MORPHOGENESIS OF THE COLLAGENOUS STROMA IN THE CHICK CORNEA

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ABSTRACT

The embryonic chick corneal epithelium produces a highly structured acellular matrix beneath its basal surface during early development. This matrix, which contains collagen, serves as a morphogenetic template for subsequent stromal development in that the three-dimensional architecture of the adult corneal stroma is initially established, in miniature, in this epithelially derived connective tissue. Examination of the early corneal epithelium and matrix in both the light and electron microscope suggests that self assembly of the matrix may be one of several important factors in the morphogenesis of this early connective tissue.

INTRODUCTION

The corneal stroma in the adult chicken is made up predominantly of water, collagen, glycosaminoglycans and stromal fibroblasts (Anseth, 1961; Coulombre, 1965; Hay and Revel, 1969). The stroma is approximately 200 microns thick and consists of layers 2 to 4 microns in thickness which lie roughly parallel to the corneal surface. The collagen fibrils within each layer are all oriented in the same direction and at approximately right angles to the collagen fibrils in the two adjacent layers. In addition to this orthogonal arrangement of adjacent layers, there is a gradual angular shift of the layers in a clockwise direction proceeding from the outer to inner layers (Coulombre, 1965). This angular shift proceeds in the same direction in both eyes and is thus asymmetric about the mid-body axis (Coulombre, 1965).

In the chick embryo the corneal stroma forms within the framework of an acellular collagenous matrix, called the postepithelial layer (Meyer and O'Rahilly, 1959) or primary corneal stroma (Hay and Revel, 1969). The primary corneal stroma is produced by the corneal epithelium (Hay and Revel, 1969; Goodfellow et al., 1969) and is deposited in an orthogonal pattern beneath the epithelial surface (Ladijenski, 1915; Laguesse, 1923; Hay and Revel, 1969). The acellular primary corneal stroma serves as a scaffold for mesenchymal cells which invade and produce the adult or secondary corneal stroma, and it has been suggested that the organization of the primary corneal stroma may contain, in miniature, the complex pattern of organization found in the adult (Ladijenski, 1915; Coulombre, 1965).

The present work is a light and electron microscope study of the organization and deposition of the primary corneal stroma during development. The results show that the fully formed primary corneal stroma is organized in a pattern identical to that present in the adult stroma and thus support the idea that the primary stroma dictates the pattern of organization of the adult stroma. Ultrastructural observations on the developing primary stroma raise the interesting possibility that the

morphogenesis of the three-dimensional architecture of this acellular matrix is, in part, a self assembly process.

MATERIALS AND METHODS

Embryos of white Leghorn chickens were incubated at 38°C and staged according to Hamburger and Hamilton (1951). Corneas at successive stages of development were fixed and impregnated with silver in order to stain the argyrophilic primary corneal stroma and thereby facilitate its observation in the light microscope. Corneas were fixed at room temperature for 4 hr in 6% formaldehyde buffered to pH 3.2 with 0.28 M glycine. After fixation the tissues were rinsed for several seconds in aqueous 1.5% $AgNO_3$ under subdued lighting and then placed in fresh 1.5% $AgNO_3$ for 4 hr in the dark. After silver impregnation the tissues were rinsed in a reducing solution, 1.5% hydroquinone in 7% aqueous formaldehyde and then placed in fresh reducing solution in the dark for a minimum of 2 hr. The tissues were dehydrated in ethanol and embedded in Araldite (Ciba 6005). Sections were cut on a Porter-Blum MT-2 ultramicrotome with glass knives and stained with 0.25% toluidine blue in 0.25% sodium borate.

Measurements of the orientation of the orthogonally arranged collagen fibrils of the corneal stroma were made on serial sections, 2 μ in thickness, which were cut in a plane parallel to the corneal surface. Before sectioning, the corneal diameter passing through the choroid fissure was oriented parallel to the cutting direction of the microtome. The serial sections were then cut, mounted on glass slides, and observed in a light microscope with a calibrated rotating stage and an eyepiece fitted with a graticule. The orientation of the orthogonally disposed collagen fibrils in the center of each section was measured in reference to a scratch in the section caused by the glass microtome knife. The orientation of the orthogonal fibrils in each section could then be related to that axis of the cornea which passes through the choroid fissure.

To define changes in cell shape which occur in the basal cell layer of the corneal epithelium during deposition of the primary corneal stroma, the intercellular cement was stained with silver. Fresh corneas at successive stages of development were dissected from the eye in an aqueous solution of 0.15% $AgNO_3$. After 10–15 min in the silver solution the corneas were transferred to 8% unbuffered formaldehyde in a Petri dish. The Petri dish was placed on a microscope stage and the tissue was exposed to a focused beam of white light of moderate intensity. After 15–30 min of photoreduction the tissues were dehydrated in ethanol, cleared in xylene, and mounted in Permount (Fisher Scientific Company, Pittsburgh, Pa.)

Radioautographic studies of the production of primary stroma were conducted with L-proline-^{3}H (New England Nuclear Corp., Boston, Mass.; SA 5.86 Ci/mmole) resuspended in Hanks' solution. The isotope was administered by dribbling the solution on the exposed cornea *in ovo*. Each animal received from 8 to 10 μCi. At timed intervals after administration of the isotope, animals were killed and fixed in either 1.3% osmium tetroxide in collidine buffer at pH 7.1 or buffered 8% formaldehyde and embedded as described above. Sections 1.5 μ thick were cut perpendicular to the corneal surface, mounted on glass slides, and dip coated in Kodak NTB-3. After exposure at room temperature in light tight boxes containing Drierite (W. A. Hammond Drierite Co., Xenia, Ohio) for 1–3 wk, the preparations were developed in D-19 at 18°C for 2 min, washed briefly in distilled water, and fixed in Kodak rapid fix for 1.5 min. After washing, the tissues were stained with 0.25% toluidine blue in 0.25% sodium borate and mounted with Permount. To quantitate the distribution of grains in the corneal stroma, sections were cut perpendicular to the corneal surface and the stroma was subdivided into bands 5 μ in width and 75 μ in length, the long axis of the band being parallel to the corneal surface. The number of grains in each 5 μ band was then counted. Grains over cells were not counted.

Tissues for electron microscopy were fixed at room temperature for 15–45 min in 2.5% acid stabilized purified glutaraldehyde (Trelstad, 1969), 4% paraformaldehyde in 0.1 M sodium cacodylate buffer at pH 7.5 (Karnovsky, 1965). Calcium was added to a final concentration of 0.008 M. After aldehyde fixation, the tissues were washed briefly in 0.1 M sodium cacodylate and postfixed in 1.3% osmium tetroxide in pH 7.1 collidine buffer at 4°C. After osmication the tissues were washed in 0.2 M collidine buffer at pH 6.1 for 20 min and then stained with 2.0% uranyl acetate in 0.2 M collidine at pH 5.1 for 1½ hr at room temperature. After staining, the tissues were washed for 20 min with 0.2 M collidine at pH 6.1, dehydrated in ethanol, and embedded in Araldite (Ciba 6005). Sections with silver interference colors were cut on a Porter-Blum MT-2 ultramicrotome with glass knives, mounted on naked 200 mesh copper grids, and stained with lead citrate. Sections were examined in an RCA-3E or AEI EM6 electron microscope.

RESULTS

Outline of Corneal Stromal Development

The primary corneal stroma begins to be deposited beneath the basal surface of the corneal epithelium on day 3 (stage 18), and during the subsequent 2½ days of development increases to about 10 μ in thickness (Fig. 1). Late in the 5th

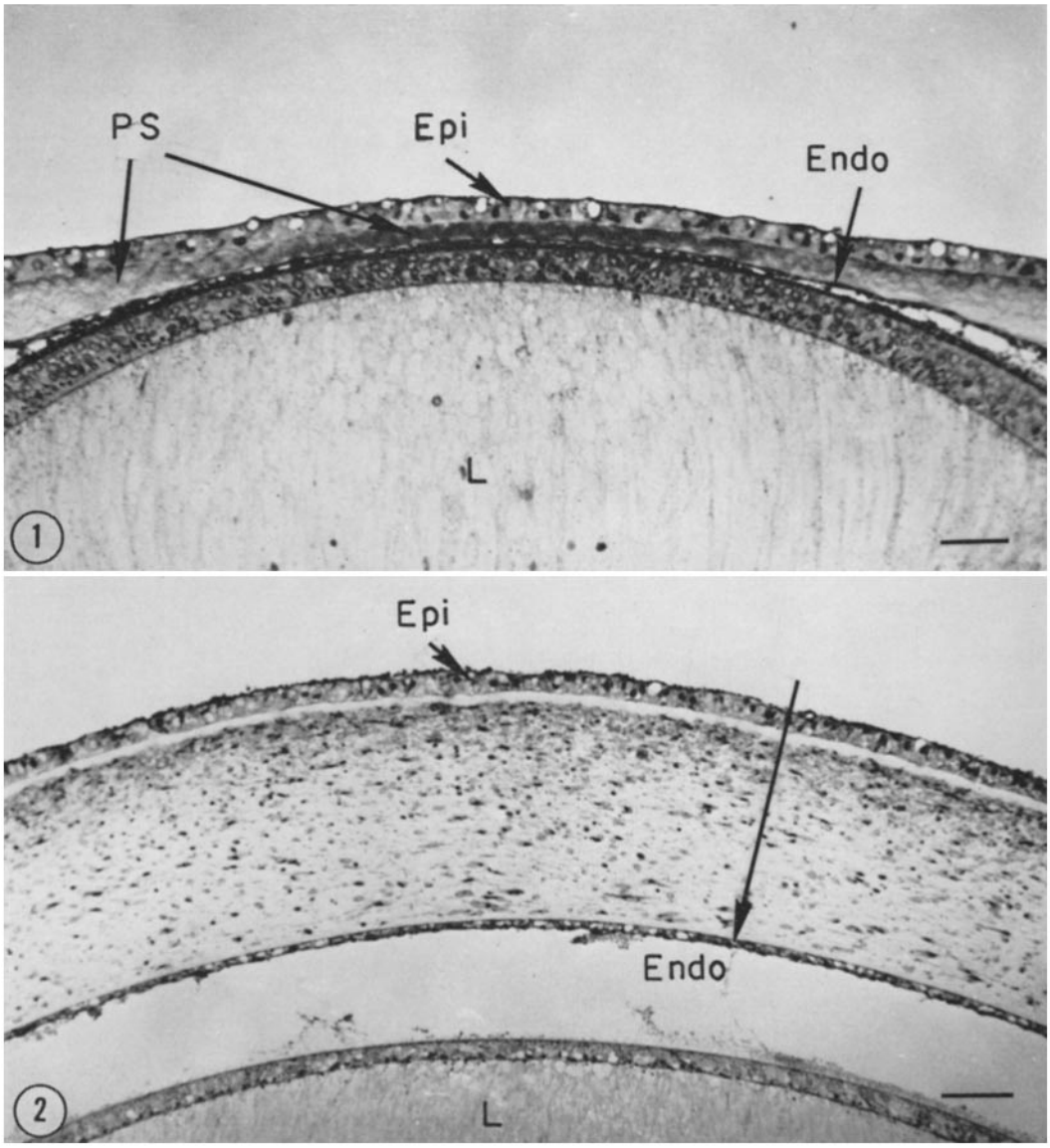


FIGURE 1 Stage 27. Plane of section: perpendicular to the corneal surface. The cornea consists of the two-cell layered outer epithelium (*Epi*), the acellular primary corneal stroma (*PS*), and the flat one-cell layered endothelium (*Endo*). The lens (*L*) is contiguous with the endothelium. The primary corneal stroma in the center of the cornea is more densely packed than at the periphery. The stroma is completely devoid of cells. The collagen fibrils in the stroma are not aligned strictly parallel to the corneal surface, but are present in a wavy or sinusoidal pattern. × 230. Mark, 40 μ.

FIGURE 2 Stage 31. Plane of section: perpendicular to the corneal surface. The cornea consists of the outer epithelium (*Epi*), stroma, and inner endothelium (*Endo*). The stroma shows two distinct regions: the narrow acellular portion beneath the outer epithelium, and the cellular portion. The cellular portion represents the primary corneal stroma which is being transformed by the mesenchyme into the secondary or adult stroma. The acellular portion beneath the epithelium is the recently synthesized primary corneal stroma which will eventually be invaded by mesenchyme. *L*, lens. × 240. Mark, 40 μ.

day of development (stage 28) the primary corneal stroma rapidly swells to about 60 μ in thickness. Shortly after this stromal swelling, mesenchymal cells invade the middle and posterior layers of the primary stroma (Fig. 2) and begin producing the adult or secondary corneal stroma (Hay and Revel, 1969). Before day 5 the corneal stroma thus consists exclusively of the primary corneal stroma, a cell-free collagenous matrix produced by the corneal epithelium. After day 5 the corneal stroma consists of two different regions: a posterior cellular region which is being transformed into the secondary corneal stroma by the mesenchyme, and an anterior uninvaded region which is a more recently deposited portion of the acellular primary corneal stroma (Fig. 2). The anterior acellular region is continuously invaded from its posterior face by mesenchyme over the subsequent 4½ days of development so that by day 10 (stage 36) it measures less than 1.0 μ in thickness. The formation of the secondary stroma by day 14 is essentially complete in regard to the number and orientation of its layers, and subsequent development consists of addition of more collagen to the existing layers and the continued deposition of glycosaminoglycans (Anseth, 1961; Coulombre and Coulombre, 1964; Coleman, Herrmann and Bess, 1965; Hay and Revel, 1969).

Orientation of Collagen in the Primary and Secondary Corneal Stroma

Serial thick sections cut parallel to the corneal surface through the primary corneal stroma at all stages before day 5½ (stage 28) reveal that the layers of collagen fibrils at all levels through the primary stroma are organized in an orthogonal pattern. These orthogonal layers are all in register at successive levels through the stroma; no angular displacement is evident between the outer and inner layers (Fig. 3, 5, and 6). The absolute orientation of the orthogonal layers in respect to a fixed anatomic point in the developing optic cup, the choroid fissure, is constant: one axis of the orthogonal stroma is parallel to, and the other axis is perpendicular to, the corneal diameter which passes through the choroid fissure (choroid fissure axis). The first layers of the primary corneal stroma which form during days 3–5 of incubation are therefore all deposited in an orthogonal pattern which shows no angular displacement and which has a fixed spatial orientation relative to the rest of the developing eye.

On day 5½ (stage 28) the primary corneal stroma rapidly swells from 10 to about 60 μ in thickness and is invaded by mesenchyme. By day 6 (stage 29) the entire corneal stroma is about 110 μ thick. The deeper 100 μ portion of the stroma has been invaded by mesenchyme and is becoming the secondary corneal stroma; the anterior 10 μ of stroma has not been invaded by mesenchyme. The orthogonal layers of collagen in the posterior 100 μ of invaded stroma show no angular displacement in respect to each other and they all lie in the same absolute orientation in respect to the choroid fissure as the earlier uninvaded primary corneal stroma, i.e., one axis of the invaded stroma is parallel to the choroid fissure axis, the other is perpendicular. On the other hand, in the 10 μ thick, anterior, uninvaded portion of the primary stroma, each successive layer of collagen shows a slight angular displacement from preceding layers such that the net displacement of the most superficial layer is about 30° from the choroid fissure axis (Fig. 6). The rate of displacement in the primary stroma at this and later stages of development is 2.5°–5.0° per μ of corneal thickness and there are, on the average, about 3 layers per μ. The absolute amount of angular displacement from layer to layer can, therefore, be roughly estimated at 1°–2°. The direction of this displacement is clockwise from the outer to inner layers and is in the same direction in both eyes. The first indication of an angular displacement in the layers of the corneal stroma thus appears in the uninvaded anterior portion of the primary corneal stroma shortly after the posterior portion of the primary stroma has been invaded by mesenchyme.

By day 7 (stage 33) the corneal stroma is about 170 μ thick; the posterior 160 μ of stroma have been invaded by mesenchyme, the anterior 10 have not. The orthogonal layers of collagen in the posterior 120 μ of invaded stroma are all in register and show no angular displacement in respect to the choroid fissure axis (Figs. 5 and 6). The orthogonal layers in the remaining anterior 40 μ of invaded stroma show an angular displacement, clockwise, as the stroma is traversed from the outside inward, of about 50° in respect to the choroid fissure axis (Figs. 4 and 6). In the uninvaded primary stroma beneath the epithelium, the more posterior orthogonal layers of collagen show a net displacement of 50° from the choroid fissure axis and the more anterior layers about 75° (Figs. 5 and 6).

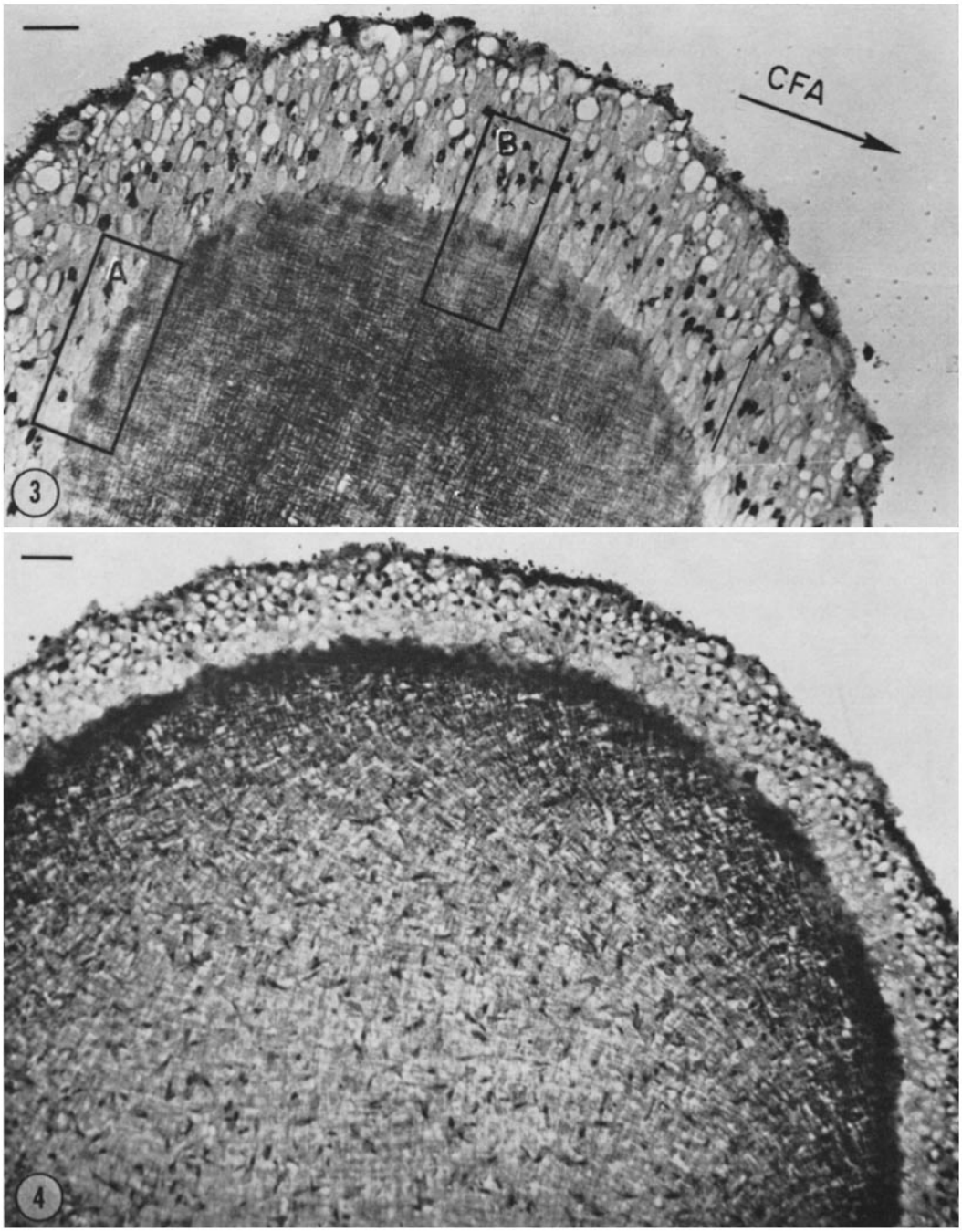


FIGURE 3 Stage 28. Plane of section: parallel to the corneal surface. The orthogonal organization of the primary corneal stroma is apparent. Note that the orientation of the matrix does not change from the center to the periphery. The matrix in the center is at a deeper level within the stroma than that at the periphery because of the corneal curvature. The basal cells in the epithelium are elongated (*narrow arrow*) in a direction perpendicular to the choroid fissure axis (*CFA, arrow*). The direction of this elongation is the same across the entire corneal surface. Electron micrographs from the regions indicated by the rectangles are presented in Figs. 14 (*A*) and 15 (*B*). × 400. Mark, 20 μ.

FIGURE 4 Stage 34. Plane of section: parallel to the corneal surface. The orthogonal orientation of successive levels of the stroma can be illustrated in one section because of the corneal curvature. Note the gradual angular change in the orientation of the matrix from the center to the periphery. The section is being viewed from the endothelial side, and the direction of the shift is therefore clockwise from the outer or more superficial levels to the inner deeper levels. When the orientation of the serial sections was measured, only the exact center of each section was used. × 370. Mark, 20 μ.

On day 9 (stage 35) the corneal stroma is about 190 μ thick; the posterior 180 μ of the stroma have been invaded by mesenchyme, the anterior 10 μ have not. The orthogonal layers of collagen in the posterior 140 μ of invaded stroma are all in register and, as before, undisplaced in respect to the choroid fissure axis. The orthogonal layers in the remaining anterior 40 μ of invaded stroma show an angular displacement, clockwise as the stroma is traversed from the outside inward, of about 80° in respect to the choroid fissure axis. In the uninvaded primary stroma beneath the epithelium, the more posterior layers show a net displacement of 80° from the choroid fissure axis, and the more anterior layers about 130° (Figs. 5 and 6).

By day 14 (stage 40) the corneal stroma is about 200 μ thick and is invaded throughout by mesenchyme except for a narrow 1–2 μ thick zone beneath the epithelium. The thickness of the corneal stroma at this stage begins to decrease because of the onset of dehydration (Coulombre and Coulombre, 1964). The compaction of stroma is most prominent in the posterior layers where the orthogonal stroma shows no angular displacement. Whereas at day 9 the posterior 140 μ of stroma showed no angular displacement, at day 14 only the posterior 50 μ of stroma show no angular displacement (Fig. 6). Presumably, the difference of 90 μ results from compaction due to the dehydration that begins at about this time. The orthogonal layers in the remaining 150 μ of stroma show a net angular displacement of about 210° in respect to the choroid fissure axis (Fig. 6). The rate of angular displacement is thus about 1.4° per μ. Since each layer is from 2 to 4 μ thick, the angular displacement per layer is from 2.8° to 5.6°. This rate is slightly greater than that calculated for the primary corneal stroma. The direction of displacement at day 14 is clockwise from the outer to inner layers and is in the same direction in both eyes. The orthogonal geometry of the collagen layers in the 14 day cornea is the same as that found in the adult in both extent and direction of displacement.

Ultrastructure of the Primary Corneal Stroma

Electron microscope examination of the uninvaded primary corneal stroma in several different planes of section confirms both the orthogonal pattern of the fibrils and their collagenous nature. The collagen fibrils are uniformly about 250 A in diameter and have an average macro period of about 600 A. The subbanding pattern of the collagen fibrils is similar to that described in other collagens (Figs. 8 and 9). Surrounding the collagen fibrils are aggregates of filamentous and amorphous materials (Figs. 9 and 10) which have

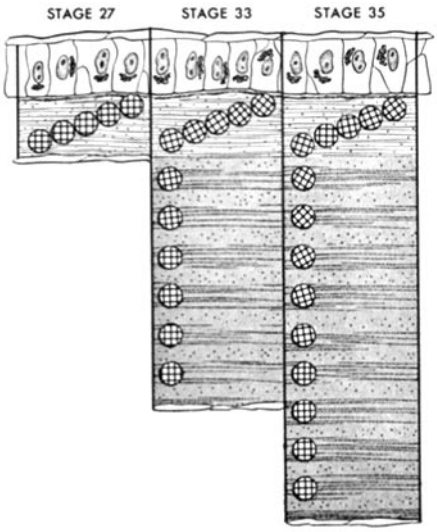


FIGURE 5 Three stages in the development of the corneal stroma are illustrated. Two planes of section have been diagrammed. The circles with orthogonal grids, representing a plane parallel to the corneal surface, have been superimposed on the diagram representing a section perpendicular to the corneal surface. Each circle illustrates the orthogonal orientation of the stromal matrix at that particular level in the stroma. The vertical direction corresponds to the choroid fissure axis. The light stroma is the uninvaded primary stroma; the gray stippled stroma is the invaded stroma.

At stage 27 all levels of the acellular primary stroma are in register and one axis of the matrix is parallel to the choroid fissure axis.

At stage 33 the posterior levels of the invaded stroma are in register with each other and with the choroid fissure axis. In the anterior levels of invaded stroma a slight shift in the orientation of the matrix is apparent. The stroma in the uninvaded region now reveals a gradual angular shift.

At stage 35 the posterior levels again are in register with each other and with the choroid fissure axis. At successively more superficial levels, the invaded stroma shows a gradual angular displacement. In the uninvaded stroma an angular displacement between successive levels is also present. Note that the angular displacement of successive levels from the invaded to uninvaded portions is a smooth continuum.

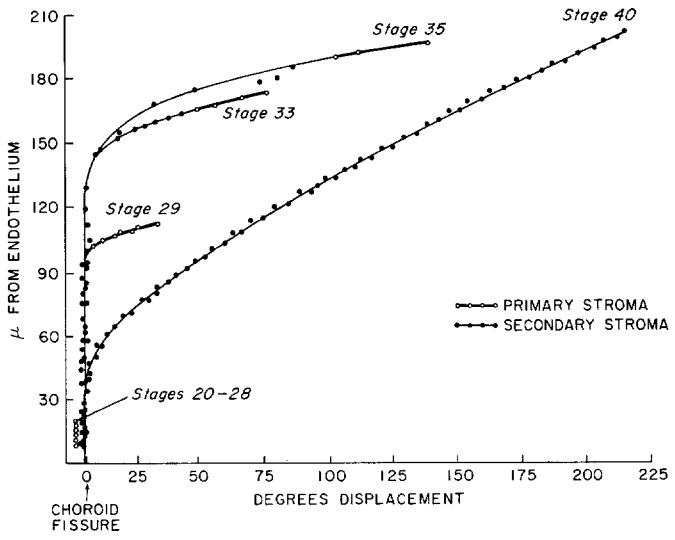


FIGURE 6 Serial sections through the corneal stroma were cut at different stages of development. The orientation of the orthogonal matrix in each section was measured at the center of the section and related to the corneal diameter which passes through the choroid fissure. The position of one axis of the matrix in each section has been plotted in respect to its distance from the posterior endothelium. During the early stages of development (20–28) (days 3–5) when only the primary stroma is present, all levels are in register with each other and with the choroid fissure axis. At stage 29 (day 6) the primary stroma has thickened, due to swelling, and has been invaded in its posterior portion by mesenchyme which begins the production of the secondary stroma. The posterior levels of the stage 29 stroma show no displacement from each other or from the choroid fissure axis. The primary stroma at stage 29 shows about 30° of displacement. At stages 33 (day 7.5) and 35 (day 8.5) a greater proportion of the invaded secondary stroma shows an angular displacement. Note that the curves joining the primary and secondary stroma are smooth and continuous. At stage 40 (day 14) the configuration of the adult has been achieved. No primary stroma is present beneath the epithelium. The change in shape of the curves between stages 35 and 40 is caused by compaction of the posterior levels due to stromal dehydration.

previously been shown to be digestible with trypsin (Hay and Revel, 1969).

The collagen fibrils are not organized into continuous sheets or layers, but instead form bundles which are generally 1–3 fibrils thick and 5–25 fibrils wide (Figs. 7, 8, and 9). The layers of primary stroma consist of these belt-shaped bundles of fibrils which are laterally associated at similar levels within the stroma. It seems likely that it is these bundles of fibrils which are seen in the silver-impregnated light microscope preparations (Fig. 3).

In sections cut perpendicular to the corneal surface, the bundles or layers of bundles are not aligned strictly parallel to the epithelial basal surface, but describe a fairly regular sinusoidal pattern (Fig. 1). Each layer of the primary stroma, however, maintains an orthogonal orientation relative to the adjacent layers. This sinusoidal pattern of the primary stroma is present both before and after mesenchymal cells invade.

The distance between fibrils *within a bundle* is quite uniform, resulting in a parallel arrangement of the fibrils (Figs. 9 and 10). In magnitude this interfibrillar (center-to-center) spacing is strikingly similar to the periodicity of the collagen fibrils, i.e. 600 A (Figs. 8 and 10). This is apparent in preparations cut both perpendicular (Fig. 8) and parallel to the corneal surface (Fig. 10). The average ratio of this interfibrillar distance to the collagen period from multiple preparations from stage 24 to 34 was 1.0 ± 0.1. The spacing between adjacent orthogonal fibrils, on the other hand, is not uniform and ranges from about 300 A to 1000 A (Figs. 7 and 8).

Radioautographic Localization of the Site of Deposition of Primary Stroma

Radioautograms of corneas cut perpendicular to the corneal surface were prepared from tissue sections at ½, 1, 2, and 3 day intervals after ad-

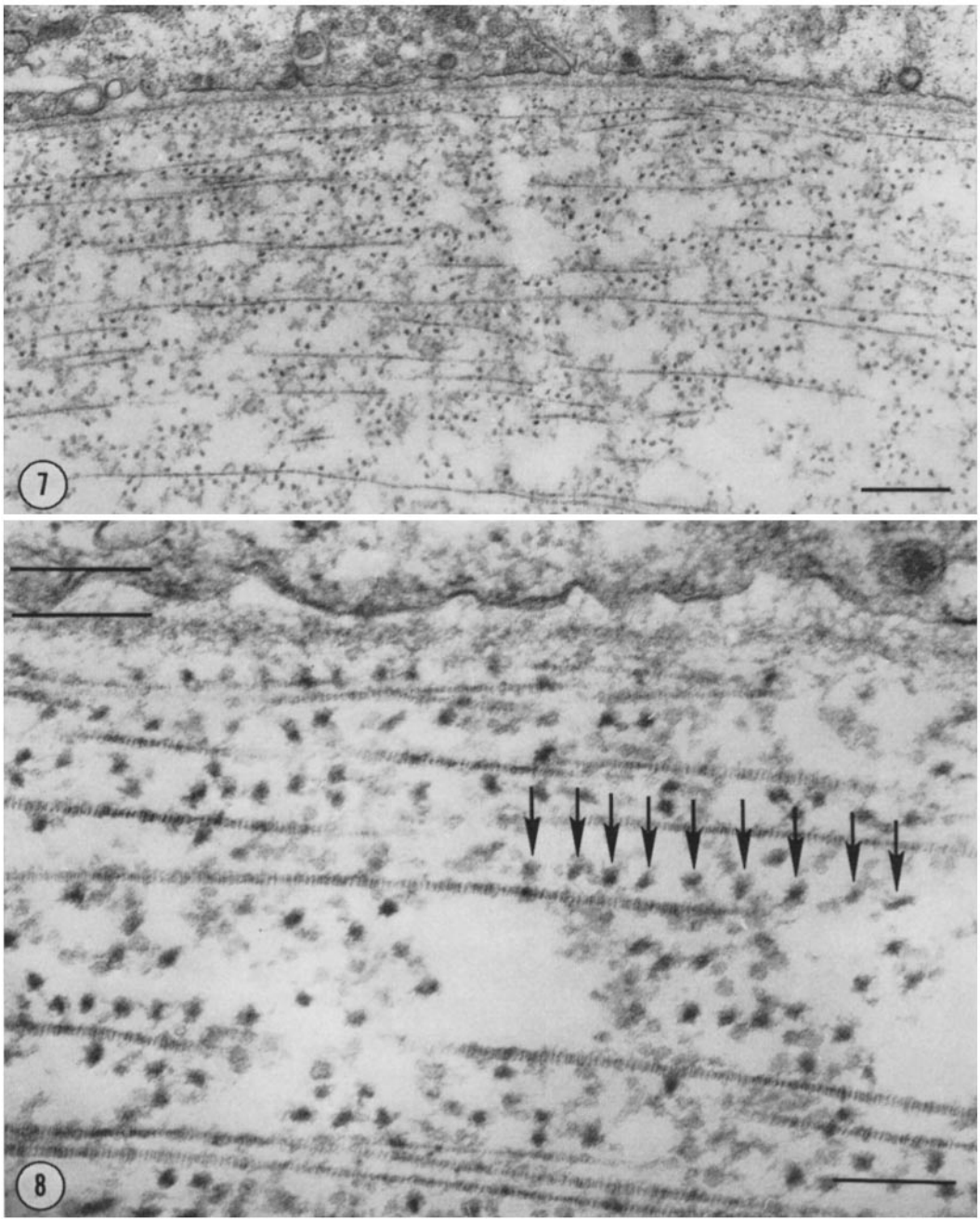


FIGURE 7 Stage 28. Plane of section: perpendicular to the corneal surface and parallel to the choroid fissure. The orthogonal matrix is cut such that one group of fibrils is parallel and the other perpendicular to the plane of section. The fibrils are grouped in small flattened bundles of from 5 to 20 fibrils. Groups of bundles which are at the same level in the stroma are the equivalent of layers. It is apparent that such layers are not continuous sheets. Within bundles the spacing between fibrils is quite uniform. × 25,100. Mark, 0.5 μ.

FIGURE 8 Stage 28. Plane of section: the same as Fig. 7. The grouping of fibrils into thin, flat bundles is illustrated. A typical bundle is indicated by the arrows. The distance between fibrils in the bundles corresponds closely with the periodicity of the collagen fibrils. The ratio of interfibrillar spacing to collagen fibril periodicity in this example is 1.15. Note the dense vacuole at the upper right which probably is an elongated vacuole (Fig. 16) cut in cross section. The two horizontal lines indicate the plane of section of Fig. 16. × 86,500. Mark, 0.2 μ.

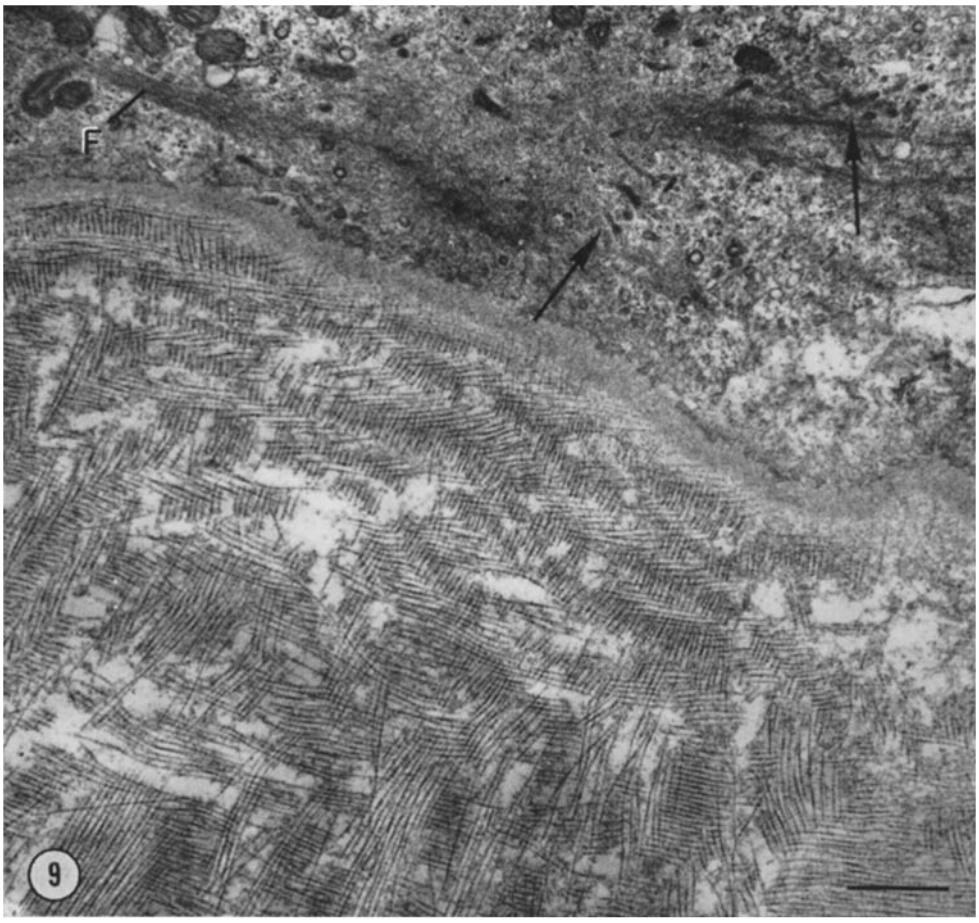


FIGURE 9 Stage 34. Plane of section: parallel to the corneal surface. Several features of the organization of the primary stroma are illustrated. The fibrils are not organized as continuous sheets, but rather as broad bundles. The bundles are arranged in an orthogonal pattern. The uniform interfibrillar spacing of the fibrils within the bundles is illustrated by the parallel alignment of the fibrils. Within the basal cell cytoplasm, bundles of 80 A filaments (*F*) and numerous elongated vacuoles (*arrows*) are present. × 13,400. Mark, 1.0 μ.

ministration of proline-^{3}H to 4½ day (stage 25) embryos. By 12 hr after administration of the isotope the 5 μ wide band of stroma with the highest grain density (50% of total grains) was present in the primary stroma within 5 μ of the outer epithelium (Figs. 11 and 13). The entire stroma of the 12 hr preparation (stage 27 at fixation) consisted only of the uninvaded primary stroma and measured 30 μ in thickness. The band of grains was continuous across the entire stroma at this and later stages, and the distribution of grains within a band often reflected the sinusoidal pattern of the primary stroma described above. By 27 hr after administration of the isotope the 5 μ wide band of stroma with the highest grain density (19% of total stromal grains) was present 30 μ from the basal surface of the outer epithelium (Fig. 13). The stroma in the 27 hr preparation (stage 29 at fixation) had thickened to about 70 μ and mesenchymal invasion was just beginning. By 51 hr after administration of the isotope (stage 30 at fixation) the stroma was well invaded and the 5 μ wide band of stroma with the highest grain density (16% of total stromal grains) was located 50 μ from the basal surface of the outer epithelium (Fig. 13). Finally, by 71 hr (stage 34 at fixation) the 5 μ wide band with the highest grain density (15% of total stromal grains) was located 100 μ

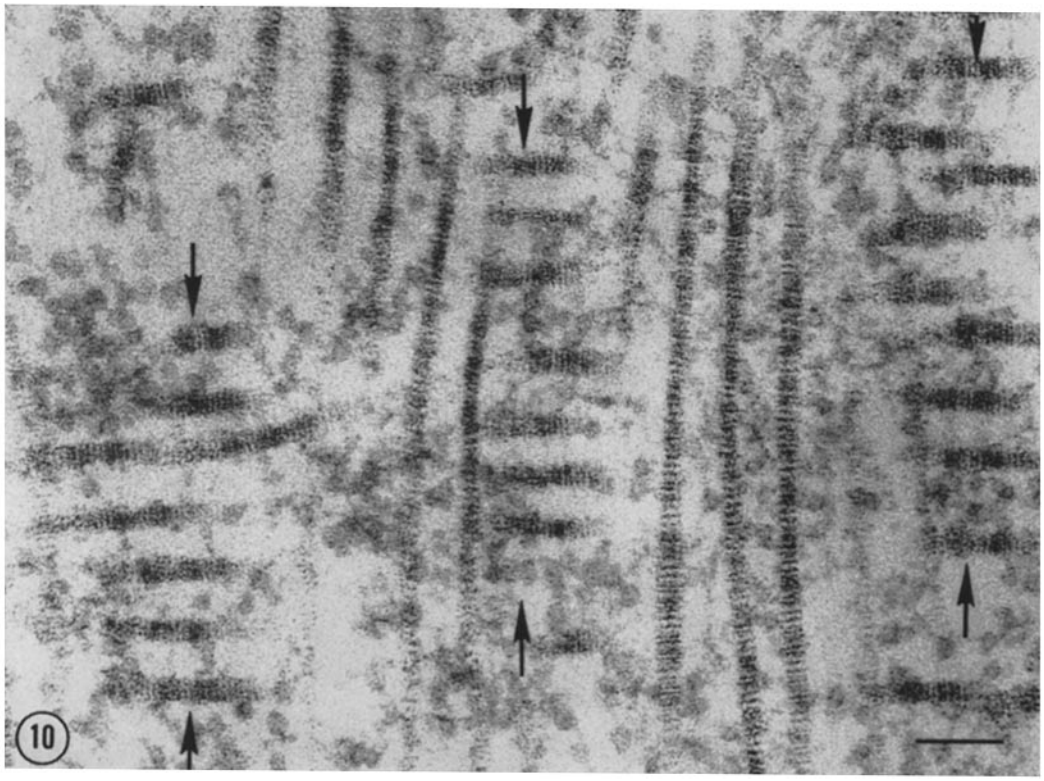


FIGURE 10 Stage 32. Plane of section: parallel to the corneal surface. The similarity of the interfibrillar spacing and the collagen periodicity is illustrated. The average interfibrillar distance of the fibrils within the bundles between the arrows divided by the macro period of the collagen fibrils is 1.09. × 113,000. Mark, 0.1 μ.

from the outer epithelium, and the entire stroma had grown to about 170 μ in thickness (Fig. 13). The radioautographic data from three separate sets of experiments were nearly identical.

Basal Epithelial Cell Shape and Orientation

The shape of the cells in the basal layer of the two-layered corneal epithelium changes rather dramatically during early development as followed both in whole mounts stained with silver and in tissue sections viewed by light or electron microscopy. At stage 18 when the primary corneal stroma is first beginning to be deposited beneath the epithelium, the basal cells viewed along the optic axis of the eye are elongated perpendicular to the choroid fissure axis (Figs. 3, 12, 14, and 15) and are therefore in register with one group of orthogonal fibrils in the primary corneal stroma. The size and orientation of the basal cells are quite uniform across the entire corneal surface, and the cells measure 22.7 × 7.6 μ in the plane of the cornea. The cells in the periderm or outer squamous layer at stage 18 are polygonal, usually 5 sided, and roughly 50 μ in greatest diameter. At stage 23, the basal cells continue to be elongated perpendicular to the choroid fissure axis. The cells have remained approximately the same length, but have become narrower measuring 21.1 × 4.2 μ. The size and shape of the periderm are unchanged. By stage 28 the basal cells have become more elongated in the plane of the cornea and measure 53.2 × 3.9 μ. The shape of the periderm continues unchanged. At stage 29 there is a rapid shortening of the elongated basal cells, and the cells become 16.8 × 4.8 μ. By stage 30 the basal cells have become isodiametric in the plane of the cornea with an average diameter of 6–8 μ. The basal cells remain this shape and size for the rest of development.

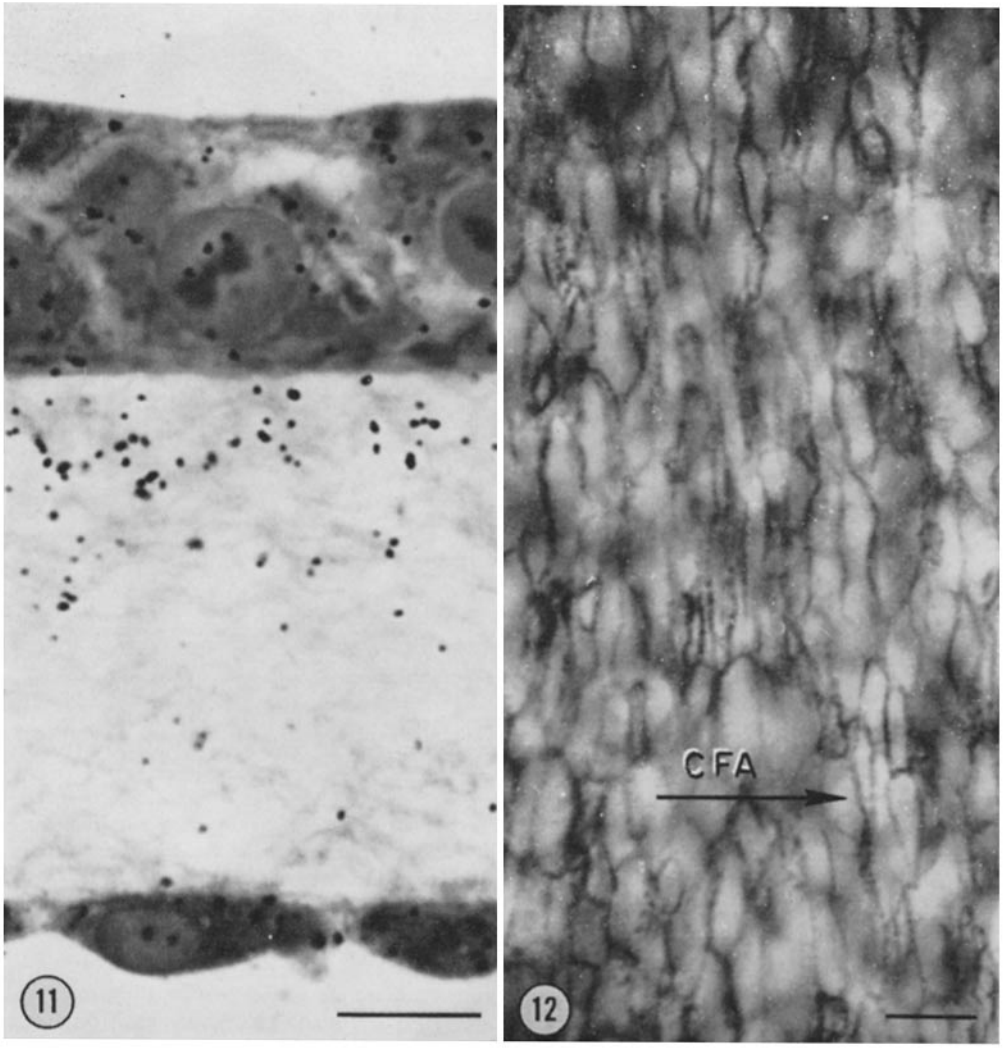


FIGURE 11 Stage 27. Plane of section: perpendicular to the corneal surface. Radioautogram 12 hr after administration of proline-^{3}H. Within the acellular primary stroma the majority of grains are present within 7 μ of the basal surface of the epithelium. × 1920. Mark, 10 μ.

FIGURE 12 Stage 27. Whole mount of the cornea stained with silver to outline the intercellular space and viewed along the optic axis. The plane of focus is at the level of the basal epithelial cells. The cells are elongated in a direction which is perpendicular to the choroid fissure axis (*CFA, arrow*). The same orientation of the cells is present across the entire cornea. × 600. Mark, 20 μ.

Basal Cell Ultrastructure

The ultrastructure of the basal epithelial cells shows many features typical of a secretory epithelium including a well developed endoplasmic reticulum and Golgi apparatus (see Hay and Revel, 1969 for review). Of particular interest to the present study is the finding, near the basal surface of the epithelium, of at least three different oriented structures within the cytoplasm. These three structures are: filaments 80 A in diameter which are organized into bundles; microtubules; and elongated vacuoles which contain a dense, slightly fibrillar material.

The bundles of filaments 80 A in diameter are

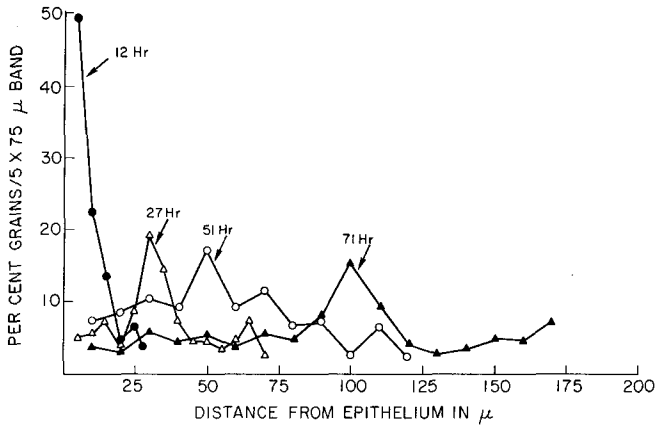
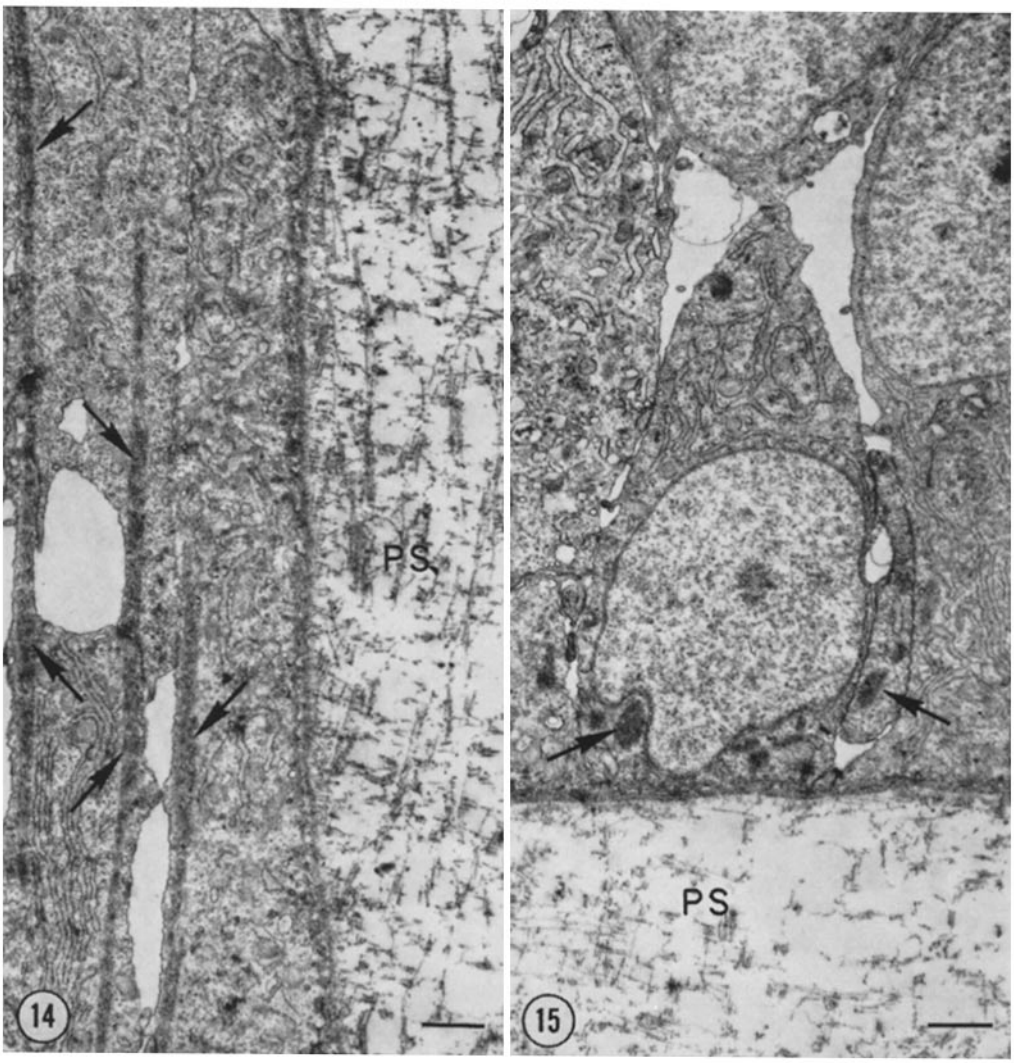


FIGURE 13 Radioautograms were prepared of corneas at timed intervals after administration of proline-^{3}H to stage 25 (4.5 day) embryos. In sections cut perpendicular to the corneal surface the stroma was subdivided into bands 5 μ deep and 75 μ wide. The number of grains in each band were counted and expressed as a per cent of total grains. The position of the band with the highest grain density lies deeper in the stroma at successive time intervals in both absolute and relative terms.

most frequently seen in the basal half of the basal epithelial cell (Fig. 14). They are quite prominent from the fourth through sixth days of development during the period when the basal cell is undergoing changes in shape. The bundles range from 2000 to 6000 A in diameter and generally lie parallel to the basal cell surface. The bundles of filaments terminate at the cell membrane in a *macula adhaerens*, and bundles of filaments in adjacent cells are apposed to such contact specializations (Fig. 14). At the later stages of development when the basal cells have assumed an isodiametric shape, the bundles of filaments continue to be present (Fig. 9). The intracellular orientation of the filamentous bundles is often such that they parallel the axes of the subjacent collagenous matrix (Figs. 9, 14).

Microtubules are found in the basal cells in a variety of different orientations as judged in sections cut in different planes. In sections cut in the plane of the basal surface of the elongated basal epithelial cell, the majority of the microtubules are oriented parallel to the axis of cell elongation (Fig. 16). This distribution of the microtubles is most striking during the early stages of corneal development when the basal cells are undergoing changes in shape. At the later stages of development when the basal cells are isodiametric, microtubules continue to be present near the basal cell membrane, but in much smaller number and with no apparent pattern.

The dense elongated vacuoles are found almost exclusively in the basal half of the basal epithelial cells. Their exact source and content are unknown although it has been suggested that they may contain collagen (Trelstad, 1971). The vacuoles average about 1000 A in diameter and about 6000 A in length (Fig. 16). They consist of a limiting membrane within which there is a dense, slightly fibrillar material which is not detectably cross striated. Near the basal cell surface the majority of elongated vacuoles is parallel to the basal cell membrane and in an orientation similar to that of the subepithelial orthogonal collagen fibrils. During the early stages of formation of the primary stroma, when the basal cells are long and narrow, the dense elongated vacuoles near the basal cell surface are predominantly oriented parallel to the same axis as the cell (Fig. 16). Vacuoles oriented perpendicular to the long axis of the cell, however, are also seen, and such vacuoles are often adjacent to a like-oriented microtubule (Fig. 16). During the later stages of formation of the primary stroma when the basal cells are isodiametric and the layers of primary stromal collagen are being deposited with an angular displacement, the orientation of elongated vacuoles which are immediately adjacent to the basal cell surface is usually in close register with the orthogonal orientation of the collagen fibrils in the immediately subjacent subepithelial stroma.



FIGURES 14 and 15 Stage 28. Plane of section: parallel to the corneal surface. These electron micrographs were taken from the same section, and their relative position to each other is indicated in Fig. 3. × 8380. Mark, 1.0 μ.

In Fig. 14 (3*A*) the prominent elongation of the cells is illustrated. The axis of cell elongation parallels the basal cell surface and one axis of the adjacent orthogonal primary stroma (*PS*). Large bundles of 80 A filaments are present in the basal cell cytoplasm (*arrows*). Such bundles terminate at the cell membrane in *maculae adhaerentes*.

In Fig. 15 (3*B*), the axis of cell elongation is perpendicular to the basal cell surface. The cells appear less elongated than those in Fig. 14, owing to corneal curvature. The absolute orientation of the cells in Figs. 14 and 15 is the same. Note again that the axis of the cell parallels one axis of the adjacent stroma (*PS*). The 80 A filament bundles have been cut obliquely (*arrows*).

DISCUSSION

The present study indicates that the morphological organization of the collagenous stroma in the chick cornea develops in several distinct steps during embryogenesis. The first step is the formation of the acellular primary corneal stroma. This collagenous matrix organizes into an orthogonal pattern as it is deposited beneath the corneal

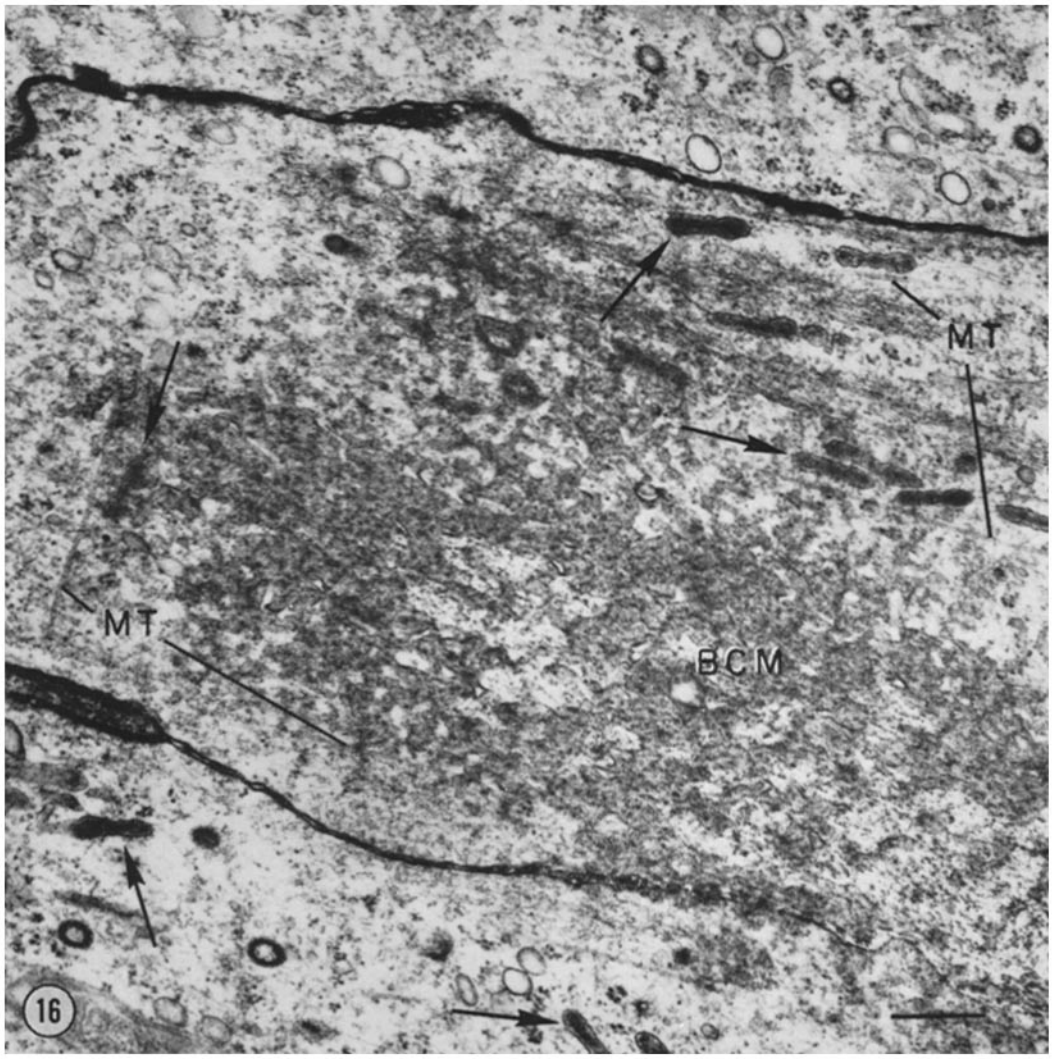


FIGURE 16 Stage 28. Plane of section: parallel to the corneal surface. This section is one of a series of serial thin sections taken near the basal cell surface. The approximate level of this section is indicated by the two horizontal lines in Fig. 8. The elongation of the cell is apparent. Elongated vacuoles are prominent within the cell (*arrows*). These vacuoles contain a dense, slightly fibrillar, nonstriated material. A majority of the vacuoles are aligned parallel to the axis of the cell. Microtubules (*MT*) are also present and are oriented both parallel and perpendicular to the axis of the cell. The basal cell membrane (*BCM*) has a granular substructure. × 25,400. Mark, 0.5 μ.

epithelium. The first portion of the matrix is deposited with all levels of the orthogonal matrix in register, whereas the subsequently deposited, more superficial levels of the matrix are deposited with a gradual angular displacement from the previously deposited levels. The second step in the development of the stroma is the invasion of the acellular primary stroma by mesenchyme. This step actually begins before the formation of the primary corneal stroma is completed. The fusiform mesenchymal cells use the primary stroma as a scaffold and tend to be oriented in a pattern similar to that of the orthogonal layers of the primary stroma. The next step is the deposition of collagen by the mesenchyme to form the secondary or adult stroma. This collagen is deposited by the

mesenchymal cells in orthogonal layers which show a pattern identical to that seen at earlier stages in the primary corneal stroma. On the basis of these observations it seems reasonable to conclude that the primary corneal stroma serves as a morphogenetic template within which the adult corneal stroma forms. A consideration of the factors leading to the formation and organization of the primary corneal stroma is thus clearly of central importance in understanding one aspect of the morphogenesis of the corneal stroma in the domestic fowl.

Production and Deposition of the Primary Corneal Stroma

The collagen of the primary corneal stroma is formed by the corneal epithelium. Evidence in support of this view is derived from a variety of morphological studies (Kessler, 1877; Laguesse, 1926; Hay and Revel, 1969; Trelstad, 1970; Dodson and Hay, 1971) and from recent biochemical investigations (Goodfellow et al., 1969; Conrad, 1970). Taken together, these studies clearly indicate that the collagen of the primary corneal stroma is synthesized by the corneal epithelium and then excreted into the subepithelial stroma.

The most recently synthesized primary corneal stroma is deposited in the region immediately beneath the epithelial basal surface. This is indicated in the radioautograms presented here in which, at timed intervals after the administration of proline-^{3}H to the embryo, a band of radioactivity in the stroma is first detected immediately beneath the epithelium and later at progressively greater distances, in both absolute and relative terms, from the basal surface of the epithelium. As a consequence of this pattern of deposition, the deeper layers of the primary stroma are those which are formed first and the more superficial layers are those which are formed last.

The duration of primary stromal deposition is of particular importance in attempting to understand the factors which lead to the gradual angular displacement of the orthogonal matrix in its anterior regions. If the primary stroma has been completely deposited before the appearance of an angular displacement among its superficial layers, then the angular displacement which subsequently appears would have to arise by an actual physical shift or reorientation of the stromal layers. On the other hand, if the production of the primary stroma is still in progress when the angular displacement appears in the superficial zone, it would be more probable that the displacement is the result of some influence on the orientation of the collagen as it is being deposited beneath the epithelium. The available morphological data (Hay and Revel, 1969; Trelstad, 1970) and the present radioautographic data indicate that the primary corneal stroma continues to be produced after angular displacement is first detected (stage 29) in the anterior region of the primary stroma. It would seem likely therefore that the pattern of organization of each portion of the primary stroma is established as it is being deposited at the interface of the epithelium and primary stroma.

Structure of the Primary Corneal Stroma

From the present light and electron microscope observations, at least five different characteristics of the organization of the primary corneal stroma can be described. First, the primary stroma is an orthogonal collagenous matrix, a finding which is in agreement with the observations of previous investigators (Ladijenski, 1915; Laguesse, 1923; Hay and Revel, 1969). Second, in the fully formed primary stroma there is a gradual angular displacement in the anterior layers which is in the same direction in both eyes and which measures about 200° in net extent. Third, the collagen fibrils in the primary stroma are not organized as discrete sheets or layers, but rather as belt-shaped bundles. These bundles are positioned within the stroma in such a manner that, in sections cut perpendicular to the corneal surface, laterally aligned bundles can appear as layers. It is clear from tracing such groups laterally, however, that they do not form complete, uninterrupted sheets across the cornea. Fourth, the collagen bundles do not lie strictly parallel to the basal surface of the corneal epithelium, but rather describe a sinusoidal pattern. As a consequence, a bundle of fibrils at one point may lie at an angle in respect to the basal surface of the epithelium, and at another point lie parallel. Recently (Nadol et al., 1969) have shown that collagen in the developing fish dermis is organized as orthogonal shingles, consisting of parallel collagen fibrils which intersect the basal surface of the epithelium at an angle. Because of the loose packing of the collagen bundles in the primary corneal stroma as compared with that in the fish dermis, it is difficult to firmly establish a shingle pattern in the primary stroma. The bundles of collagen in the primary stroma, however, clearly

do not lie exclusively parallel to the epithelium in plywood-like sheets. Fifth, *within the bundles* the collagen fibrils are of uniform diameter, parallel to one another and quite uniformly spaced. The striking similarity between the center-to-center spacing of the collagen fibrils and their periodicity (average of 600 A) suggests the interesting possibility that orthogonally disposed bundles of fibrils determine each other's spacing (Gross, 1956; Weiss, 1957; Gross, 1961). Such an interaction between fibrils would probably depend on some kind of bridging macromolecule since the distance separating fibrils is greater than that encountered in usual macromolecular interactions. It is of interest that Weiss and Ferris (1954) have also reported a similarity in collagen fibril spacing and periodicity in the basement lamella of amphibians. Further discussion of this self assembly hypothesis will be presented in a later section of the paper.

Epithelial Cell Shape

The cause and function of the changes in shape of the basal epithelial cells which occur during the third through sixth days of development are unexplained. Both microtubules and microfilaments, two structures associated with the establishment (Gibbins et al., 1969) and change (Byers and Porter, 1964; Spooner and Wessels, 1970) in cell shape during development, are prominent in the basal cells. In addition to these intracellular structures, it should also be noted that mechanical stresses associated with the growth of the eye as a whole are probably also important (Coulombre, 1956; Coulombre, 1957). The function of these changes in cell shape is not known, but, as discussed below, they may reflect the existence of morphogenetically important oriented mechanical stresses within the epithelium and developing stroma.

Orienting Factors during Primary Stromal Development

From the preceding consideration of the source, site of deposition, and development of pattern in the primary corneal stroma, it would appear that the factors which serve to orient the stroma operate within a narrow zone near the basal surface of the epithelium. These factors must operate so that: (*a*) from days 3 to 6 the orthogonal matrix is deposited without angular displacement and with one axis in register with the choroid fissure axis; (*b*) following day 6 the matrix is deposited with a gradual angular displacement; and (*c*) the direction of the angular displacement is the same in both eyes. Several possible sources of such orienting factors, suggested by the present observations, are the basal epithelial cells themselves, physical stresses within the corneal stroma, and interactions between components of the matrix. In the following paragraphs, each of these possibilities will be discussed.

The influence of a cell on the orientation of the collagen that it excretes has been frequently noted (Stearns, 1940; Porter and Pappas, 1959), but it is not yet known whether this orientation derives, for example, from the manner in which collagen is excreted by the cell, from the manner of collagen polymerization on or near the cell surface, or from some other oriented matrix such as a basement membrane or glycosaminoglycan which the cell may also excrete into the extracellular space. The excretion of collagen by the corneal epithelium has been suggested to occur by vacuoles derived from the Golgi apparatus (Hay and Revel, 1969). Recently, evidence has been presented which suggests that the dense elongated vacuoles in the corneal epithelium contain collagen which is in the process of being excreted (Trelstad, 1971). The fact that these elongated vacuoles near the basal cell membrane tend to be aligned in register with the orthogonal collagen fibrils in the primary stroma raises the interesting possibility that the intracellular orientation of the vacuoles influences the orientation of the collagen as it is excreted by the cell. From a previous study of changes in the position of the Golgi apparatus during corneal development it is clear that the intracellular orientation of some, if not all, of the basal cell organelles is under precise control (Trelstad, 1970).

In addition to potential influences from the cell of origin, the physical state of the environment into which the collagen is excreted may have a bearing on its orientation. This is well illustrated by the orientation of collagen fibrils along lines of mechanical stress (Doljanksi and Roulet, 1934; Dueggeli, 1937; Stearns, 1940). From the changes in cell shape which occur in the basal cell layer of the corneal epithelium between days 3 and 6 it seems likely that these cells, and probably also the subjacent primary corneal stroma, are subjected to some kind of mechanical force which lies in the plane of the cornea and which is oriented perpendicular to the choroid fissure axis. This force could serve to orient the collagen as it polymerizes into

fibrils beneath the epithelium. The fact that the primary stroma which is deposited during this three day period of development shows no angular displacement and that one of its axes is in register with this apparent force supports this suggestion. Moreover, it may be important that at the point when the epithelial cells lose their elongated configuration (Stages 29–30) and when, presumably, the oriented stress in the stroma disappears, the pattern of gradual angular displacement in the primary stroma begins to appear.

Finally, there is the possibility that the primary stroma is a self assembly system in which the matrix influences the development of its own architecture. An indirect argument in support of this possibility can be raised on the basis of the sameness in direction of the angular displacement in both eyes and its consequent asymmetry about the midplane of the embryo. No other structural feature of the cornea or the eye has been described which shows such asymmetry. The patterns of positioning of the 14 scleral ossicles which surround the cornea are invariably mirror images (Coulombre et al., 1962). The general shape of the whole cornea, as judged by polarizing microscopy, is also symmetrical about the mid body axis (Coulombre, 1965). From such observations it might be argued indirectly that the orienting factors which account for the sameness in direction of the angular displacement of the stroma must reside within the cornea itself. A direct argument in support of this hypothesis can be raised from the present data. Since the primary stroma is acellular, the only components present within the matrix during its formation are the stromal macromolecules themselves. As has been shown, the center-to-center spacing of the collagen fibrils *within a bundle* of fibrils is equal to the 600 A periodicity of the fibrils. Such a similarity could be purely conincidental. Or it might indicate that some kind of interaction is occurring between the collagen fibrils such that the fibrils which lie in one direction influence the packing order of the fibrils which lie orthogonal to them. If such an interaction exists it might explain the sameness in displacement direction for both eyes. Since the collagen molecule is a right-handed super-helix (Ramachandran, 1967), the collagen fibril must also have a "handedness." If the three-dimensional architecture of the primary stroma is a result, in part, of interactions between the collagen fibrils, then it might also be expected to have a handedness. And this handedness would be the same in both eyes and the pattern of the corneal stroma would thus be asymmetric about the mid-plane of the embryo.

If such interactions between the collagen fibrils occur they must be mediated by some kind of bridging molecule since the fibrils in adjacent orthogonal layers are separated by too great a distance (300–1000 A) to interact directly. The glycosaminoglycans (Conrad, 1970; Toole and Trelstad, 1971) in the developing corneal matrix could serve as such bridging molecules and because of their asymmetry (Mathews, 1970) also contribute to the handedness of the matrix. Recent ultrastructural studies have demonstrated that the glycosaminoglycans from a variety of connective tissues, including cornea, interact with the collagen fibrils at periodic intervals along their length equal to the collagen periodicity (Serafini-Fracassini and Smith, 1966; Smith et al., 1967; Smith and Frame, 1969). It has been suggested that the glycosaminoglycan matrix in the cornea may influence the orderly packing of the collagen fibrils (Balazs, 1965; Smith and Frame, 1969).

Although attractive, the self assembly hypothesis cannot explain why the layers of primary corneal stroma deposited between the third and sixth days are undisplaced. If the oriented mechanical forces discussed above, however, are operative between days three and six as suggested, then they might override the inherent displacement tendency of the matrix during this period and cause it all to be deposited in register. The combination of oriented mechanical stress and matrix self assembly could thus satisfy the three requirements outlined at the beginning of this section. Nonetheless, it should be emphasized that these suggested morphogenetic forces are speculations which will require future experimental verification.

Stromal Collagens

Operationally there are at least two kinds of collagen present in the developing corneal stroma. One is produced by the epithelium and is present in the primary corneal stroma. Its function is that of a morphogenetic template and it probably constitutes less than 1% of the total stromal collagen at hatching (Conrad, 1970). The other kind of collagen is produced by the mesenchyme and is present in the secondary or adult corneal stroma. The two collagens also differ in their staining properties in that the primary stroma is strongly argyrophilic, i.e. stains like reticulin, whereas the

secondary stroma is not or is only weakly argyrophilic. Whether these differences in time of ontogenetic appearance, staining properties, cell of origin, and function indicate a difference in molecular composition of the two collagens as has recently been described for collagens from other connective tissues in the chick (Miller and Matukas, 1969; Trelstad, et al., 1970) is impossible to determine without further investigation. Nonetheless, it raises the interesting possibility that molecular heterogeneity exists not only between collagens from different adult tissues, but also within the same tissue at different points in development.

Formation of the Secondary Corneal Stroma

Shortly after the mesenchymal cells invade the primary corneal stroma on day 6 (stage 29) they begin to produce the collagen which will constitute the adult stroma (Coleman et. al., 1965; Hay and Revel, 1969; Conrad, 1970). The manner by which the spatial pattern which is present in the primary corneal stroma is passed on to the secondary stroma is not known. It is possible that the primary corneal stroma orients the mesenchymal cell and that the mesenchymal cell then influences the orientation of the collagen it produces. Another possibility is that the primary stroma could serve directly as a nucleus or template around which the mesenchymally derived collagen precipitated. At present there are no data which would allow selection between these or among other possibilities.

Received for publication 24 November 1970, and in revised form 29 January 1971.

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