

# FIBROBLAST-COLLAGEN INTERACTIONS IN THE FORMATION OF THE SECONDARY STROMA OF THE CHICK CORNEA

JONATHAN B. L. BARD and KAROLE HIGGINSON

From the Medical Research Council, Clinical and Population Cytogenetics Unit, Western General Hospital, Edinburgh EH4 2XU, Scotland

## ABSTRACT

Fibroblasts invade the primary corneal stroma of the 6-day-old chick embryo eye. The way in which these cells build the secondary stroma has been studied by microscope examination of the stroma during the subsequent 8 Days. Eyes were embedded in low viscosity nitrocellulose, and 30- $\mu$ m tangential sections of cornea were cut and stained with azan (giving blue collagen and red cells). These sections were sufficiently thick to include enough cells and collagen for stromal organization to be visible under Nomarski optics. Three days after invasion, the fibroblasts extend along collagen bundles in the posterior region of the stroma; surprisingly, fibroblasts near the epithelium are more rounded. The collagen itself is organized in orthogonal bundles rather than in sheets. Measurements show that posterior bundles increase in size with time while anterior stroma is similar in diameter to primary stroma. These observations confirm that the epithelium continues to deposit primary stroma up to at least the 14th day. They show, moreover, that fibroblasts deposit collagen fibrils on extant stroma and that the farther a bundle is from the epithelium, and hence the longer the period since it was first laid down, the wider it is likely to be. Analysis of the results and existing data on hyaluronic acid levels in the stroma suggests that Bowman's membrane, the region of anterior stroma that remains uncolonized by cells, is, during this period at least, primary stroma laid down but as yet unswollen.

**KEY WORDS** collagen · cornea · fibroblasts · morphogenesis · Nomarski optics

The corneal stroma of the avian eye comprises a complex, but exquisite arrangement of collagen bundles that lies between an anterior epithelium and a posterior endothelium (5). A remarkable feature of this organization is that the collagen above and below a particular level bears a well-defined relationship, usually orthogonal, to that layer. In the newly hatched chick, the collagen is very densely packed. However, the arrangement of collagen bundles permits almost all direct light

to pass through the cornea but causes destructive interference of scattered light (17). Stromal organization therefore appears to be crucial to corneal function.

Much of the work on how the stroma is formed has been reviewed by Coulombre (5) and by Hay and Revel (14). It is now known that the anterior epithelium, under the inductive influence of the lens, first lays down a primary collagenous stroma with an orthogonal arrangement beginning at around the 3rd day (9), that the posterior endothelium forms at about 4 days (3, 14), and that neural-crest fibroblasts invade the stroma, now

swollen by hyaluronic acid (HA), produced by the endothelium, at about 6 days (2, 6). While it is possible to follow some of these early events by observing the tissues directly, from the 7th day onwards the cornea is, for the purposes of direct observation, opaque, and the organization can only be studied in sectioned material. The major observations on later development have been made by the Coulombres' group (5, 6, 7, 21) who have shown that the anterior epithelium continues to deposit layers of collagen fibrils on top of extant stroma, up to at least the 14th day, and that the orientation of these new fibrils is anticlockwise in both eyes with respect to existing stroma. Both the Coulombres' and de Ladijenski have also mentioned that after 8 days the fibroblasts are aligned along the collagen axes (6, 16). With conventional light or electron microscopy, however, it is not easy to study either cell morphology or collagen structure because sections are too thin to include enough material. Thus, little is known of how the secondary stroma is built up from the primary stroma.

In this paper, we report the use of a histological technique employing thick sections that permits the direct study of cell and collagen-bundle morphology in the developing stroma. The technique requires that the eye be fixed and the water replaced with low viscosity nitrocellulose (LVN), a material that allows 30  $\mu\text{m}$  sections to be cut and that can be stained with Heidenhain's azan (giving red cells and blue collagen fibers). Viewed under Nomarski micro-interference optics which allow optical sectioning, both cellular and collagen detail can be seen, and there is sufficient depth in the section for structural relationships to be observed merely by changing focus. A preliminary report of this work has already been published (4).

## MATERIALS AND METHODS

### *Specimen Preparation*

Fertilized white Leghorn eggs were obtained from the East of Scotland College of Agriculture (Easter Howgate, Midlothian). Embryos were staged according to the tables of Hamburger and Hamilton (13). At appropriate ages, whole eyes were excised, fixed in buffered glutaraldehyde overnight, and embedded in LVN (8). The fixed eyes were first dehydrated in ethanol and allowed to stand for 24 h in a 1:1 (vol:vol) ether/methanol mixture. They were then placed for 2 days in each of 5, 10, and 30% mixtures of LVN and 2:1 ether:methanol. The standard LVN mixture contained 140 g of LVN, 210 ml of ethanol, 250 ml of ether, and 2 ml of castor oil

(8). It was prepared a week before use, kept in the dark, and discarded when it became very thick.

For embedding, eyes were placed in 2.5-cm aluminum foil bottle tops, covered with LVN, and allowed to set overnight in a dessicator containing a beaker of chloroform. The blocks were then removed from the foil and mounted on wooden stubs with the cornea uppermost. The blocks were stored for several weeks in 70% ethanol to allow the LVN to harden.

Before cutting on a sledge microtome, the block was trimmed and notched so that sections could later be matched. Great care was taken in orienting the block to ensure that sections would be cut perpendicular to the optic axis of the eye. It was important during section cutting to ensure that both blade and block were well wetted with 70% ethanol. We found that sections much less than 30  $\mu\text{m}$  thick were extremely hard to handle while the optical resolution declined rapidly in sections much thicker than 30  $\mu\text{m}$ . All the observations reported here were therefore made on 30  $\mu\text{m}$  sections.

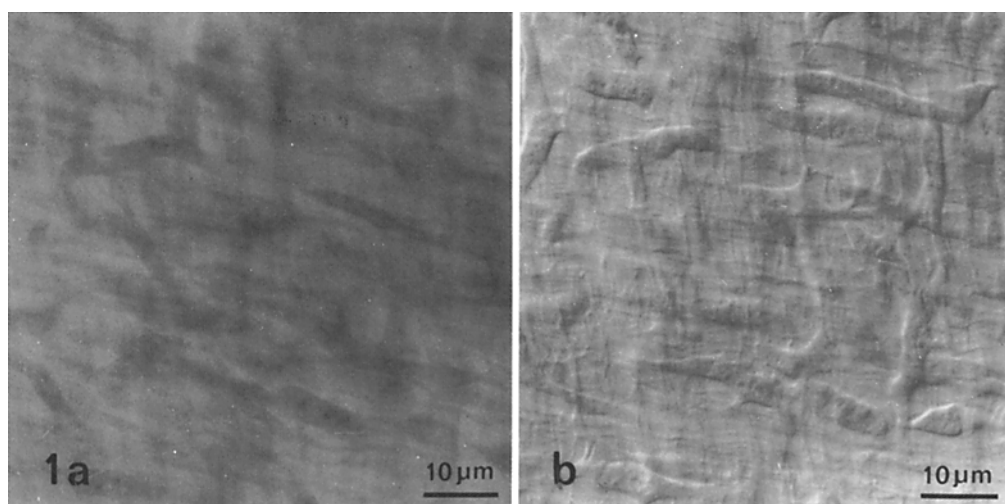
The sections were transferred through graded alcohols to water, stained with Heidenhain's azan (8), oriented on glass slides, and mounted in D.P.X. (Baird and Tatlock Ltd., Essex, England). Two to five sections (depending on the age of the embryo) were found to include the central part of the cornea from the thin, external periderm to the endothelium. The thickness of corneas after embedding in LVN is about 20% less than after embedding in Araldite. About 50 eyes, between 5 and 21 days of age, were sectioned and examined. There were no obvious differences between corneas of the same age. For stages where measurements were made, serial sections of three corneas were each extensively photographed.

### *Microscope Techniques*

Sections were viewed on a Zeiss Universal microscope fitted with Nomarski optics, an automatic camera, and a rotating stage. With a 100  $\times$  planchromat objective and an oiled condenser, the optical resolution was good in the top 10  $\mu\text{m}$  or so of the sections, but declined as the plane of focus was lowered: the depth of focus itself was about 2–3  $\mu\text{m}$ .

It is remarkable how much more detail is visible under Nomarski than under bright-field optics; in the latter case, one sees red cells with blurred outlines and barely visible nuclei and ill-defined blue strands of collagen (Fig. 1a). Under Nomarski optics (Fig. 1b), however, the outlines of the cells are clearly defined; moreover, the collagen bundles now stand out, the edges in particular being sharp; it is sometimes even possible to see detail within bundles.

Contrast in specimens viewed under Nomarski optics derives from the interference between two polarized beams of light that come from a single source, so that they pass through the specimen about 0.1  $\mu\text{m}$  apart and are then recombined (1). Where the optical paths of the



**FIGURE 1** (a) A region of 14-day-old chick corneal stroma embedded in LVN and stained with azan. The shapes of cells and collagen bundles can just be resolved. (b) The same area of the stroma viewed under Normarski optics. Cellular outlines and bundle morphology are now far clearer.  $\times 1,000$ .

two beams are different, there will be interference. The final image therefore shows how the optical path length of the specimen changes over the field. In the hydrated cornea, the refractive indices of water, collagen and cells are different, and there is sufficient space between, say, collagen bundles to permit them to stand out from the background. After the cornea condenses (15 days), two factors affect image formation. First, there is little water and hence the collagen is compacted; this causes a reduced difference between the optical paths of the two beams, resulting in poorer discrimination. Second, the material is so dense that the light is scattered and loses its polarization so that the interference effect is further lost. The optical technique therefore gives good contrast and resolution only where the specimen is hydrated.

For photography, specimens were rotated so that the Wollaston prism (that splits the beam) was at  $45^\circ$  to the collagen bundle axes. All bundle measurements were made with a Brinell eyepiece from prints magnified to  $1,000\times$ . It was estimated that the accuracy of measurement was  $0.2\ \mu\text{M}$ .

## RESULTS

The observations reported in this paper were made on axial regions of  $30\ \mu\text{m}$  thick, transverse, LVN sections of chick corneas viewed under Normarski optics. The study concentrates on the period between 6 and 14 days of development. At the start of this interval, two events occur: first, the corneal stroma swells as a result of HA secretion by the endothelium, and second, neural-crest fibroblasts migrate into this swelling stroma (18, 20). At the end of the period the cornea

condenses, presumably because water is excluded as hyaluronidase breaks down HA (20).

While observations have been made on corneas of all developmental ages beyond 4 days, most attention has been paid to the initial swelling (6 days), the last stage before condensation (14 days), and an arbitrary, intermediate stage (10 days); the results are grouped accordingly.

### *Basic Morphology of the Cornea*

The chick cornea is a complex, stratified organ (Fig. 2a). On the outside is a thin periderm (a monolayer of cells during the first 2 wk of development) that covers a thick epithelium; on the inside is another epithelium known as the endothelium (14). Sandwiched between these epithelia is the corneal stroma: organized collagen bundles in a matrix containing glycosaminoglycans (22). Fibroblasts colonize the stroma except for the anterior few micrometers, the region known as Bowman's membrane, which remains free of cells. At the interface of the stroma and the endothelium there is, from the 10th day onwards, a roughly hexagonal arrangement of nodes about  $0.1\ \mu\text{m}$  apart known as Descemet's membrane (14).

Before describing the changes that occur in the collagenous stroma and its fibroblast population, let us summarize briefly the appearance of the cellular layers of the fixed cornea which, at the resolution of the technique, remain unaltered during the period considered. Anterior peridermal

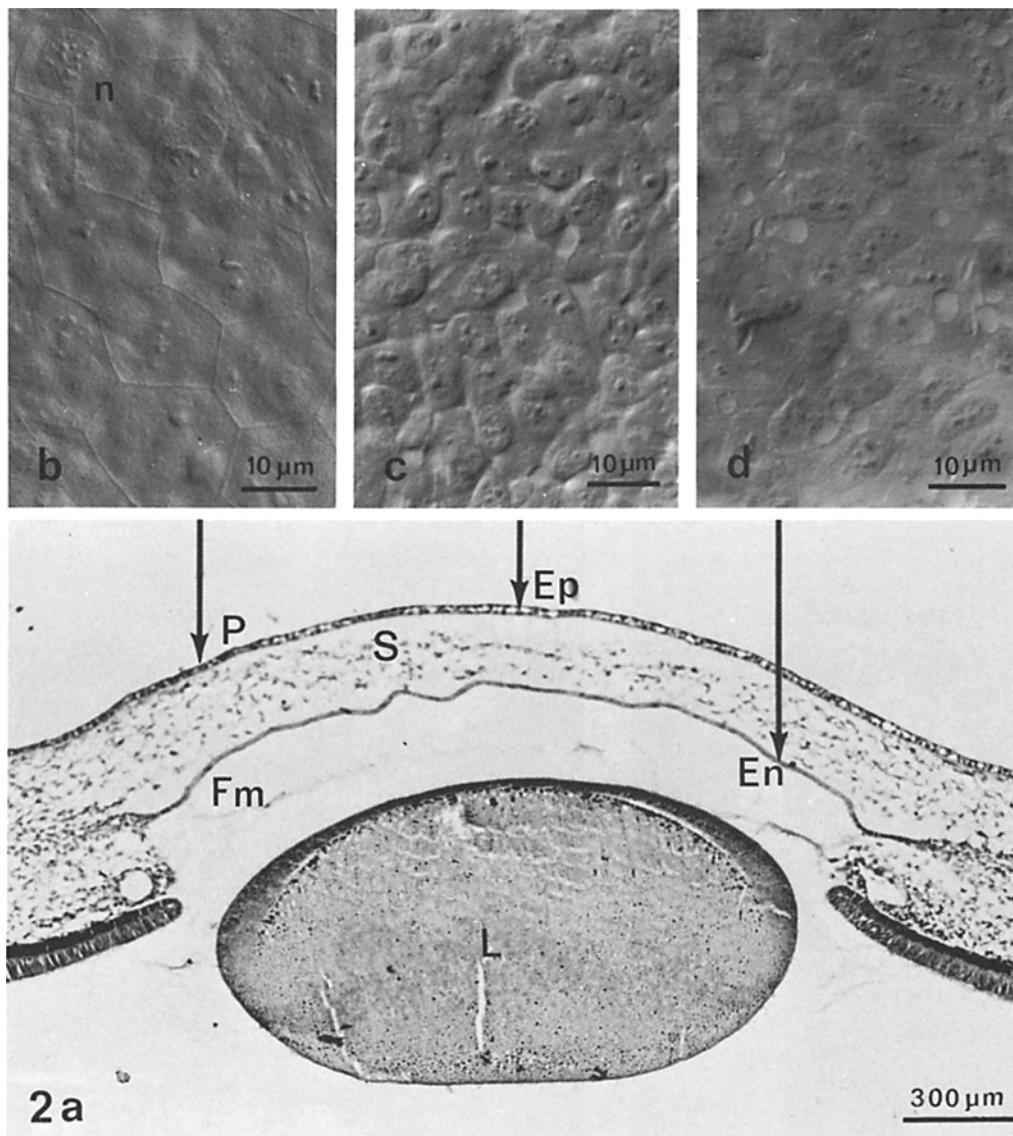


FIGURE 2 (a) A micrograph of a wax-embedded section of a 7-day-old eye stained with hematoxylin. On the exterior of the cornea is a thin periderm (*P*) covering the epithelium (*Ep*). Fibroblasts are invading the stroma (*S*); note that they first colonize basal stroma. Between the lens (*L*) and the cornea is the anterior chamber in which fibrous matrix (*Fm*) can be seen. (b-d) Transverse LVN sections of the periderm, endothelium, and epithelium viewed under Nomarski optics. Nuclei (*n*) are often very clear in the periderm cells. (a)  $\times 150$ , (b-d)  $\times 1,000$ .

cells (Fig. 2b) are very large (some 20  $\mu\text{m}$  across), thin ( $\sim 2 \mu\text{m}$ ), and roughly hexagonal or pentagonal in shape. The cells of the epithelium, in contrast, are columnar, being about 6  $\mu\text{m}$  in diameter and about 30  $\mu\text{m}$  thick (Fig. 2c). Occasional metaphase figures have been observed in the epithelium but not in the periderm. At the interface

between the epithelium and the stroma, the region of Bowman's membrane, virtually no detail is visible. Likewise, there is no feature identifiable with Descemet's membrane. The endothelium, however, is clearly visible; the cells are very thin, about 8  $\mu\text{m}$  across, and granular (Fig. 2d). Within the anterior chamber, the fibrous matrix known

to be present from scanning electron microscope observations (3) is unstained by azan (suggesting that it is not collagen) and is not visible in LVN sections. Indeed, the periderm is the only cell sheet that is better displayed by Nomarski optics than by other microscope techniques. The situation is very different when the stroma is considered.

### *Stromal Development*

**INITIAL FIBROBLAST INVASION, STAGE 28:** At around the 6th day of development, fibroblasts invade the swelling cornea, migrating from all points on the circumference (Fig. 3*a*) and initially colonizing the basal stroma. While many cells tend to be parallel to the epithelial surface and point radially inwards, some cells are observed in all orientations. Fibroblasts, as reported earlier (2), show a variety of forms in the stroma. The majority are elongated with a long anterior process that may split at its leading point into small filopodia. The posterior trailing end may also be long but is often short, giving the cells a pear-shaped appearance. Within cells, organelles can be seen. In addition to extended fibroblasts, there are many rounded-up, dividing cells whose metaphase plates are often strikingly clear. (An example of such a cell in a 10-day-old cornea is shown in Fig. 4*c*).

At higher magnification, with a 100 × objective, the organization of the collagen bundles can be seen. Uncolonized and apparently unswollen stroma consists of closely packed collagen bundles,<sup>1</sup> all of which are either parallel or perpendicular to the choroid fissure (Fig. 3*b*). In colonized

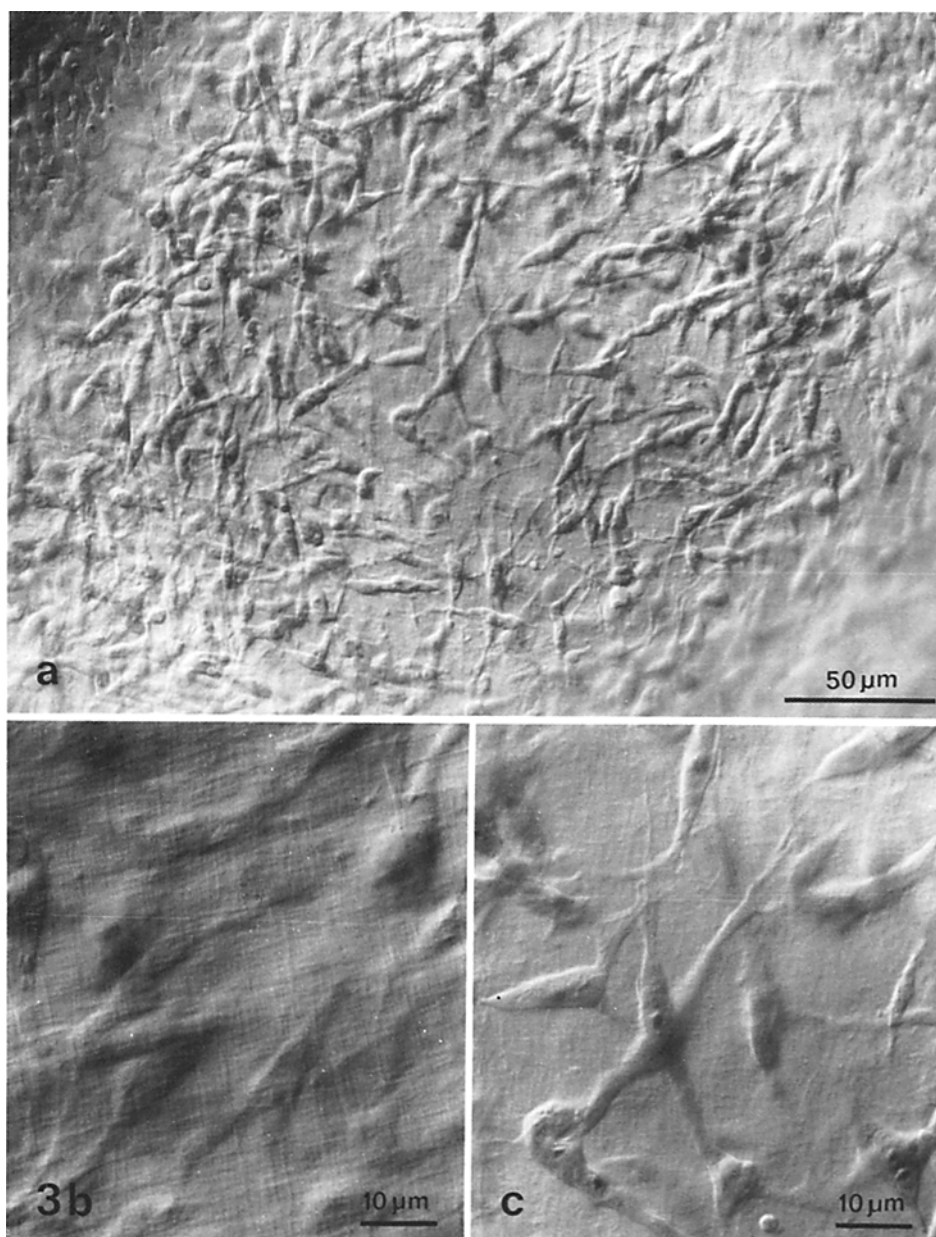
stroma, the collagen bundles are farther apart, and the array appears less regular than before (Fig. 3*c*). This difference could derive both from the swelling and from cell movement that disturbs the lattice. The diameters of the collagen bundles are the same before and after colonization. Almost all the fibrils are 0.3–0.5 μm thick as measured from photographic prints (Fig. 5). This value is, however, near the theoretical resolution for the thick specimen, as a single bundle has two edges each of which is a source of contrast under the micro-interference optics. As the Wollaston prism splits the beam by about 0.1 μm (1), it is possible, first, that up to 0.2 μm of the measured diameter is due to image spreading from the optical system and, second, that bundles of diameter less than 0.1 μm are not resolved. For these reasons, the true diameters of these fine bundles may be a little less than those measured and the proportion of bundles at the narrow end of the histogram may be too low.

**INTERMEDIATE DEVELOPMENT 8–10 DAYS:** The first clear evidence that the cells do not remain passive within the stroma comes on the 9th day: fibroblasts now take up an orthogonal arrangement that is clearly different from the initial radial distribution. Indeed, on the 8th day there are indications of this in the basal section of the stroma. By 10 days, the change in cellular organization is striking: in sections near the endothelium almost all the fibroblasts remain elongated, are parallel to the epithelium, and extend along the collagen bundles that are parallel and perpendicular to the choroid fissure (Fig. 4*a* and *c*).

Surprisingly, the morphology of a fibroblast in the 10-day-old cornea depends on its position in the stroma. While basal fibroblasts are elongated, cells near the epithelium are more rounded and seem to lack processes (Fig. 4*b*). Furthermore, the orientation of cells rotates anticlockwise as one focuses upward in the specimen towards the epithelium. At this stage, all of the rotation is in the top LVN section of the stroma and the change in cellular orientation are seen merely by focusing up and down. Earlier work (6, 7, 20) has shown that the orientations of collagen bundles deposited by the epithelium at this stage show such rotations; clearly, the cells are aligning themselves along these bundles.

Collagen organization in superficial sections of 10-day-old eyes is not, however, readily resolvable as the material seems to be very closely packed

<sup>1</sup> The term *bundle* is used to describe the separate morphological units seen in Nomarski micrographs. Superficially, they appear to be strands, but electron micrographs (14, 21) show that this word is inappropriate as even the finest unit in the primary stroma is a bundle of five or so fibrils, each about 22 nm in diameter and another 20 nm apart (a total of about 0.2 μm). In older eyes, there may be many hundreds of fibrils grouped together in a single parallel array. In 14-day-old eyes, viewed under Nomarski optics, the wider bundles often seem to be made of several finer ones, and there is even occasional branching. For the purpose of measurement, such a bundle is considered to be a single unit as it is not possible to resolve ground substance between the strands. Moreover, since there has been no condensation to encourage bundles to coalesce, there is no reason to suppose that these strands were originally separate. It is not, however, clear how such bundles were formed.

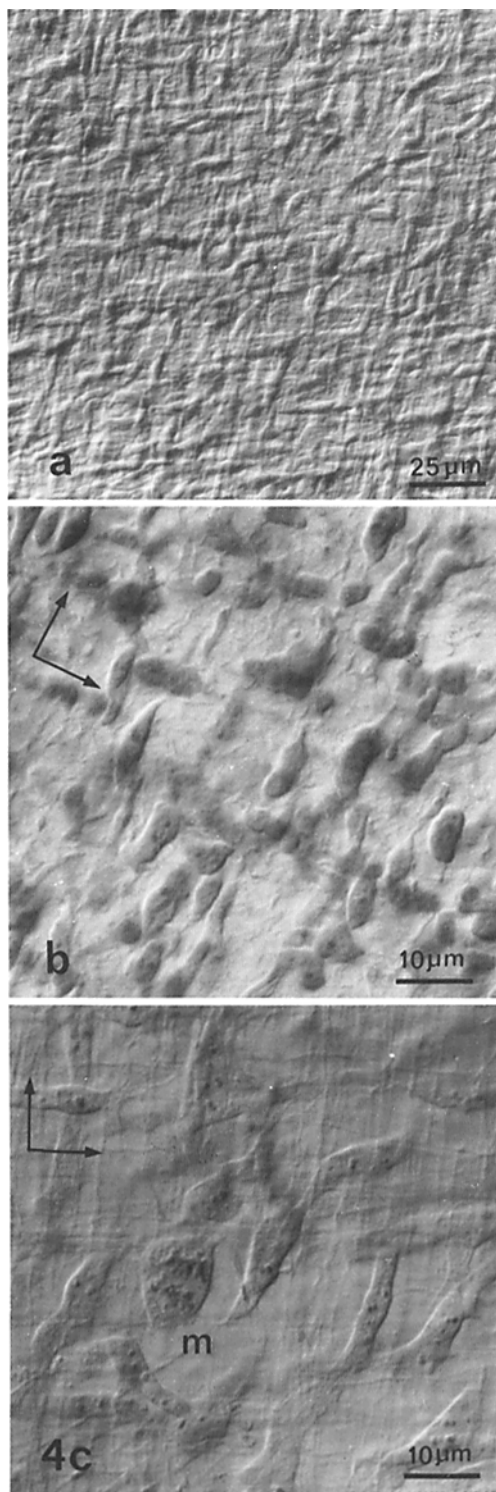


**FIGURE 3** (a) A low power micrograph of an LVN section of a 6-day-old (stage 28) stroma viewed under Nomarski optics. Fibroblasts are migrating into the stroma from all points of the periphery. (b) A high power view of the unswollen, anterior stroma of a stage-28 eye. Collagen bundles are thin and closely packed. Note that the fibroblasts are below the plane of focus. (c) A high power micrograph of swollen stroma of a stage 28 cornea that is colonized by fibroblasts. The collagen bundles are still thin but are now less densely packed. (a)  $\times 400$ , (b and c),  $\times 1,000$ .

(Fig. 4b). Those bundles that can be seen are relatively fine ( $<0.8 \mu\text{m}$ ).

Considerably more detail of the stromal collagen is visible in basal sections of 10-day-old eyes (Fig.

4c). Bundle diameters are much wider than they were in 6-day eyes. They now range from  $0.4$  to  $3.0 \mu\text{m}$  with a mean of  $0.83 \mu\text{m}$  (Fig. 5). There are fewer bundles of diameter less than  $0.4 \mu\text{m}$



and roughly the same proportion in the range 0.4–0.6  $\mu\text{m}$  in 10-day as compared to 7-day eyes. About 60% of the 10-day bundles are wider than those seen in 7-day eyes.

In these basal sections, the majority of fibroblasts extend along collagen bundles (Fig. 4c). A minority of cells, however, lie at an angle to the collagen axes and appear to adhere to bundles in both directions. Occasional fibroblasts are L-shaped: half of a cell may extend on collagen bundles in one direction while the other half adheres to nearly orthogonal strands. In 8–10-day eyes, frequent mitoses are seen (Fig. 4c).

**STROMAL MORPHOLOGY BEFORE CONDENSATION:** At 14 days, the appearance of the stroma is similar to that of the 10-day eye. Fibroblasts near the epithelium are rounded (Fig. 7a) while those in the center (Fig. 7b) and those near the endothelium (Fig. 7c) are elongated. Basal fibroblasts still tend to be parallel and orthogonal to the axis of the choroid fissure, but there is a greater region of stroma over which the axes of orientation of the cells move anticlockwise (as one approaches the epithelium). The changes in direction are in accord with those described for collagen bundles by Trelstad and Coulombre (21). One noticeable difference, in contrast to the 10-day corneas, is that only occasional dividing cells are seen in the 14-day stroma. A further difference is that in the anterior sections of the 14-day stroma (Fig. 7a), collagen bundles can be resolved far more clearly than in the same region of a 10-day eye (for reasons that are not known).

It turns out that virtually all of the 14-day stroma is included in three thick sections; and as collagen bundles can be resolved in each of them, it has been possible to see how their diameter

**FIGURE 4** (a) A low-power micrograph of the basal section of a 10-day-old stroma. The fibroblasts are now oriented parallel and perpendicular to the choroid fissure axis (the vertical axis of the picture for all three micrographs). (b) A high power micrograph of the anterior section of the same eye. The cells are oval and lack processes, and the collagen of the stroma is not visible. Note that the axes of the cellular orientations are rotated some 60° anticlockwise with respect to the axis of the choroid fissure (arrows). (c) The basal section of the same cornea. Basal fibroblasts are elongated and tend to align along the collagen bundles which are now parallel and orthogonal to the choroid fissure. A dividing cell (m) shows groups of anaphase chromosomes. (a)  $\times 400$ , (b–c)  $\times 1,000$ .

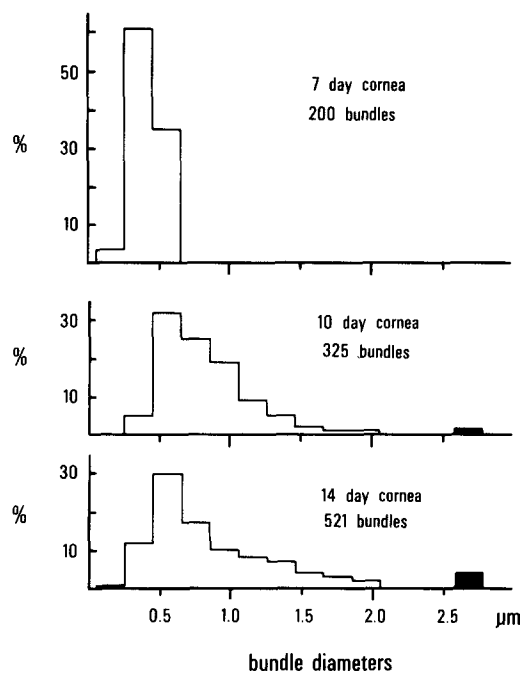


FIGURE 5 A histogram of the diameters of collagen bundles from three basal sections of 7-, 10- and 14-day-old stromas. Bundles of diameter greater than  $2.0\ \mu\text{m}$  have been grouped. This peak is centered about the mean diameter of such bundles and is shown in black. In 10-day-old corneas, about 40% of the bundles are in the range found for 7-day-old stromas, most of the remaining bundles are less than  $1.0\ \mu\text{m}$  across. In 14-day-old stroma, some 40% of the bundles are again in the range covered by 7-day-old stroma. Over half of the larger bundles have diameters greater than  $1.0\ \mu\text{m}$ . The widest bundles were  $4.0\ \mu\text{m}$  across.

distribution varies over the stroma (Fig. 6). Narrow bundles, typical of primary stroma, comprise about 83%, 60%, and 42% in the outer, middle, and inner sections, respectively. In the outer sections, there are virtually no bundles wider than  $0.8\ \mu\text{m}$ , showing that there is little difference between this stroma and 7-day primary stroma. This is an expected result as the endothelium continues to deposit new primary stroma on top of older stroma, and the outer section thus contains this recently deposited collagen. The middle and inner populations differ in that there are many more thick bundles in the latter than in the former: some 15% of the inner bundles are more than  $1.5\ \mu\text{m}$  wide as compared to 5% in the middle sections. As the inner section contains the original primary stroma, a simple conclusion may be

drawn: the older the bundle, the wider it is likely to be.

It is noticeable that there are considerably more thick bundles in the basal section of the 14-day stroma than in the corresponding section of the 10-day stroma (Fig. 5). Bundles in the 14-day stroma may be up to  $4.0\ \mu\text{m}$  wide, and it is sometimes possible to resolve detail within them. Large bundles ( $<2.5\ \mu\text{m}$ ) often appear to be composites of two or more finer bundles (see arrows on Fig. 7c), rather than a single wide uniform band. This impression is confirmed by the occasional branching that is seen when a large bundle becomes two smaller ones.

## DISCUSSION

The observations presented in this paper concern the interaction between primary collagen stroma and the fibroblasts that colonize it. After this invasion the secondary adult stroma is laid down. The discussion focuses first on the growth of collagen bundles and, in turn, on the development of the stroma and the morphological significance of

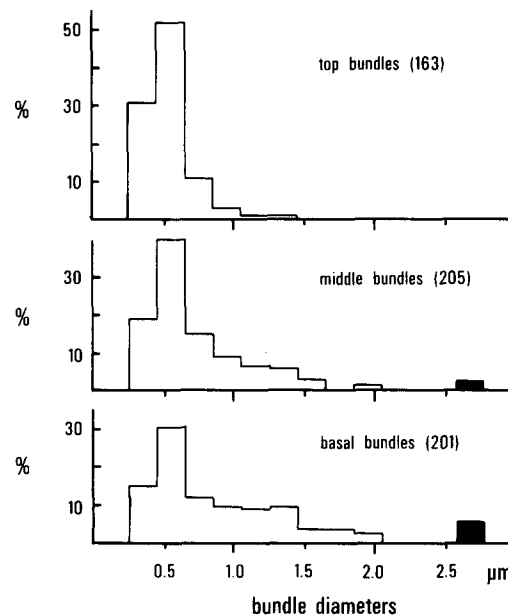


FIGURE 6 A histogram of the diameters of collagen bundles from a single 14-day stroma. Bundles of diameter greater than  $2.0\ \mu\text{m}$  are grouped and shown in black. In the diameter range of primary bundles ( $<0.6\ \mu\text{m}$ ) are found 85%, 60%, and 42% of outer, middle, and inner bundles, respectively. Many more inner than middle bundles have a diameter greater than  $1.5\ \mu\text{m}$ .



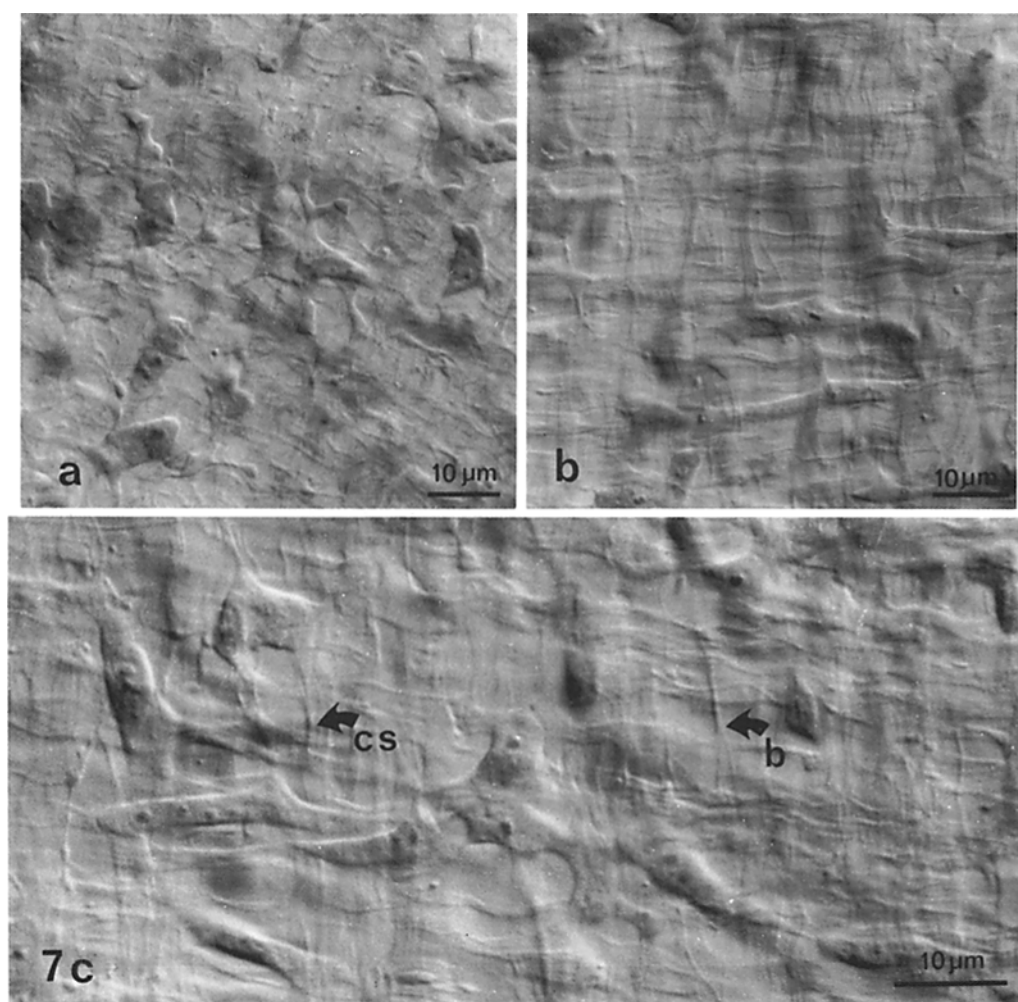


FIGURE 7 Micrographs of the top (*a*), the middle (*b*) and the basal section (*c*) of a 14-day-old cornea. Bundle diameters increase in size and cells become more elongated as one approaches the endothelium. Particularly thick bundles may have a composite structure (*cs*) and bundles may also bifurcate (*b*). All micrographs are oriented so that the choroid fissure axis is parallel to the vertical axis of the figure. (*a-b*)  $\times 1,000$ ; (*c*)  $\times 1,500$ .

Bowman's membrane. The latter part of the discussion is concerned with the behavior of the cells and, in particular, with how their morphology is influenced by the collagen organization.

#### *Growth of Secondary Stroma*

The results show that, after fibroblast invasion of the cornea, the width of the collagen bundles increases and that, in basal sections particularly, the older the eye the wider the bundles are likely to be. It is not possible to determine unequivocally from morphological considerations alone whether

the epithelium or the fibroblasts are the source of the new collagen that is deposited on the existing bundles. (There is evidence that the only other candidate, the endothelium, does not produce collagen [18].) However, several considerations make it likely that the majority of this collagen is produced by the fibroblasts. Were the collagen produced by the epithelium, the very long molecules would have to diffuse from the anterior of the stroma towards the endothelium through a dense mass of bundles before aggregating preferentially on basal rather than anterior collagen.

This is most unlikely to occur and, in fact, the newly synthesized epithelial collagen continues to form primary stroma (7). Fibroblasts, on the other hand, are ubiquitous within the stroma, and no significant diffusion of collagen molecules is necessary for their assembly into fibrils and deposition on existing bundles.

Evidence that no new bundles are produced within the stroma comes from the histograms of bundle diameters (Figs. 5 and 6). The fact that the proportion of fine bundles is greater in middle than in basal 14-day-old stroma suggests that, as time passes, primary bundles are increasingly likely to have secondary stroma deposited on them. As the same proportions of fine bundles are present in 10- and 14-day-old basal stroma, it is probable that no new fine bundles are laid down in this region in the intervening 4 days. The observations thus imply that the collagen synthesized by the fibroblasts is deposited on and enlarges about 60% of the original primary bundles.

It therefore seems likely that the fibroblasts lay down collagen on the primary stroma rather than depositing new bundles. If so, this confirms a suggestion put forward by de Ladijenski over 60 yr ago. The measurements on bundle diameters also confirm a prediction implicit in the work of Coulombre and Coulombre (7) who showed that the epithelium continues to deposit new primary stroma up to the 14th day of development at least. Assuming that fibroblasts lay down collagen on extant bundles at a rate independent of stromal location, one would expect that the diameters of posterior (older) collagen bundles will be greater than those of the anterior (recent) stroma. The measurements (Fig. 6) confirm this expectation.

More unexpected was the observation that, up to 14 days at least, the collagen fibrils are grouped in bundles rather than in continuous sheets. Clearly, the stroma is not initially laid down in layers of closely packed thin fibrils as in, for example, the amphibian cornea (5). As bundles of both orientations are seen within a single plane of focus ( $\sim 2 \mu\text{m}$ ), it is not possible to decide from examination of LVN sections whether all the bundles in a single plane are parallel or not.

### *Stromal Development and Bowman's Membrane*

One can reconstruct the development of the post 7-day-old stroma by following the progress of a hypothetical layer of primary stroma immedi-

ately after it has been laid down by the epithelium. This layer, initially tightly packed like unswollen 4-day-old stroma viewed in the electron microscope (14, 21), will swell as HA made by the endothelium (18, 20) slowly reaches it; as it swells, fibroblasts will colonize this newly available substratum. In due course, the bundles in the layer widen as new collagen fibrils are deposited on them by the fibroblasts. Meanwhile, the epithelium has continued to deposit primary stroma on top of the original layer. What was primary stroma has now become a layer of secondary stroma.

There is a corollary to this dynamic analysis that concerns the region of stroma known as Bowman's membrane. This view predicts that there should be an area of primary stroma, just below the epithelium, that is slowly swelling and is not yet invaded by cells. Bowman's membrane is between the epithelium and the swollen stroma; it is, at this stage, composed of dense, orthogonal fibrils and is uninvaded by fibroblasts. One may therefore suggest that it is a transition phase for collagen laid down by the epithelium but as yet unswollen and uncolonized by cells.

Further evidence consistent with this view comes from the work of Hay and Revel (14). They have measured the thickness of Bowman's membrane with considerable care and find that it narrows from  $20 \mu\text{m}$  at stage 28 to about  $2 \mu\text{m}$  at stage 35 (9 days). The latter thickness is maintained until stage 40 (14 days), just before condensation. During this period, it is composed mainly of orthogonal collagen fibrils. After condensation occurs at around stage 40, Bowman's membrane starts to thicken again. Such behavior correlates directly with the synthesizing activity of the endothelium which starts to produce HA at around stage 28, the stage at which Bowman's membrane starts to narrow. This activity ceases at around stage 40 when hyaluronidase breaks down the stromal HA (20) and the membrane starts to thicken again. It therefore seems that when HA is being synthesized, Bowman's membrane has constant narrow thickness, but that when HA is absent, Bowman's membrane widens. This widening after condensation, incidentally, suggests that the epithelium continues to deposit primary stroma.

The morphology of Bowman's membrane, as hatching approaches, changes a little (14) as oblique collagen fibrils are laid down within the orthogonal matrix. It is not clear whether such fibrils are deliberately oriented or whether there is no room within the highly condensed stroma for

normal morphogenesis to occur. It is, however, noteworthy that in the human eye the post-epithelial orthogonal collagen organization is largely maintained (15). It therefore seems reasonable to suggest that Bowman's membrane is uninvaded, unswollen primary stroma and that, in the adult, it was mainly laid down by the epithelium after condensation.

### Cellular Behavior

Turning now to the morphology and morphogenetic activity of the fibroblasts that invade the cornea, the data presented here confirm the original observation of de Ladijenski (16) and Coulombre and Coulombre (6) that fibroblasts align along the axes of the collagen bundles. The observations raise additional questions as to why the cells take so long to orient themselves and why the morphology of a fibroblast depends on its location in the stroma.

Consider the first problem. It takes some 48 h or more for the cells migrating in from the corneal circumference to align themselves orthogonally along the bundles. This is a surprisingly long time, considering that fibroblasts plated in vitro onto aligned collagen, collagen far less well aligned than that in vivo, elongate themselves along the orienting axis in about 12 h (10, 11).

One possibility for the delay is that cells may fail to distinguish the two aligning axes in densely packed stroma, and may remain randomly oriented until the bundles become separated enough for an individual cell to sense directed bundles on which to extend. In addition, single primary bundles may be too fine to take the strain of a cell's adhesion or to accommodate all of the fine filopodia on the cell's leading process until further collagen has been deposited to widen and strengthen them.

Such explanations suggest reasons why fibroblasts take some time to align themselves along collagen bundles. They fail, however, to explain the differences in morphology between extended, posterior, and rounded anterior cells in the 10- and 14-day-old stroma. Assuming that the fibroblasts form a single population, the explanation of this phenomenon cannot depend on bundle diameters alone, as the cells are elongated both in the 6-day-old eye with its fine, stromal bundles and in the basal part of the 10-day-old stroma which has broad, collagen bundles.

The morphology of cells in different parts of the stroma from 10 days onward may, however, de-

pend on the density of bundle packing rather than on bundle diameters. While the fine 7-day-old bundles are relatively loose when fibroblasts colonize them, those in the anterior 10- and 14-day-old stroma are so tightly packed that it is hard to distinguish them in LVN sections. It seems likely that late primary stroma is, when first laid down by the epithelium, too densely packed for the cells to migrate into it. Only after a small amount of swelling has occurred will cells insinuate themselves and colonize this stroma. The compact morphology of anterior fibroblasts could well derive from this stroma still remaining too dense for cells to extend long processes. The fibroblasts will only take up a bipolar morphology when more HA has diffused in, causing further loosening of the stroma (cf. anterior and middle micrographs of 14-day-old stroma, Fig. 7a and b). It is significant that the HA responsible for the swelling is produced by the endothelium (18, 19) and has therefore to diffuse across the cornea before the anterior stroma will swell.

### Cellular Morphogenesis

Perhaps the simplest aspect of stromal morphogenesis is the question of why the fibroblasts take up an orthogonal array. The simplest possible explanation, that cells use collagen bundles as a substratum for extending, is likely to be correct, accepting, as discussed earlier, that some deposition of fibroblast-synthesized collagen on the primary stroma may first be necessary to strengthen the bundles. Aligned collagen will certainly orient cells in vitro (11), and one need invoke no more than the concept of "contact guidance" (23) to understand how the cellular pattern derives from the template of the primary stroma. The question of how the orthogonal organization of the cells is created thus reduces to the question of how orthogonal primary stroma is laid down by the epithelium in the first place.

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