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Development of the iris in the chicken embryo

I. A study of growth and histodifferentiation utilizing immunocytochemistry for muscle differentiation

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SUMMARY

The development of the iris was studied in chicken embryos from 4 days of incubation through hatching and in chicks up to 17 days posthatching. Serial sections of the eye were stained routinely or by immunocytochemical methods using myosin antiserum to enhance detection of differentiating muscle by light microscopy. Eyes from embryos at each stage were also examined by scanning electron microscopy.

Outgrowth of the iris occurs in a sequential pattern around the eye, beginning at 6½ days of incubation in the region temporal to the choroid fissure and proceeding around the eye. Since differentiation of the iris follows the same pattern, similar areas of the iris were compared at each stage of development.

At 6½ days of incubation, pigment granules can be seen in the anterior epithelium at the root of the iris, but it is not until 8 days of incubation that pigment is identified in the same area of the posterior epithelium. As development proceeds, the pigmentation of both epithelial layers progresses from the root of the iris to the pupillary margin.

Early features of the development of the sphincter muscle can first be recognized in the 7-day embryo, when epithelial buds form from the anterior epithelium adjacent to the pupillary margin. Cells from these buds detach from the epithelium and populate the entire irideal stroma; these cells are the precursors of the sphincter muscle. The epithelial buds increase in size and distribution, and coalesce into a continuous ridge of cells around the iris by 10 days of incubation. The first muscle cells differentiate at 11 days of incubation when striated fibres can be identified by immunocytochemistry. The first striated fibres of the dilator muscle are seen in the 13-day embryo. Differentiation of the dilator muscle begins near the root of the iris and proceeds toward the pupillary margin, ultimately arising from the entire anterior epithelium except near the pupillary margin. Further differentiation of the sphincter and dilator muscles continues throughout incubation and for several weeks beyond hatching.

INTRODUCTION

The iris consists of two pigmented epithelial layers associated with a vascular stroma containing the sphincter and dilator pupillae muscles. The epithelial layers of the iris, and the sphincter and dilator muscles develop from the epithelium of the optic cup, while the vascular and connective tissues of the stroma are derived from mesenchyme.

There have been relatively few studies on the development of the irideal muscles. The origin of the sphincter and dilator muscles from the neuroepithelium of the optic cup was first established in birds and mammals by

Nussbaum in the 1890's (Lewis, 1903). A study in the chicken embryo by Lewis (1903) described the early differentiation of the sphincter muscle from epithelial buds arising from the anterior epithelial layer of the iris. Lewis' descriptions were based upon light microscopic observations of serial sections, and he noted that epithelial buds appeared by 7 days of incubation and accumulated faster on one side of the iris than on the other. In an ultrastructural study on the development of the irideal muscles, Brini, Porte & Stoeckel (1964) gave a more detailed outline of similar developmental features, but with discrepancies in the timing of developmental events. They described bud formation and differentiation of the sphincter muscle beginning in the 11-day embryo and were unable to establish the onset of differentiation in the dilator muscle. Another ultrastructural study (Narayanan & Narayanan, 1981) sampled tissues of the developing iris at only 2-day intervals. It is difficult to survey extensive areas of a large developing organ by electron microscopy and if regional variations exist in the developing iris as Lewis first implied, then an accurate timetable of development cannot be obtained unless identical areas or serial sections are compared at different ages.

The early recognition of tissue differentiation is also a problem in developmental studies of the iris. One solution has been to employ cytochemical methods to enhance detection of cell products which characterize the specialization of the cells or tissue. In their studies on the distribution of pigment in the iris anlagen, El-Hifnawi & Hinrichsen (1975) used enzyme cytochemistry to correlate tyrosinase activity with the ultrastructural features of melanogenesis. Studies of muscle differentiation in the iris have been based solely upon the histological features of myogenesis, but in other embryonic systems, immunocytochemical methods have been successfully employed to study developing muscle (Finck, Holtzer & Marshall, 1956; Holtzer, Marshall & Finck, 1957). In the present study we use the immunoglobulin-enzyme double bridge technique of Ordronneau & Petrusz (1980) with antiserum against chicken muscle myosin to study the differentiation of the irideal muscles.

The purpose of this study was to accurately describe irideal development *in vivo* with particular emphasis upon the development of the sphincter and dilator muscles. Serial sections of the iris viewed by light microscopy allowed survey of the large organ rudiment, and the immunocytochemistry permitted earlier recognition of muscle differentiation than was possible by simple light microscopy. Observations at each stage of development were also made by scanning electron microscopy.

MATERIALS AND METHODS

Fertile chicken eggs were obtained from a commercial hatchery and were stored up to seven days at room temperature before incubation at 37·7 °C in a humidified forced-draft incubator.

1. *Preparation of tissues for light microscopy*

Chicken embryos were staged according to the method of Hamburger & Hamilton (1951). The eyes were removed from embryos of 4 through 21 days incubation and from chicks up to 17 days posthatching and fixed by immersion in Bouin's fluid (Lillie, 1954) or 2·5 % paraformaldehyde and 0·5 % glutaraldehyde in a phosphate buffer (Pearse, 1961). The Bouin's-fixed tissues were dehydrated through a series of ethanols, cleared in toluene, and embedded in Paraplast. The blocks were sectioned either parallel to or perpendicular to the optical axis of the eye, so that transverse or frontal sections, respectively, were obtained. Serial sections 5 μm thick were cut and stained with haematoxylin and eosin. Individual sections from these same blocks were stained immunocytochemically and counterstained in toluidine blue, or stained in toluidine blue alone.

The aldehyde-fixed tissues were dehydrated through a graded series of ethanols and embedded in glycol methacrylate (GMA) according to the method of Bennett, Wyrick, Lee & McNeil (1976). Sections 1–2 μm thick were cut and stained with methylene blue-basic fuchsin or toluidine blue.

2. *Immunocytochemistry*

The immunocytochemical staining method employed in this study was the immunoglobulin-enzyme double bridge technique of Ordronneau & Petrusz (1980). The primary antiserum used for the procedure was rabbit antiserum to chicken skeletal muscle myosin (rA-cMyosin; Antibodies Inc., Davis, CA). Incubation of tissue sections in the primary antiserum was followed by two series of sequential incubations in sheep antiserum to rabbit gamma globulin (Antibodies, Inc.) and rabbit anti-horseradish peroxidase (gift of Dr Peter Petrusz, University of North Carolina at Chapel Hill). The slides were then treated with a solution of horseradish peroxidase and finally with a solution of 3, 3′-diaminobenzidine tetrahydrochloride and hydrogen peroxide. Positive staining was termed muscle protein immunoreactivity (MPI).

Method specificity (Petrusz, Ordronneau & Finley, 1980) was evaluated by correlating the intensity of the staining with increasing dilutions of the primary antiserum and with omission of the primary antiserum. The specificity of the primary antiserum was assayed by the staining of morphologically distinct myotubes and muscle fibres, and by evaluation of staining intensity using absorbed primary antiserum. Absorbed antiserum was prepared by adding increasing amounts of purified antigen to a constant dilution of the primary antiserum (Petrusz, DiMeo, Ordronneau, Weaver & Keefer, 1975). Either one of the following purified antigens was used: chicken skeletal muscle myosin (Finck *et al.* 1956, Method A) or chicken muscle actin (Sigma Chemical Co., St. Louis, MO). Sodium dodecyl sulfate (SDS) gel electrophoresis (Laemmli, 1970) of the chicken myosin and actin was carried out to assess the purity of those proteins (Porzio & Pearson, 1977).

3. *Preparation of tissues for scanning electron microscopy (SEM)*

Eyes from chickens of 5½ days incubation through 17 days posthatching were removed and fixed by immersion overnight in 2·5 % paraformaldehyde and 0·5 % glutaraldehyde in phosphate buffer. The tissues were then fixed for one hour in 1 % osmium tetroxide in phosphate buffer, dehydrated in a graded series of ethanols, and dried from liquid carbon dioxide in a Samdri-790 critical-point dryer. The specimens were sputter coated with gold or gold–palladium. A limited number of specimens were prepared without sputter coating by using thiocarbohydrazide–osmium binding (Malick & Wilson, 1975). All specimens were examined in a JSM-35 scanning electron microscope operated at 20 or 25 kilovolts.

RESULTS

At early stages, the size and differentiation of the iris were found to vary in different regions of the eye. Outgrowth of the iris begins in the quadrant adjacent and temporal to the choroid fissure, close to the temporal branch of the long ciliary artery. Development proceeds around the eye, so that usually outgrowth and differentiation lag in the region of the iris adjacent and rostral to the choroid fissure. In the descriptions of irideal development that follow, the most advanced features of the iris have been described at each stage.

Between 4 and 6 days of incubation, the region of the optic cup which will contribute to the iris and ciliary body can be recognized only by an attenuation or thinning of the epithelium near the margin of the cup. The iris rudiment is not morphologically distinct until 6½ days of incubation (stage 30) when it appears as a small extension of the epithelium and mesenchyme projecting from the margin of the optic cup (Fig. 1). The two epithelial layers of the iris are continuous with each other at the pupillary margin, and with the epithelial layers of the ciliary body at the root (Fig. 2). The anterior epithelial layer is composed of short

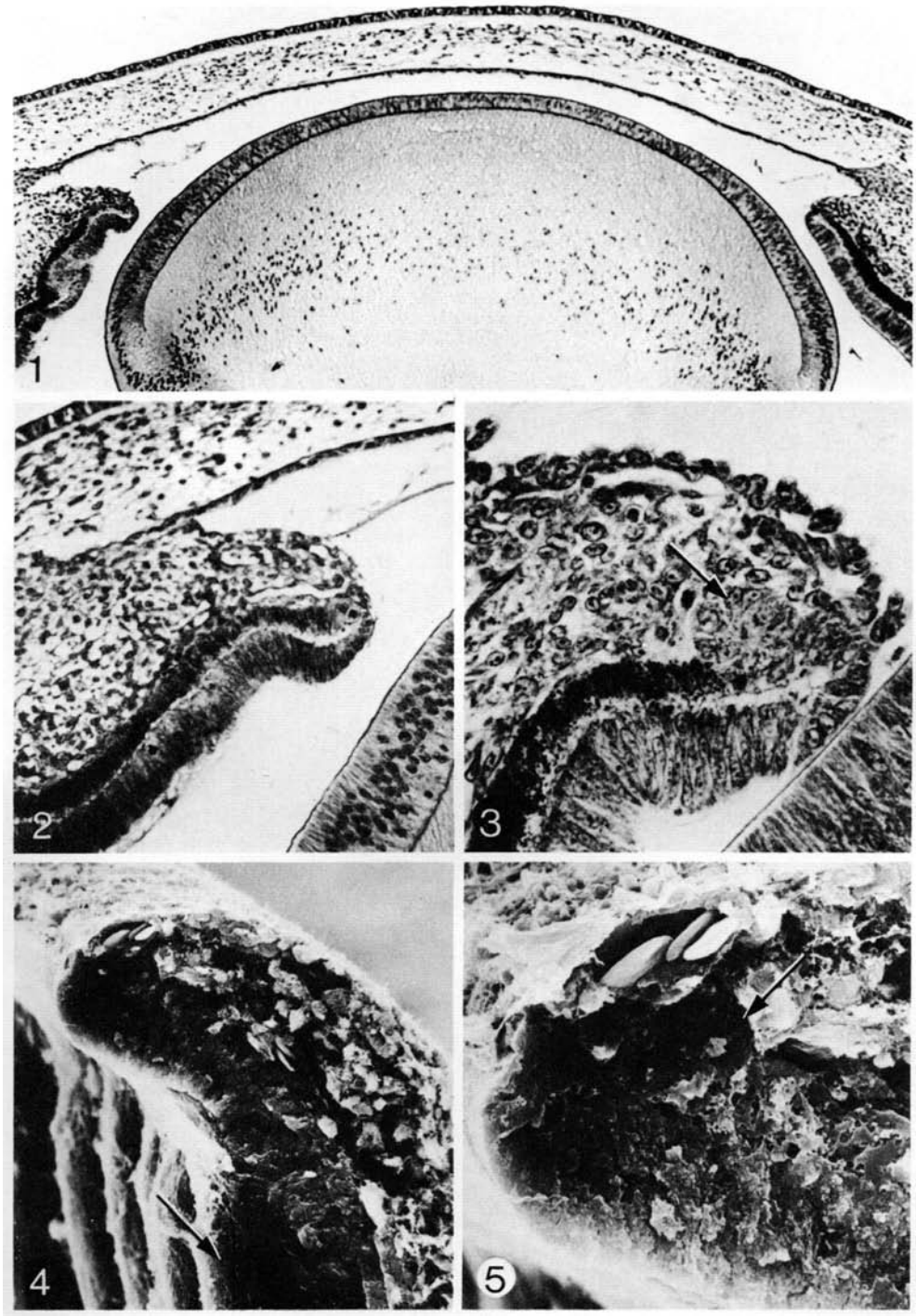
Fig. 1. Transverse histological section of the anterior segment of the eye of a 6½-day (stage-30) embryo. The iris anlage is a small projection of epithelium and mesenchyme at the rim of the optic cup. The large central structure is the lens; the cornea is at the top of the field. ×100.

Fig. 2. Histological section of the iris of a 7-day (stage-31) embryo. Note the mitotic figure in the marginal epithelium. ×240.

Fig. 3. Histological section of the iris of a 7½-day (stage-32) embryo. An epithelial bud extends into the stroma (arrow). ×500.

Fig. 4. Scanning electron micrograph of the iris in a 7½-day (stage-32) embryo. The pupillary margin is at the left of the field. The arrow indicates a developing ciliary process. ×570.

Fig. 5. Higher magnification of the pupillary region of the iris shown in Fig. 12. A small bud (arrow) from the anterior epithelium projects into the stroma. ×1570.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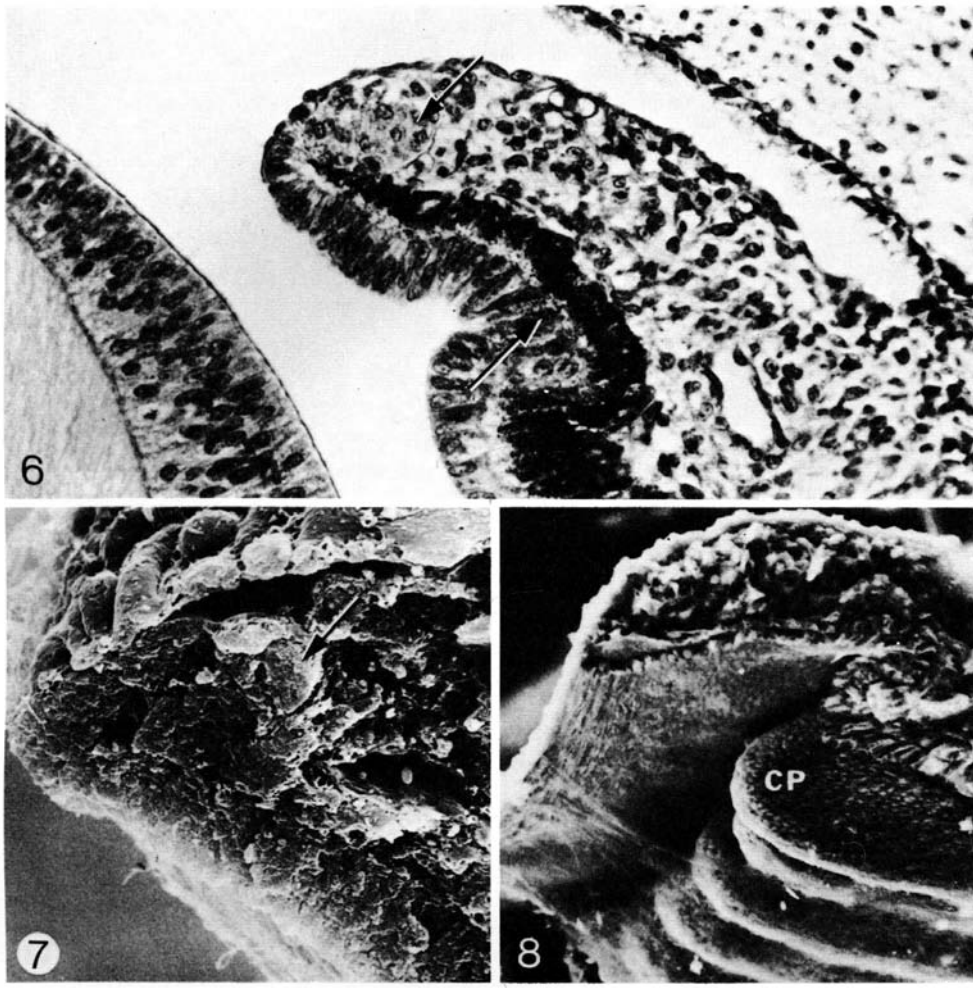


Fig. 6. Transverse section of the iris of an 8-day (stage-34) embryo. A large epithelial bud projects into the stroma (upper arrow). Note the accumulation of pigment granules in the posterior epithelium at the root of the iris (lower arrow). ×460.

Fig. 7. Scanning electron micrograph of the pupillary region of the iris of a 9-day (stage-35) embryo. The view is of the cut surface of the iris, and the pupil is at the left of the field. There is a large epithelial bud (arrow) arising from the anterior and marginal epithelium. ×1400.

Fig. 8. The iris and ciliary body of a 10-day (stage-36) embryo. This scanning electron micrograph shows the ciliary processes (CP) which are situated posterior to the iris. The wispy material at the lower left and bottom of the field are remnants of the vitreous body. The pupil is at the upper left of the field. ×175.

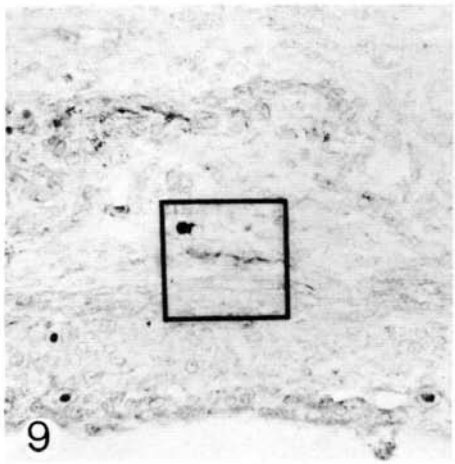
columnar cells and is associated with mesenchyme – the stroma of the iris. The posterior epithelium at this stage is a pseudostratified columnar epithelium. Both layers are unpigmented except for sparse melanin granules present in the anterior epithelium.

In the 7-day embryo, focal thickenings appear in the anterior epithelium, and by 7½ days (early in stage 32) these thickenings have enlarged to form distinct epithelial buds (Fig. 3). The buds are also seen by scanning electron microscopy (Figs 4, 5) which better demonstrates their continuity with the anterior and marginal epithelium. The epithelial buds at this stage vary in size; in some areas the bud nearly fills the stroma at the pupillary margin, in other areas, only a small cluster of cells is present. In subsequent stages (Fig. 6), the epithelial buds become progressively larger and more numerous. By 9 days (Fig. 7), large epithelial buds have coalesced into a nearly continuous ridge of cells around the iris. In the 10-day embryo (stage 36), clusters of cells have separated from the epithelial ridge and can be seen within the stroma surrounded by mesenchymal cells. Up to 10 days of incubation, immunocytochemical staining does not reveal muscle protein immunoreactivity (MPI) in any cells of the iris.

During the period of 7 to 10 days of incubation, the relationship of the iris to the ciliary processes has changed with the growth of both structures (compare Figs 4, 8). The ciliary processes in the 10-day embryo now underlie the peripheral half of the posterior epithelium. During this same period, there is also a gradual increase in the pigmentation of the iris. In the 7½-day embryo, the anterior epithelium is lightly pigmented from the root of the iris approximately to its midpoint. Pigmentation of the posterior epithelium begins at the root of the iris in the 8-day embryo (Fig. 6), and progresses toward the pupillary margin as in the anterior epithelium. By 10 days, nearly all of the posterior epithelium is pigmented, though the melanin granules are primarily limited to the basal cytoplasm, adjacent to the posterior chamber.

In the 11-day embryo (stage 37), clusters of epithelial cells are present within the stroma nearly to the root of the iris. The stroma is richly vascularized and blood vessels surround and separate groups of epithelial cells. The anterior epithelium is not yet densely pigmented, and the posterior epithelium has changed from pseudostratified to simple columnar. In frontal section of the iris, small clusters of pigmented cells are seen within the stroma (Fig. 10; clump cells, c.f. Wobmann & Fine, 1972). The epithelial cells within the stroma have become elongated; a few are multinucleated and represent myotubes of the developing sphincter muscle (Figs 9, 10). These myotubes are of very small diameter, and some display light immunoreactive staining which is occasionally seen in a banded pattern (Fig. 10). The 11-day embryo is the youngest in which MPI is seen in the iris, and also represents the earliest time at which myotubes can be identified in sections or by SEM.

Similar features are seen in the developing iris at 12 days of incubation (stage 38). In the 13-day embryo (stage 39), the irideal stroma contains many cells which demonstrate MPI. In transverse sections of the iris, most of the positive cells are located in the middle third of the stroma and are clustered into small groups (compare Figs 11, 12). The distribution of the immunoreactive cells corresponds precisely with the population of the stromal epithelial cells and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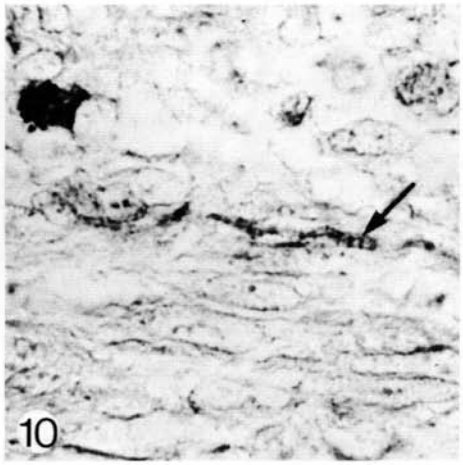
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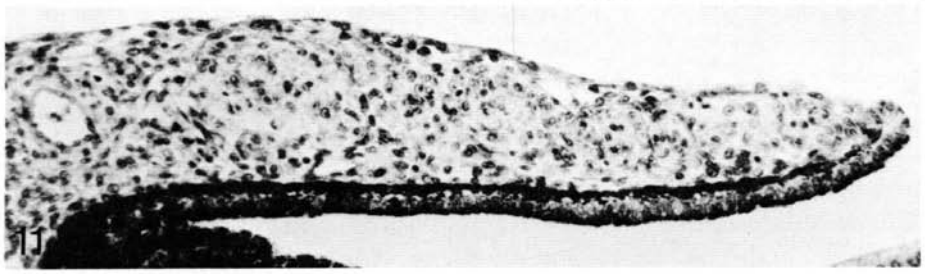
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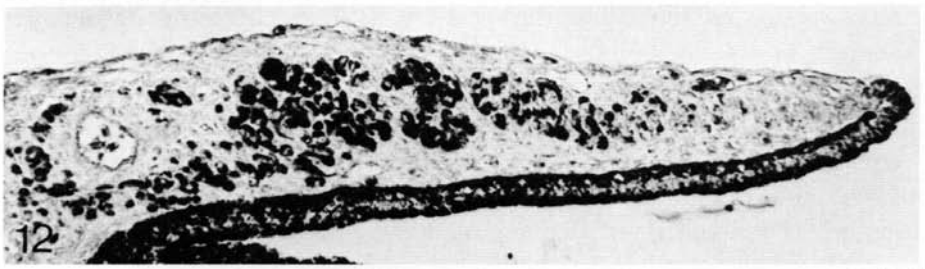
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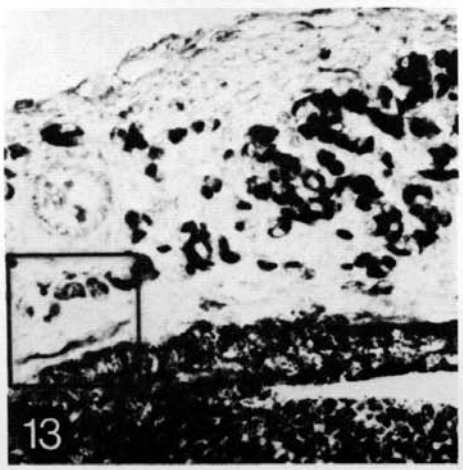
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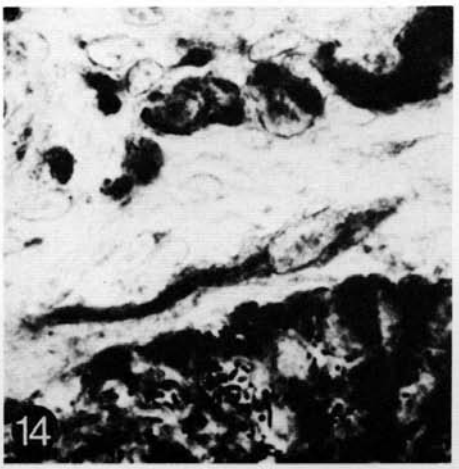
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developing myotubes of the sphincter muscle. Occasionally, small elongated cells displaying MPI can be identified in close proximity to the anterior epithelium in the peripheral third of the iris, near the root (Figs 13, 14). Striations are sometimes present within these cells, which represent fibres of the developing dilator muscle. In the 13-day embryo, both epithelial layers are pigmented, and occasional pigment granules are seen in the marginal epithelium as well. Thus by 13 days, all of the features of the adult iris are present, and further development involves the continued differentiation of these structures.

At very early stages, the iris is a nearly planar ring, but as it enlarges its shape more closely resembles the lower segment of a blunt cone. Consequently, frontal sections of the eye are not parallel to the iris and result in oblique sections through the stroma and epithelial layers. Oblique sections of the iris in the 12-day embryo reveal a few myotubes of the sphincter muscle with narrow regions of striations, though at this age, they are especially difficult to resolve in conventionally stained sections. In the 13-day embryo, myotubes are frequently seen; occasionally, thin striated fibres may also be observed (Figs 15, 16). However, adjacent sections stained immunocytochemically reveal abundant myotubes and myofibres (Fig. 17). Some fibres show differential accumulation of the reaction product, resulting in light and dark bands (Fig. 18). Immunoreactive cells of the sphincter muscle are seen throughout the irideal stroma in the 15-day embryo (stage 41), except in the area closest to the pupillary margin, where the stromal epithelial cells are densely pigmented. Precursor cells of the sphincter muscle arise from the anterior and marginal epithelium until at least 17 days of incubation, and probably until 19 or 20 days.

Fig. 9. The iris of an 11-day (stage-37) embryo after immunocytochemical staining. The plane of section is frontal and passes through the irideal stroma. The area outlined is enlarged in Fig. 10. Antiserum: rA-cMyosin; dilution: 1: 10 000. ×340.

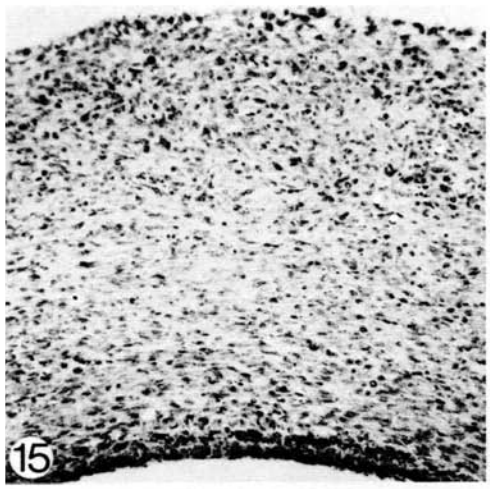
Fig. 10. Higher magnification of the section shown in Fig. 9. A small fibre (arrow) demonstrates bands of immunoreactive staining. The pigmented cell in the upper left portion of the field is a clump cell. ×1320.

Fig. 11. Transverse histological section of the iris of a 13-day (stage-39) embryo. ×275.

Fig. 12. Immunoperoxidase-stained section of the iris of a 13-day (stage-39) embryo. Compare with Fig. 11 and note the deeply stained muscle cells within the irideal stroma. Antiserum: rA-cMyosin; dilution: 1: 10 000. ×255.

Fig. 13. Transverse section of the iris of a 13-day (stage-39) embryo stained by the immunoperoxidase method. Only the peripheral half of the iris is shown. When viewed under the microscope, the dark brown reaction product in the stained stromal cells is diffuse and thus easily distinguished from the black melanin granules of the pigmented epithelium. Antiserum: rA-cMyosin; dilution: 1: 10 000. ×390.

Fig. 14. Higher magnification of the immunoreactive cells shown in Fig. 13. The cell which parallels the pigmented epithelium is a fibre of the developing dilator muscle. Differentiating cells of the sphincter muscle are seen in cross section at the top of the field. ×1420.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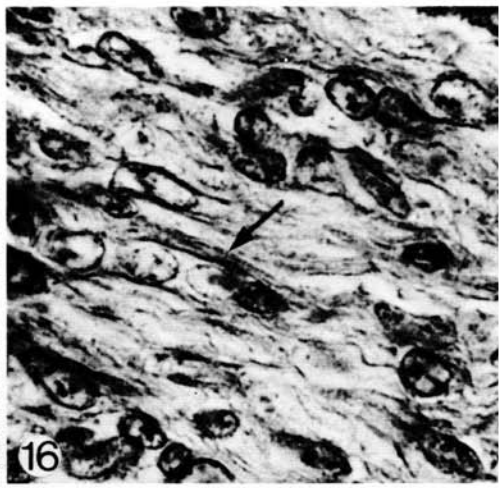
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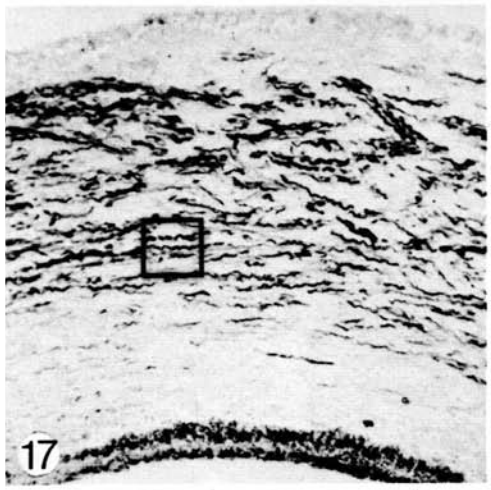
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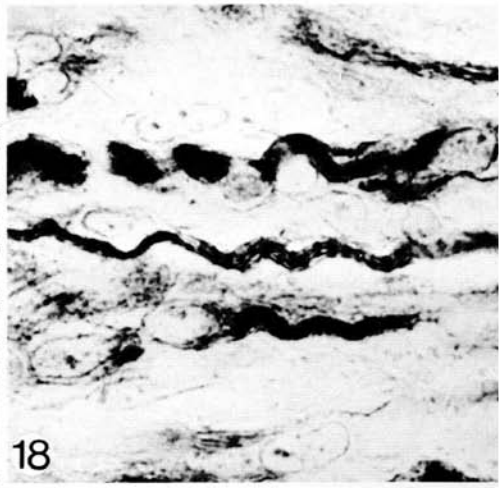
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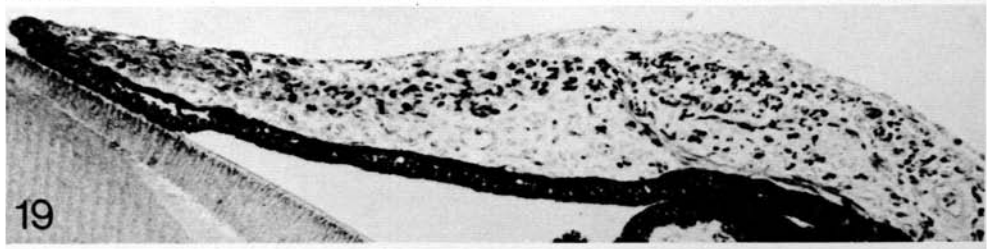
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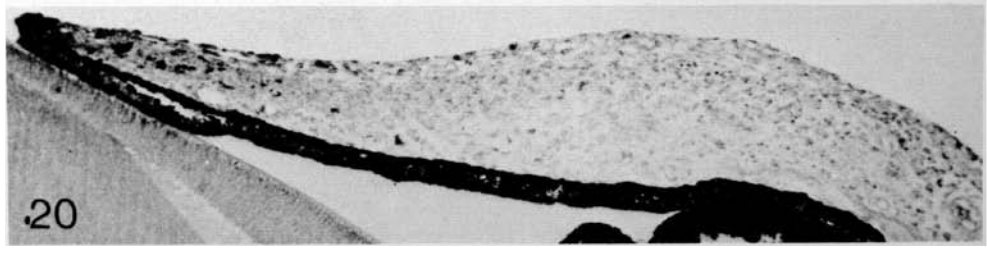
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Fibres of the dilator muscle originate from the peripheral third of the anterior epithelium at 12 and 13 days of incubation (Figs 13, 14). Cells of the anterior epithelium develop long muscular processes which extend into the stroma; other epithelial cells separate completely from the anterior epithelial layer as they differentiate into muscle fibres. There are large numbers of dilator cells which demonstrate MPI at 13 days, but myotubes of the dilator are not observed in conventionally stained sections until 14 days of incubation (stage 40). As differentiation proceeds, more of the anterior epithelium participates in the formation of the dilator muscle, and in the 15-day embryo, developing dilator fibres are seen in association with the peripheral half of the anterior epithelium. One day after hatching, fibres of the dilator muscle are present along the entire surface of the anterior epithelium, except in the region near the pupil where the epithelial buds arise as precursor cells of the sphincter muscle.

Further differentiation of the sphincter and dilator muscles occurs throughout the remaining period of incubation, and for several days and weeks beyond hatching. In the fully differentiated iris, the sphincter muscle fills much of the stroma. Fibres of the dilator muscle are less abundant, and many are obscured by their close proximity to the densely pigmented epithelium.

Immunocytochemistry Controls. The immunoperoxidase staining sequence resulted in a brown reaction product at sites of muscle protein immunoreactivity (MPI). Positively stained cells were distinguished from the pigmented cells of the iris by the colour and distribution of the reaction product. The reaction product in positively stained cells imparts a diffuse brown colour to the cytoplasm, while the melanin of pigmented cells is black in colour and situated within granules.

The specificity of the immunocytochemical method was assessed by diluting the primary antiserum until staining was abolished, and by omitting the primary

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Fig. 15. Oblique histological section of the marginal half of the iris of a 13-day (stage-39) embryo. ×130.

Fig. 16. Oblique histological section of the irideal stroma of a 13-day embryo. A small striated fibre is indicated (arrow). ×1260.

Fig. 17. Section adjacent to that shown in Fig. 15 after immunoperoxidase staining. Developing fibres of the sphincter muscle are stained. The area outlined is enlarged in Fig. 18. Antiserum: rA-cMyosin; dilution: 1:10000. ×130.

Fig. 18. Higher magnification of the area outlined in Fig. 17. Note the conspicuous striations in the middle fibre due to heavy and light accumulations of the reaction product. ×1260.

Fig. 19. Transverse histological section of the iris of a 15-day embryo stained by the immunoperoxidase method. The absorption procedure was carried out without the addition of antigen to the primary antiserum. Antiserum: rA-cMyosin; dilution: 1:20000. ×190.

Fig. 20. Section adjacent to that shown in Fig. 19, stained in the same manner except for the addition of 87 μg/ml of chicken myosin to the primary antiserum. Immunoreactive staining is abolished. The stromal cells near the margin of the iris are pigmented. Antiserum: rA-cMyosin; dilution: 1:20000. ×190.

antiserum from the staining sequence. Increasing dilutions of the primary antiserum resulted in a stepwise decrease in staining intensity, and abolishment at a dilution of 1: 1 000 000. Specific staining was also abolished when the primary antiserum was omitted from the staining sequence. Nonspecific staining, i.e. staining which persisted in the absence of the primary antiserum, was seen only in erythrocytes.

The specificity of the primary antiserum was assessed by the staining of morphologically distinct muscle cells, and by the absorption of the antiserum with homologous and heterologous antigens. The rA-cMyosin serum stained only striated muscle fibres, myotubes, and oval or round cells which were presumed to be myoblasts. The staining was usually diffuse throughout the cytoplasm of these cell types, though in striated fibres the staining was most intense within the A band of the sarcomere. No staining was seen in the smooth muscle cells of the blood vessels, nor were any other cell types stained. Staining was abolished after absorption of the diluted antiserum with chicken muscle myosin at a concentration of 8·7 μg/ml (Figs 19 and 20). The antiserum was also absorbed with actin from chicken muscle; this was effective in eliminating the staining at a concentration of 1000 μg/ml.

Protein profiles of the chicken myosin and chicken actin used for the absorption were obtained by SDS gel electrophoresis. The electrophoretic pattern of the chicken myosin showed one intense band corresponding to the heavy chain of the myosin molecule, and three dark bands corresponding to the light chain of the myosin molecule. A faint band indicating C-protein, a component of thick filaments (Offer, Moos & Starr, 1973), was also present. Electrophoresis of the chicken actin resulted in an intense band corresponding to actin, and faint bands indicating C-protein and the myosin heavy chain. The levels of the contaminating proteins in either the myosin or actin solutions were not considered significant.

DISCUSSION

Our observations indicate that the development of the iris occurs sequentially around the eye. Initial outgrowth of the iris from the optic cup occurs in the region temporal to the choroid fissure and proceeds around the eye; the onset and progression of histological differentiation follows the same pattern. Though earlier studies have noted asymmetry in the distribution of the epithelial buds (Lewis, 1903) and morphological asymmetry of the iris and ciliary body during development (Townes-Anderson & Raviola, 1981), the *sequential* pattern of differentiation around the eye has previously been reported only in the ciliary body (Bard & Ross, 1982). The sequential pattern of differentiation of the iris described here has important implications for most developmental studies in which random sectors of the iris are compared at different ages. During early irideal development, especially between 7 and 13 days of incubation in the chicken embryo, there are significant variations in differentiation among the

various quadrants of the iris at any given stage. In this study, comparable areas were examined at different ages, and descriptions were based on the most advanced features of differentiation at each stage.

Outgrowth of the iris was seen at 6½ days of incubation, slightly earlier than in other studies which first identified the iris at 7 days of incubation (Hamilton, 1952; Renard, Hirsch, Savodelli & Pouliquen, 1980) or at 7 to 8 days of incubation (Romanoff, 1960). Reference to the 'irideal' epithelium as early as 3 days of incubation (El-Hifnawi & Hinrichsen, 1975; El-Hifnawi, 1977) have been based upon the ultrastructural identification of melanosomes in the inner epithelium of the optic cup. This terminology may be misleading, since pigment develops in the inner epithelium of both the iris and the first ciliary process. At 3 days of incubation, pigment in the inner epithelial layer of the optic cup can distinguish only the iris-ciliary body rudiment from the neural retina; the distinction between iris and ciliary body cannot be made with certainty until outgrowth of the iris at 6½ days.

This study supports most of Lewis' observations on the origin of the sphincter muscle. The marginal epithelial buds originate at 7 days of incubation, gradually increase in size and distribution around the iris, and coalesce into a single ridge of cells by 10 days. Cells of this ridge populate the irideal stroma and differentiate into muscle. However, our findings do not support Lewis' observations that pigmented epithelial buds also contribute to the sphincter muscle. Pigmented buds were observed infrequently in the middle and peripheral portions of the iris and probably give rise to clump cells.

The single ultrastructural study on the development of the irideal muscles in the chicken embryo conducted by Brini *et al.* (1964) also described a single zone in the marginal epithelium in which precursors of the sphincter muscle arise. However, their timetable of developmental features does not coincide either with Lewis' observations or our own. They first noted myofilaments in cells of the developing sphincter muscle at 11 days of incubation and striated fibres at 12 to 13 days. In the present study, epithelial buds were demonstrated by light microscopy at 7 days of incubation and striated fibres of the sphincter muscle identified by immunocytochemical methods at 11 days. The discrepancies may result from the limited survey afforded by transmission electron microscopy combined with a failure to consider regional variations around the eye. Brini and associates studied the differentiation of the sphincter muscle near the pupillary margin where the precursor cells arise, but our results indicate that the first epithelial cells to differentiate into muscle are in the stroma, in the peripheral half of the iris. Similar reasons probably account for their inability to establish the onset and pattern of differentiation of the dilator muscle. Their studies do show that the dilator muscle develops along the entire surface of the anterior epithelium, except at the margin where the sphincter muscle originates. However, in the present study, we have established both the time of origin of the dilator muscle and the progression of its differentiation from the root of the iris toward the pupillary margin.

This is also the first description of the development of the sphincter and dilator muscles by immunocytochemistry. The specificity of the immunocytochemical method verified that the staining was due to the primary antiserum. The specificity of the primary antiserum was evaluated by the morphological features of the stained cells and by the absorption of the antiserum with the two predominant proteins in muscle, myosin and actin. Positive staining occurred only in muscle cells, including morphologically distinct myofibres and myotubes, and presumptive myoblasts. The reaction product within striated fibres was most intense in the A bands where the myosin filaments are located, but was also present in the I bands which contain actin filaments. The results of the absorption with chicken muscle myosin and actin indicate that the primary antiserum was specific for both proteins. However, the purpose of the immunocytochemistry was to facilitate the light microscopic identification of differentiating muscle, and since all staining was confined to muscle cells, the dual staining of myosin and actin actually enhanced identification.

In fact, the immunocytochemical staining method permitted earlier recognition of muscle differentiation than had been feasible using conventional staining methods alone. This, combined with careful survey with respect to regional variations around the eye, allowed a more accurate chronology of irideal development, and in particular, of the differentiation of the sphincter and dilator pupillae muscles. A subsequent report (Ferrari & Koch, 1984) also utilizes these methods in the study of iris development *in vitro*.

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