary epithelium. This would be expected to increase IOP, which could result in the ocular growth spurt seen by Bard and Ross (1982a) just prior to fold formation. A second implication is that, if an increase in cell size (width and height) is an intrinsic property of the epithelial cells themselves, this may generate forces that could contribute to the formation of the ciliary folds. Both of these speculations raise the possibility that, in a sense, the ciliary epithelium may contribute to, or cause, its own morphogenesis.

Finally, the excessive cell volume in the PE and NPE of eyes with reduced IOP must be considered. This raises the possibility that fluid transport by the ciliary epithelium is regulated in a feedback manner by IOP. In the face of reduced pressure, inhibition is released and the cells increase their rate of transport. This hypothesis is consistent with the findings of Langham (1959a,b) that increased IOP in adult rabbits decreased production of aqueous humor. If increased secretion resulted in increased cell volume, the paradox of increased cell volume in the face of reduced pressure might be explained. These conjectures could be tested if IOP could be measured and regulated by external intervention during the sixth day of embryonic life. Such experiments are beginning in the laboratory of Dr. Beebe.

An estimate of extracellular space in the ciliary epithelia was considered particularly important, because Bard and Ross (1982a) reported increases in "lateral detachment" between these cells prior to fold formation. No evidence was seen for significant changes in lateral cell associations during the period of fold formation, based on the measurements of extracellular space and on detailed study of cell morphology by electron microscopy. These observations suggest that the role of lateral cell detachment in ciliary fold formation may need to be reevaluated.

The source of IOP in the early embryonic eye is presently unknown. The Coulombre's assumed that IOP in the embryo was generated by the synthesis and hydration of the proteoglycans and glycosaminoglycans of the vitreous body (Coulombre and Coulombre, 1957). In recent studies, Fitch and co-workers (Fitch, et.al., 1988) have shown that the majority of vitreal collagen synthesis in the embryonic chicken eye occurs in the anterior portion of the optic cup, the region that is forming the ciliary epithelium. Smith and Newsome (1978) showed that the ciliary epithelium incorporates and secretes more [^{35}S] sulfate in the form of glycosaminoglycans than any other ocular tissue. Fine and Zimmerman (1963) showed that, in monkeys and man, mucopolysaccharides secreted by the nonpigmented layer of the ciliary epithelium flowed into the vitreous body. Therefore, the ciliary epithelium may be the most important source for the components of the

vitreous body.

It has been suggested that the ciliary epithelium may secrete aqueous humor and generate IOP in the embryonic eye (Porte, Stoeckel, Brini, and Metais, 1968; Beebe, 1986; Beebe, et.al., 1986; Beebe and Reichman, 1988). By E5 or E6, the ultrastructure of the cells of the ciliary epithelium resemble that of cells specialized for ion and protein secretion. In previous studies in which our laboratory measured the protein concentration in the embryonic vitreous humor and the rate of exchange of proteins between the vitreous humor and the blood (Beebe, et.al., 1986), it was concluded that plasma proteins entering the eye were being constantly diluted by influx of a fluid of low protein content, similar to aqueous humor. The recent demonstration of carbonic anhydrase in the prospective ciliary epithelium just after formation of the optic cup (Linser and Moscona, 1984) and the delay in eye growth caused by inhibitors of this enzyme (Linser and Plunkett, 1989) suggest that aqueous humor secretion in the embryonic eye may begin soon after its formation. Taken together, these data suggest that the source of IOP in the early embryo is probably a combination of fluid secretion by the ciliary epithelium as well as the synthesis and hydration of the components of the vitreous body (figure 58). If correct, the epithelial layers of the ciliary epithelium may indirectly regulate their own mitotic activity and contribute to their own morphogenesis through the generation of IOP in the embryo.

SUMMARY

Many factors have been shown to be involved in the development of the ciliary epithelium (figure 58). This includes the lens, vitreous body and IOP.

The cells in the PE and NPE begin to differentiate ultrastructurally and histochemically from the adjacent retinal pigmented epithelium and neural retina, respectively, by E4. The ultrastructural morphology of the PE and NPE cells shows the presence of gap junctions and tight junctions at the apical and apicolateral cell membranes. The presence of these tight junctions on the apicolateral surfaces of the NPE cells suggest that the blood aqueous-barrier at E4. By E6, the PE and NPE cells have acquired the ultrastructural morphology of cells actively involved in fluid and electrolyte transport. This includes apicolateral tight junctions, basal cell membrane infoldings, lateral intercellular interdigitations, and numerous mitochondria. The NPE cells have also developed the large dilated cisternae of the RER characteristic of cells actively involved in protein synthesis and secretion. Together, these data suggest that the cells of the ciliary epithelium may be involved in embryonic aqueous humor secretion and the generation of IOP in the chicken embryo by E6.

Specific histochemical staining for AChE was initially detected in the PE and NPE cells at E4; whereas it did not

appear in the retina until E6. On this basis, it was possible to differentiate the ciliary epithelium from the adjacent retina early in development. From E9 through hatching, the ora serrata, the cellular transition zone between the ciliary epithelium and the retina, could be used to distinguish the AChE stained ciliary epithelium anteriorly and the layered AChE stained retina posteriorly.

The changes in cellular dynamics during the development of the ciliary epithelium (E4-E8) were examined. Striking changes were observed in the labelling index, cell packing density, cell height and cell volume of the PE and NPE. The labelling index of the NPE rapidly increased between stages 30 and 31, during the time of the initial formation of folds. Concurrently, cell density decreased and cell height increased resulting in an apparent three-fold increase in cell volume. In the PE, decreases in cell density and increases in cell height led to a four-fold increase in cell volume. The increase in the volume of the PE cells began at stage 28, 24 hours prior to the changes seen in the NPE. The apparent reduction of IOP, by intubation of the eye with a hollow tube, affected some of the above mentioned parameters. These data raise the possibility that active transport by the cells of the ciliary epithelium caused the increase in cell volume preceeding the formation of the ciliary folds. If this interpretation is correct, the ciliary epithelial cells may indirectly regulate their own mitotic activity and may

contribute to their own morphogenesis through the generation of IOP in the embryo.

Table 1

Summary of the Major Morphological Changes in the Ciliary Epithelium, Neural Retina, and RPE

	DAYS 4-6	DAYS 6-7	DAYS 7-19
PE	-increase in RER & stacks of Golgi apparatus -increase in basolateral membrane specializations -accumulation of pigment -development of gap junctions between adjacent cells	-increase in RER, SER cytoplasmic vesicles & cell membrane specializations	increase in SER, pigment, & cell membrane specializations
RPE	-appearance of apical RER -increase in number of polysomes & free ribosomes	none	-development of cell membranes specializations -increased pigment
NPE	-development of basal RER & mitochondria -increase in SER, large RER & mitochondria apically -increase in membrane specializations	increase in RER with large cisternae & basal mitochondria	-distribution of RER & SER throughout the cell -increase in cell membrane specializations
NEURAL RETINA	-development of RER, stacks of Golgi apparatus & mitochondria	none	-the distinct layering pattern of the neural retina is visible

TABLE 2

Labelling Index (%)

PE

STAGE	FOLDED	UNFOLDED
31	15.21 ± 0.43	14.71 ± 0.64
32	12.20 ± 0.87	13.09 ± 0.62
33	9.80 ± 0.46	10.02 ± 0.35

NPE

STAGE	FOLDED	UNFOLDED
31	37.64 ± 0.87	37.18 ± 0.69
32	37.57 ± 0.40	37.32 ± 0.53
33	34.93 ± 0.57	35.28 ± 0.86

Labelling index of the folded and non-folded regions of the PE and NPE during the time of fold formation. There was no significant difference between the folded and non-folded regions. Therefore, the increase in the labelling index in the NPE does not immediately cause fold formation.

TABLE 3

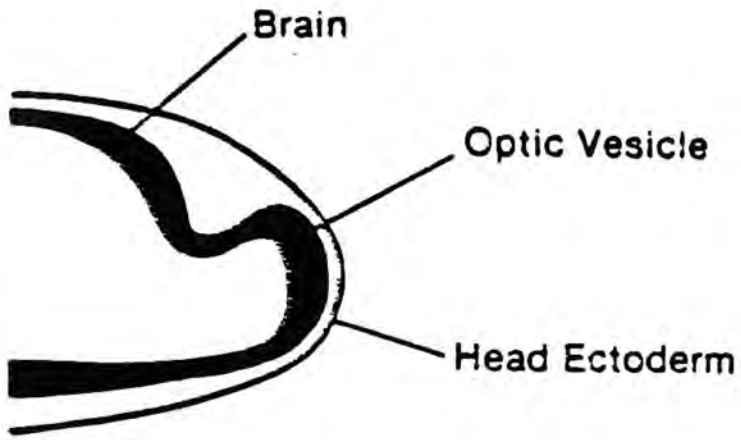
Extracellular Volume (%)

DAY	PE	NPE
4	9.23 ± 0.48	7.53 ± 0.58
5	5.85 ± 0.59	15.12 ± 4.73
6	2.39 ± 0.13	7.83 ± 0.64
7	5.51 ± 0.54	6.86 ± 2.01

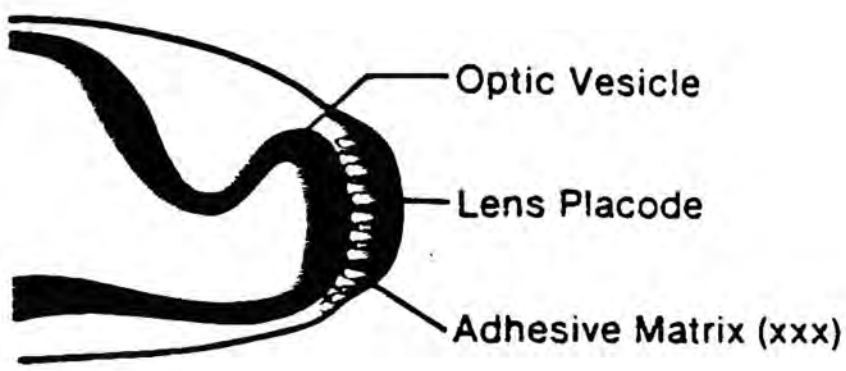
Percent extracellular volume in the PE and NPE during their development. The changes in extracellular volume were not sufficient to cause the large changes in tissue volume observed during the development of the PE and NPE.

Figure 1. Diagram of the development of the chicken eye (from Beebe, 1986).

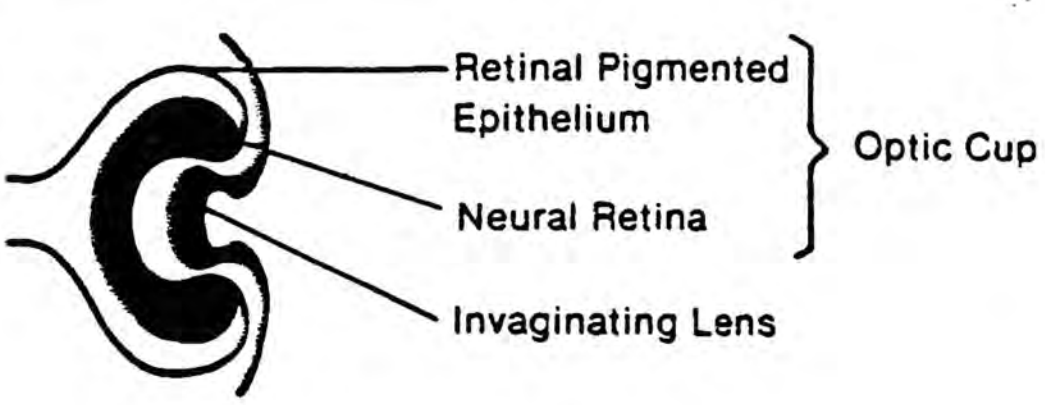
A.


Brain
Optic Vesicle
Head Ectoderm

B.


Optic Vesicle
Lens Placode
Adhesive Matrix (xxx)

C.


Retinal Pigmented Epithelium
Neural Retina
Invaginating Lens
Optic Cup

D.

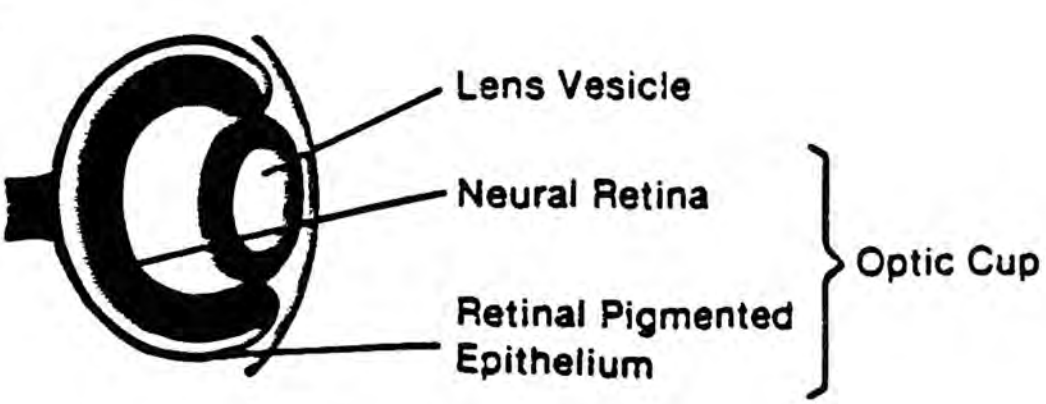
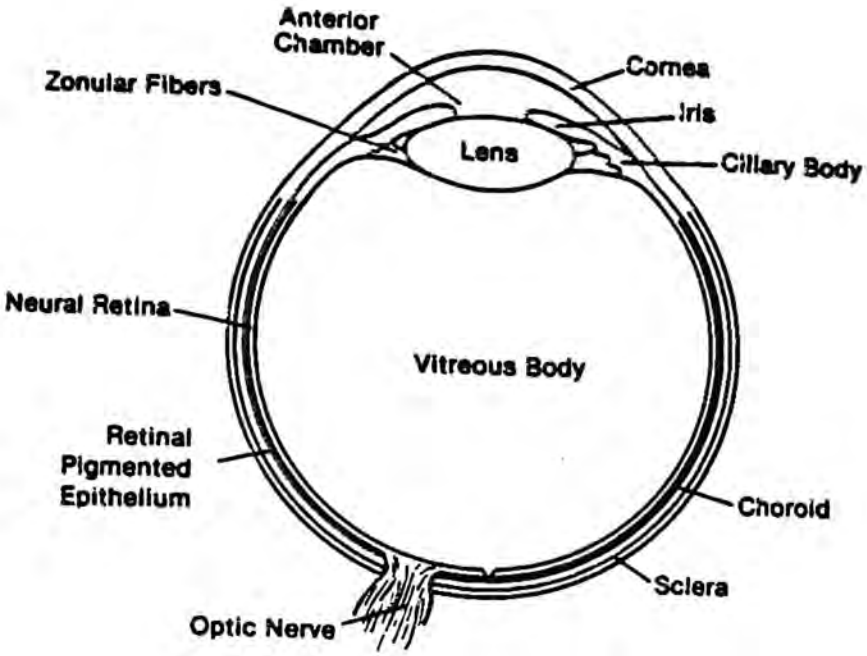

Lens Vesicle
Neural Retina
Retinal Pigmented Epithelium
Optic Cup

Figure 2A. The structure of the adult vertebrate eye.

Figure 2B. Diagram of the anterior portion of the eye. (2A & 2B from Beebe, 1986).

A.


Anterior Chamber
Zonular Fibers
Cornea
Iris
Lens
Ciliary Body
Neural Retina
Vitreous Body
Retinal Pigmented Epithelium
Choroid
Sclera
Optic Nerve

B.

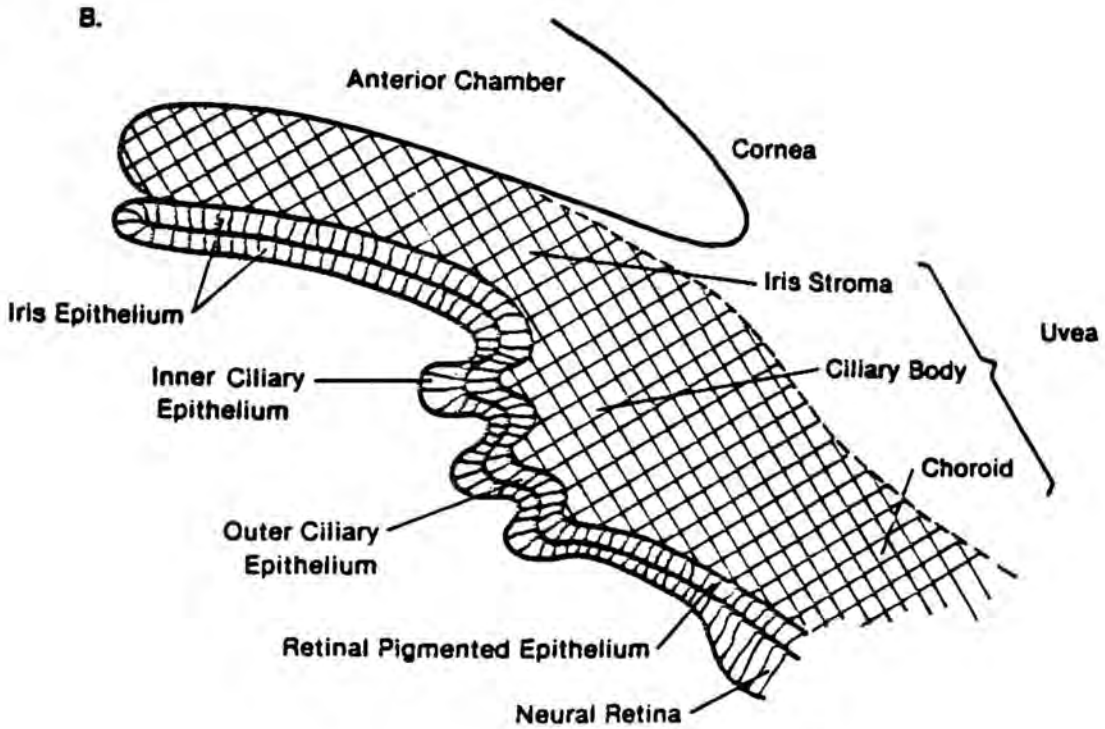

Anterior Chamber
Cornea
Iris Stroma
Iris Epithelium
Uvea
Inner Ciliary Epithelium
Ciliary Body
Choroid
Outer Ciliary Epithelium
Retinal Pigmented Epithelium
Neural Retina

Figure 3. Anterior segments of embryonic chicken eyes viewed from inside the vitreous chamber to observe the formation of the ciliary folds. The formation of the folds begins at the stage 31 and continues through the end of stage 33. (numbers represent the stage of embryonic development, 3x).

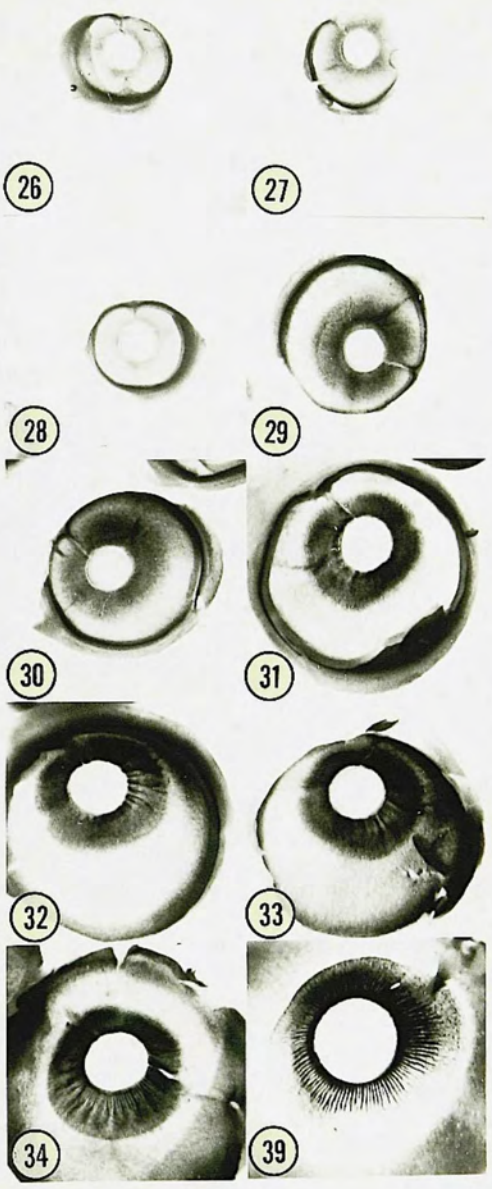

26
27
28
29
30
31
32
33
34
39

Figure 4. Electron micrograph of a PE cell from an E4 chicken embryo. The cell contains mitochondria (M) and RER (arrowheads) scattered throughout the cytoplasm and occasional stacks of Golgi apparatus (G) and pigment granules (P). The PE cells are connected to each other by densely stained junctional complexes (arrows). (12,500x).

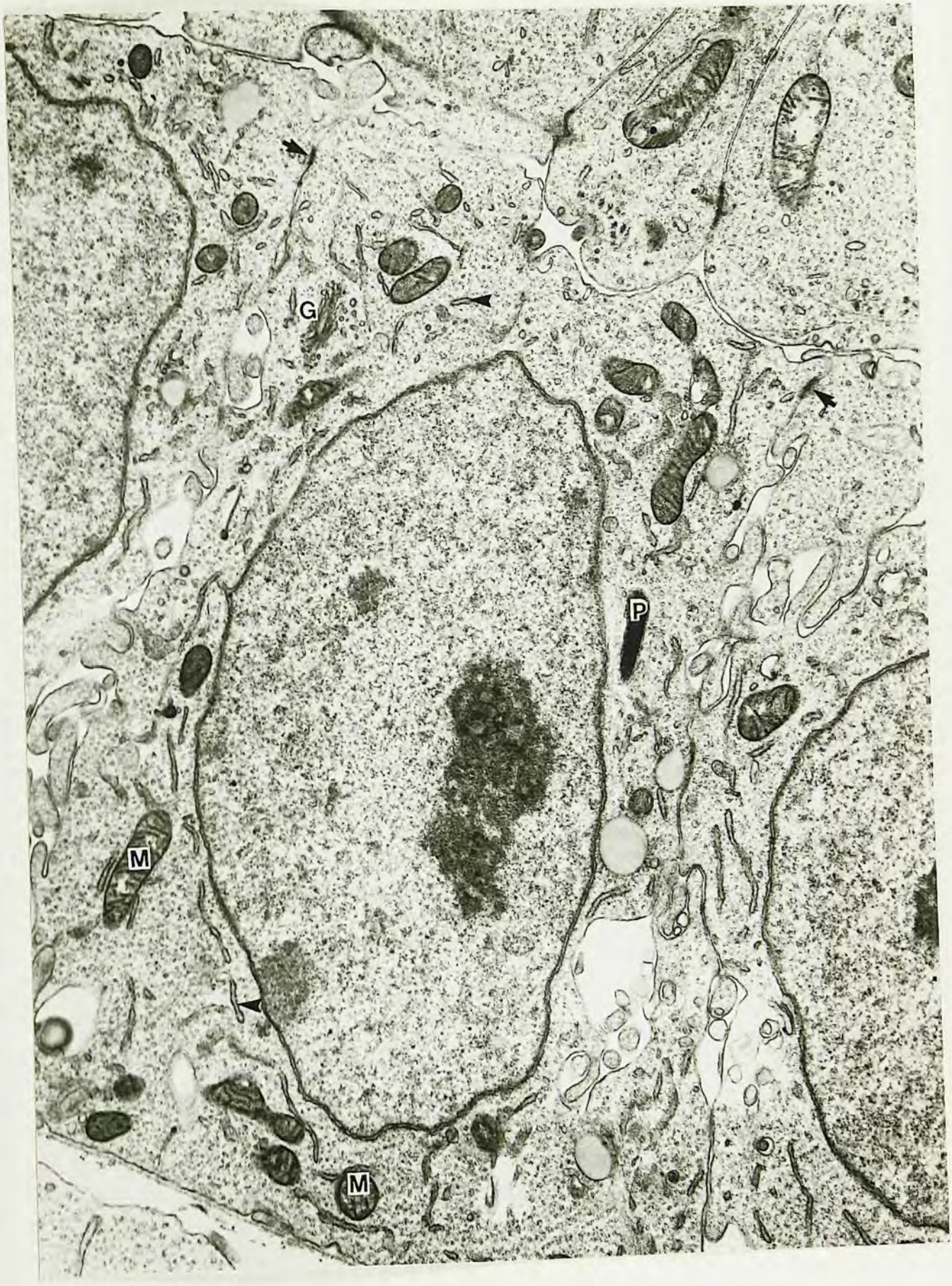

G
P
M
M

Figure 5. Electron micrograph of the apical portion of two RPE cells from an E4 chicken embryo. Scattered mitochondria (M), RER (arrowhead) and smooth vesicles (arrows) can be observed. (25,000x).

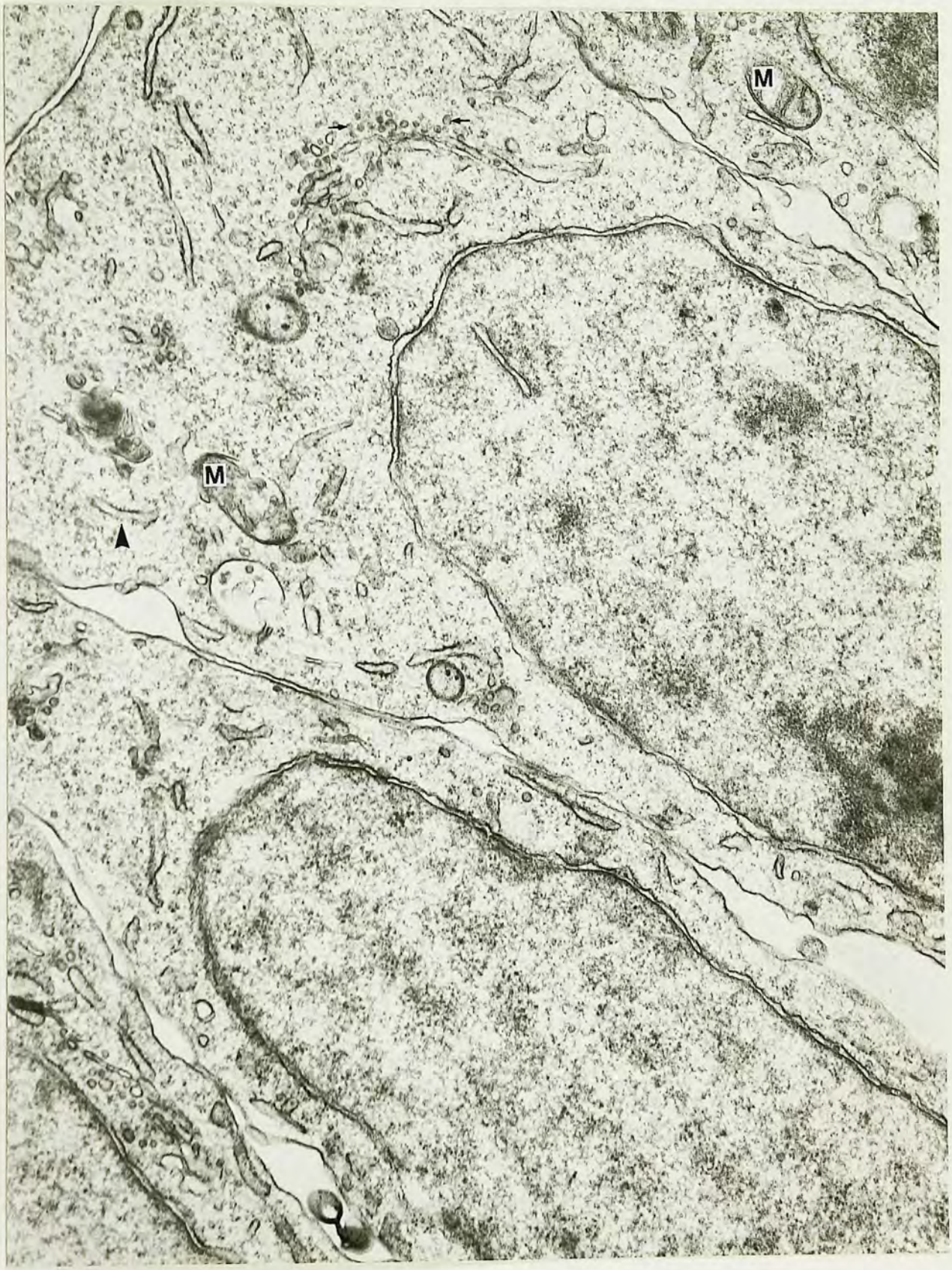

M
M

Figure 6. Electron micrograph through the basal portion of three RPE cells from an E4 chicken embryo. Mitochondria, RER, and numerous polysomes can be seen. (25,000x).

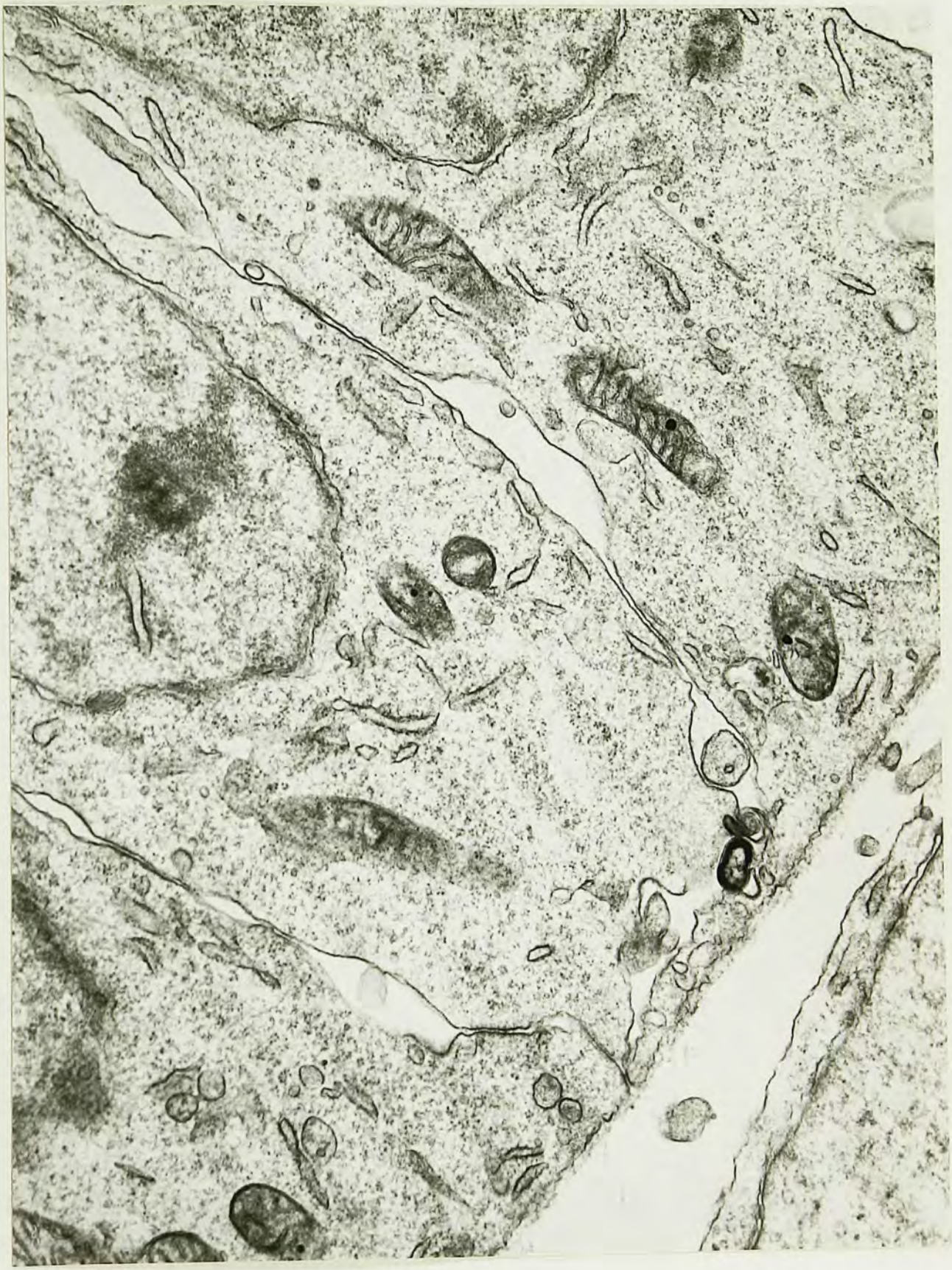


Figure 7. Electron micrograph of the apices of several PE & NPE cells from an E4 embryonic chicken. An occasional basal body (asterisk) or a cilium (large arrow) may be present. Junctional complexes, including what appear to be gap junctions, connect the apices of the NPE cells together (small arrow) and the apices of the NPE cells to those of the PE (arrowheads). (25,000x).

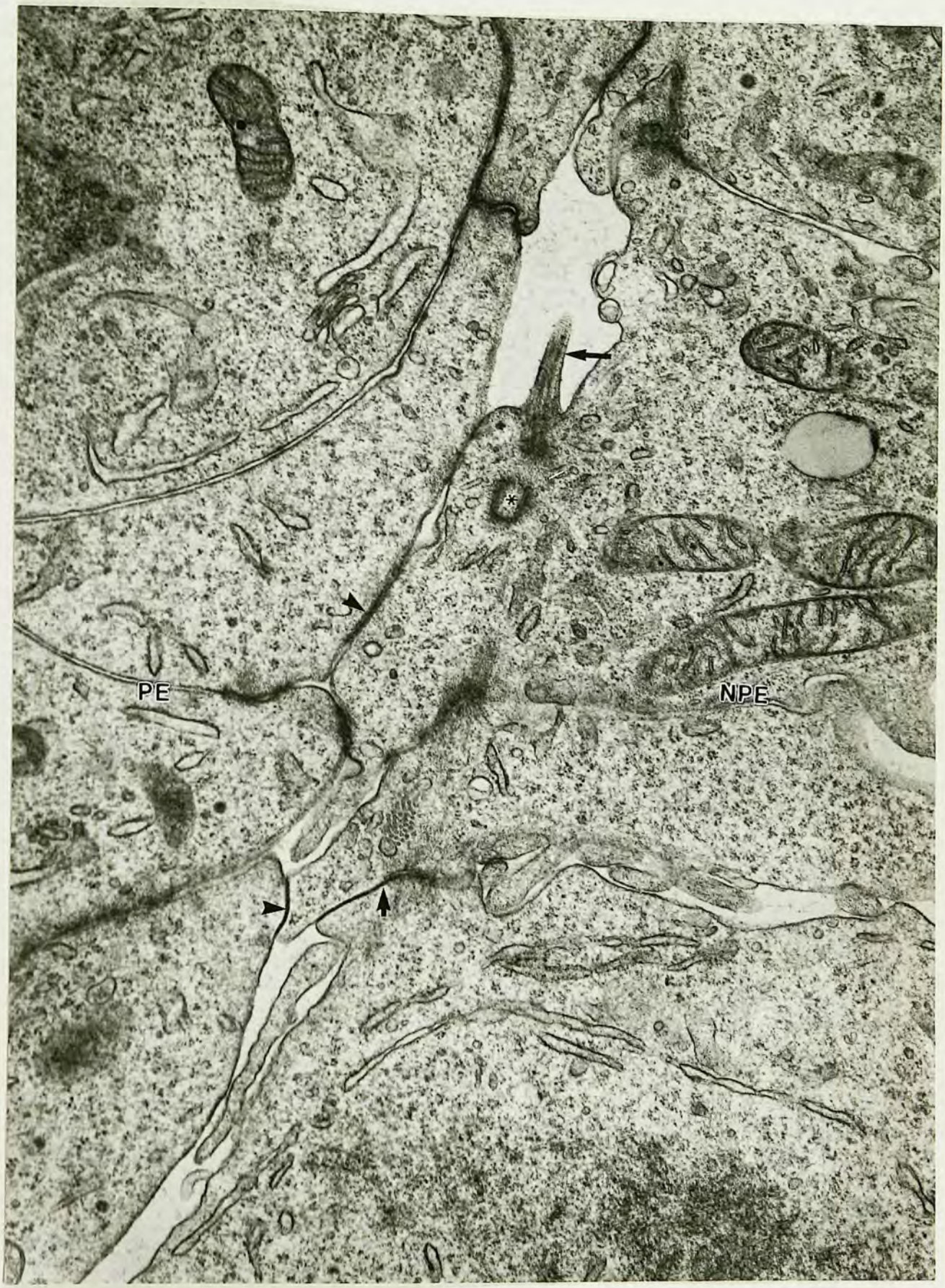

PE
NPE

Figure 8. Electron micrograph of the lower apical region of several NPE cells from an E4 chicken embryo. Occasional mitochondria & RER are seen as well as Golgi apparatus (G), numerous smooth vesicles (arrows) and occasional coated pits or vesicles (arrowhead). (25,000x).


G

Figure 9. Electron micrograph of the midsections of several NPE cells from an E4 chicken embryo. Mitochondria, RER, Golgi apparatus (G) and numerous vesicles (arrowheads) can be seen. (25,000x).

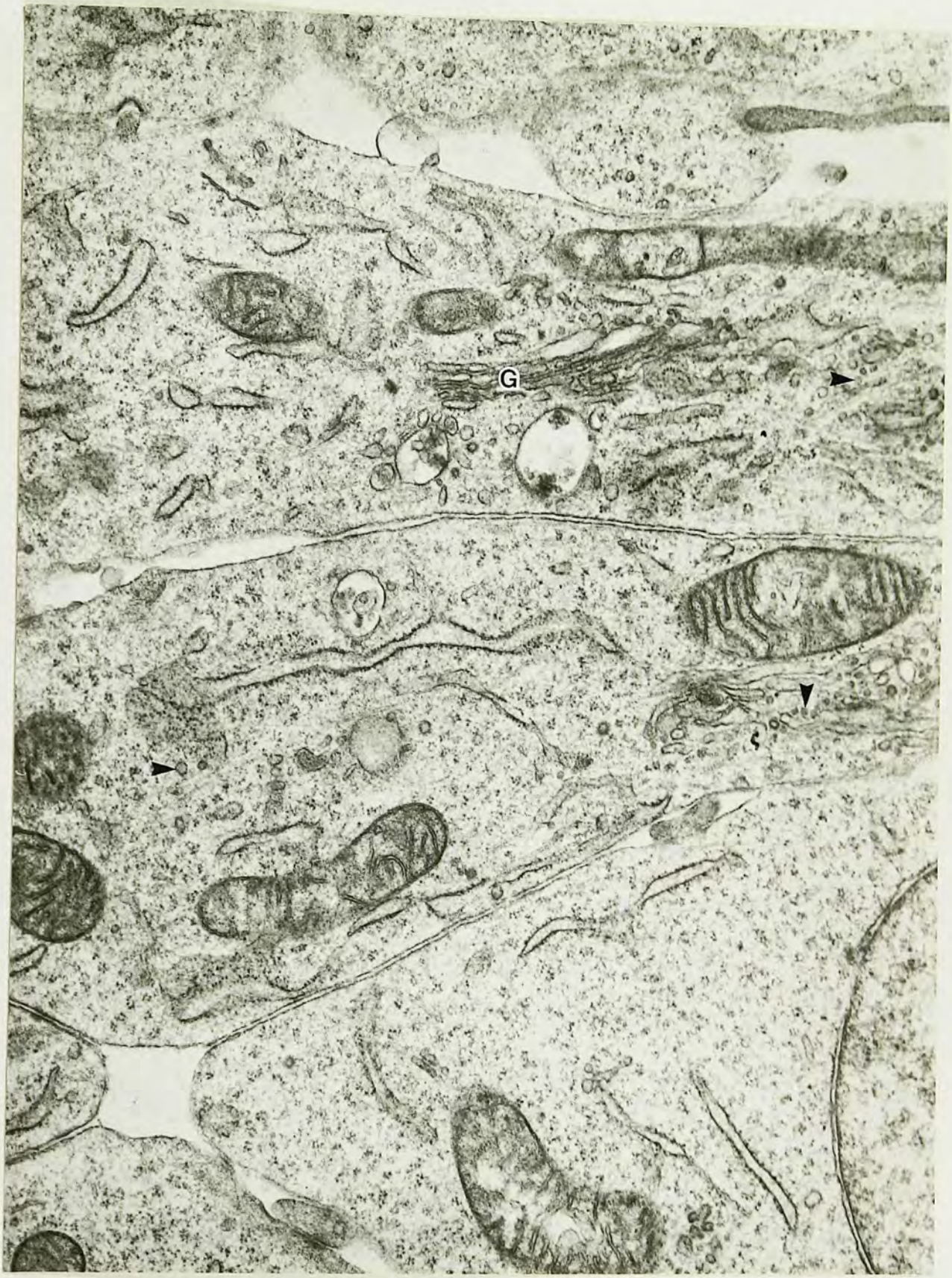

G

Figure 10. Electron micrograph through the basal portions of several NPE cells from an E4 embryonic chicken. Relatively few mitochondria & sparse RER are seen. The cytoplasm contains numerous polysomes & free ribosomes. (12,000x).

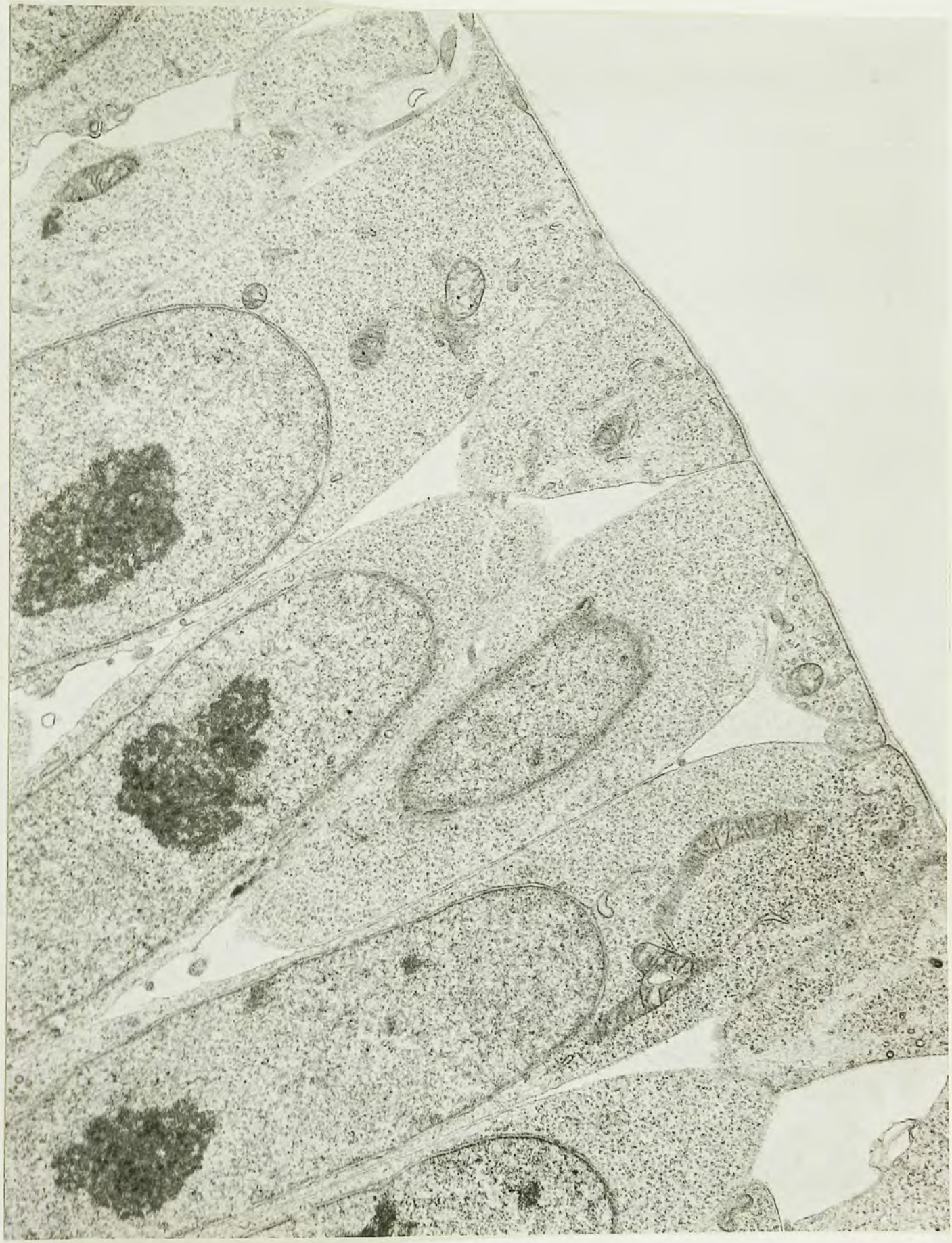


Figure 11. Electron micrograph of two cells representative of those in the inner third of the neural retina from an E4 embryonic chicken. The cytoplasm contains numerous free ribosomes & polysomes with occasional mitochondria. (25,000x).

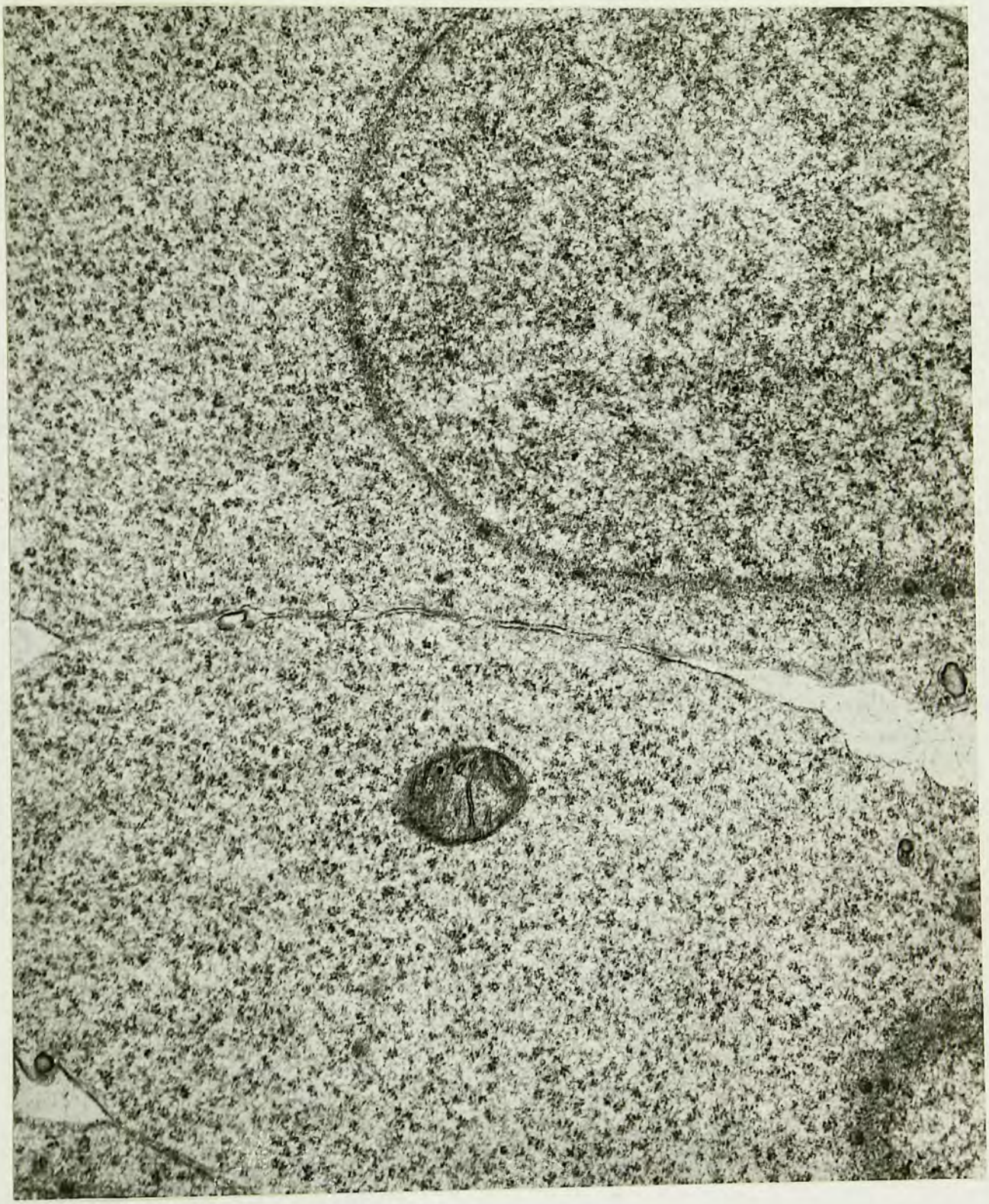


Figure 12. Electron micrograph of cells representative of those in the middle third of the neural retina from an E4 chicken embryo. Sparse mitochondria & RER are seen in a cytoplasmic matrix rich in polysomes & free ribosomes. An occasional coated pit (arrowhead) or smooth vesicle may be found. (25,000x).

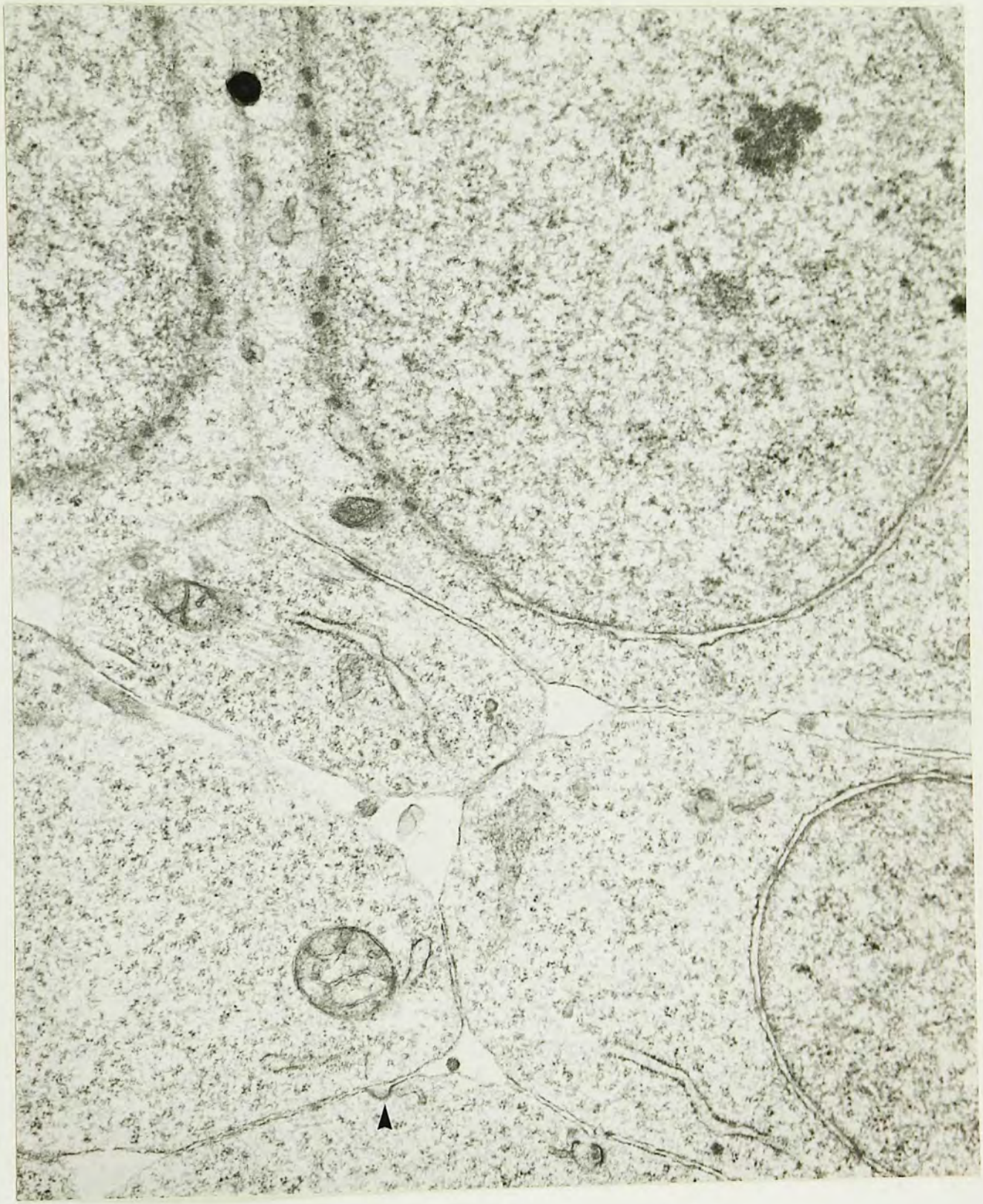


Figure 13. Electron micrograph representative of cells in the outer third of the neural retina from an E4 chicken embryo. Numerous smooth vesicles & occasional coated vesicles, mitochondria, & RER can be seen. (25,000x).

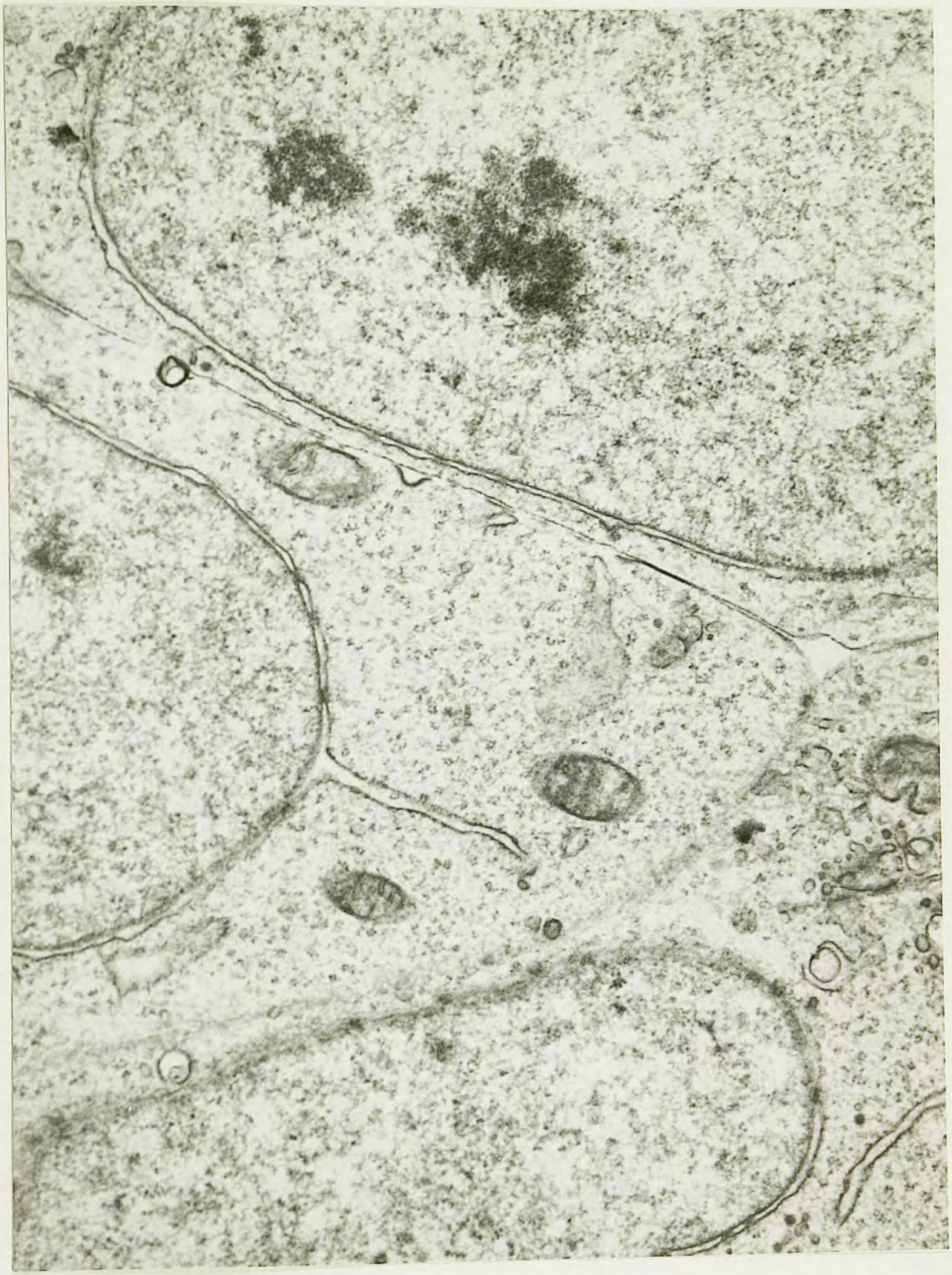


Figure 14. Electron micrograph of the apical portions of several PE cells from an E6 chicken embryo. The number of mitochondria has increased relative to E4 and the cells have an extensive, well-developed Golgi apparatus (G). (12,500x).

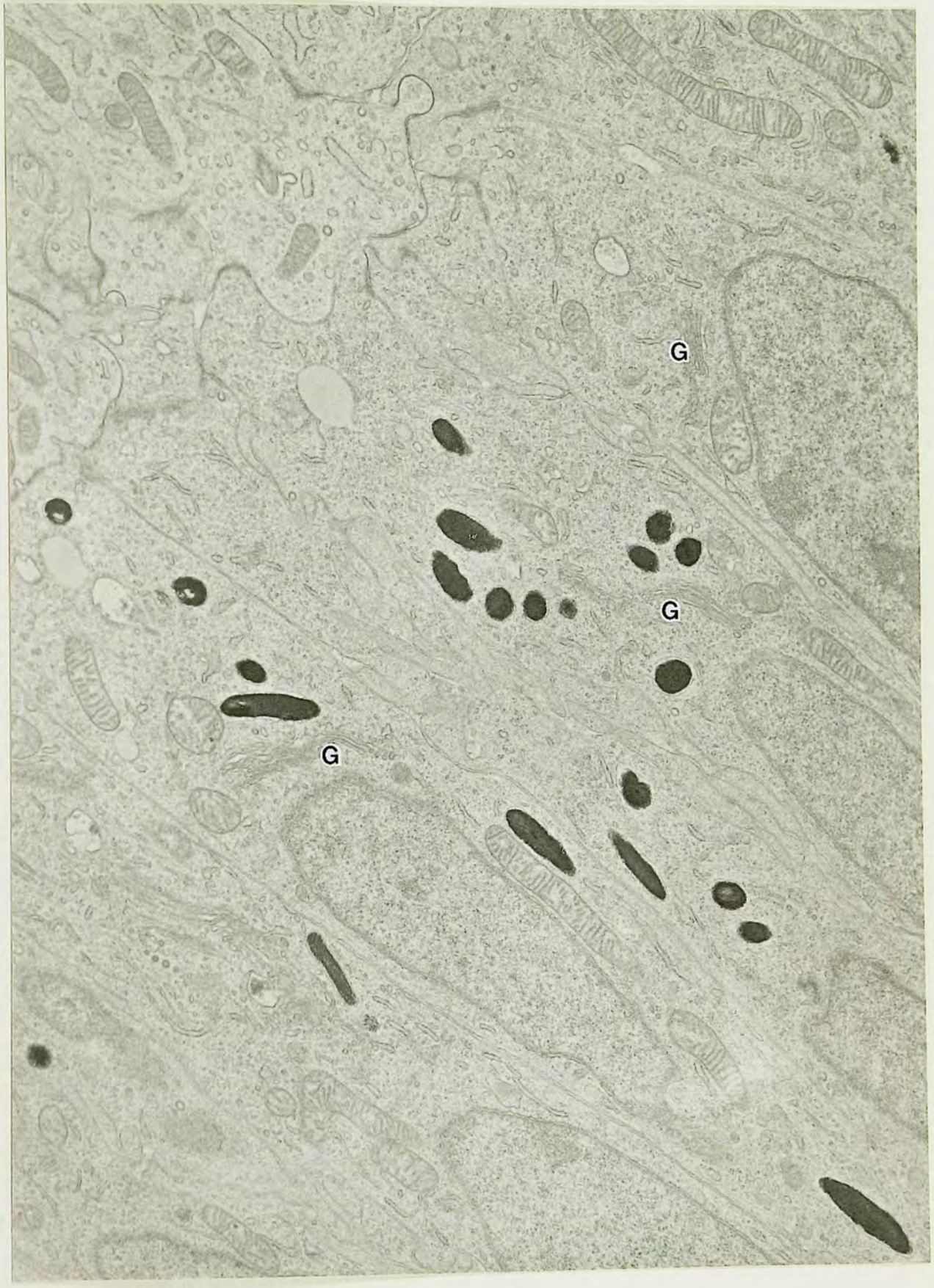

G
G
G

Figure 15. Electron micrograph of the basal portions of several PE cells from an E6 chicken embryo. The basal cell membrane shows numerous infoldings, coated pits (arrowheads) and lateral intercellular interdigitations. Well-developed Golgi apparatus (G) and basolateral, intercellular gap junctions (arrow) have formed. (25,000x).

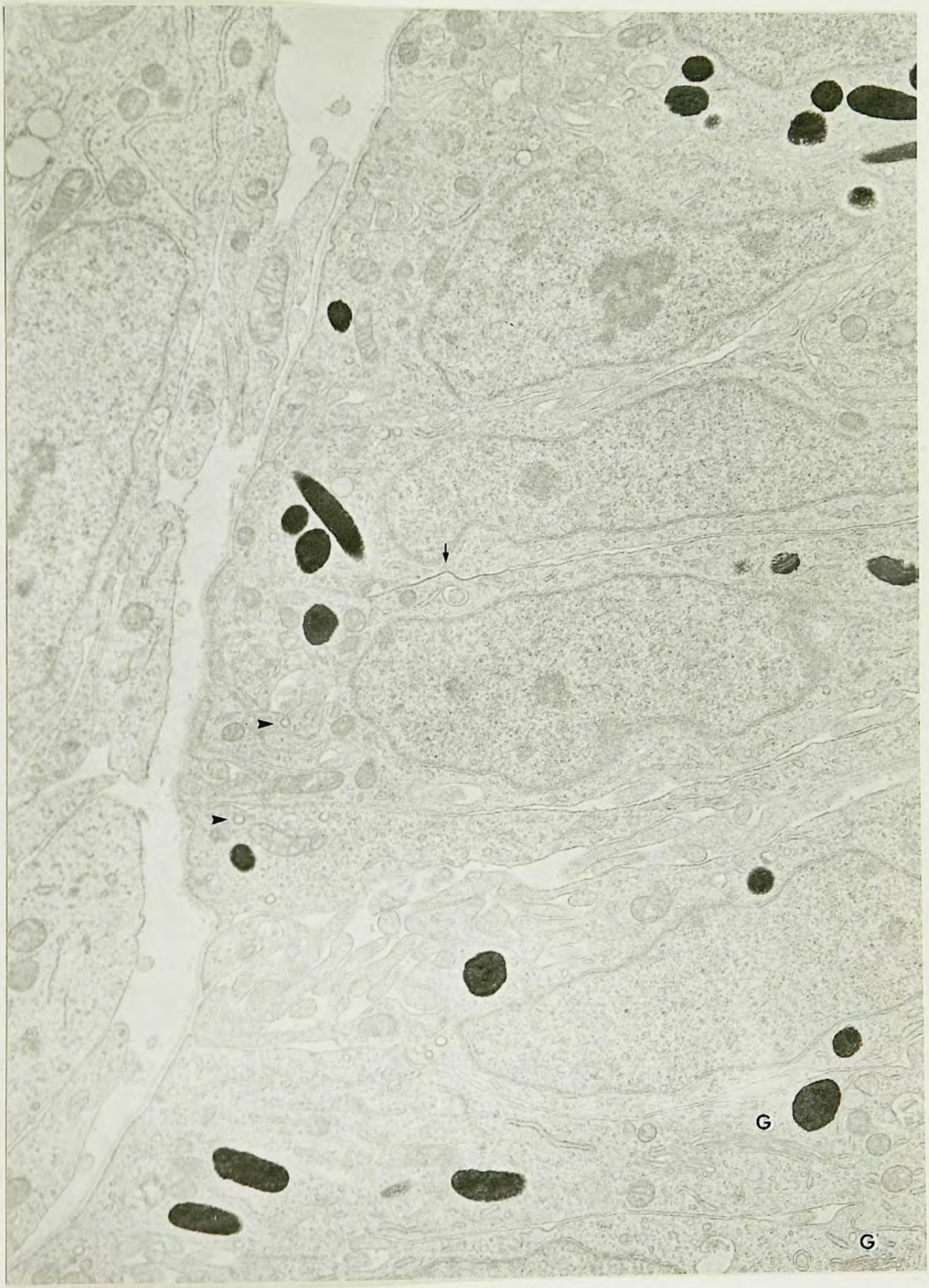

G
G

Figure 16. Electron micrograph of the apical portions of two RPE cells from an E6 chicken embryo. The cells have numerous vesicles with small amounts of RER and mitochondria. (28,000x).

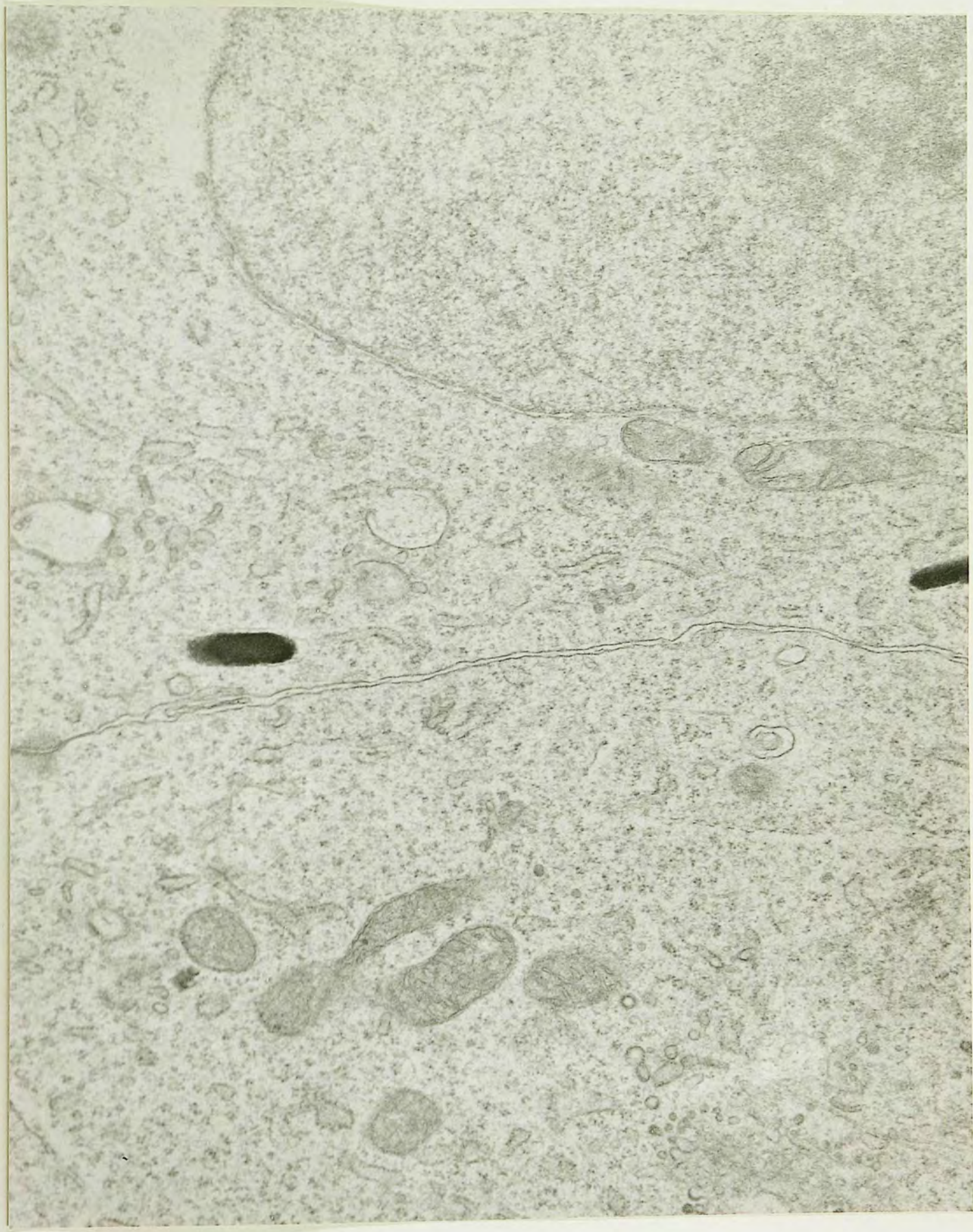


Figure 17. Electron micrograph of the basal portion of several RPE cells from an E6 chicken embryo. The cell does not have the extensive membrane specializations seen in the PE. (21,200x).

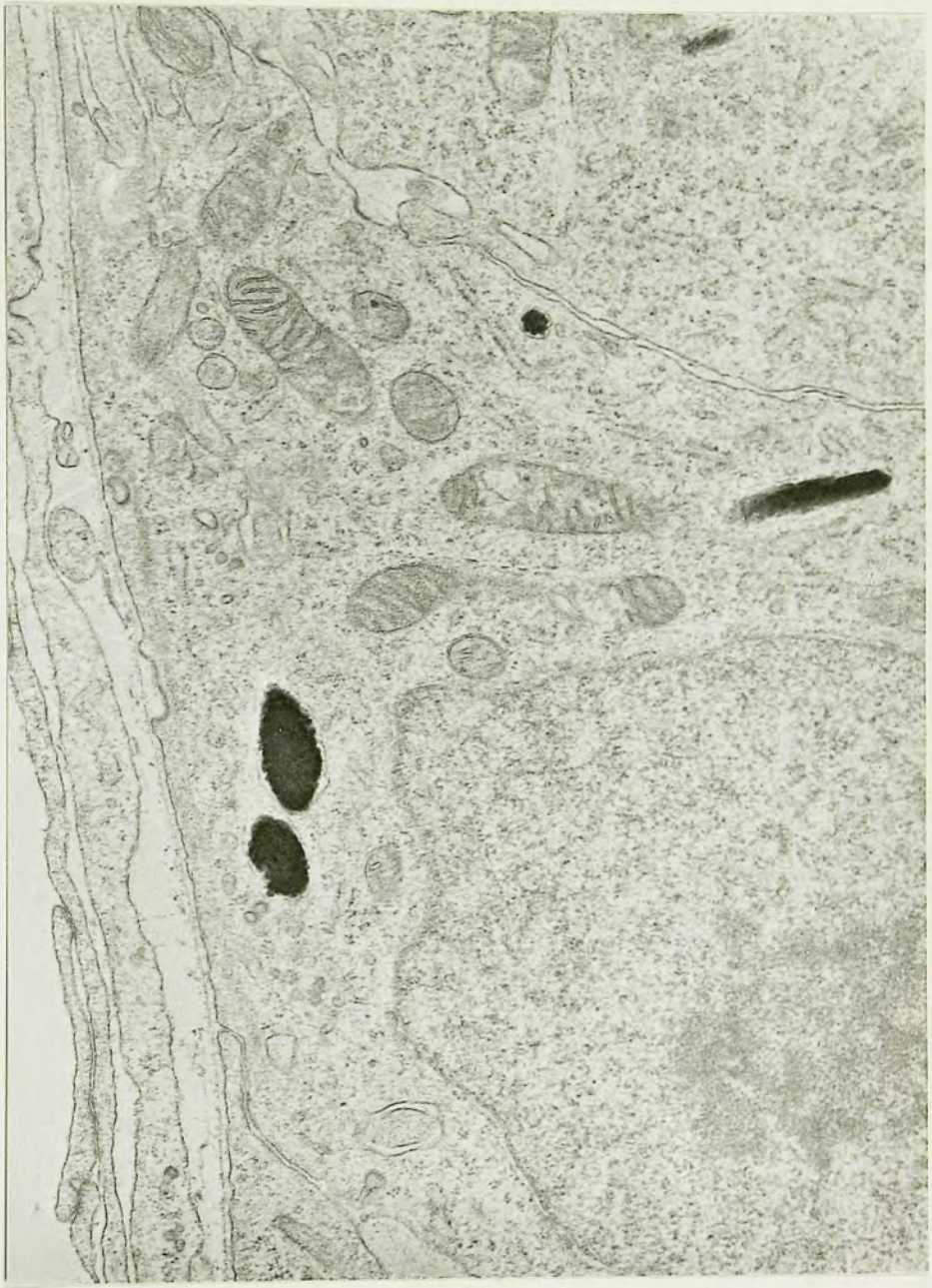


Figure 18. Electron micrograph of the apical portion of several PE and NPE cells from an E6 chicken embryo. The NPE cells have extensive, dilated RER (Asterisks) and large numbers of mitochondria. Numerous junctional complexes, including gap junctions, connect adjacent NPE cells (large arrowheads) and NPE cells to PE cells (small arrowheads). (15,800x).

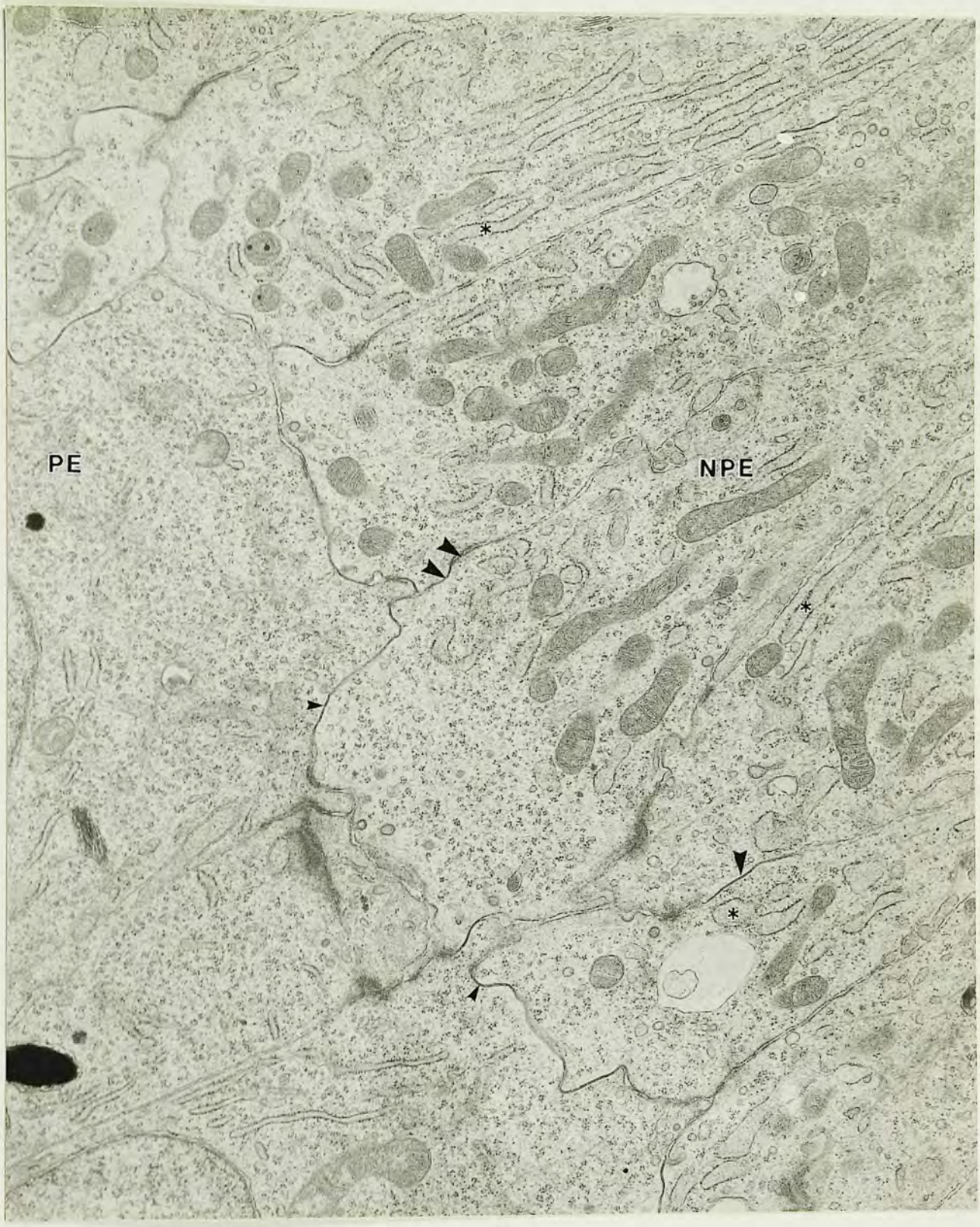

PE
NPE

Figure 19. Electron micrograph of the full thickness of the NPE from an E6 chicken embryo. The extracellular spaces have increased in size & the lateral cell membrane specialization are more extensive than those at E4. (P=posterior chamber, 6,500x).

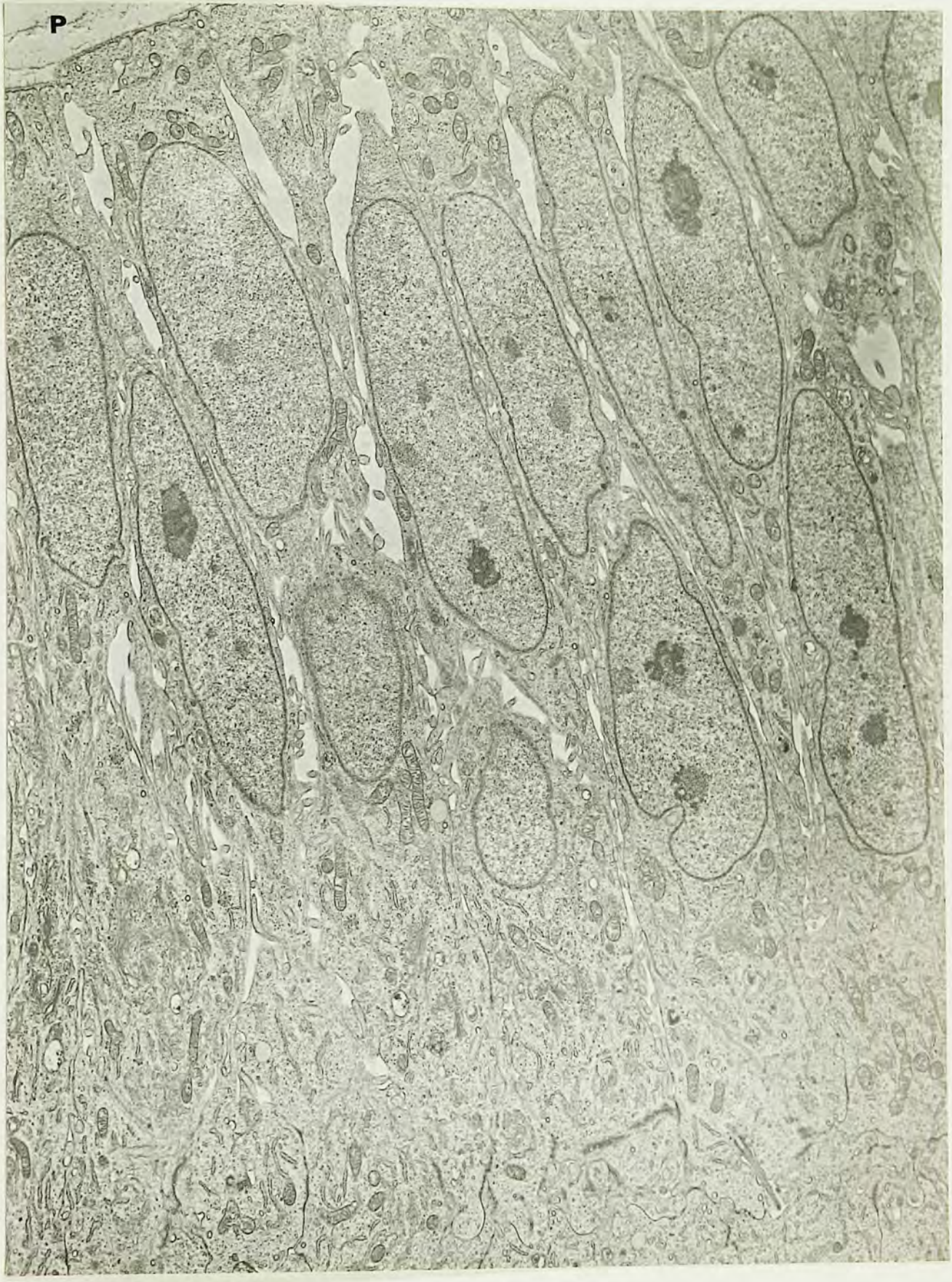

P

Figure 20. Electron micrograph of the basal portion of several NPE cells from an E6 chicken embryo. The cells have more mitochondria and vesicles (arrows) than seen at E4. (12,500x).

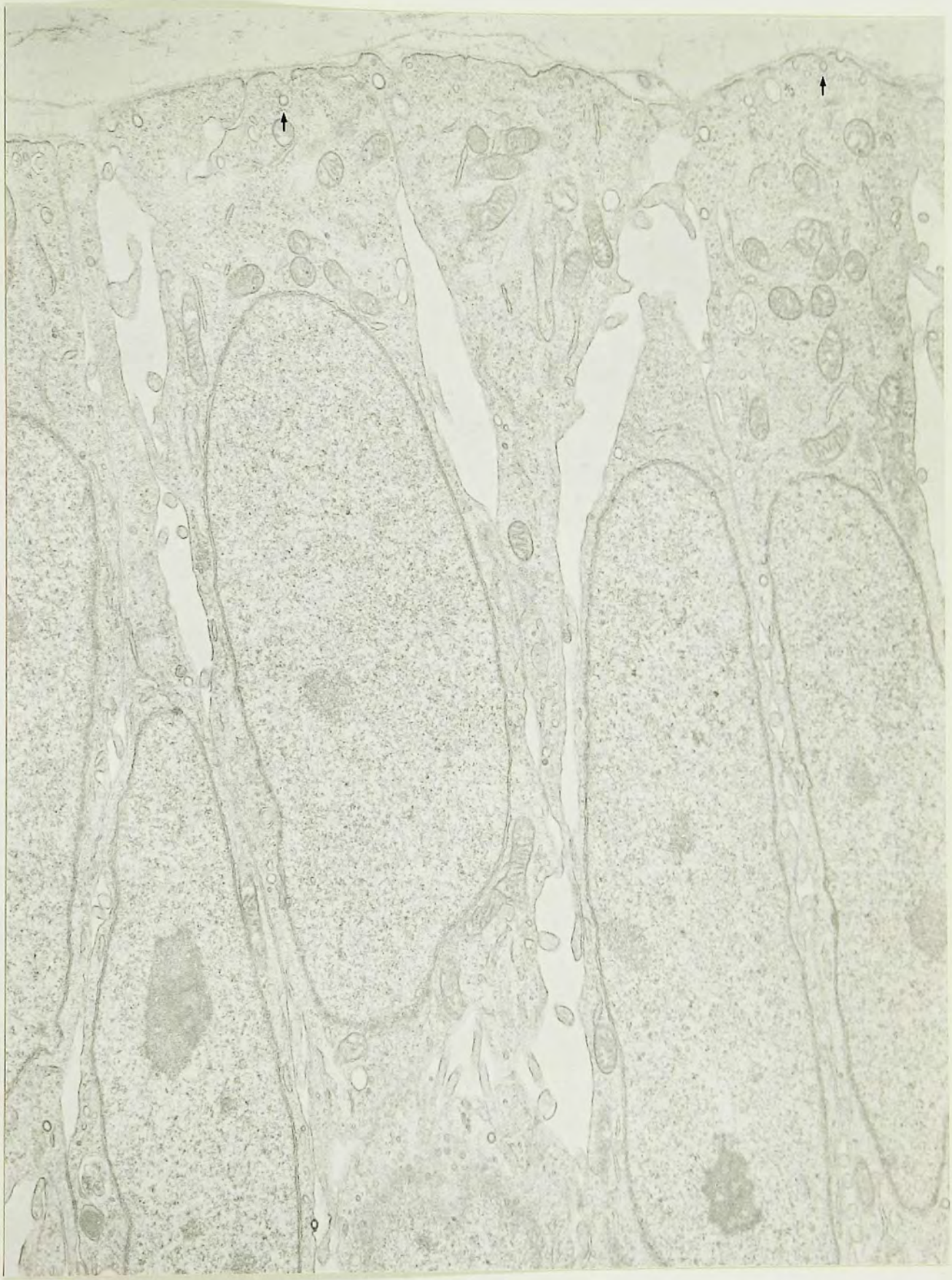


Figure 21. Electron micrograph representative of cells in the inner third of the neural retina from an E6 chicken embryo. The cytoplasm contains numerous polysomes & free ribosomes with interspersed mitochondria. (25,000x).

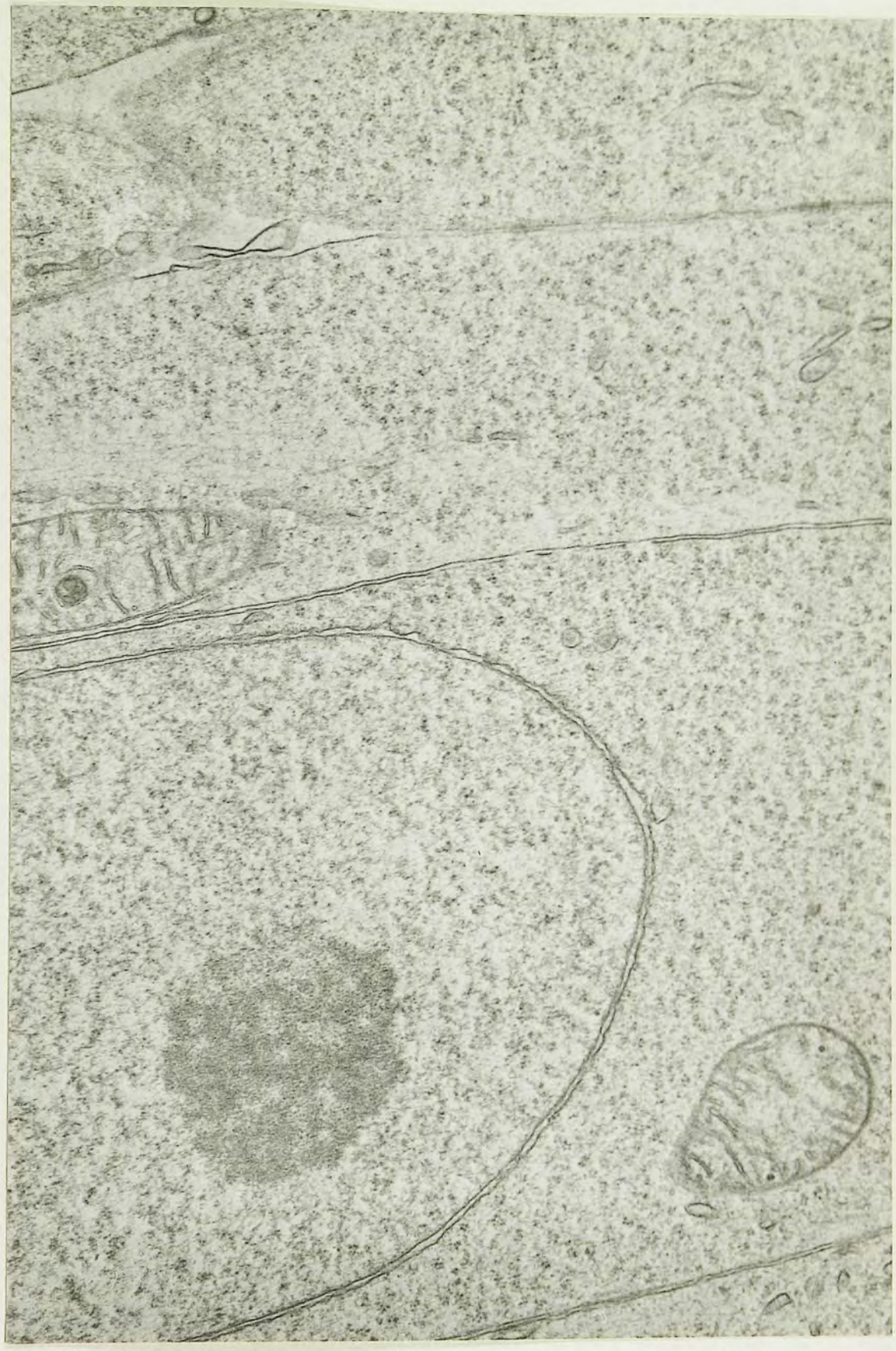


Figure 22. Electron micrograph of a cell representative of those in the middle third of the neural retina from an E6 chicken embryo. The cytoplasm contains polysomes & free ribosomes with sparse mitochondria. (25,000x).

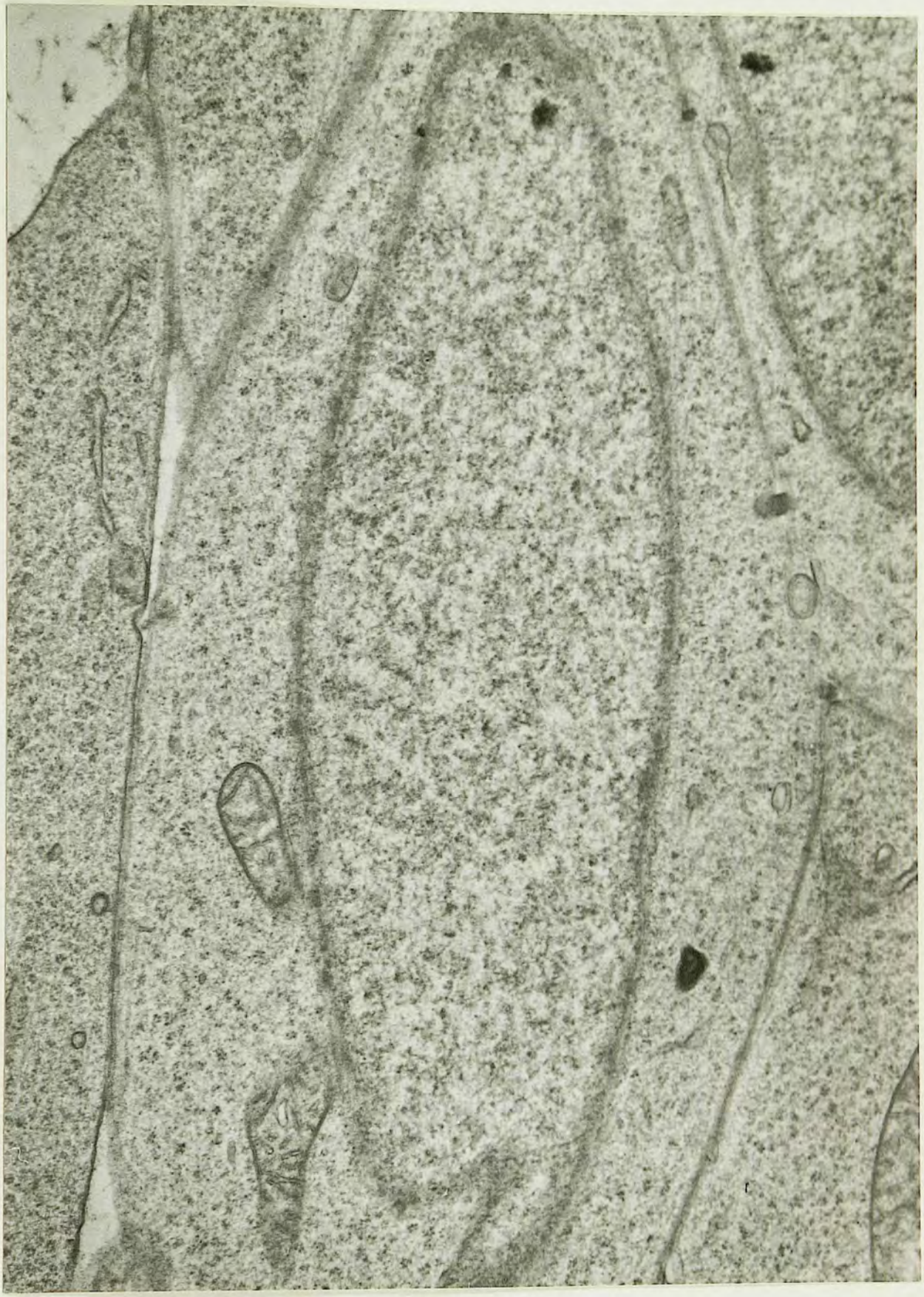


Figure 23. Electron micrograph of cells representative of those in the outer third of the neural retina from an E6 chicken embryo. The cells have developed an extensive Golgi apparatus (G), and more RER (asterisks) than on E4 but not as extensive as the NPE. (M=mitochondria, 25,000x).

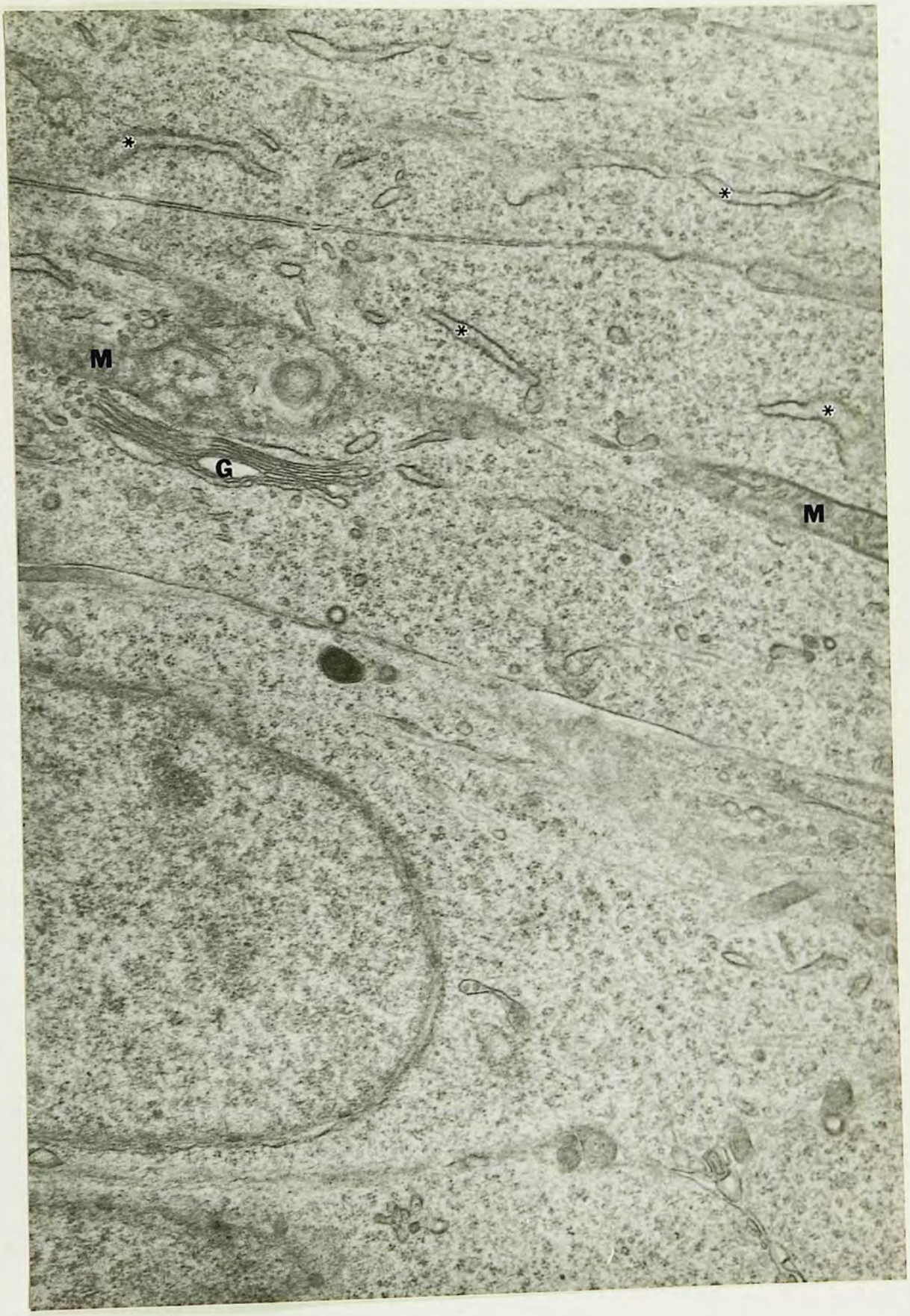

M
G
M

Figure 24. Electron micrograph of the basal portion of several PE cells from an E7 chicken embryo. The basolateral cell membrane infoldings have increased in size & number. These membrane specializations are associated with numerous mitochondria. (23,700x).

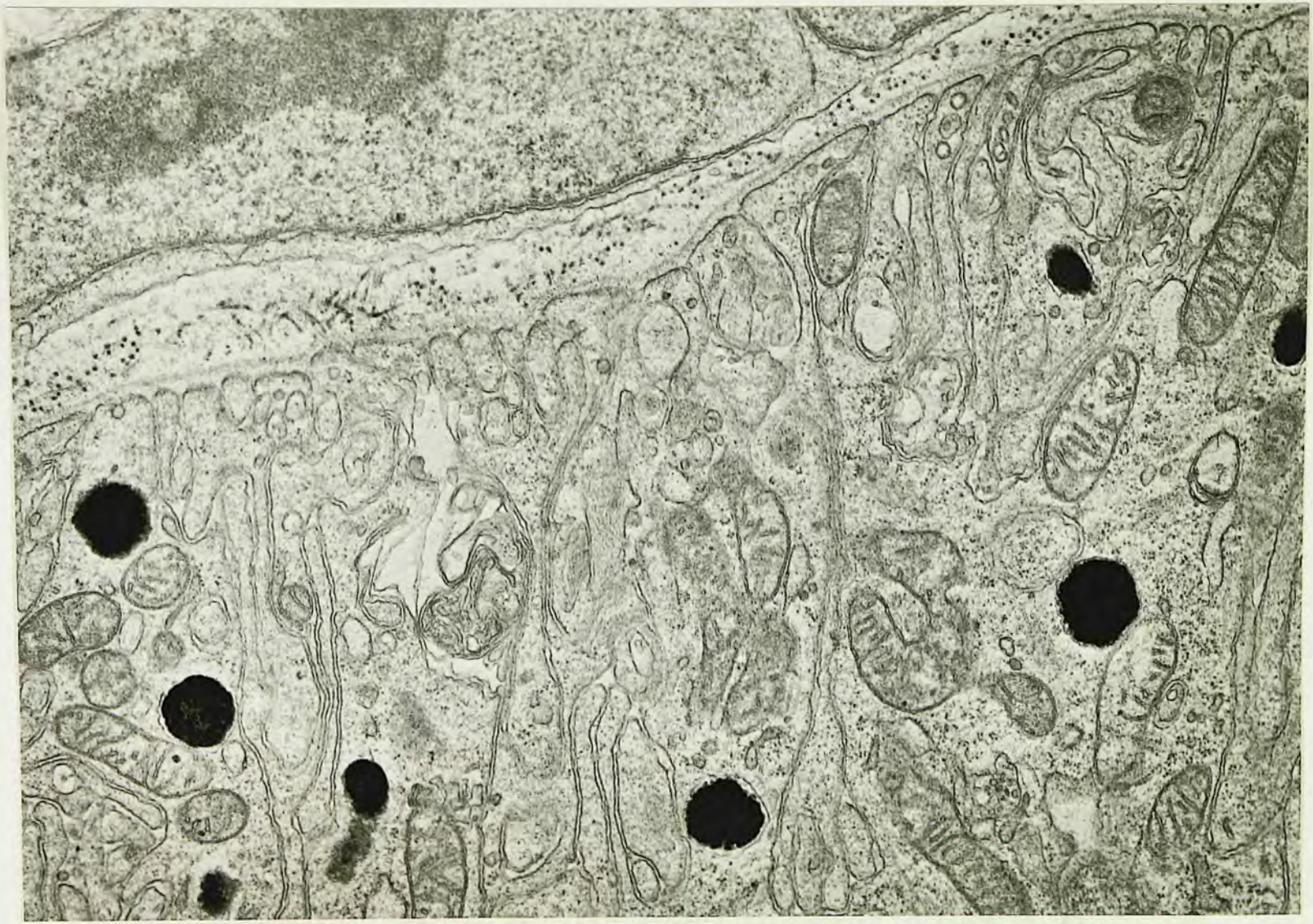


Figure 25. Electron micrograph of the apical portion of several PE cells from an E7 chicken embryo. The cytoplasm has developed more mitochondria, RER, and pigment granules than at E6. (25,000x).

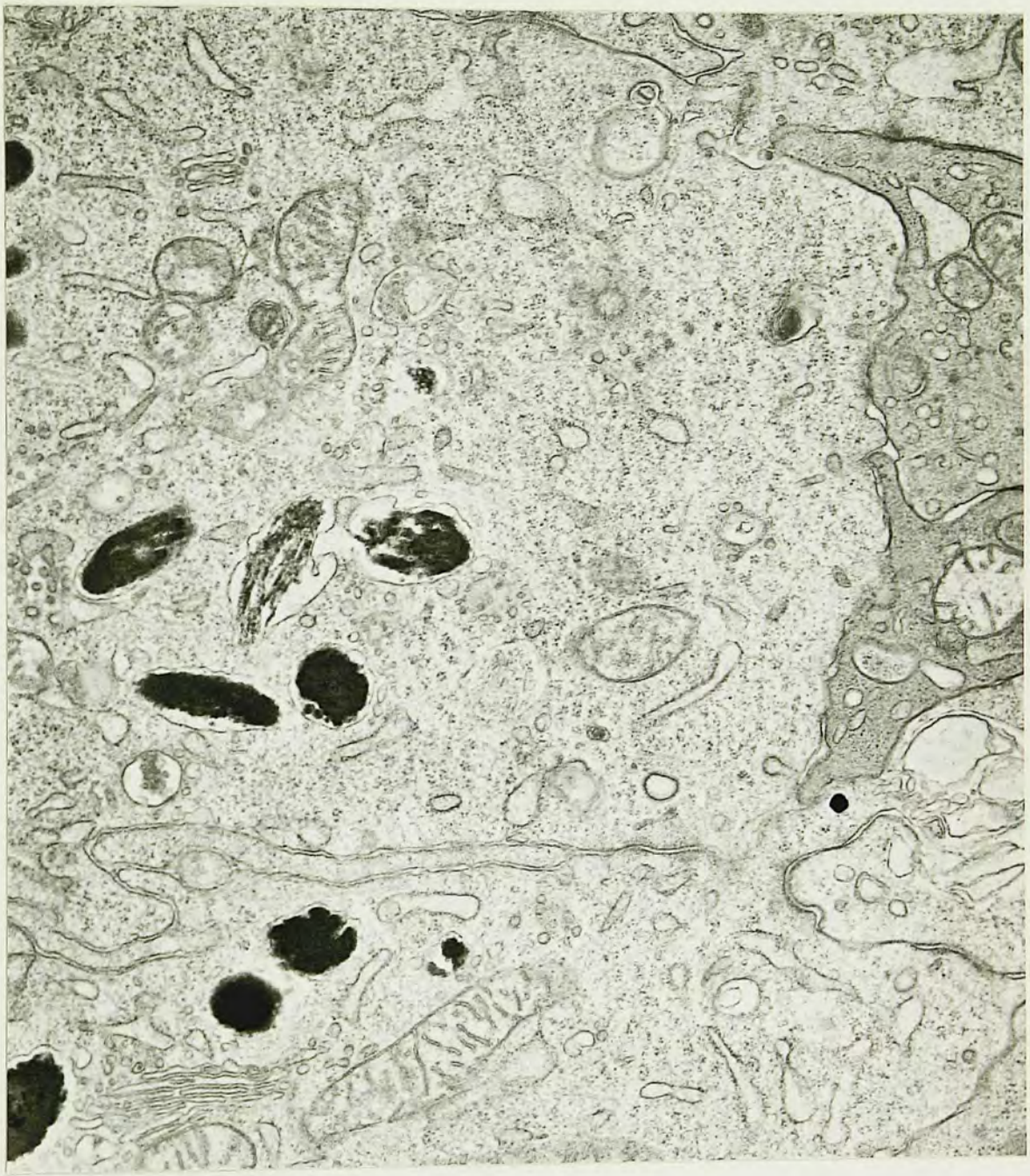


Figure 26. Electron micrograph of the apices of several PE and NPE cells from an E7 chicken embryo. The cells of the NPE have a larger amount of dilated RER (asterisks) than those at E6. (25,000x).

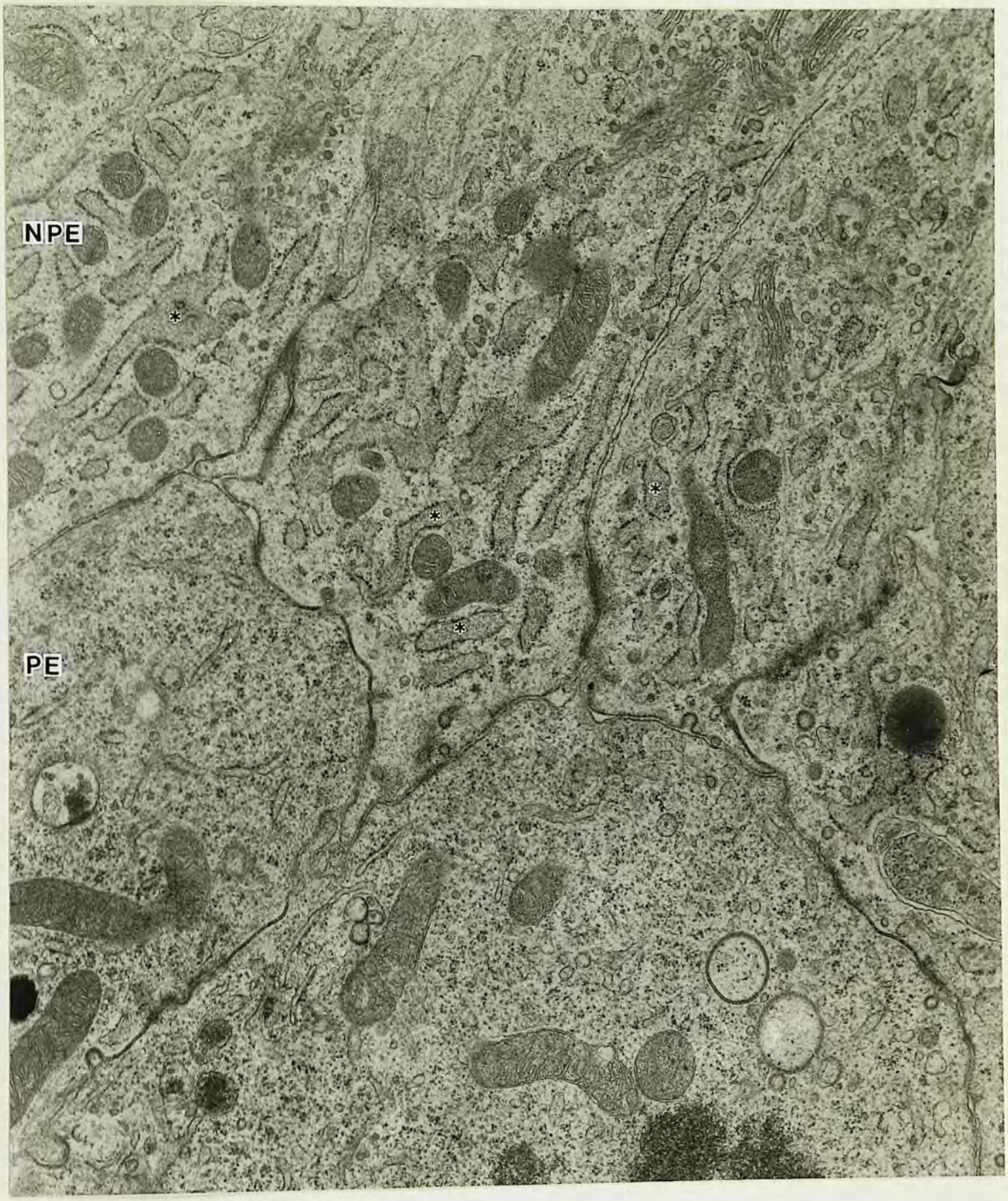

NPE
PE

Figure 27. Electron micrograph of the full length of several PE cells and the apical portions of several NPE cells from an E19 chicken embryo. The PE cells have a more extensive system of basolateral cell membrane infoldings than seen at E7. (12,500x).

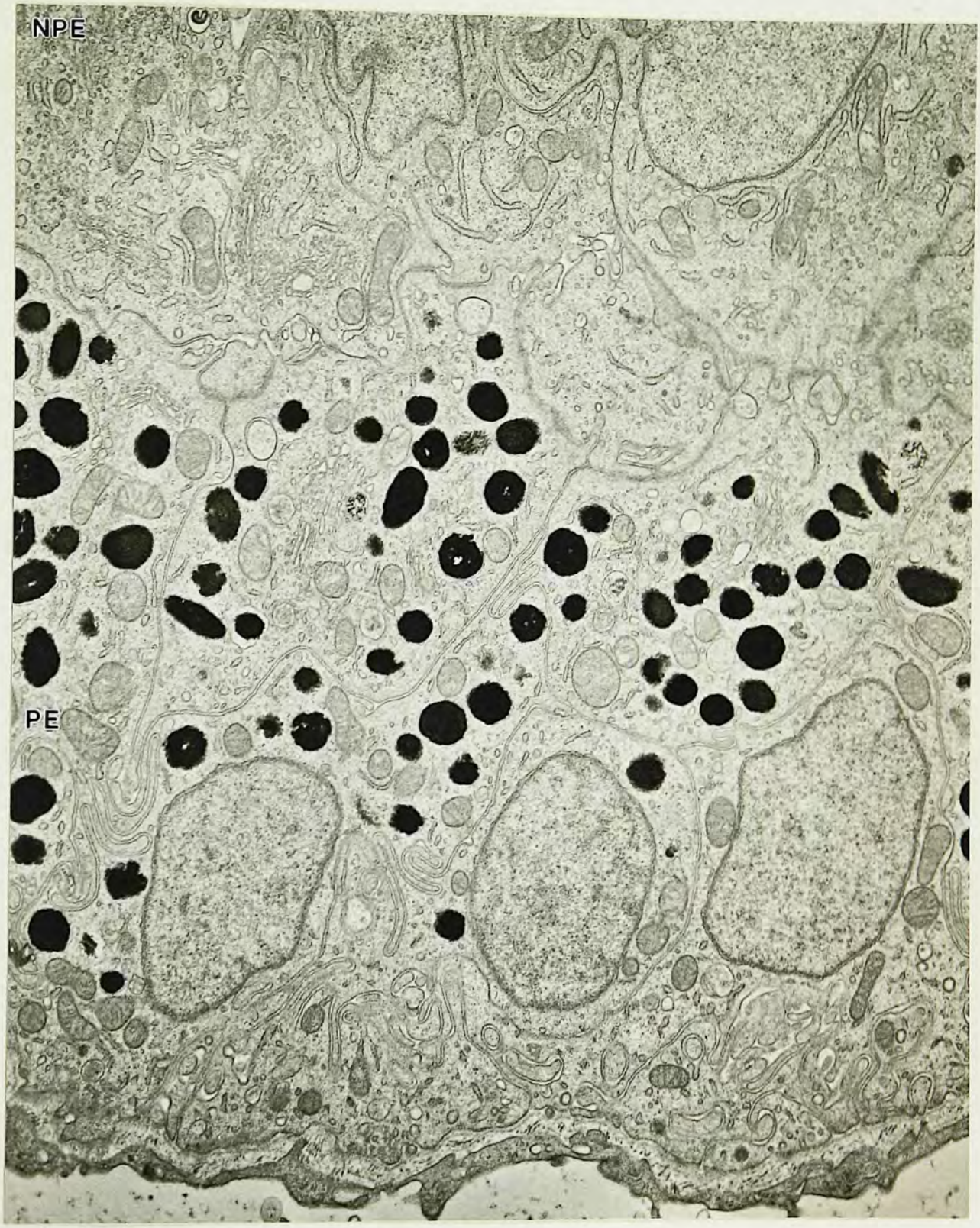

NPE
PE

Figure 28. Electron micrograph of the basal portion of several NPE cells from an E19 chicken embryo. The cells have developed an extensive network of RER throughout the cytoplasm. (P=posterior chamber, 13,000x).

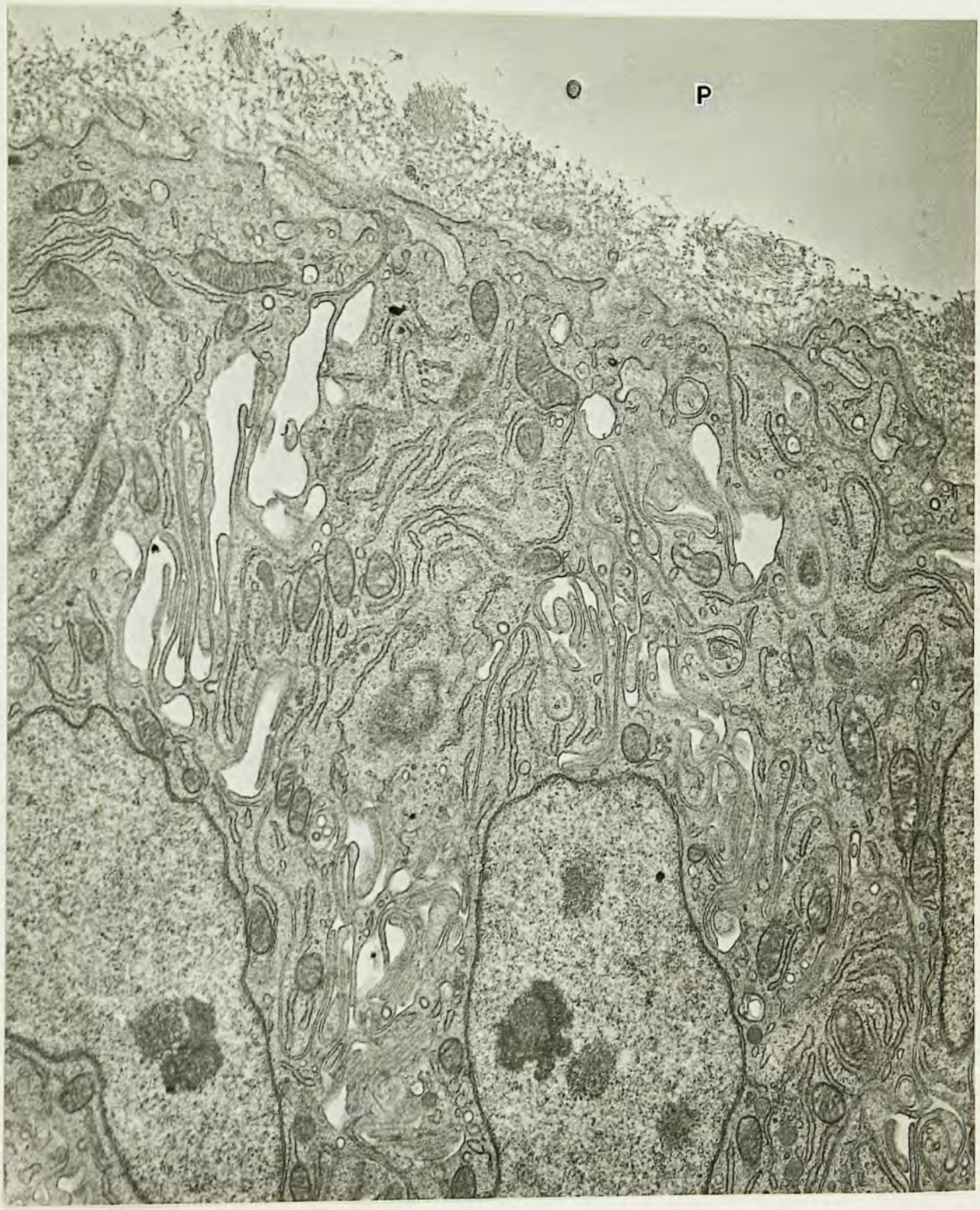

P

Figure 29. Light micrograph of the anterior segment of an E4 (stage 25) chicken eye stained for AChE. The reaction product is first visible in the prospective ciliary epithelium at this stage. The staining is more intense in the nasal half of the ciliary epithelium (arrowhead) than the temporal half. Faint staining is visible in the lens (L). (200x).


L

Figure 30. Light micrograph of a transversely sectioned whole eye from an E5 chicken embryo stained for AChE. The increased intensity of the AChE staining in the ciliary epithelium (arrows) and the lens (L) is clearly visible. (100x).

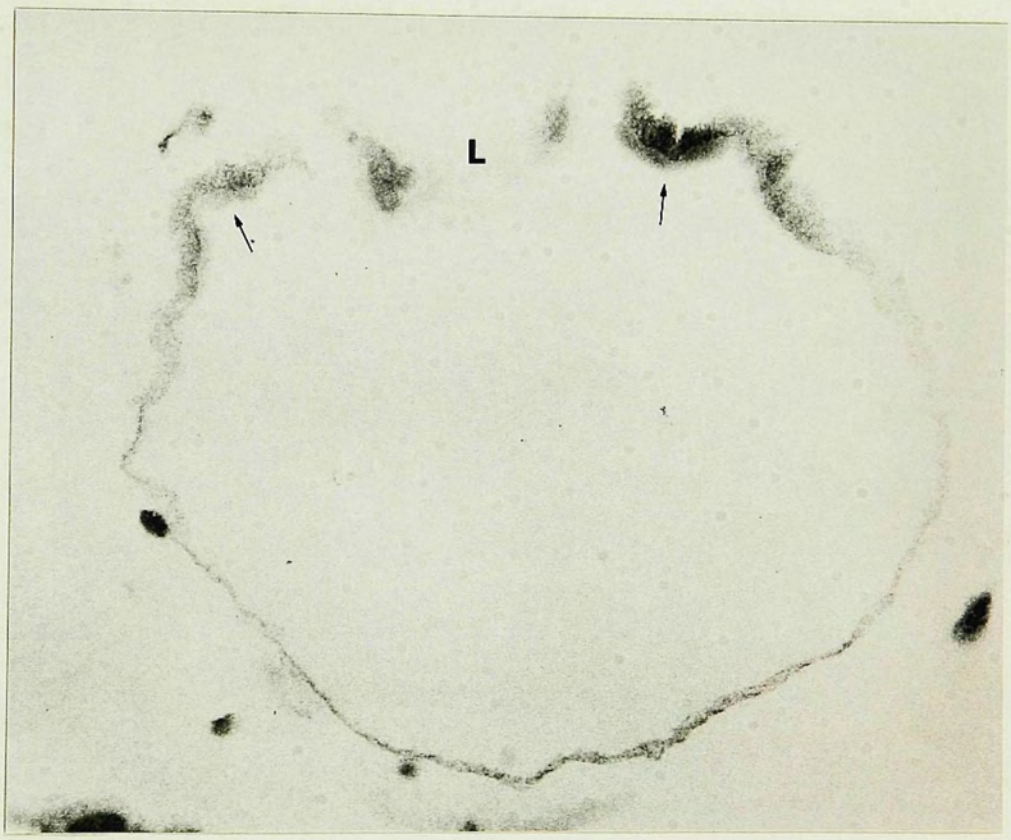

L

Figure 31. Light micrograph of a transversely sectioned whole eye from an E6 chicken embryo stained for AChE. The reaction product was observed in the ciliary epithelium (arrow), Lens (L), and ganglion cell layer of the posterior retina (arrowheads). (75x).


L

Figure 32. Light micrograph of a transversely sectioned whole eye from an E7 chicken embryo stained for AChE. The ciliary epithelium shows intense staining in both the nasal & temporal halves (arrows). The AChE reaction product is proceeding anteriorly in the ganglion cell layer & is also visible in other layers of the retina (R). (L=lens, 75x).

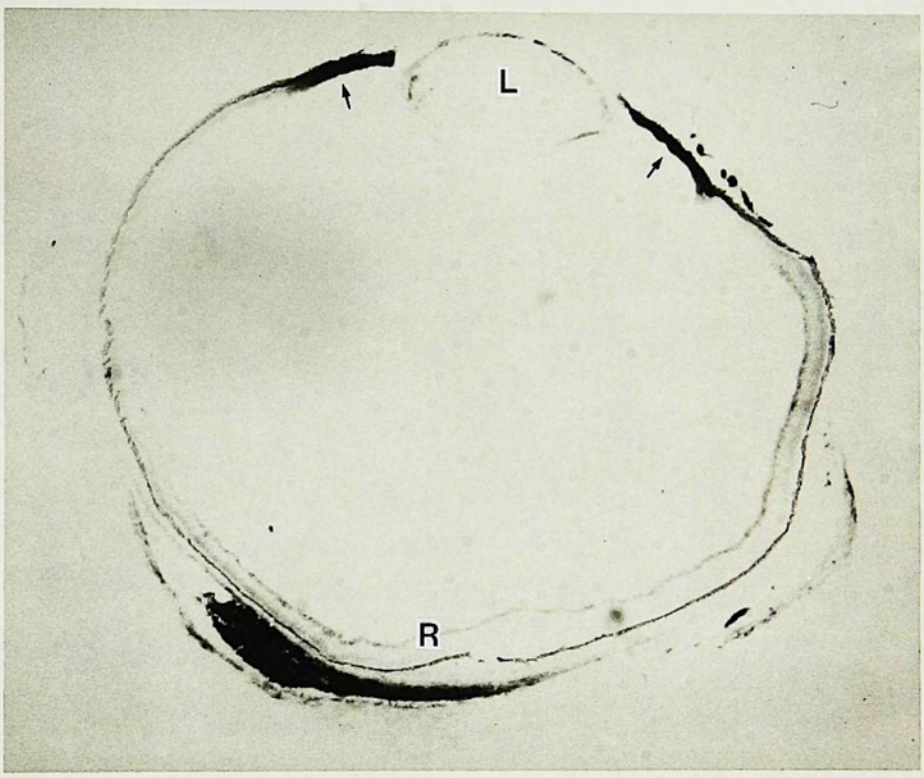

L
R

Figure 33. Light micrograph of a transversely sectioned whole eye from an E8 chicken embryo stained for AChE. The ciliary epithelium stains intensely (arrows) and the staining in the retina (R) extends towards the ora serrata. (L=lens, 45x).

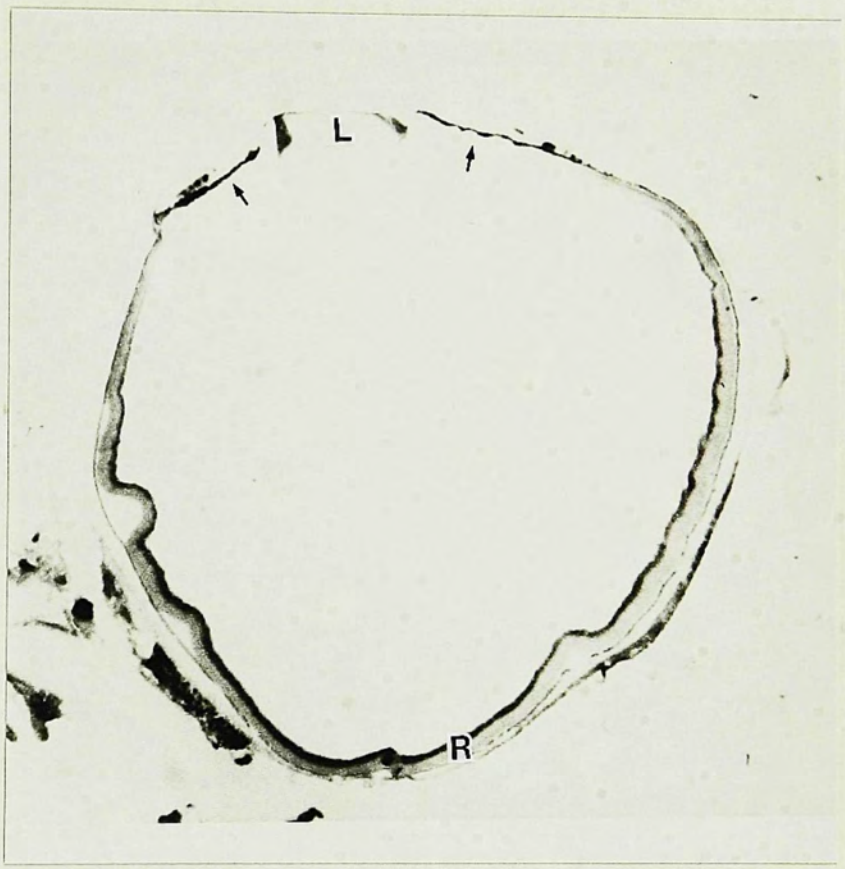

L
R

Figure 34. Light micrograph of a transversely sectioned whole eye from an E9 chicken embryo stained for AChE. The ciliary epithelium is seen on both sides of the lens (L). The AChE staining in the retina (R) has increased and reached the ora serrata (arrowhead). (40x).

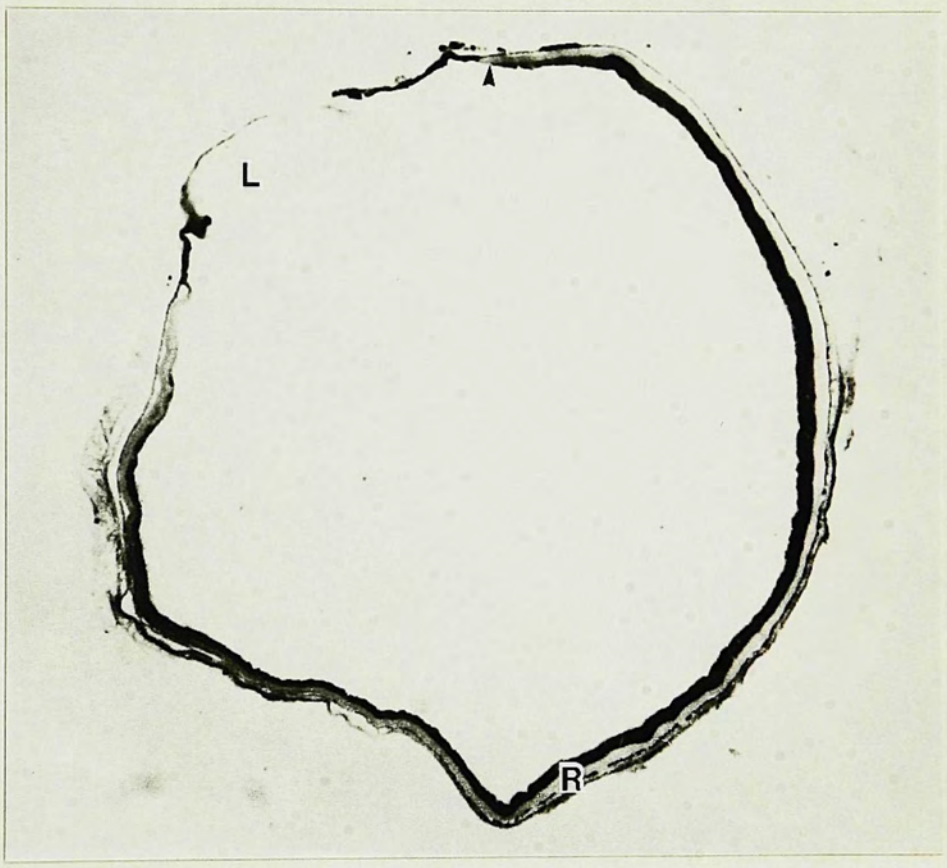

L
R

Figure 35. Light micrograph of the ora serrata from an E9 chicken embryo eye reacted for AChE. The ora serrata (arrow) is the transition zone between the retina (left) and the ciliary epithelium (right). (200x).

Figure 36. Electron micrograph of AChE staining from an eye of an E6 chicken embryo. The reaction product is found in the space between the two layers of the nuclear envelope (arrowheads) and in the cisternae of the RER (large arrow) in both the PE and NPE; and the extracellular spaces of the NPE (small arrows). (7,150x).

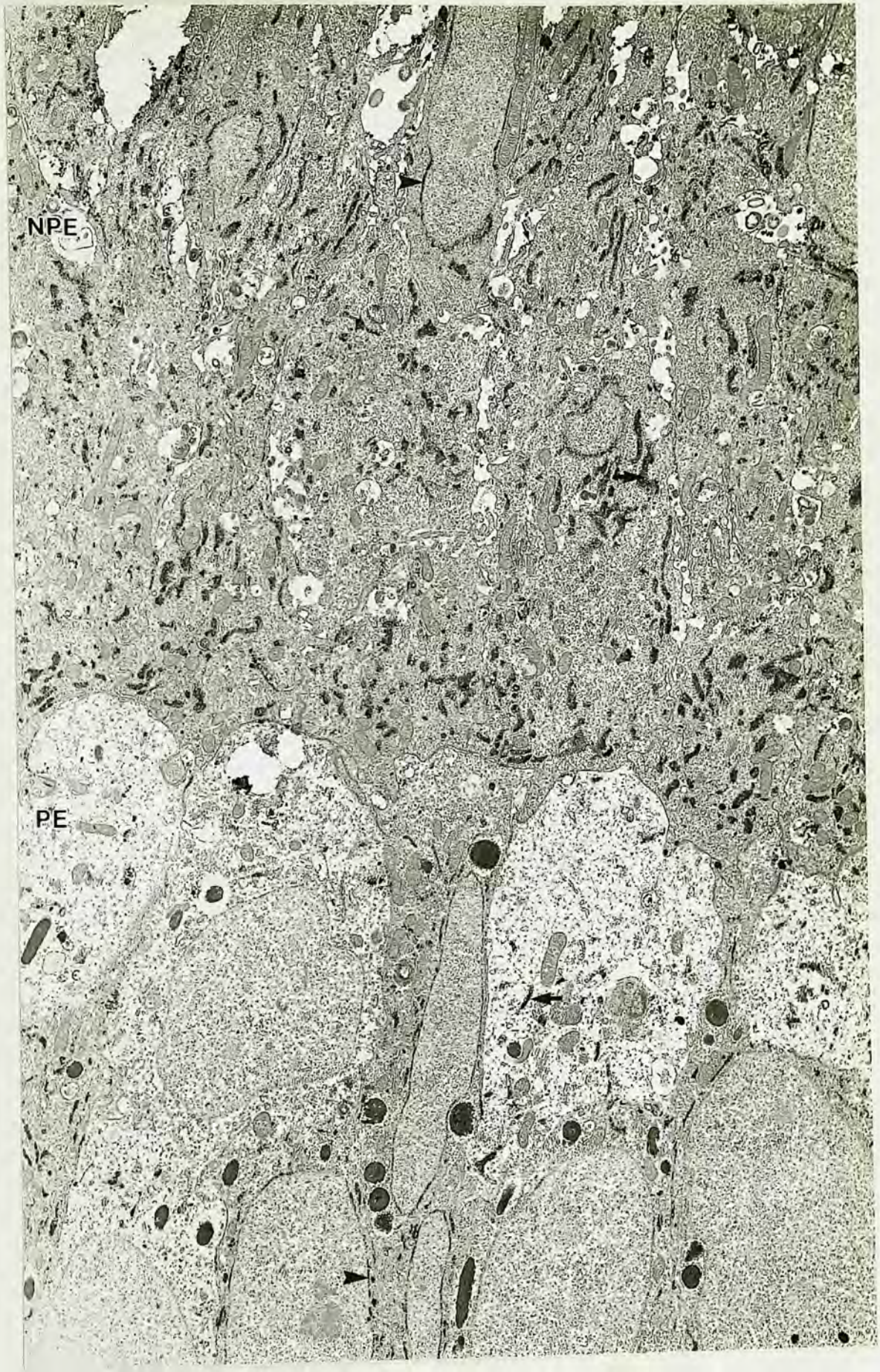

NPE
PE

Figure 37. Electron micrograph showing the localization of AChE staining in an E6 NPE cell. The reaction product is visible in the space between the two layers of the nuclear envelope (arrowheads) and in the cisternae of the RER (asterisks). (Nu=nucleus, 38,000x).

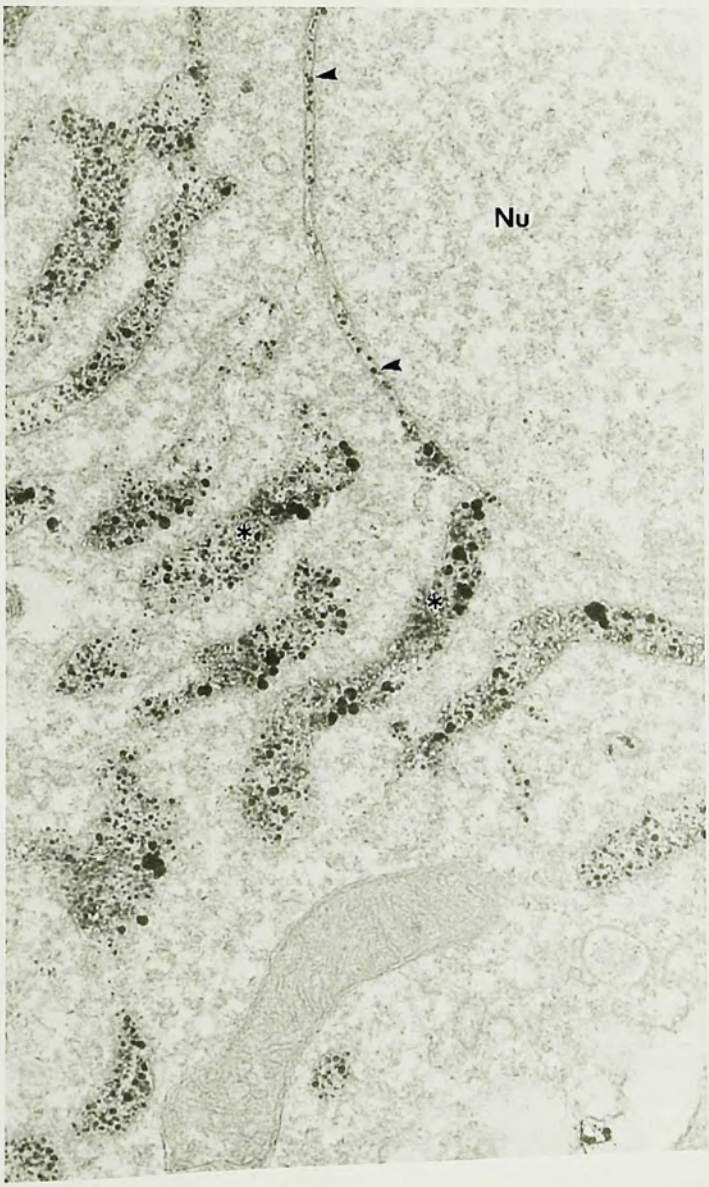

Nu

Figure 38. Light micrograph of a transversely sectioned whole eye from an E16 rat embryo stained for AChE. Due to the accumulation of pigment in the PE & RPE, it is not possible to tell whether this layer has reacted for AChE. However, the AChE reaction product is visible in the retina & extraocular muscles. No AChE staining is visible in the NPE (arrows). (50x).

Figure 39. Light micrograph of a ciliary process from a 15 day postnatal rat. Punctate staining is visible in the NPE (asterisk). It is not possible to determine whether the reaction product is also present in the PE due to the heavy pigmentation present in this layer. (250x).

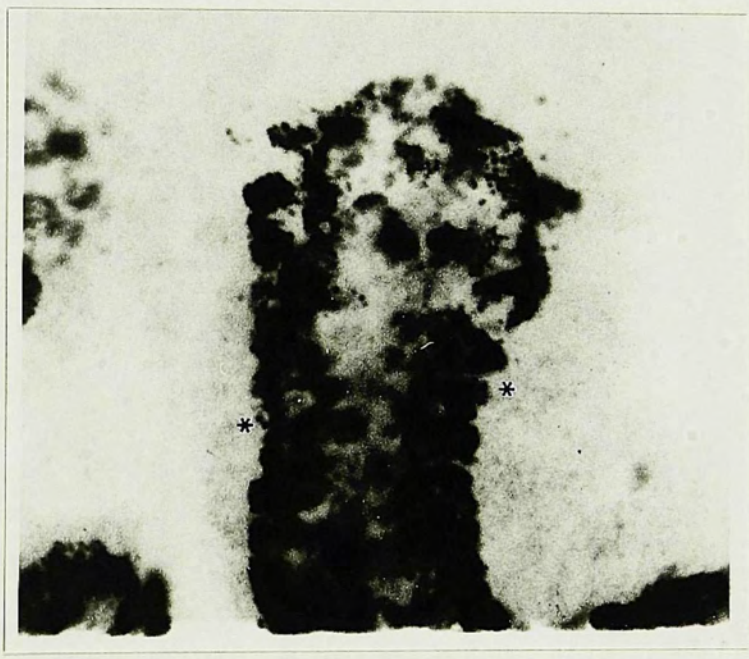
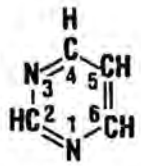
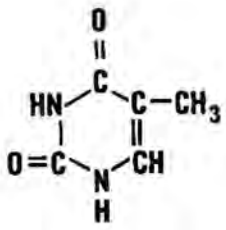


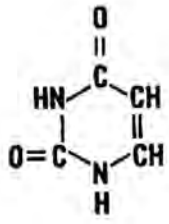
Figure 40. Diagram showing the chemical structure of ^{3}H-thymidine and BrdU, a closely related analog.



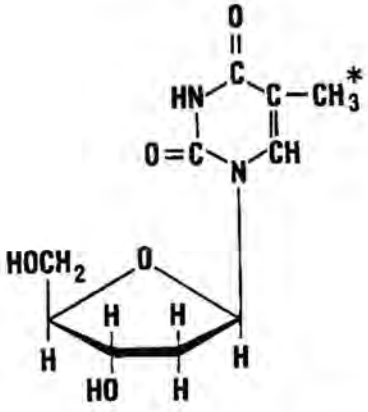
PYRIMIDINE
(PARENT COMPOUND)



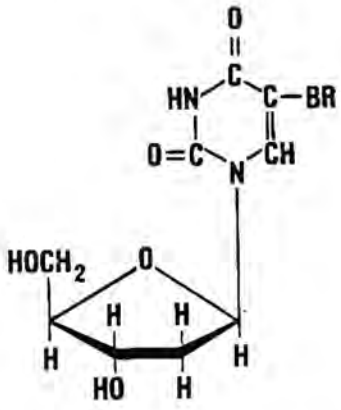
Thymine



Uracil



TRITIATED (^{3}H)—THYMIDINE



BRDU
(5—BROMO—2'—DEOXYURIDINE)

Figure 41. Graph showing variations in the labelling index of the pigmented (PE) and nonpigmented (NPE) layers of the chicken ciliary epithelium during development. The progress of fold formation in the ciliary epithelium is also shown for reference. Error bars represent the standard error of the mean.

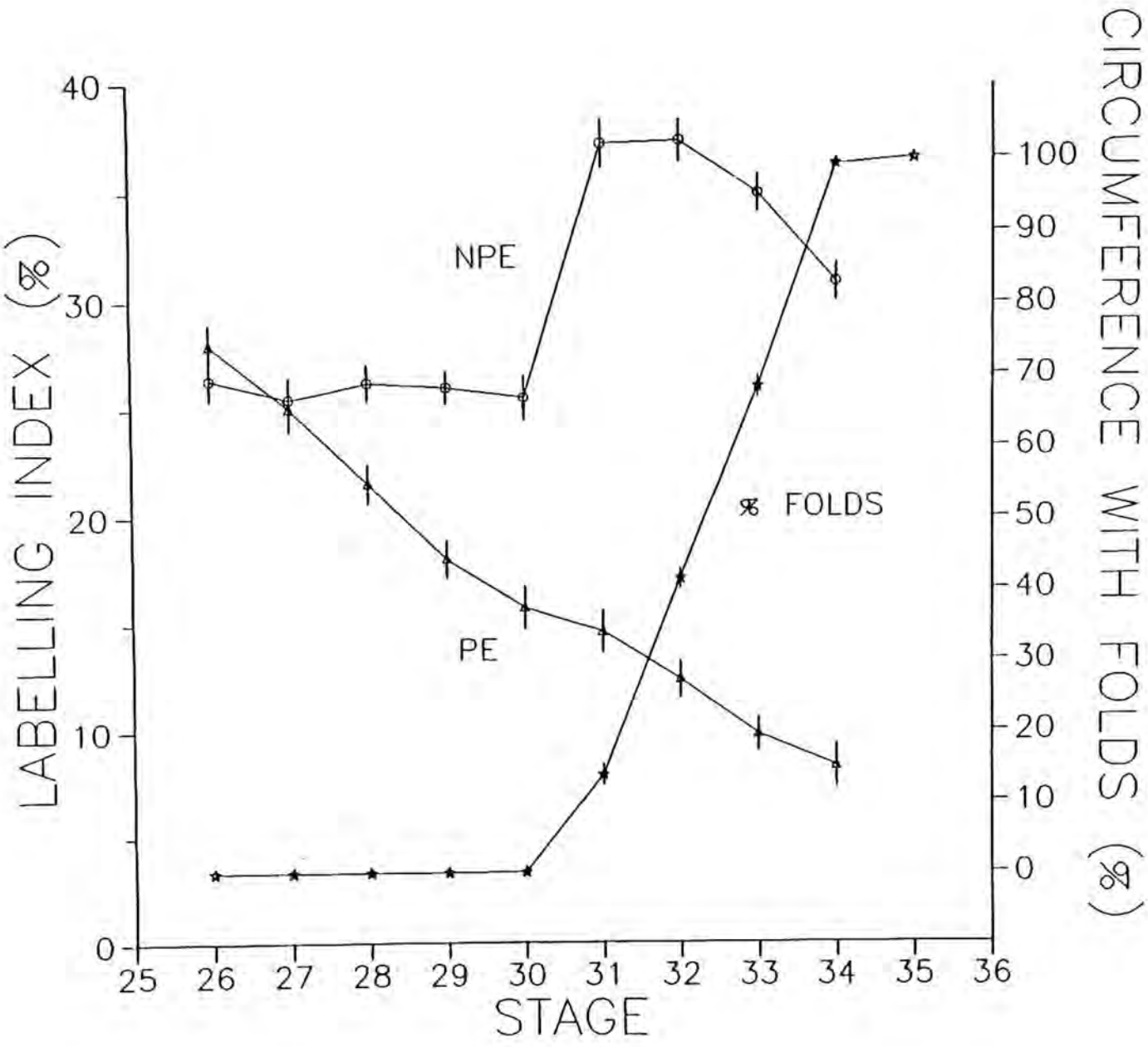

LABELLING INDEX (%)
CIRCUMFERENCE WITH FOLDS (%)
STAGE
NPE
PE
FOLDS
0
10
20
30
40
0
10
20
30
40
50
60
70
80
90
100
25
26
27
28
29
30
31
32
33
34
35
36

Figure 42. Light micrograph of the head from an E7 chicken embryo; the right eye of which was intubated with a hollow tube on E5.5. The eyes are the dark masses on either side of the head. The intubated eye was one-third to one-half the size of the contralateral control eye. (6x).

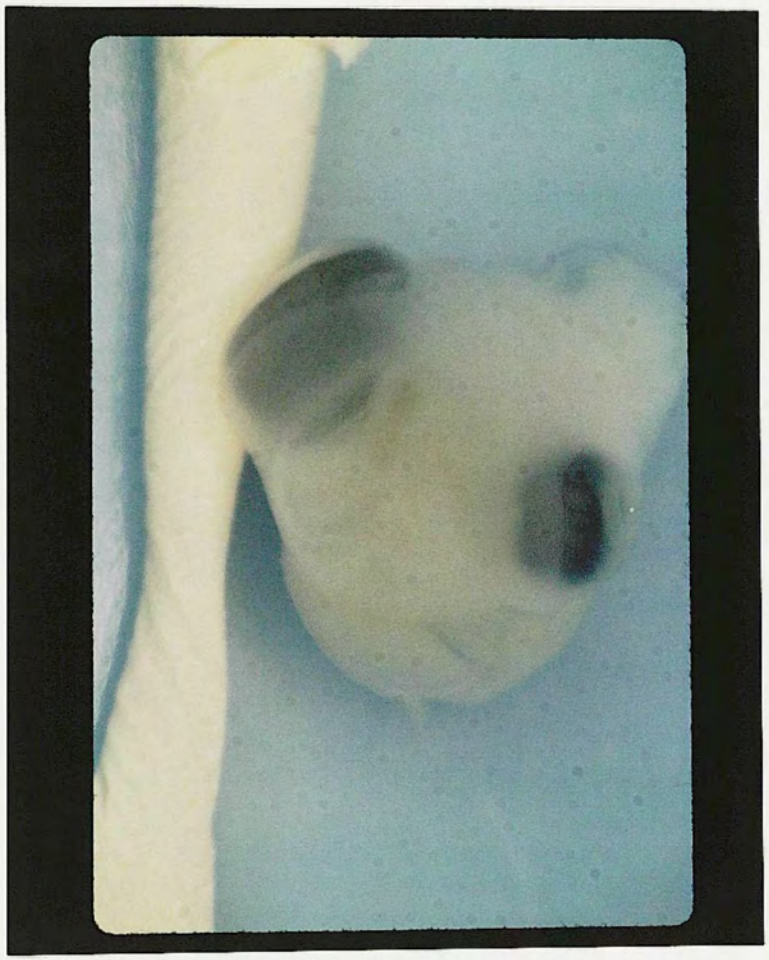


Figure 43. Light micrograph of a tangential section through an E7 chicken eye two days after intubation with a hollow tube. The neural retina continues to grow and is thrown into folds which have filled the vitreous cavity. The neural retina not directly adjacent to the RPE is only lightly stained. At higher magnification, tissue necrosis is evident in the central region. (60x).

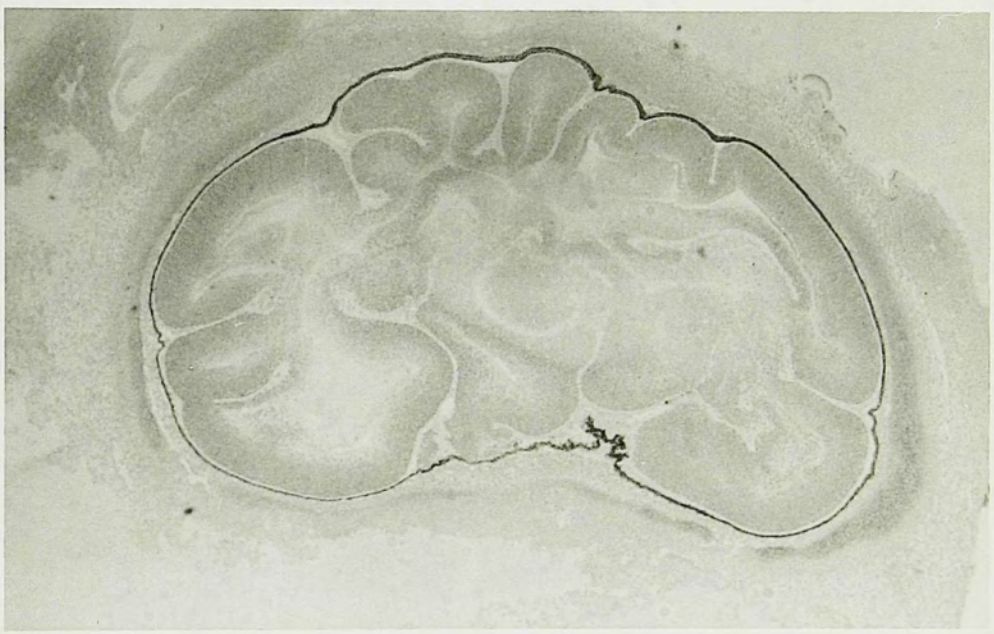


Figure 44. Graph of the labelling index versus stage of development for the PE. The data for the unoperated eye are redrawn from figure 41 for comparison with the experimental treatments.

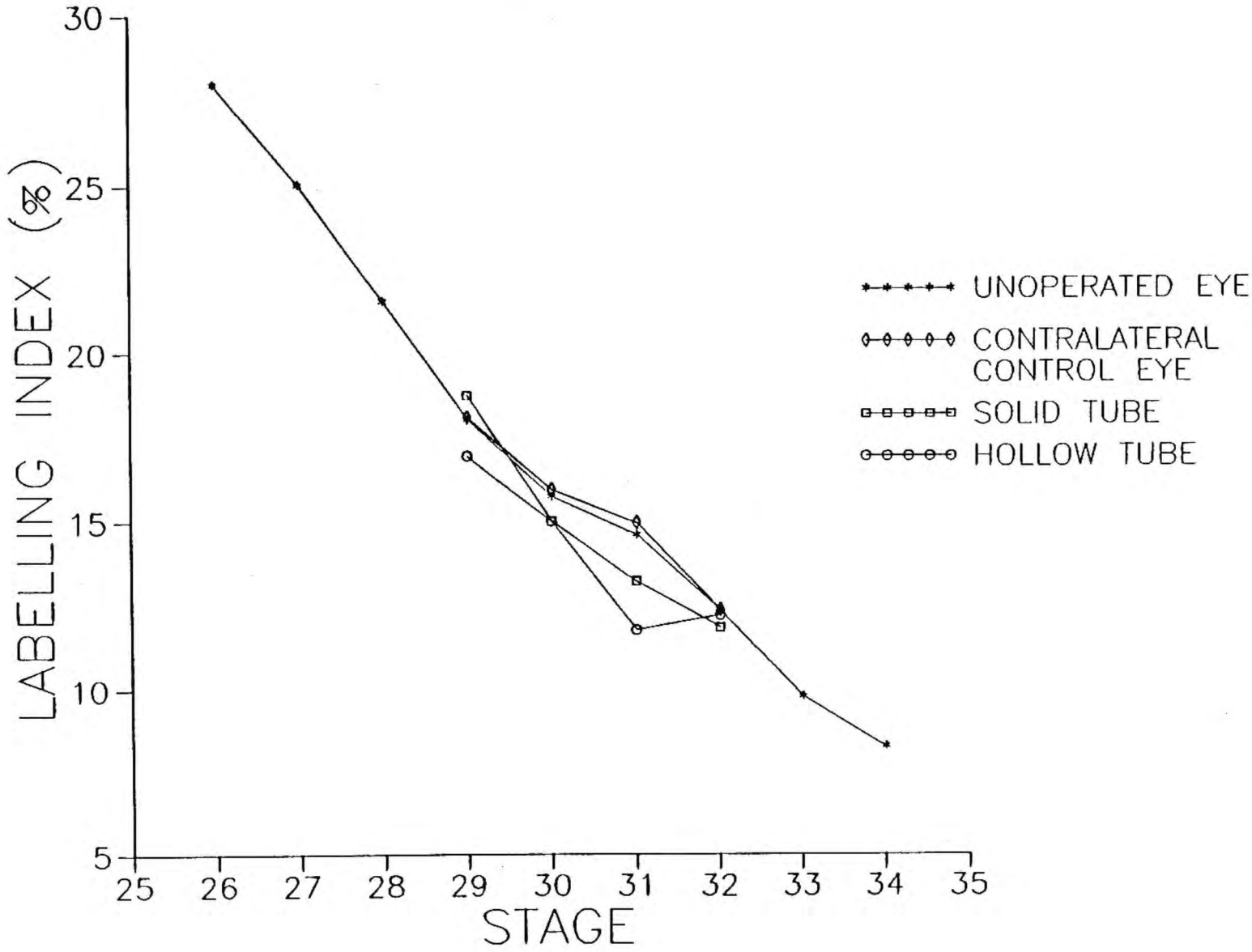

30
25
20
15
10
5
LABELLING INDEX (%)
25
26
27
28
29
30
31
32
33
34
35
STAGE
UNOPERATED EYE
CONTRALATERAL CONTROL EYE
SOLID TUBE
HOLLOW TUBE

Figure 45. Graph of the labelling index versus stage of development for the NPE. Stars represent significant changes ($p \leq 0.05$) between successive stages. The data for the unoperated eye are redrawn from figure 41 for comparison with experimental treatments.

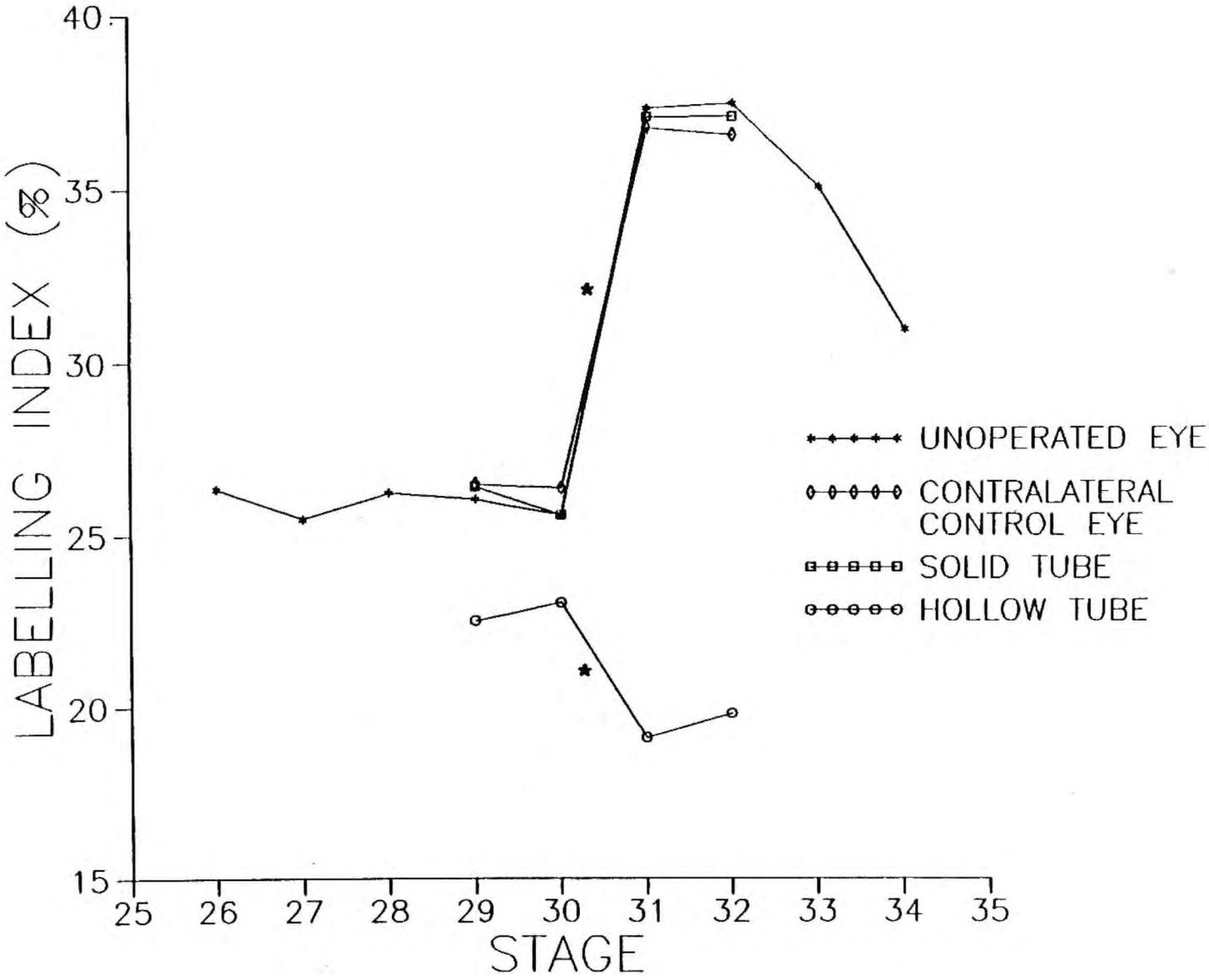

40
35
30
25
20
15
LABELLING INDEX (%)
25
26
27
28
29
30
31
32
33
34
35
STAGE
UNOPERATED EYE
CONTRALATERAL CONTROL EYE
SOLID TUBE
HOLLOW TUBE

Figure 46. Graph of the labelling index versus stage of development for the PE and NPE prior to and during fold formation. Asterisks represent significant differences ($p \leq 0.05$) between the solid & hollow intubated treatments for each layer. Error bars represent the standard error of the mean.

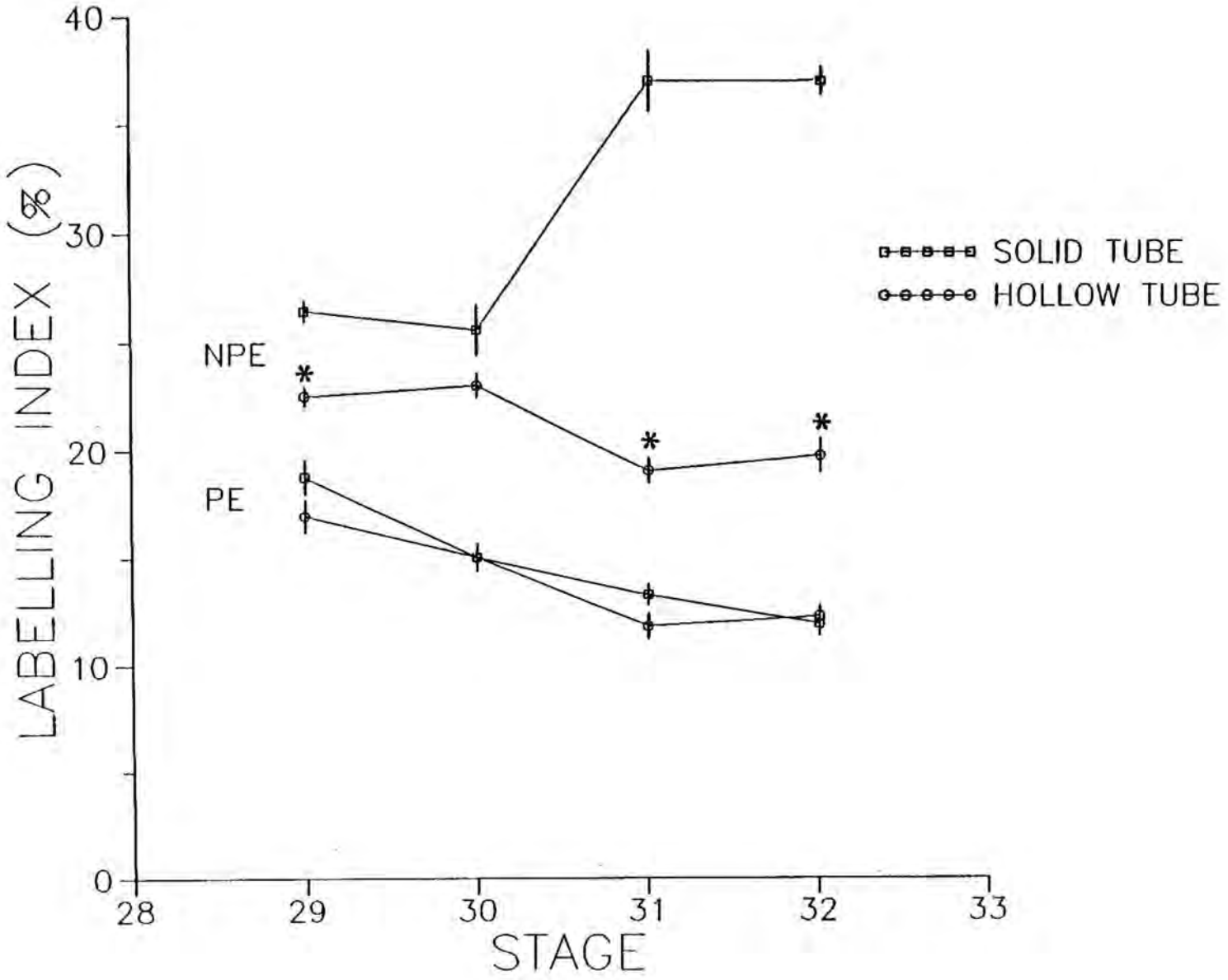

40
30
20
10
0
LABELLING INDEX (%)
NPE
PE
*
*
*
28
29
30
31
32
33
STAGE
SOLID TUBE
HOLLOW TUBE

Figure 47. Graph of the labelling index versus stage of development for the neural retina & retinal pigmented epithelium (RPE) prior to and during the time of ciliary fold formation. Error bars represent the standard error of the mean.

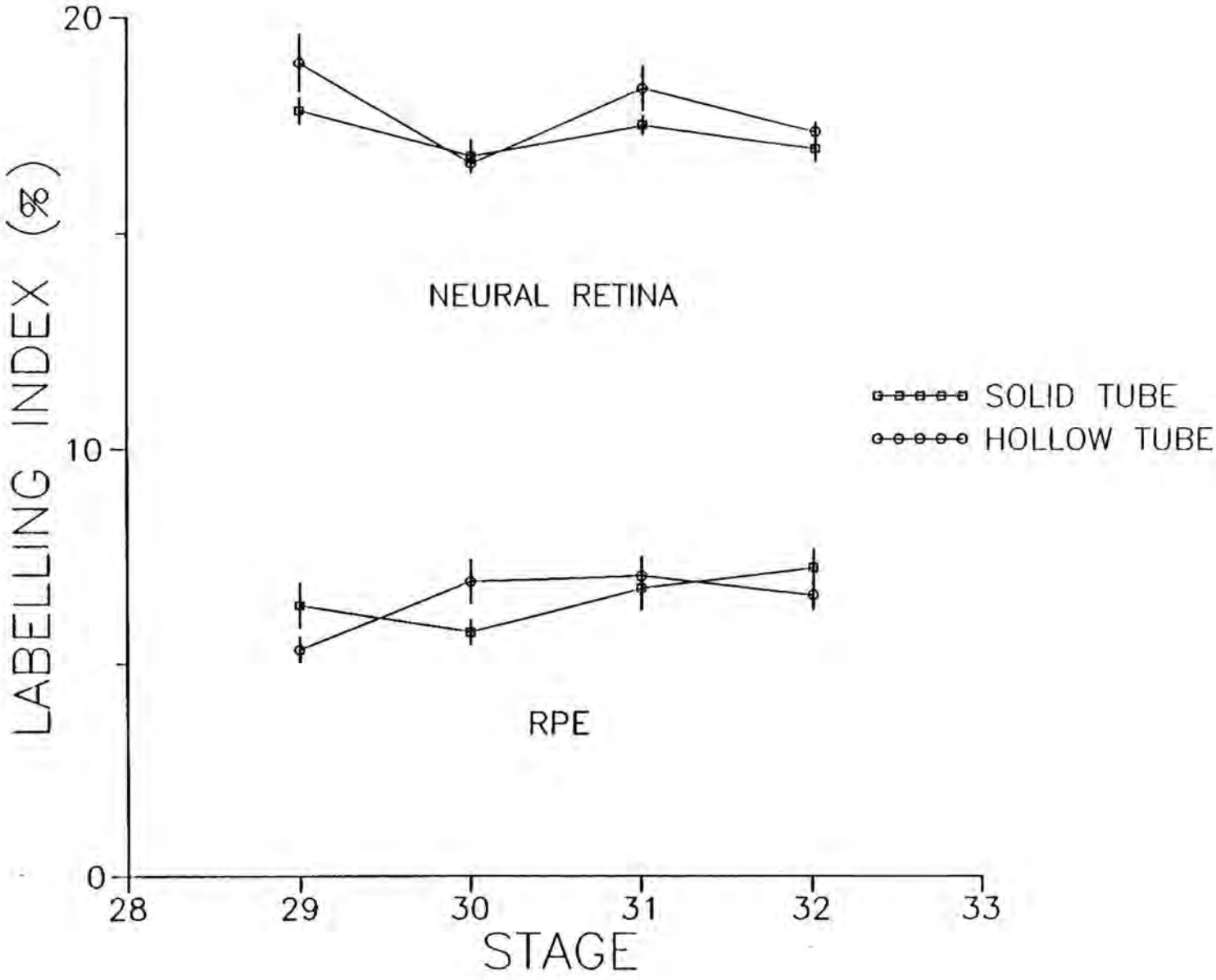

20
10
0
LABELLING INDEX (%)
NEURAL RETINA
RPE
SOLID TUBE
HOLLOW TUBE
28
29
30
31
32
33
STAGE

Figure 48. Graph of cell density versus stage of development for the PE & NPE in unoperated eyes. Stars represent significant changes ($p \leq 0.05$) between successive stages. Error bars represent the standard error of the mean.

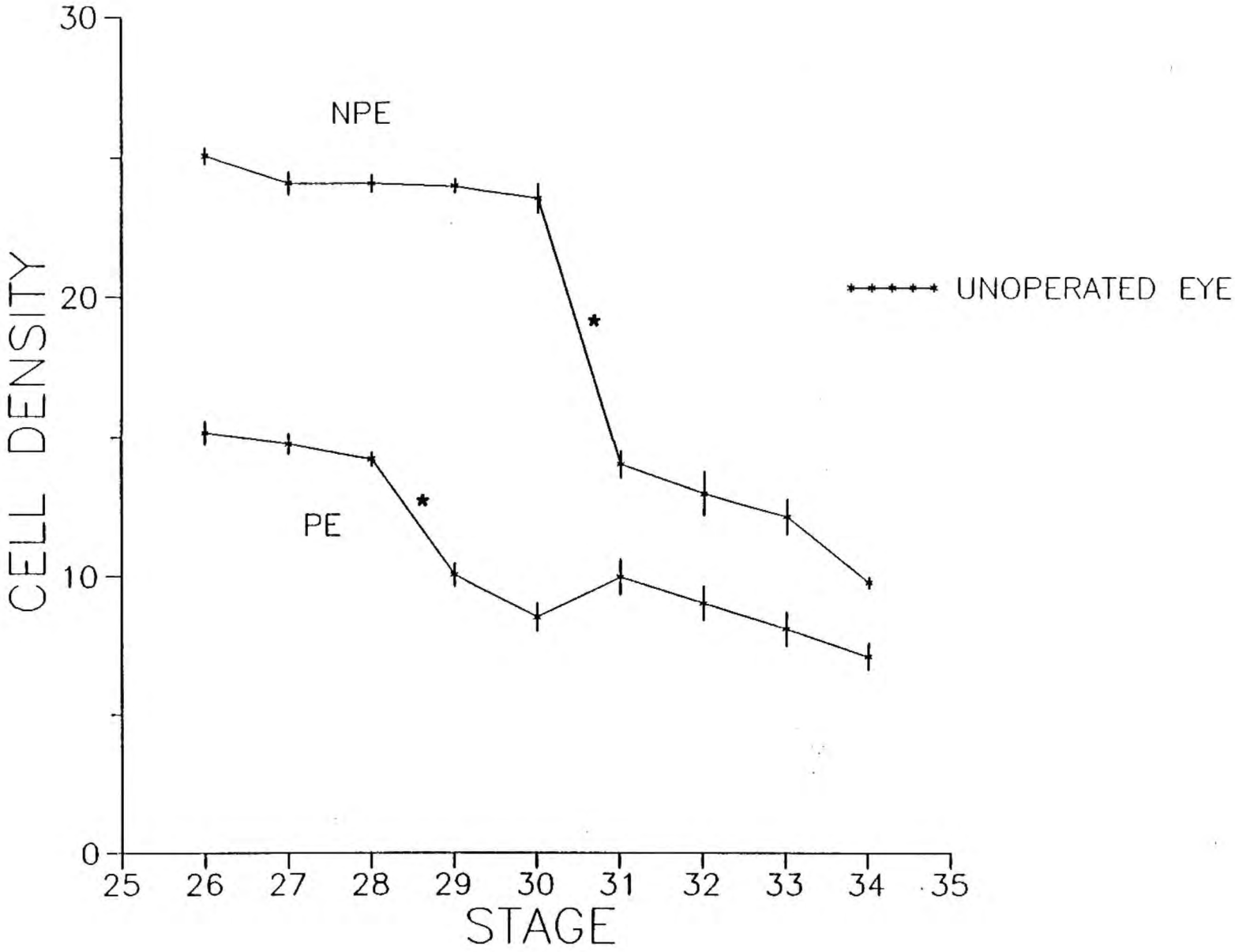

30
20
10
0
CELL DENSITY
NPE
PE
*
*
UNOPERATED EYE
25
26
27
28
29
30
31
32
33
34
35
STAGE

Figure 49. Graph of cell density versus stage of development for the PE. Asterisks represent significant differences ($p \leq 0.05$) between experimental groups. The data for the unoperated eye are redrawn from figure 48 for comparison with the experimental treatments.

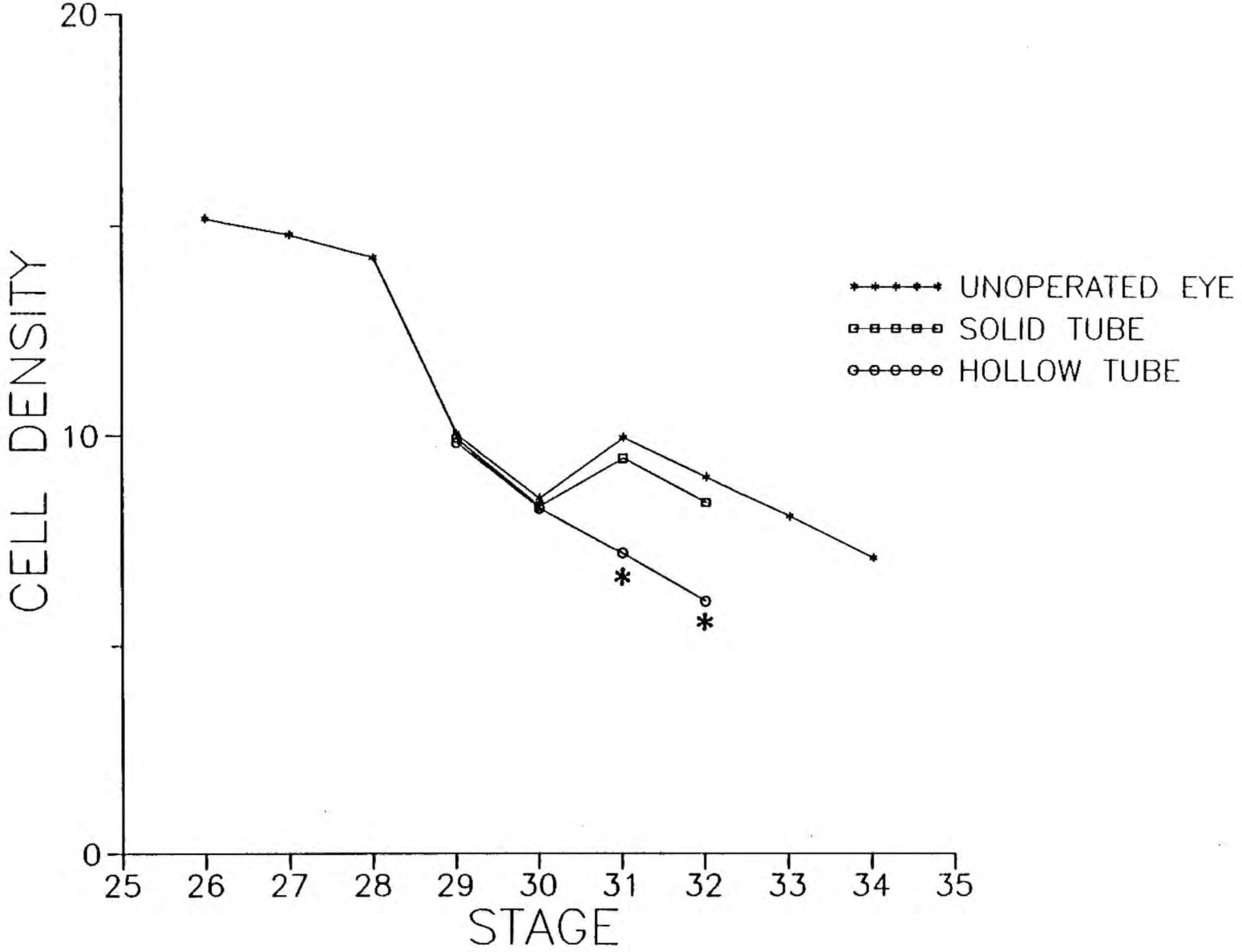

20
10
0
CELL DENSITY
25
26
27
28
29
30
31
32
33
34
35
STAGE
UNOPERATED EYE
SOLID TUBE
HOLLOW TUBE
*
*

Figure 50. Graph of the cell density versus stage of development for the NPE. Asterisks represent significant differences ($p \leq 0.05$) between experimental groups. The data for the unoperated eye are redrawn from figure 48 for comparison with the experimental treatments.

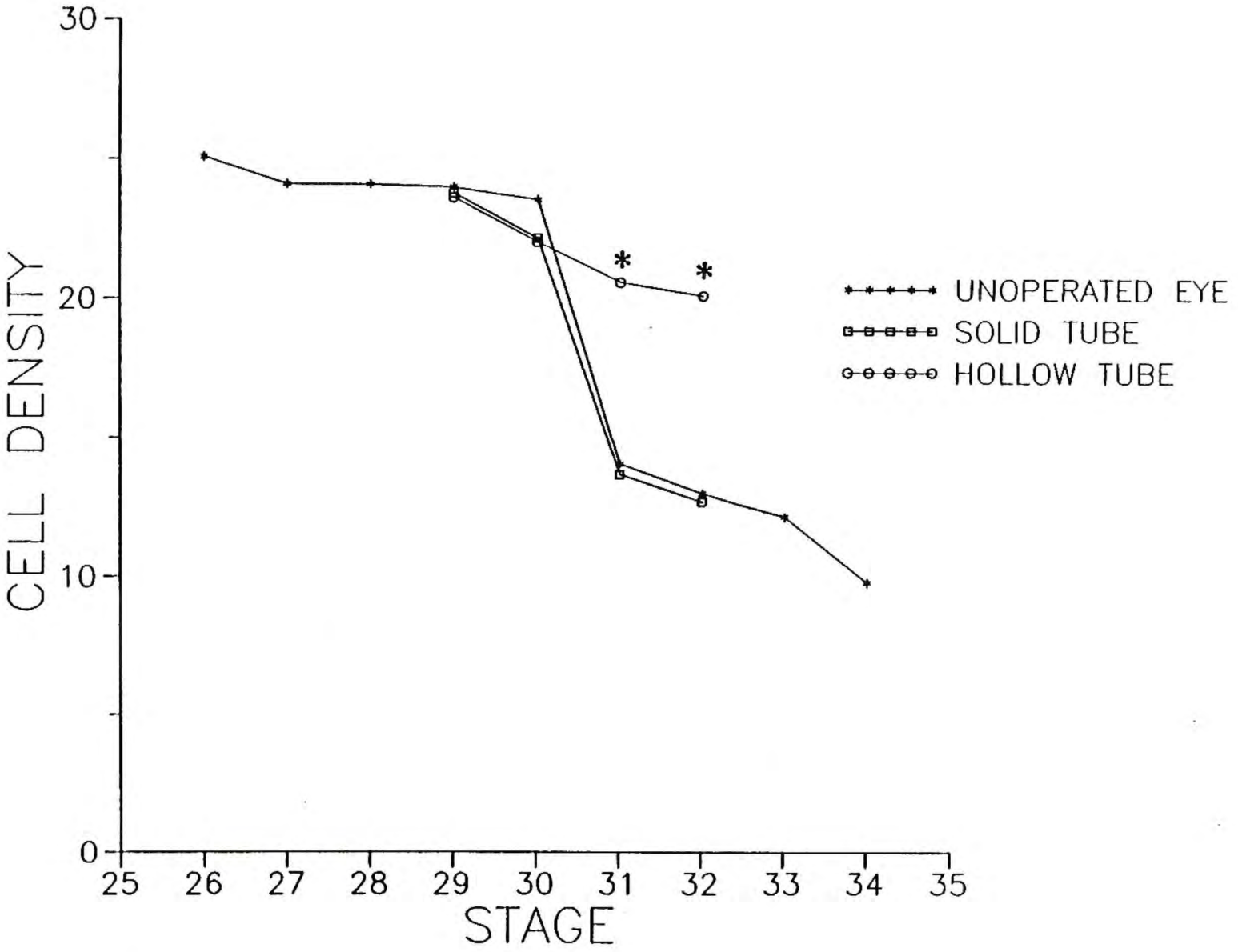

30
20
10
0
CELL DENSITY
25
26
27
28
29
30
31
32
33
34
35
STAGE
*
*
UNOPERATED EYE
SOLID TUBE
HOLLOW TUBE

Figure 51. Graph of cell density versus stage of development for the PE and NPE prior to and during fold formation. Asterisks represent significant differences ($p \leq 0.05$) between solid & hollow intubated treatments for each layer. Error bars represent the standard error of the mean.

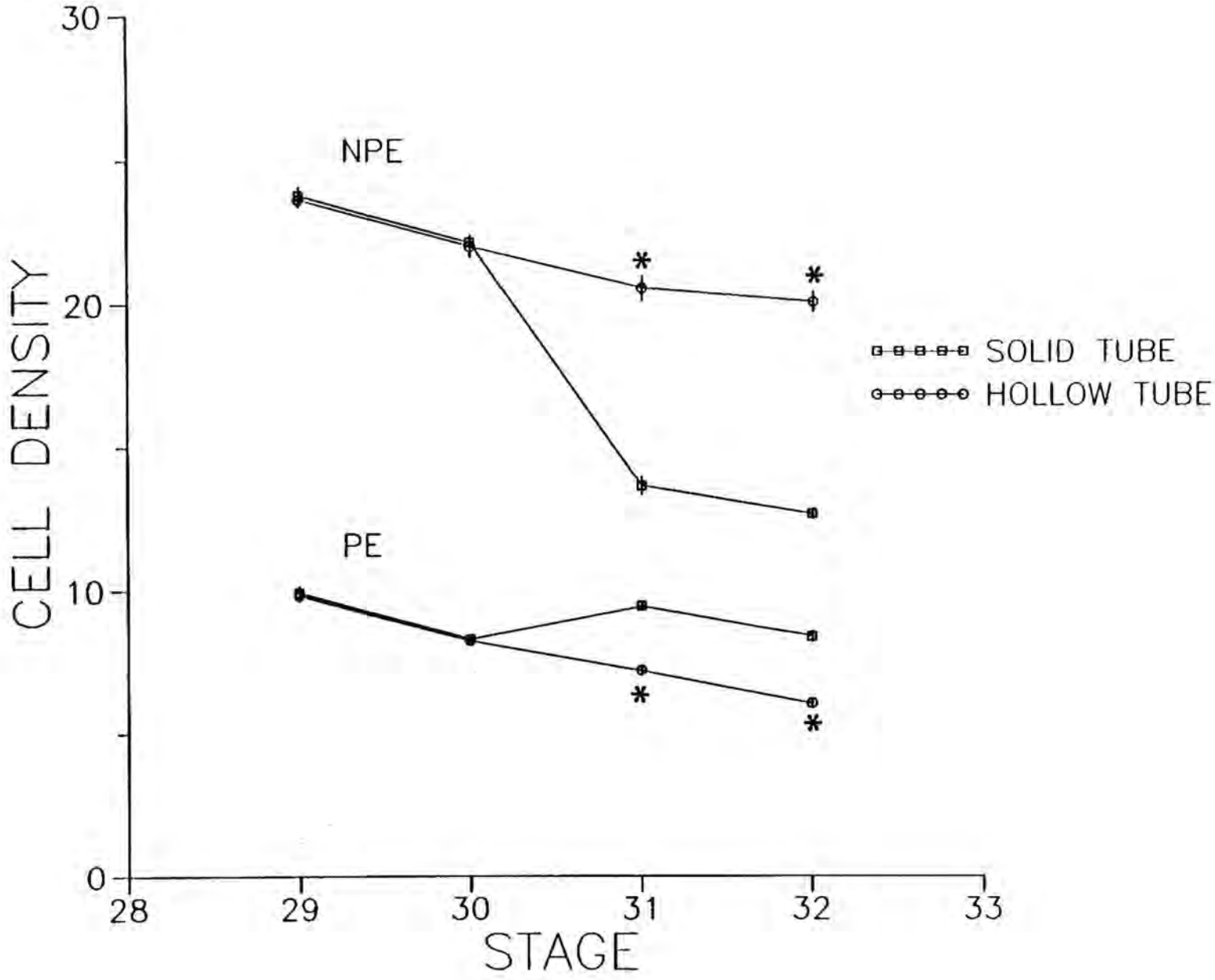

30
20
10
0
CELL DENSITY
NPE
PE
*
*
*
*
SOLID TUBE
HOLLOW TUBE
28
29
30
31
32
33
STAGE

Figure 52. Graph of cell height versus stage of development for the PE and NPE in unoperated eyes. Stars represent significant changes ($p \leq 0.05$) between successive stages. Error bars represent the standard error of the mean.

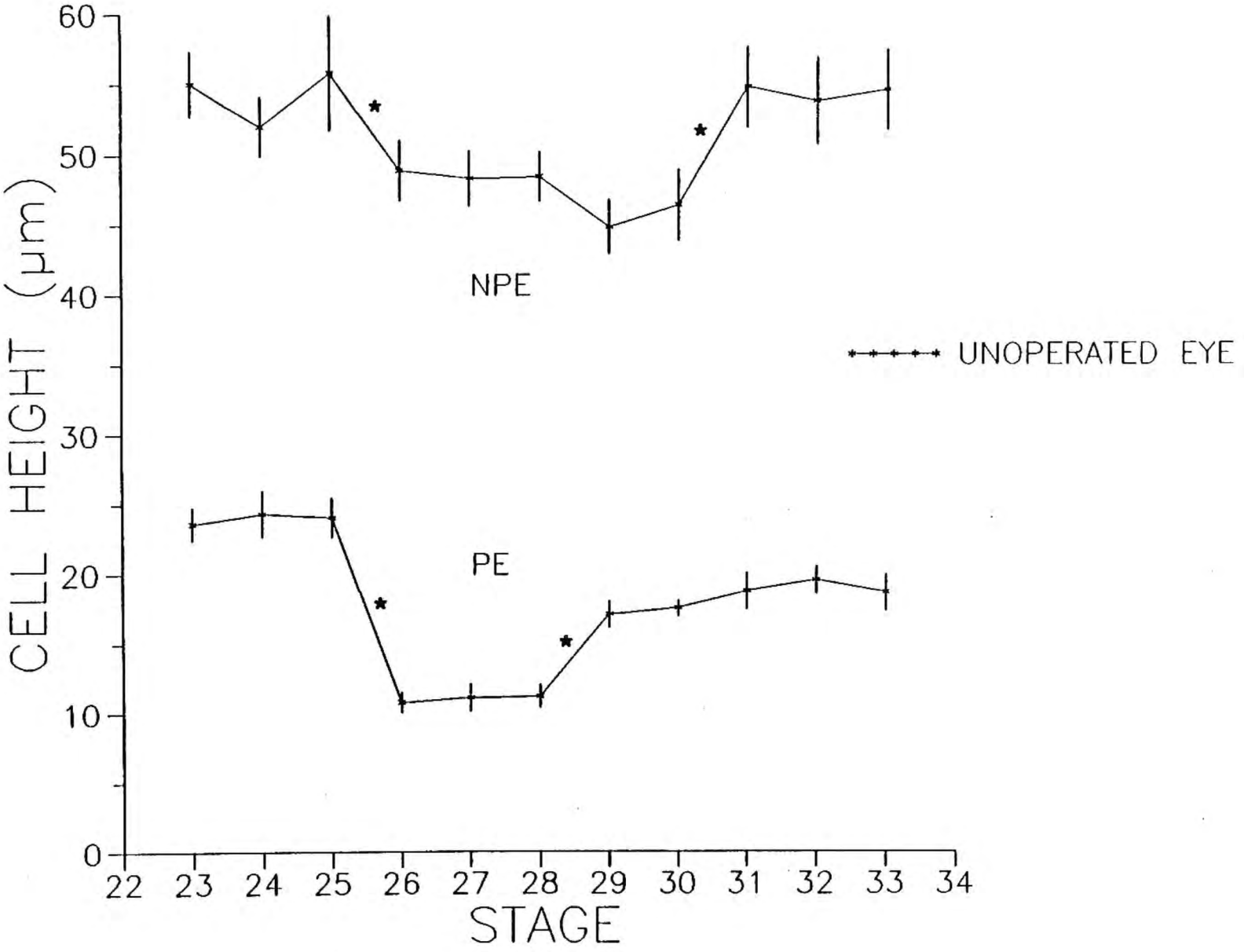

60
50
40
30
20
10
0
CELL HEIGHT (µm)
NPE
PE
UNOPERATED EYE
22
23
24
25
26
27
28
29
30
31
32
33
34
STAGE

Figure 53. Graph of the cell height versus stage of development for the PE. The data for the unoperated eye are redrawn from figure 52 for comparison with the experimental treatments. Asterisks represent significant differences ($p \leq 0.05$) between experimental groups. Error bars represent the standard error of the mean.

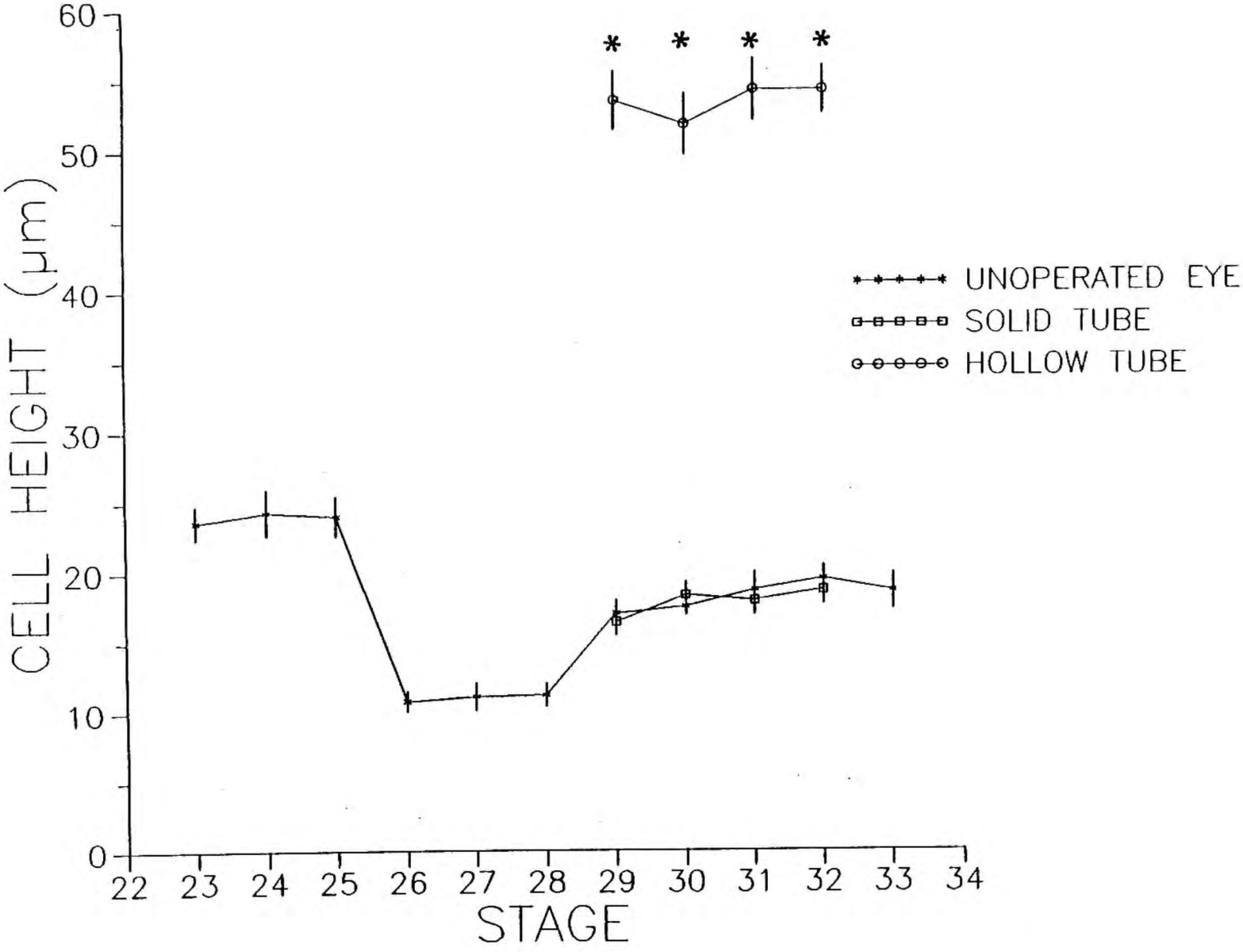

60
50
40
30
20
10
0
CELL HEIGHT (μm)
22
23
24
25
26
27
28
29
30
31
32
33
34
STAGE
*
*
*
*
UNOPERATED EYE
SOLID TUBE
HOLLOW TUBE

Figure 54. Graph of the cell height versus stage of development for the NPE. The data for the unoperated eye are redrawn from figure 52 for comparison with the experimental treatments. Asterisks represent significant changes ($p \leq 0.05$) between experimental groups. Error bars represent the standard error of the mean.

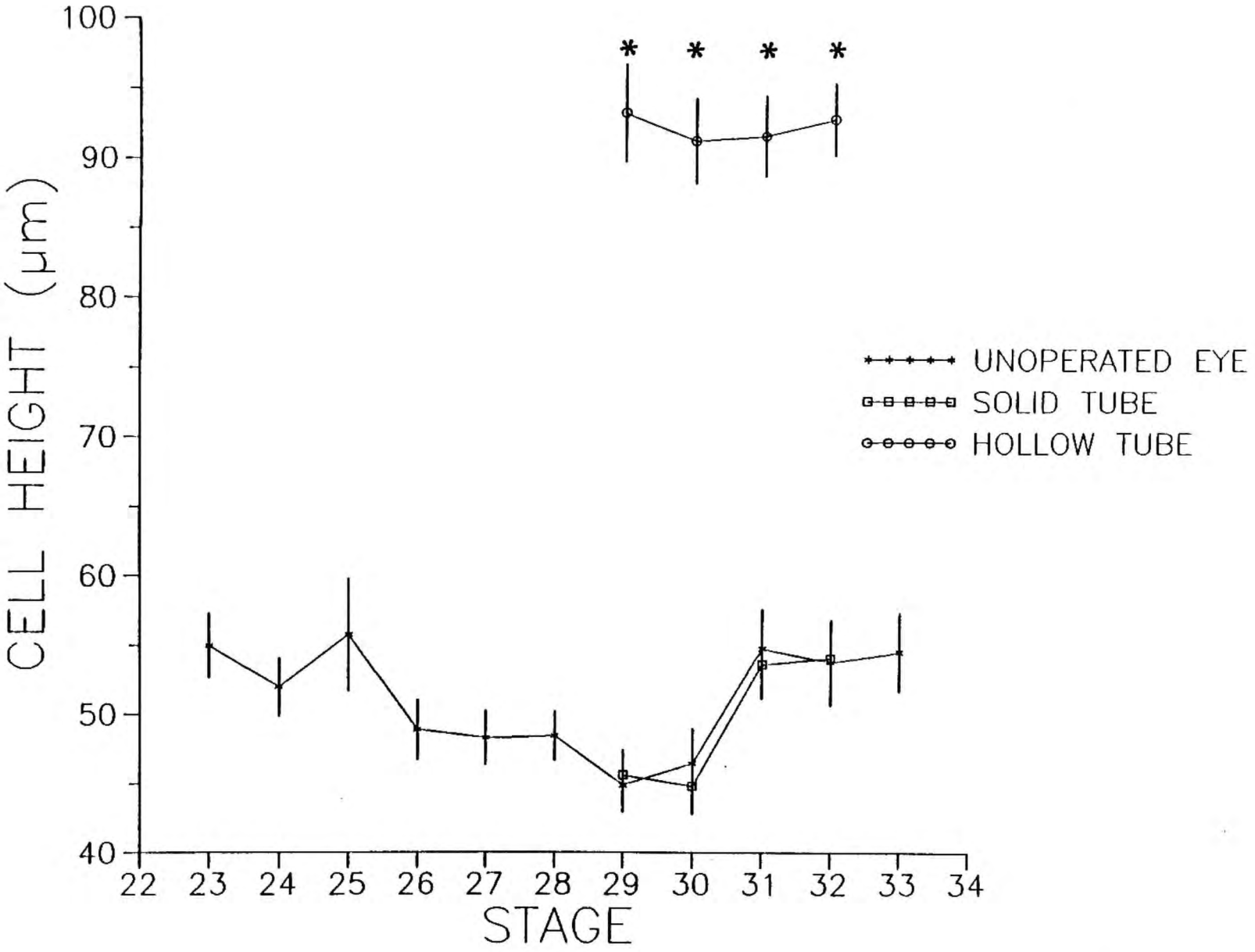

100
90
80
70
60
50
40
CELL HEIGHT (µm)
22
23
24
25
26
27
28
29
30
31
32
33
34
STAGE
*
*
*
*
UNOPERATED EYE
SOLID TUBE
HOLLOW TUBE

Figure 55. Graph of the average cell volume versus stage of development for the PE and NPE in unoperated eyes. The cell volume was calculated from the average cell height and average cell packing density at each stage for the PE and NPE.

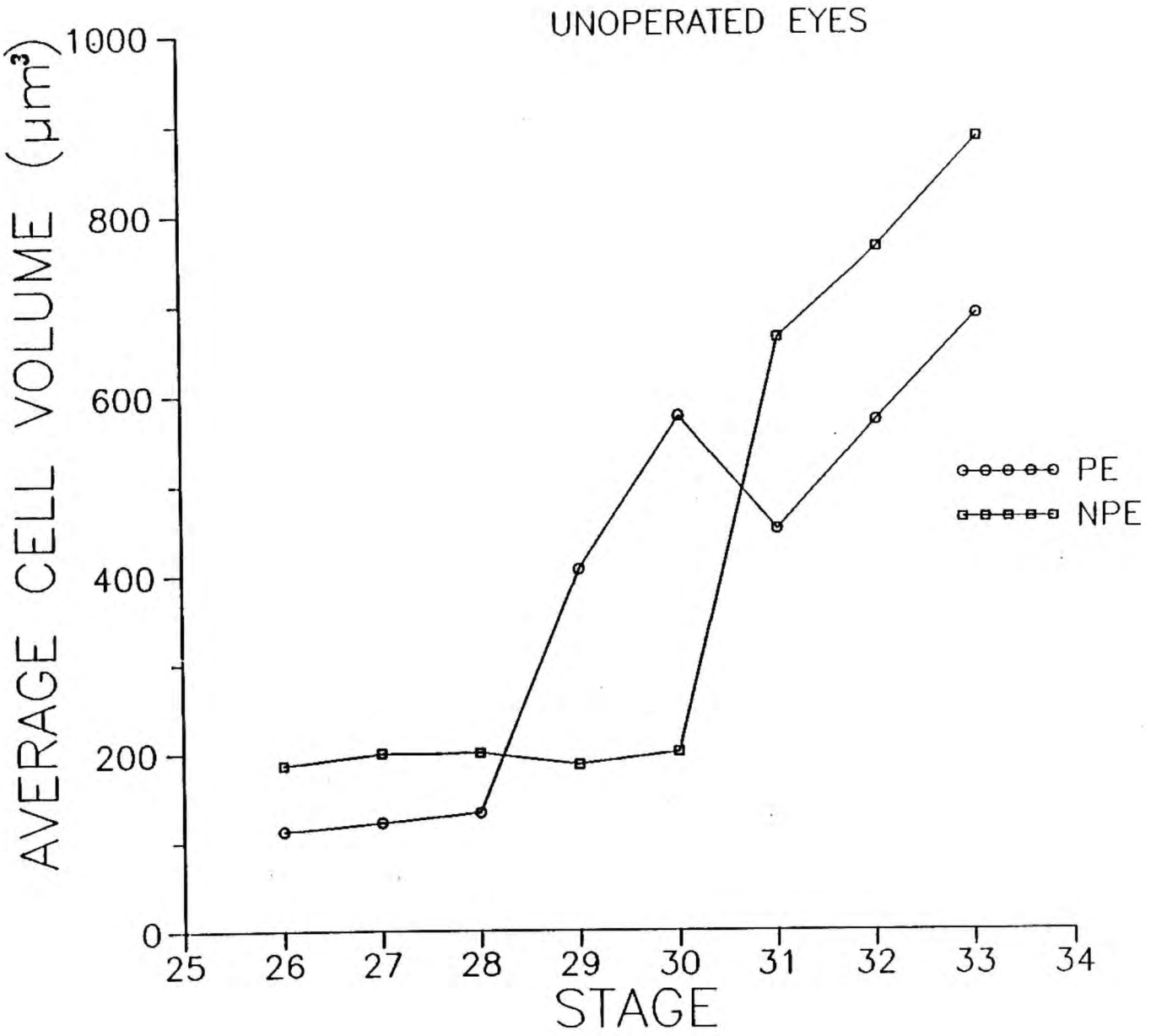

UNOPERATED EYES
AVERAGE CELL VOLUME (μm³)
1000
800
600
400
200
0
25
26
27
28
29
30
31
32
33
34
STAGE
PE
NPE

Figure 56. Graph of the average cell volume versus stage of development for the PE. The data for the unoperated eyes are redrawn from figure 55 for comparison with the experimental treatments.

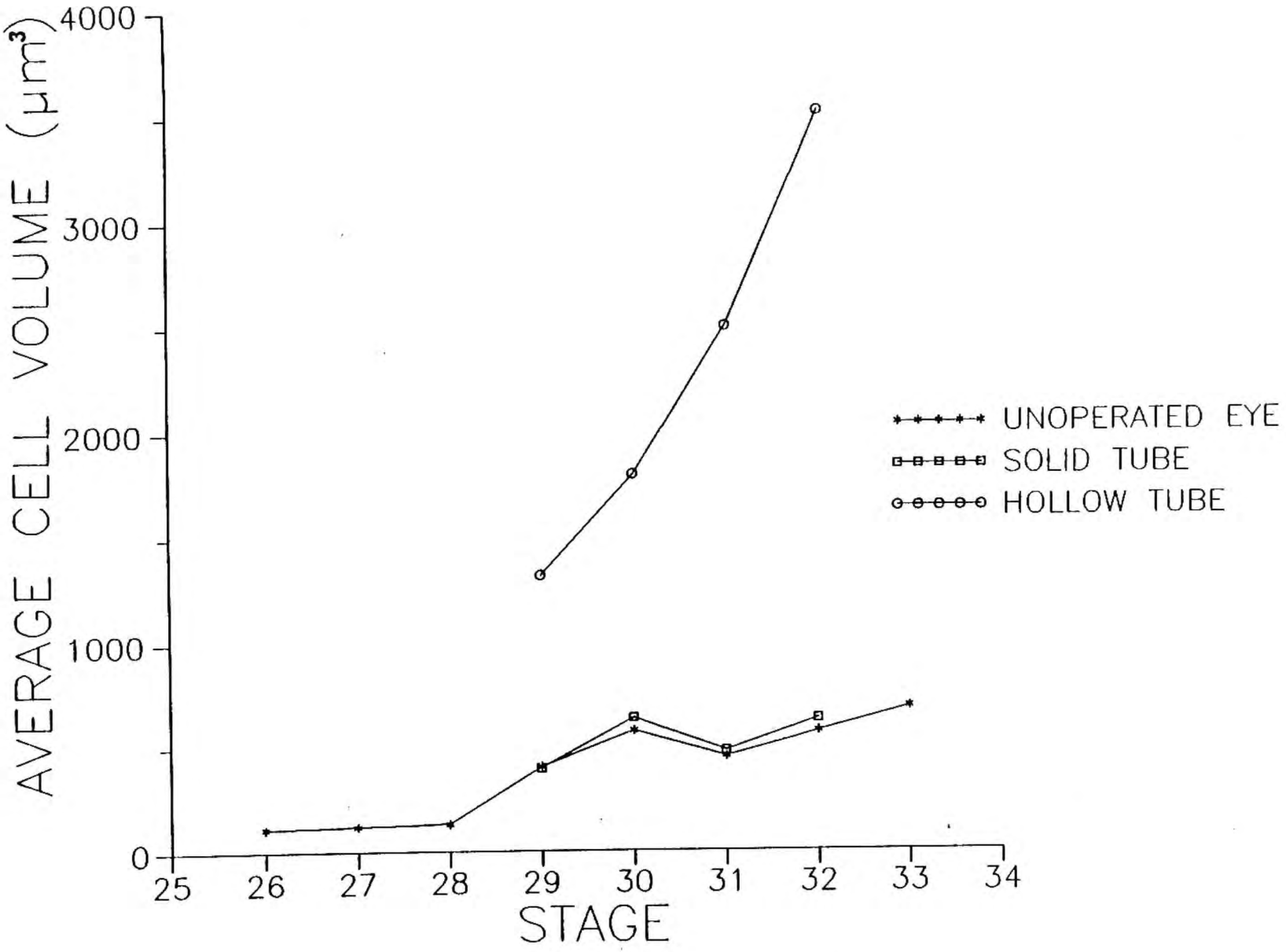

AVERAGE CELL VOLUME (μm³)
4000
3000
2000
1000
0
25
26
27
28
29
30
31
32
33
34
STAGE
UNOPERATED EYE
SOLID TUBE
HOLLOW TUBE

Figure 57. Graph of the average cell volume versus stage of development for the NPE. The data for the unoperated eye are redrawn from figure 55 for comparison with the experimental treatments.

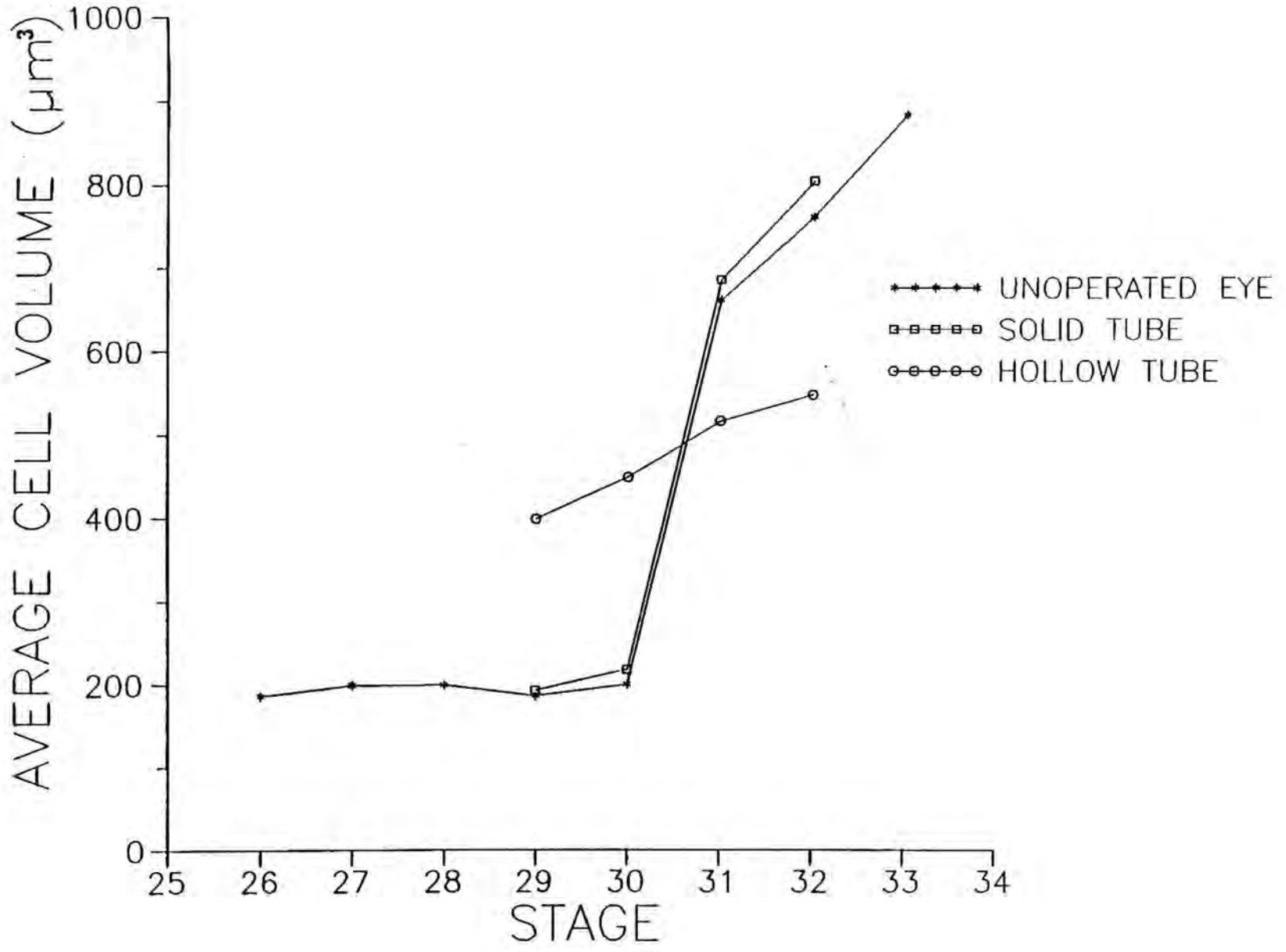

AVERAGE CELL VOLUME (μm³)
1000
800
600
400
200
0
25
26
27
28
29
30
31
32
33
34
STAGE
UNOPERATED EYE
SOLID TUBE
HOLLOW TUBE

Figure 58. Tissue interactions & factors involved in the development of the ciliary epithelium.

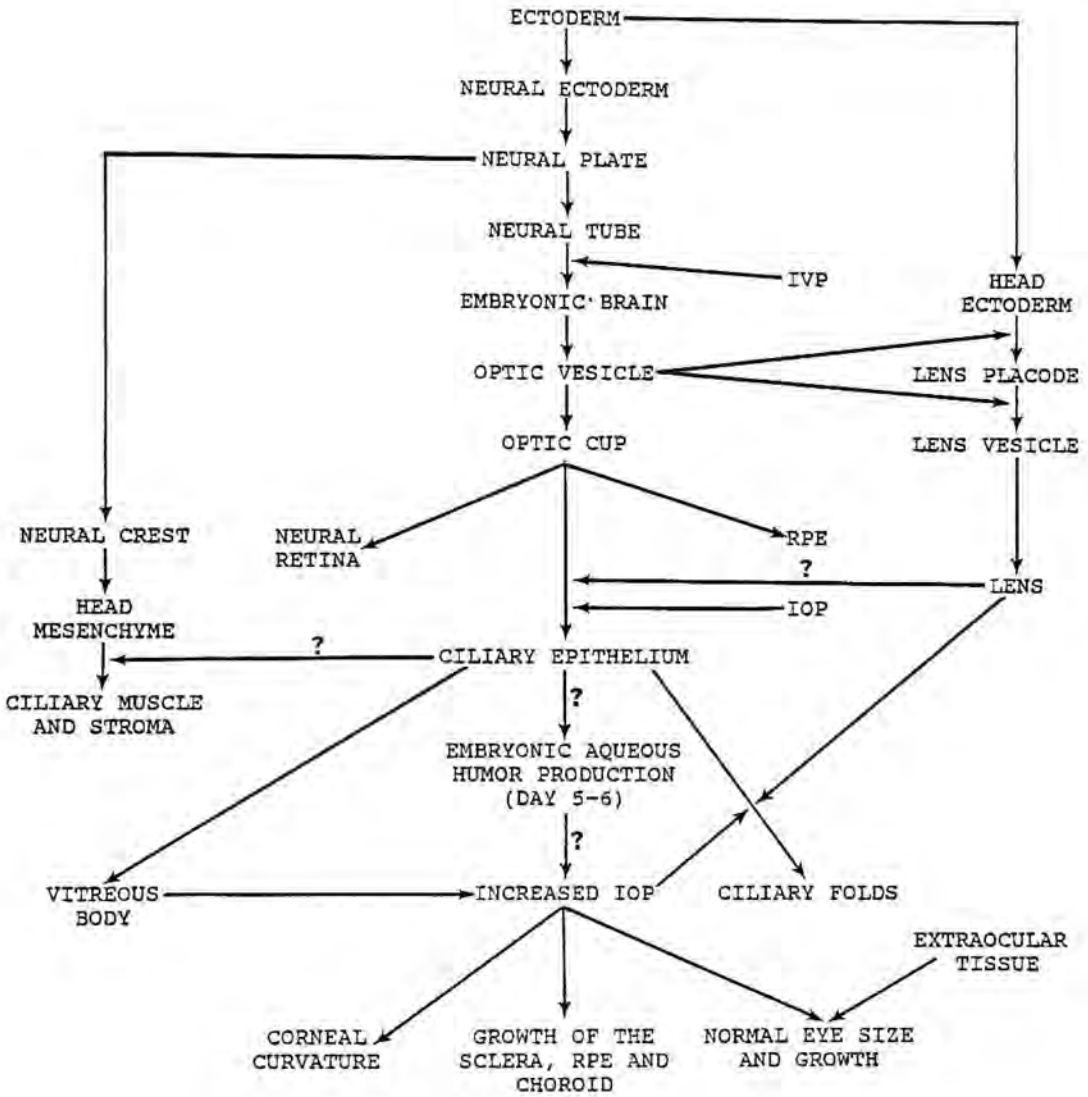

ECTODERM
NEURAL ECTODERM
NEURAL PLATE
NEURAL TUBE
IVP
EMBRYONIC BRAIN
HEAD ECTODERM
OPTIC VESICLE
LENS PLACODE
OPTIC CUP
LENS VESICLE
NEURAL CREST
NEURAL RETINA
RPE
?
LENS
IOP
HEAD MESENCHYME
?
CILIARY EPITHELIUM
CILIARY MUSCLE AND STROMA
?
EMBRYONIC AQUEOUS HUMOR PRODUCTION (DAY 5-6)
?
VITREOUS BODY
INCREASED IOP
CILIARY FOLDS
EXTRAOCULAR TISSUE
CORNEAL CURVATURE
GROWTH OF THE SCLERA, RPE AND CHOROID
NORMAL EYE SIZE AND GROWTH

Figure 59. Autoradiograph of S^{35}-methionine labelled proteins in the E6 chicken embryo. The prominent band in the lens lane represents delta crystallin, the major chicken lens protein (MW=49,000). (L=lens, ciliary epithelium=ciliary epithelium, R=retina).

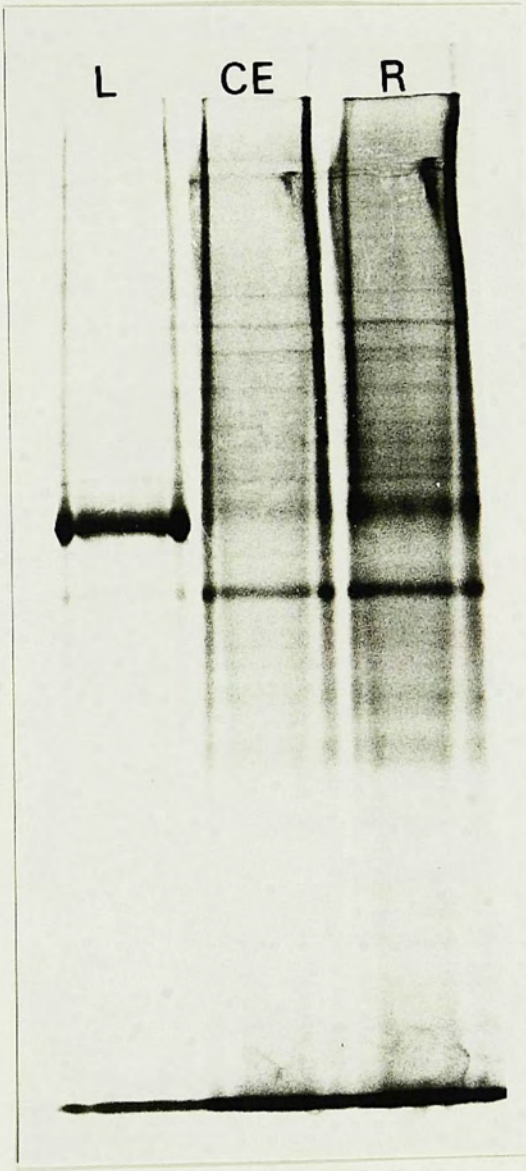

L
CE
R

Figure 60. Diagram of the procedure developed to remove the lens from the embryonic chicken eye. A. The cornea is cut 180° in the area of the limbus. B. The cornea is reflected. C. & D. The lens is carefully grasped with fine forceps and removed. E. The Cornea is replaced over the eye.

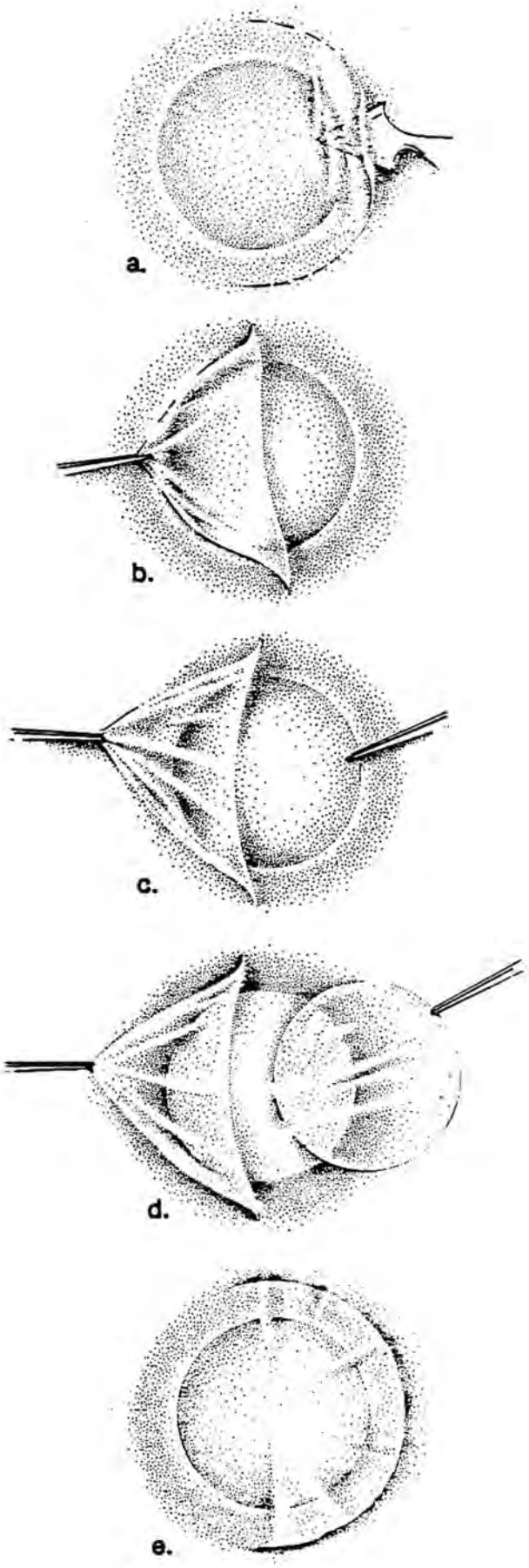
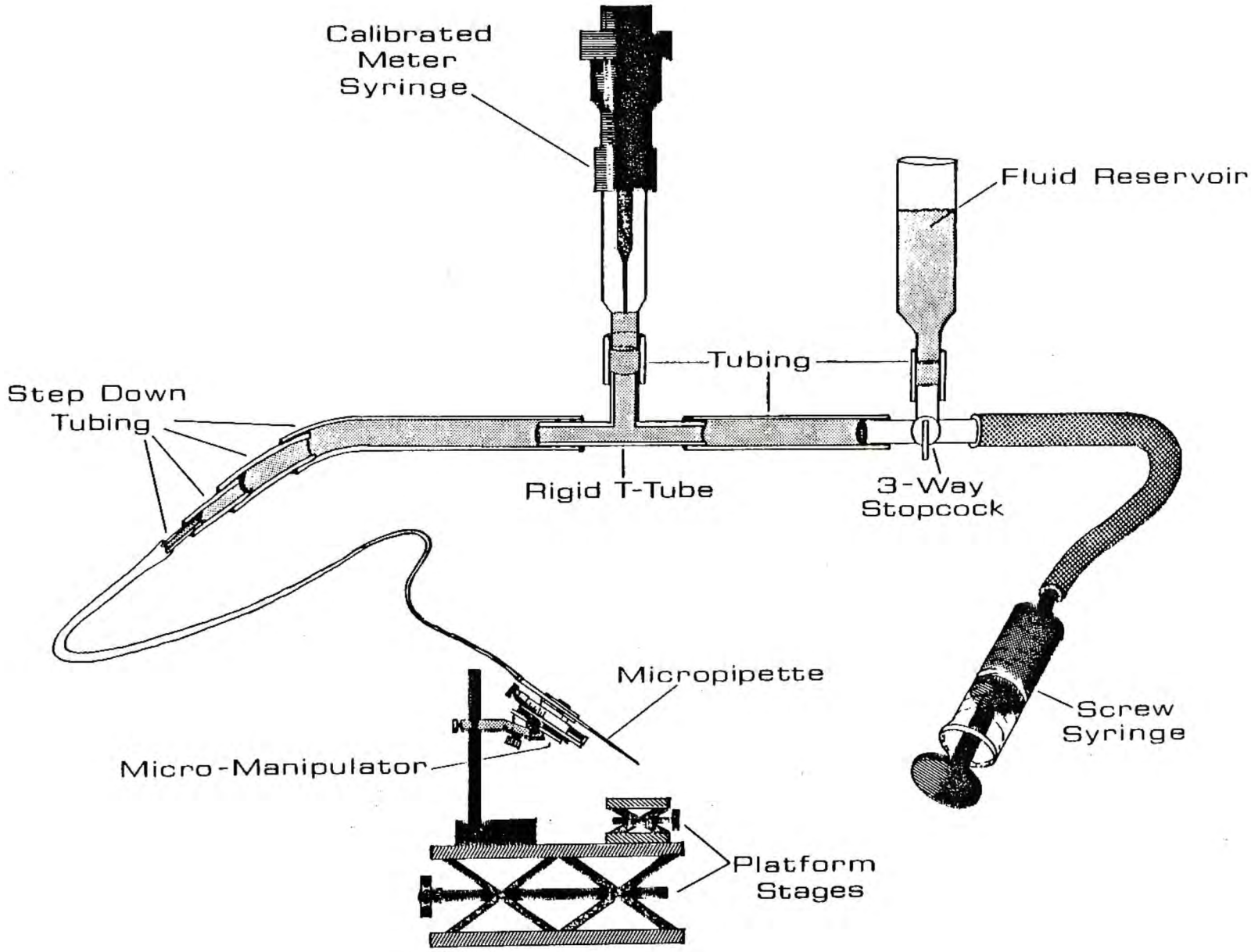

a.
b.
c.
d.
e.

Figure 61. Diagram of the manometer constructed to measure IOP in the chicken embryo.


Calibrated
Meter
Syringe
Fluid Reservoir
Tubing
Step Down
Tubing
Rigid T-Tube
3-Way
Stopcock
Micropipette
Screw
Syringe
Micro-Manipulator
Platform
Stages

APPENDIX

Stages in the development of the chicken embryo as described by Hamburger and Hamilton*.

STAGE	AGE	STAGE	AGE	STAGE	AGE
1	0-6h	17	52-64h	32	7.5d
2	6-7h	18	65-69h	33	7.5-8d
3	12-13h	19	68-72h	34	8d
4	18-19h	20	70-72h	35	8-9d
5	19-22h	21	3.5d	36	10d
6	23-25h	22	3.5d	37	11d
7	23-26h	23	3.5-4d	38	12d
8	26-29h	24	4d	39	13d
9	29-33h	25	4.5d	40	14d
10	33-38h	26	4.5-5d	41	15d
11	40-45h	27	5d	42	16d
12	45-49h	28	5.5d	43	17d
13	48-52h	29	6d	44	18d
14	50-53h	30	6.5d	45	19-20d
15	50-55h	31	7d	46	20-21d
16	51-56h				

*Hamburger, V. and Hamilton, H.L. (1951). A series of normal stages in the development of the chick embryo. J. Morph. 88, 49-92.

List of Abbreviations

ACh	acetylcholine
AChE	acetylcholinesterase
BrdU	5-bromo-2'-deoxyuridine
BuChE	butyrylcholinesterase
cAMP	cyclic adenosine monophosphate
ChAT	choline acetyltransferase
En	n=days of embryonic development
EM	electron microscopy
G	Golgi apparatus
IOP	intraocular pressure
LM	light microscopy
M	Mitochondrion
NBF	neutral buffered formalin
NPE	nonpigmented layer of the ciliary epithelium
Nu	nucleus
PBS	0.01M phosphate buffered saline
PE	pigmented layer of the ciliary epithelium
RER	rough endoplasmic reticulum
RPE	retinal pigmented epithelium
SER	smooth endoplasmic reticulum
TBS	0.5% polyoxyethylenesorbitan monolaurate in PBS

Experiments attempted which were inconclusive:

1) S^{35}-methionine protein labelling of the developing ciliary epithelium, retina and lens (figure 59) was examined to determine if any differences existed early in development. There were no differences seen in labelled proteins when comparing the ciliary epithelium and retina. The lens showed the typical, prominent delta crystallin band.

2) The effects of intraocular injection of aphidocholine, an inhibitor of DNA polymerase and thus an inhibitor of cell division, on the growth and development of the ciliary epithelium could not be determined. The injection site never closed over and the effects of the drug could not be distinguished from that caused by the reduction of intraocular pressure.

3) A sample of the monoclonal antibody GB11 was obtained (Hsi, et.al., 1986; Fredj-Reygrobellet, et.al., 1987). This antibody specifically recognizes the developing ciliary epithelium and not the retina in rabbits. The antibody did not react with the cells of the ciliary epithelium in the chicken.

4) The effects of lens removal (figure 60) in chicken embryos growing in shell-less culture was examined. The morphology of the eye was similar to that seen by reducing IOP by intubation with a hollow tube. Since the cornea did not reseal after the operation, it could not be determined if the effects were due to reduction of IOP, lens removal, or both.

AChE staining in these eyes was inconclusive. The staining was not as intense as in the ciliary epithelium region of the contralateral control eyes, yet did not show the retinal pattern either. It is possible that the ciliary epithelium was transdifferentiating into neural retina and the AChE staining pattern would later resemble that of the retina.

5) Attempts were made to measure IOP in developing chicken embryos. The manometer constructed (figure 61) did not have the required sensitivity.

Potential directions for future study:

1) Ciliary Epithelial Secretion
   a) repeat S^{35}-methionine protein labelling studies to increase the labelling and increase resolution.
2) AChE
   a) determine if muscarinic and/or nicotinic receptors for acetylcholine exist in the chicken embryonic ciliary epithelium.
   b) examine the effects of AChE inhibitors on eye development.
3) Intraocular Pressure
   a) measure IOP at developmental stages 25-34.
   b) decrease IOP with drugs and examine the eye morphology and cellular dynamics.
   c) decrease IOP mechanically and examine recovery.
   d) increase IOP to attempt to cause premature fold formation and examine the cellular dynamics.
   e) examine IOP during the lens manipulations indicated below.
   f) determine the onset of secretion by the ciliary epithelium using epithelial vesicles.
4) The Role of the Lens
   a) determine the effects of lens removal on the morphology, AChE histochemistry, and cell dynamics of the developing ciliary epithelium.
   b) determine the effects of transplantation of a

supernumerary lens on the morphology, AChE histochemistry, and cell dynamics of the developing ciliary epithelium.

5) Antibody Development

a) isolate a factor and develop a monoclonal or polyclonal antibody specific for the developing ciliary epithelium.

6) Ciliary Muscle Development

a) examine the LM and EM morphology, AChE histochemistry, monoclonal antibodies, and cell dynamics.

b) determine the effects of the ciliary epithelium on ciliary muscle development.

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