EPITHELIAL COLLAGENS AND GLYCOSAMINOGLYCANS IN THE EMBRYONIC CORNEA

Macromolecular Order and Morphogenesis in the Basement Membrane

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ABSTRACT

The embryonic corneal epithelium synthesizes both collagen and chondroitin sulfate and excretes them across the basement membrane into the subepithelial space where they assemble into a spiraling orthogonal matrix of fibrils. The assembly of collagen into fibrils is first apparent at the outer face of the basement membrane in a region of ordered chondroitin sulfate molecules. Hyaluronate, another morphogenetically important corneal macromolecule, is produced at these early stages only by the inner endothelium. These correlated biosynthetic and ultrastructural data demonstrate discrete macromolecular products of the two corneal epithelia with differing morphogenetic properties and functions.

INTRODUCTION

The collagen fibrils in the adult chick cornea are organized into orthogonal layers which are gradually displaced from each other a few degrees in a clockwise direction when viewed from the outer layers. This angular displacement of the matrix involves a net shift of over 200° and has the same clockwise handedness in both eyes (Coulombre, 1965). The development of this orthogonal, spiral-like pattern is dependent on an acellular connective tissue which the embryonic corneal epithelium deposits beneath its basal surface during early development. From ultrastructural evidence it was suggested that one of the factors responsible for the morphogenesis of this matrix is its capacity for extracellular self-assembly near the epithelial cell surface (Trelstad and Coulombre, 1971), and subsequent experimental work using inhibitors of both collagen and glycosaminoglycan (GAG) production supports this suggestion (Coulombre and Coulombre, 1972; 1974). Previous studies of the GAG present in the developing cornea also have demonstrated the early predominance of hyaluronate and several important morphogenetic events, including the invasion of mesenchyme and the onset of transparency, have been shown to correlate temporally with the presence and removal of this macromolecule (Toole and Trelstad, 1971).

In continuing the study of the morphogenesis of

the chick cornea we now report (*a*) a partial characterization of the matrix macromolecules synthesized by the epithelium, mesenchyme, and endothelium, and (*b*) the ultrastructure of the basement membrane separating the basal surface of the epithelial cell from the most recently formed orthogonal collagen fibrils. It is into this narrow zone that the macromolecules are excreted from the epithelial cell, either to form the basement membrane or to traverse it and then precipitate into fibrils with an orthogonal orientation. The results indicate that the outer corneal epithelium produces, in addition to collagen, sulfated GAG, chiefly chondroitin sulfate, which is distributed within the basement membrane in an ordered pattern. The endothelium, on the other hand, does not synthesize sulfated GAG but appears to be the major source of hyaluronate during early corneal morphogenesis.

MATERIALS AND METHODS

Fertilized White Rock eggs were incubated in a humidified incubator at 37.5° to the desired stages. Embryos were removed after various times of incubation and staged according to Hamburger and Hamilton (1951).

Electron Microscopy

ROUTINE FIXATION: Tissues for routine electron microscopy were fixed in 4% paraformaldehyde-2.5% glutaraldehyde in 0.1 M sodium cacodylate at pH 7.5 with 8 mM calcium (Karnovsky, 1965) for 10–15 min at room temperature. The tissues were then washed in cacodylate buffer for 20 min and fixed in 1.3% osmium tetroxide buffer with 0.2 M collidine at pH 7.2 for 1 h at 4°C. After fixation the tissues were dehydrated in a graded series of cold ethyl alcohol, infiltrated with an equal mixture of the final embedding mixture and propylene oxide, and embedded in Araldite (6005 Ciba Products, Summit, N. J.). Tissues were sectioned on a Porter-Blum MT-1 (Ivan Sorvall, Inc., Newtown, Conn.) and examined, after staining with uranyl acetate and/or lead citrate, in an RCA EMU 3G or JEM 100B electron microscope.

RUTHENIUM RED STAINING: Tissues were fixed in the 4% paraformaldehyde-2.5% glutaraldehyde described above to which ruthenium red (Polysciences, Inc., Warrington, Pa.) was added at a concentration of 1 mg/ml (Luft, 1971). In some cases the fixative also contained 0.3% Alcian blue 8GX (Allied Chemical Corp., Morristown, N. J.). The tissues were fixed for 15–20 min at room temperature and then washed with the cacodylate buffer containing ruthenium red (1 mg/ml). Tissues were postfixed in the 1.3% osmium tetroxide fixative described above to which ruthenium red was added at 1 mg/ml, and then some tissues were additionally stained en bloc with uranyl acetate as follows: the fixed tissues were washed in a 0.2 M collidine buffer, pH 6.1, for approximately 20 min at room temperature and stained in a 2% uranyl acetate solution in 0.2 M collidine, pH 6.1 (final pH 5.0), for 1.5 h at 4°C. After staining, the tissues were washed again with the 0.2 M pH 6.1 collidine buffer with two changes, dehydrated, and embedded.

LANTHANUM STAINING: Tissues were fixed in the 4% paraformaldehyde-2.5% glutaraldehyde fixative described above plus 0.3% cetyl pyridinium chloride or 0.3% Alcian blue (Shea, 1971). Tissues were fixed for 15 min at room temperature, washed after fixation in the cacodylate buffer, and postfixed in 1.3% osmium tetroxide in 0.1 M collidine pH 8.0 containing 1% lanthanum nitrate (Polysciences, Inc.) for 1.5 h at room temperature (Revel and Karnovsky, 1967). After the lanthanum staining, the tissues either were dehydrated immediately and embedded or were stained en bloc with uranyl acetate as described above and then dehydrated and embedded.

ENZYME-TREATED TISSUES: In order to establish the nature of the material staining with ruthenium red, dissected corneas from 6-, 9-, and 12-day embryos were digested with either Chondroitinase ABC (Miles Laboratories, Inc., Kankakee, Ill.) or leech Hyaluronidase (Biotrics, Inc., Arlington, Mass.) before processing. The digestions with both enzymes were done in Hanks' balanced salt solution at pH 7.2 for 3 h at 37°C. After digestion, the tissues were fixed in ruthenium red-containing fixatives and processed as described above.

Biochemistry

PREPARATION AND INCUBATION OF TISSUES: The collagens and GAG synthesized by the isolated corneal epithelium, mesenchyme, and endothelium were studied in corneas removed from stage 25 to stage 32 embryos. The epithelia of stages 25–29 were removed by treatment of dissected corneas with 0.04% EDTA in balanced salt solution at room temperature for 30 min (Dodson and Hay, 1971) followed by microdissection.

The biosynthetic activities of the endothelial cells in respect to GAG synthesis were evaluated in stage 26 corneas in which the only cellular components are epithelium and endothelium. The epithelium was removed with EDTA, thus leaving only endothelium attached to the acellular primary stroma.

The collagen synthesized by corneal mesenchyme was evaluated in stage 30–32 organ cultures of corneal stromas prepared by removal of epithelia and endothelia by both EDTA treatment and mechanical scraping.

The tissues were labeled in 1 ml of F-12 medium plus 10% fetal calf serum containing either 50 μCi of [^{3}H]acetate for GAG or 50 μCi of [^{3}H]proline for collagen. Ascorbic acid (100 μg/ml) and β-aminopropionitrile (β-APN) (50 μg/ml) were added along with the [^{3}H]pro-

line. The tissues were incubated at 37°C in 5% CO_2, 95° air for 16 h. The tissues and medium were harvested together and analyzed for GAG (Toole and Gross, 1971) and collagen (Trelstad et al., 1973) as previously described and as outlined in abbreviated form below.

GLYCOSAMINOGLYCANS: Analysis of the GAG synthesized involved incorporation of [^{3}H]acetate followed by digestion with papain to degrade protein, dialysis, and precipitation along with carrier GAG by addition of cetyltrimethyl ammonium bromide, with or without prior digestion by streptococcal or testicular hyaluronidases selectively to remove specific GAG. The final precipitates were then counted in Aquasol (New England Nuclear, Boston, Mass.) in a Beckman liquid scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.). This method readily distinguished labeled acetate in hyaluronate, chondroitin sulfate, and the mucopolysaccharidase-resistant GAG, keratan, dermatan, and heparan sulfates (Toole and Gross, 1971).

COLLAGENS: After incubation of the tissue with [^{3}H]proline, the collagens synthesized by either epithelium or stromal mesenchyme were extracted with neutral phosphate buffer overnight at 4°C, combined with nonradioactive previously purified lathyritic chick skin collagen as carrier, and subjected to a two-step purification involving one precipitation at low ionic strength and neutral pH and one at high ionic strength and acid pH. The collagens were heat denatured and chromatographed at 40°C on a 0.9 × 5.0-cm column of CM-cellulose equilibrated with 0.02 M sodium acetate, pH 4.8, plus 1.0 M urea. The collagen α chains were eluted with a superimposed linear salt gradient of 0–0.13 M NaCl. Portions of the effluent were counted, whereas others were desalted, lyophilized, hydrolyzed in 6 N HCl under N_2 for 24 h at 110°C, and analyzed on a Jeolco 5AH amino acid analyzer (JEOL USA, Analytical Instrument Div., Cranford, N. J.) for radioactivity in the 3- and 4-hydroxyproline and proline residues (Trelstad et al., 1973).

RESULTS

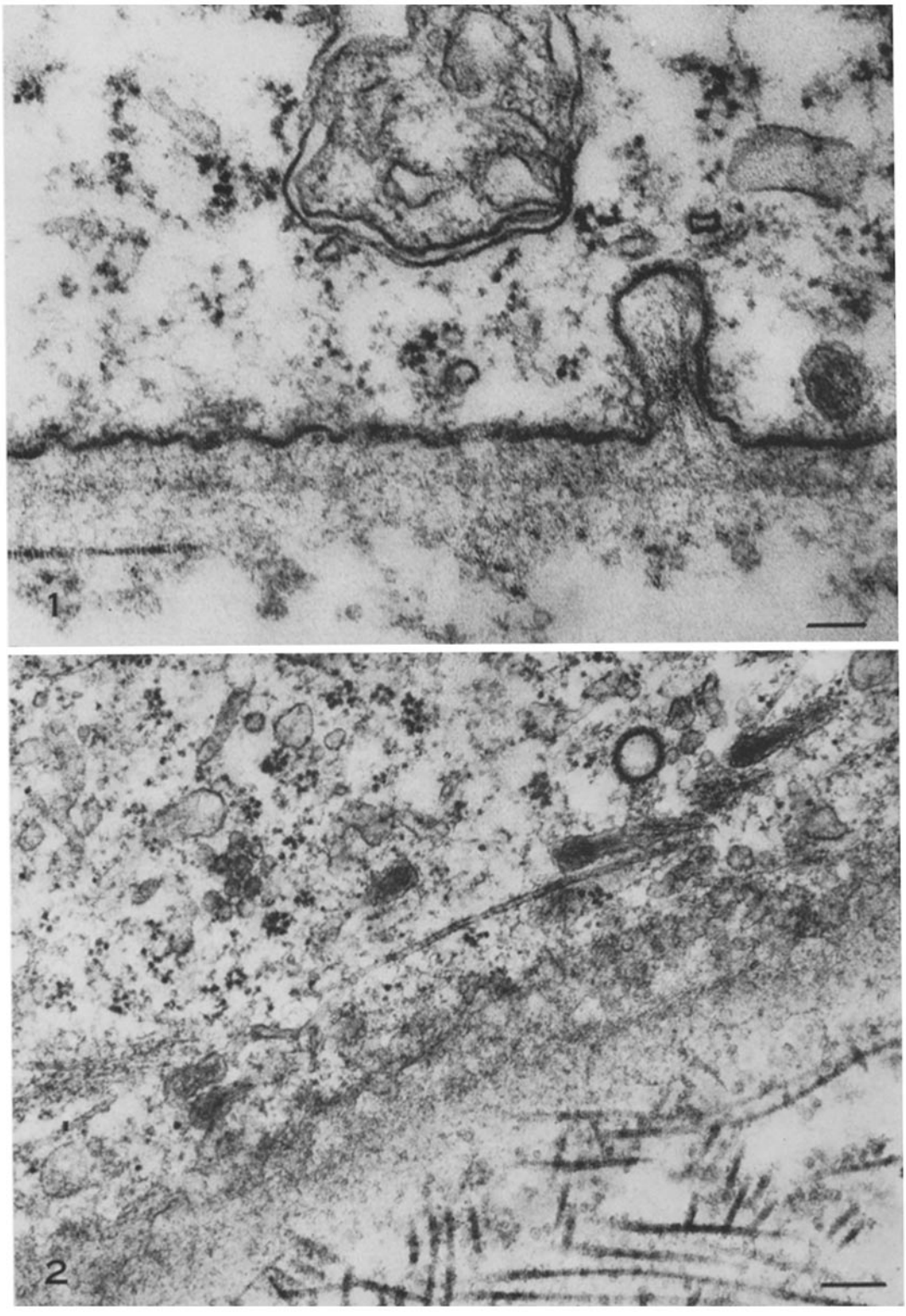
Electron Microscopy

The ultrastructure of the basal epithelial cells of the early embryonic chick cornea has been previously described (Hay and Revel, 1969; Trelstad and Coulombre, 1971). Figs. 1 and 2 illustrate the appearance of the basal surface of the epithelial cell, the basement membrane, and the subjacent collagenous stroma in preparations cut both parallel and perpendicular to the corneal surface. These preparations have not been specially stained and illustrate the ultrastructural appearance of the region of collagen excretion and orthogonal fibril formation in routinely prepared materials.

The basement membrane viewed in cross section is a continuous amorphous cell coat occupying an approximately 1,300-Å wide zone beneath the basal cell plasma membrane. Three distinct regions in the basement membrane are apparent: an inner electron-lucent layer, a central dense layer, and an outer lucent layer. The electron-dense central layer of the basement membrane, also called the basement lamina (Hay and Revel, 1969), is approximately 400 Å in thickness and the center of the lamina is approximately 600 Å from the basal plasma membrane. The zone between the basement lamina and cell surface is about 300 Å wide and contains small filamentous structures, some of which appear to be in direct contact with the cell membrane. An organized pattern of this filamentous material is not apparent, but filaments both parallel and perpendicular to the cell membrane are present. Between the basement lamina and the fibrillar stroma there exists another zone of about 300 Å in which little substructure other than some filamentous material is apparent using routine preparative methods. Striated collagen fibrils are present immediately adjacent to the outer layer.

Viewed en face the continuous but nearly amorphous nature of the basement lamina is again demonstrated (Fig. 2). No apparent pattern is present in any of the three layers although the central layer is clearly more dense. The orthogonal pattern of the fully formed collagen fibrils is present near the outer layer of the basement membrane.

In a previous study it was suggested that collagen excretion by the corneal epithelial cell occurs via membrane-limited vacuoles (Trelstad, 1971; Trelstad and Coulombre, 1971). Such collagen condensation vacuoles are again illustrated in Figs. 1 and 2, as is their close association with microtubules (Fig. 2). In Fig. 1 there is a possible example of a condensation vacuole, fused with the basal cell membrane and open to the extracellular space with portions of its contents exteriorized. The vacuole membrane contains, on its cytoplasmic surface, small electron-dense projections similar to those described on coated vesicles (Roth and Porter, 1964). These projections are clearly smaller and less dense than ribosomes, arguing against this being a profile of endoplasmic reticulum fused with the cell surface. The content of the vacuole which is apparently being discharged into the basement membrane and possibly into the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subjacent stroma consists of aggregates of fibrillar material. Of interest is the fact that the length of the fibrillar material illustrated is approximately 3,000 Å, the length of a collagen molecule.

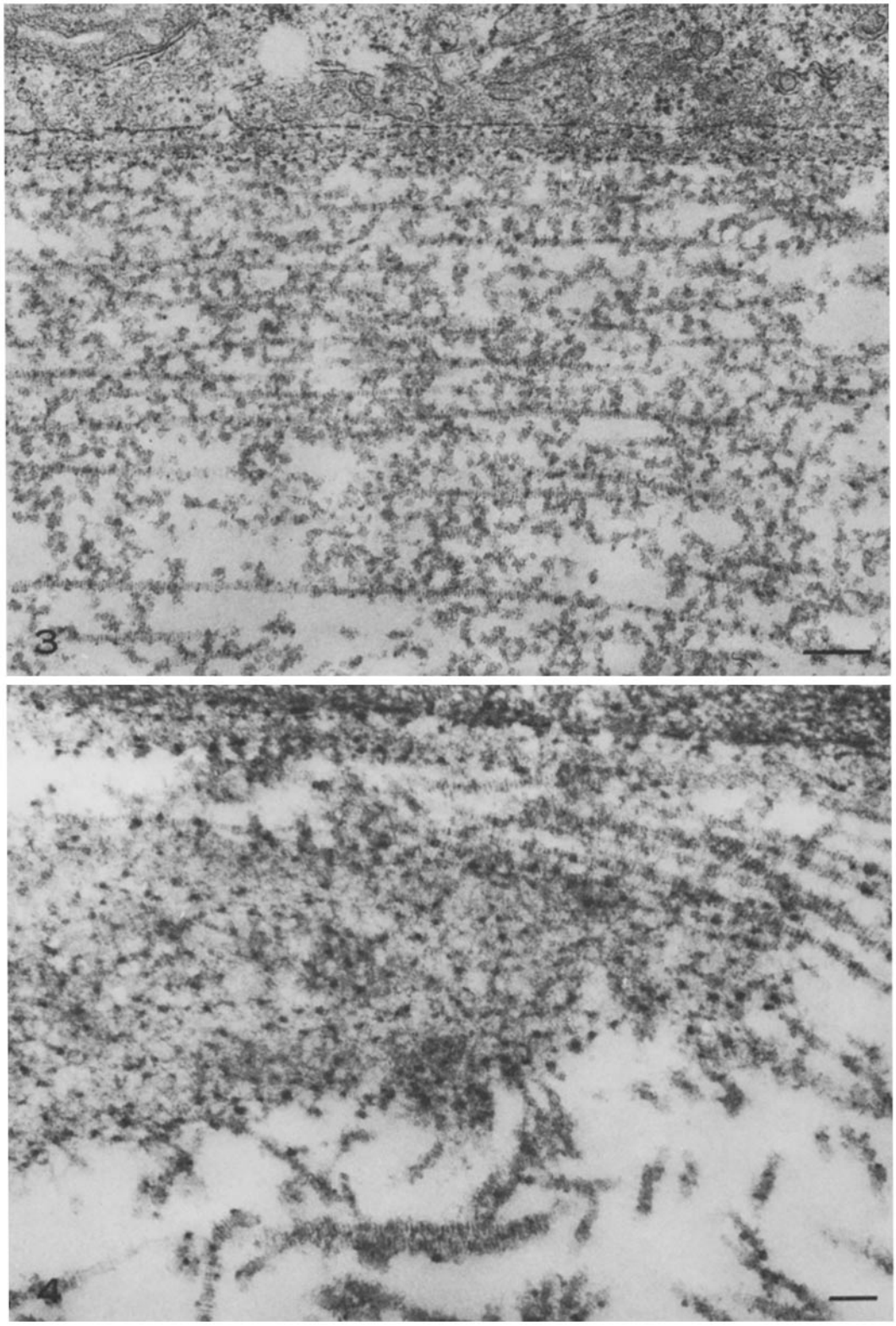
Ruthenium Red Staining

Preparations treated with ruthenium red demonstrate a well-ordered pattern of staining material in both the basement membrane and the collagen fibrils of the primary stroma (Figs. 3, 4, 5). The individual stain deposits are uniform in size, roughly spherical in shape, and average 100 ± 20 Å in diameter. In preparations cut perpendicular to the corneal surface the ruthenium red is distributed in the basement membrane in a distinct bilaminar pattern: one layer of staining is present on the inner face of the basement lamina and the second layer is present on its outer face (Figs. 4, 5). In each layer the stained material is not distributed in an absolutely perfect plane, but at numerous points appears to consist of overlapping planes, usually separated by less than 100 Å (Figs. 3, 4). This overlapping pattern is more prominent in the layer on the outer face of the basement lamina. The average distance between the two layers of ruthenium-staining material is approximately 600 Å and the distance from the inner layer to the basal cell membrane is 250 Å. In addition to the bilaminar spatial pattern of ruthenium red a regular spacing within each layer seemed to be present in which the ruthenium red-staining material was observed at periodic intervals of approximately 550–600 Å (Fig. 4). This regular spacing, however, is better visualized in sections cut parallel to the corneal surface.

In preparations cut parallel to the corneal surface the ruthenium red-stained material shows a fairly uniform distribution (Figs. 4, 5). In such sections both laminae are usually included and their images may be superimposed on each other since the distance between the two laminae (600 Å) is less than the section thickness (700–800 Å). In order to evaluate the spacing pattern of the ruthenium red-staining material in such preparations, electron micrographs such as illustrated in Fig. 5 were printed and a pin was driven through the micrograph at the point of each ruthenium red-staining site. The punched out micrograph was then placed on photographic paper in the dark room and exposed. This provided a photograph of the distribution of the ruthenium red-stained material (Fig. 6). The spacing pattern of the pinholes was then evaluated by two methods. Multiple copies of the pinhole photograph at appropriate magnifications were made on a standard photocopier. Random lines were drawn through the pattern and the distance between adjacent dots which touched that line was measured. Measurements were also made in specific axes defined by the subjacent orthogonal collagen fibrils. These data were indistinguishable from those obtained using randomly drawn reference lines. A frequency histogram of the spacing pattern between adjacent staining sites revealed a maximum at 550 Å. There is a possible repeat at even integers of this spacing distance so that at 1,100, 1,650, 2,200, and 2,750 Å there are also peaks in the frequency histogram (Fig. 7). The spacing pattern in the pinhole photograph was also analyzed by optical diffraction (with the kind assistance of Dr. Henry S. Slater, Children's Hospital Medical Center, Boston, Mass.). This analysis supported the nonrandom

FIGURE 1 The basement membrane, viewed in a plane perpendicular to the corneal surface, is a continuous coat associated with the cell surface. Three zones or layers are apparent: (1) an inner electron-lucent zone approximately 300–500-Å wide; (2) a central electron-dense zone (also called the basement lamina) which is approximately 300–400-Å wide and whose central portion lies approximately 600 Å from the cell plasma membrane; and (3) a poorly defined electron-lucent layer on the outer face of the basement lamina. Well-formed collagen fibrils are present immediately adjacent to the outer layer of the basement membrane. A possible collagen condensation vacuole which has fused with the cell surface and is excreting its contents into the subepithelial space is illustrated. The material in the vacuole appears to be fibrillar aggregates which measure approximately 3,000 × 50 Å. × 91,000. Mark = 0.1 μm.

FIGURE 2 The basement membrane viewed in a plane oblique to the corneal surface shows little substructure. The electron-dense basement lamina is discernible between the less dense inner and outer layers of the basement membrane. The orthogonal collagen fibrils are present at the outer face of the basement membrane. Intracellular collagen condensation vacuoles are present. Several are aligned adjacent to microtubules. × 49,000. Mark = 0.2 μm.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distribution of the staining sites; however, the approximate value obtained for the D spacing was 15–20% less than that obtained manually.

The striated collagen fibrils of the stroma often were stained by the ruthenium red in a periodic pattern at 600-Å intervals as has been described previously for other connective tissues (Fig. 3) (Myers et al., 1969). The interfibrillar matrix, presumably rich in hyaluronate (Toole and Trelstad, 1971), did not stain nor did intracellular structures in either of the epithelia or the mesenchyme.

Enzyme-Digested Tissues and Ruthenium Red Staining

The ruthenium red-staining material in the 9- and 12-day corneas was completely removed from the basement membrane by treatment with Chondroitinase ABC (Fig. 8), whereas strong staining persisted after treatment with leech Hyaluronidase (Fig. 9). In the 6-day cornea a small amount of ruthenium red staining remained after Chondroitinase ABC treatment, but again was undiminished by treatment with leech Hyaluronidase. The enzymatic treatment did not significantly affect the cell ultrastructure.

Alcian Blue and Alcian Blue-Ruthenium Red Staining

The apparent presence of sulfated GAG in the basement membrane would suggest that fixatives containing Alcian blue, which tends to precipitate protein polysaccharides (Shea, 1971), would enhance ruthenium red staining. This occurs, and the results using Alcian blue with and without ruthenium red are illustrated in Figs. 10, 11, and 12. Alcian blue alone preserves fibrillar material on both the inner and outer faces of the basement lamina (Figs. 10, 11). This fibrillar material is best seen on the outer face and appears to lie almost parallel to the basement lamina and then incline slightly toward the underlying stroma. It measures approximately 50 Å in diameter and has a discontinuous profile in the plane of the membrane. The presence of these fibrillar structures, especially on the outer face, is enhanced by ruthenium red (Fig. 12). The overlapping pattern of adjacent regions of this fibrillar material in a shingle-like pattern is particularly apparent in the outer layer. The slight inclination of this fibrillar material toward the subjacent stroma is suggested in the ruthenium red-stained preparations (Fig. 12).

Lanthanum Nitrate Staining

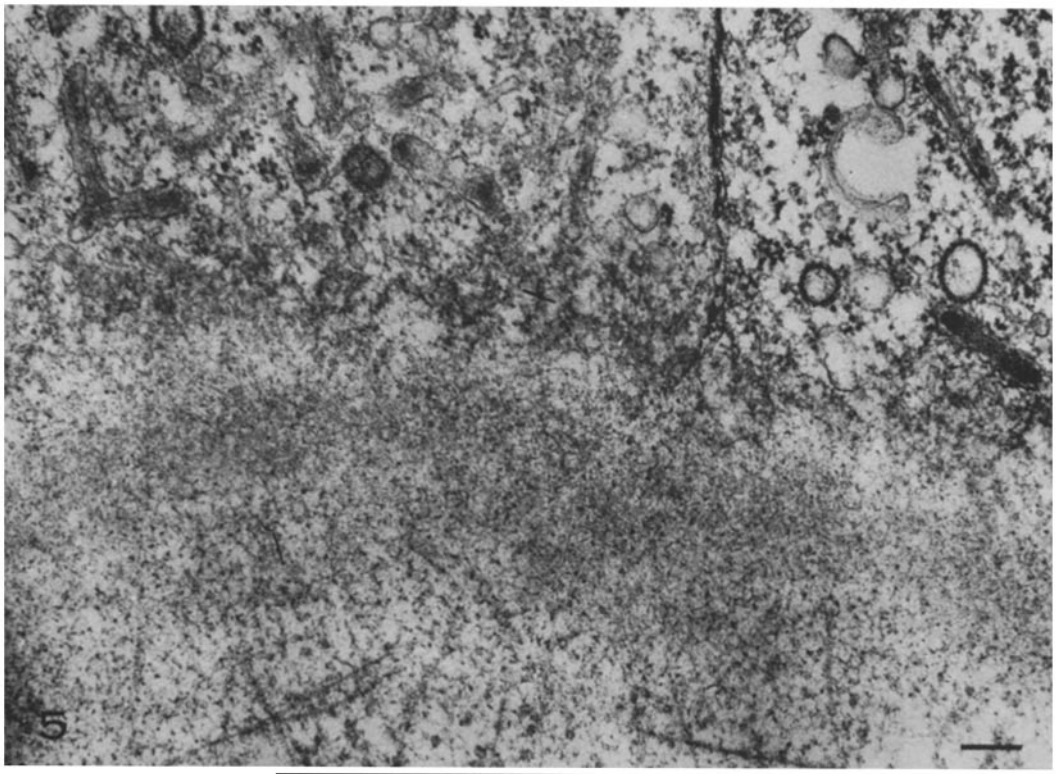
Staining with lanthanum nitrate revealed a pattern distinctly different from that seen with the ruthenium red. The lanthanum-stained preparation showed a more globular pattern of electron-dense material within the plane of the basement membrane (Fig. 13). The electron-dense material was approximately 250 Å in diameter and a precise spacing pattern could not be clearly identified. In sections cut perpendicular to the corneal surface, the heavily electron-dense, stained material was principally in the basement lamina proper and on its outer face.

Epithelial and Mesenchymal Collagen Synthesis

Characterization of the neutral salt-extractable collagens synthesized by the epithelium from stages 25–29 after labeling in vitro for 16 h was accomplished by chromatography on CM-cellulose. It revealed that the isolated epithelial cells or the intact cornea before invasion by mesenchyme is making approximately five to six times as

FIGURE 3 Ruthenium red-stained corneal epithelium cut perpendicular to the corneal surface. Electron-dense deposits of stain are present in the basement membrane and on the stromal collagen fibrils. In the basement membrane a bilaminar pattern of staining on the inner and outer faces of the stain-free basement lamina is apparent. In the stroma, staining sites are present along the fibrils at 600-Å intervals. × 51,000. Mark = 0.2 μm.

FIGURE 4 Ruthenium red-stained epithelium cut obliquely to the corneal surface. Owing to some curvature of the epithelium the basement membrane is viewed both perpendicular (top) and oblique (center) to its plane. The bilaminar pattern of staining is present in the region cut perpendicularly. At the outer face of the membrane the layer of staining material is not continuous but appears at points to overlap. The fairly uniform distribution of staining sites in the membrane sectioned obliquely is apparent. × 79,500. Mark = 0.1 μm.



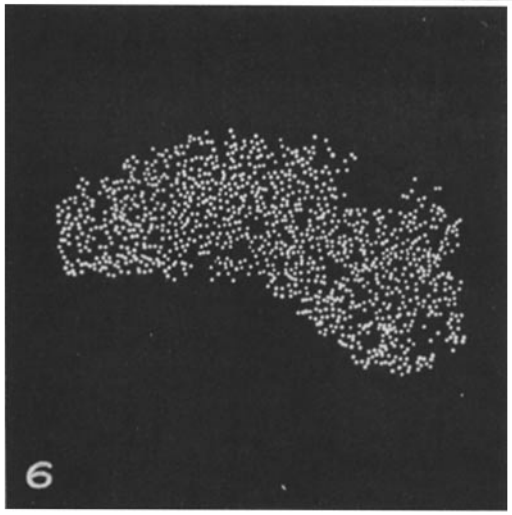


FIGURE 5 Ruthenium red-stained epithelium cut obliquely to the corneal surface. This low-magnification electron micrograph illustrates typical fields selected for analysis of the spatial distribution of the staining sites. Many collagen condensation vacuoles are again illustrated within the cells. × 41,500. Mark = 0.2 μm.

FIGURE 6 Pattern of ruthenium red-staining sites derived from electron micrographs such as Fig. 5 in which each site was punched with a pin and rephotographed. Site-to-site spacing was evaluated in such preparations by measuring distances between holes touching randomly drawn lines. Actual photographs used in such measurements were four to five times larger than illustrated.

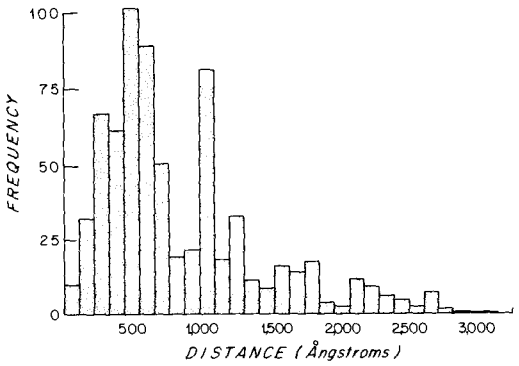


FIGURE 7 Frequency histogram of the distance between ruthenium red-staining sites in basement membranes evaluated as illustrated in Figs. 5 and 6. The values represent the center-to-center spacing between adjacent pinholes lying on randomly drawn lines through a preparation such as that illustrated in Fig. 6.

many $\alpha 1$ chains as $\alpha 2$ (Fig. 14). This result was confirmed in 12 different experiments and suggests that the epithelium is making approximately equimolar amounts of $(\alpha 1)_2\alpha 2$- and $(\alpha 1)_3$-type collagens.

Experiments performed simultaneously on corneas from slightly later staged embryos (30–32), in which the corneal epithelium and endothelium had been removed and the mesenchyme in the stroma labeled, revealed a ratio of $\alpha 1/\alpha 2$ of 2. This is consistent with that found in type I collagen, $[\alpha 1(I)]_2\alpha 2$, and confirms previous observations that the collagen in the adult chick cornea consists of this species (Trelstad and Kang, 1974).

Efforts to separate the apparently heterogeneous set of $\alpha 1$ chains made by the epithelial cells into discrete components by chromatography on DEAE-cellulose as previously described (Trelstad

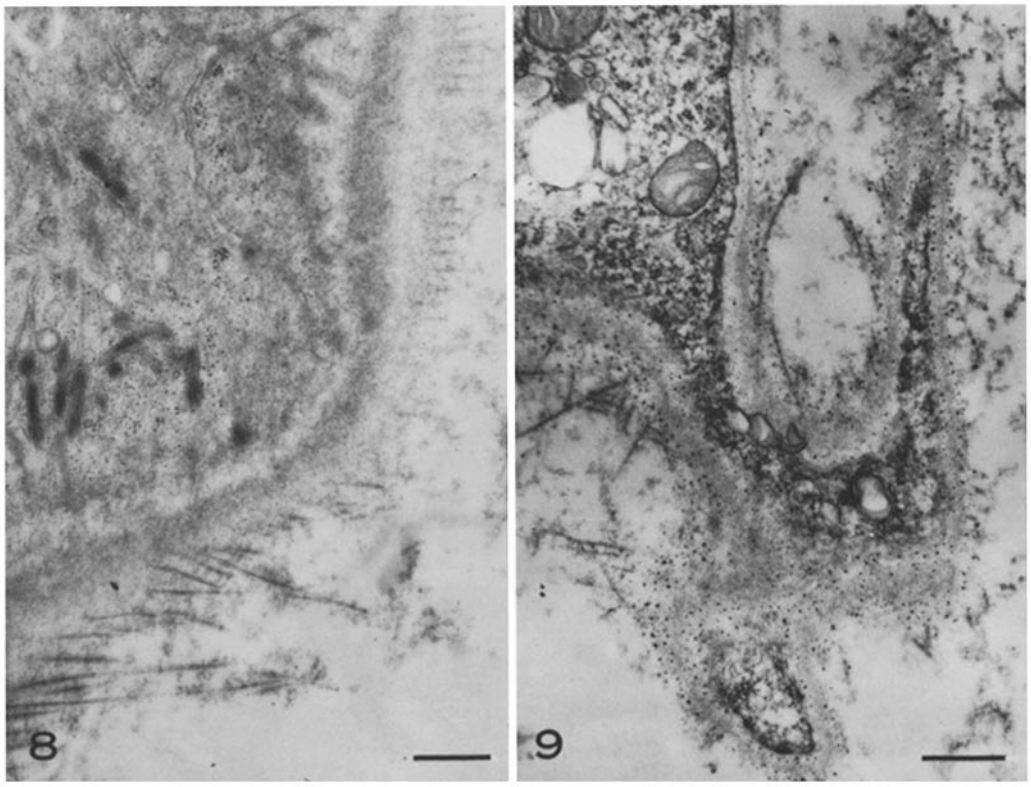
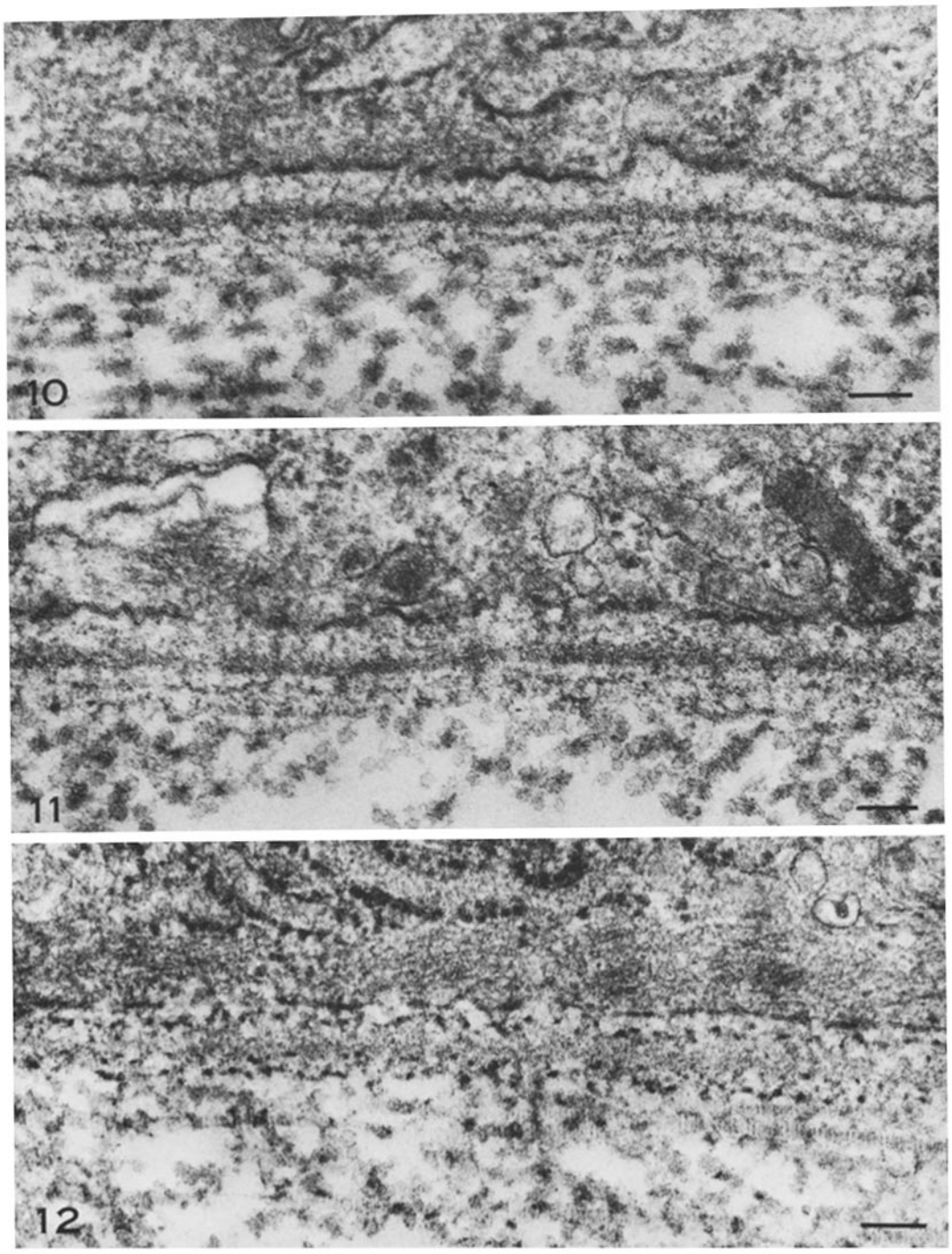


FIGURE 8 9-day corneal epithelium treated with Chondroitinase ABC, fixed and stained with ruthenium red, and sectioned parallel to the corneal surface. All staining in the basement membrane and along fibrils has been eliminated. Note the close association of the outer face of the basement membrane with the formed collagen fibrils. Densely stained collagen condensation vacuoles are particularly prominent. × 25,000. Mark = 0.4 μm.

FIGURE 9 9-day corneal epithelium treated with leech Hyaluronidase, fixed and stained with ruthenium red, and sectioned parallel to the corneal surface. Intense staining of the basement membrane by ruthenium red persists despite enzyme treatment. × 25,000. Mark = 0.4 μm.



FIGURES 10 and 11 Corneal epithelial cells fixed with Alcian blue added and sectioned perpendicular to the corneal surface. As contrasted with that in Fig. 1, the basement membrane reveals additional fibrillar structure especially in the outer layer. The basement lamina is notably electron dense and more prominent than in Fig. 1. The fibrillar profiles in the outer layer do not appear continuous but are interrupted at numerous points. × 85,000. Mark = 0.1 μm.

FIGURE 12 Corneal epithelial cells fixed with Alcian blue and ruthenium red and sectioned perpendicular to the corneal surface. The fibrillar material at the outer face of the basement membrane illustrated in Figs. 10 and 11 appears well stained with ruthenium red. The overlapping pattern of this fibrillar material is illustrated as well as its slight inclination toward the underlying stroma of collagen fibrils. × 85,000. Mark = 0.1 μm.

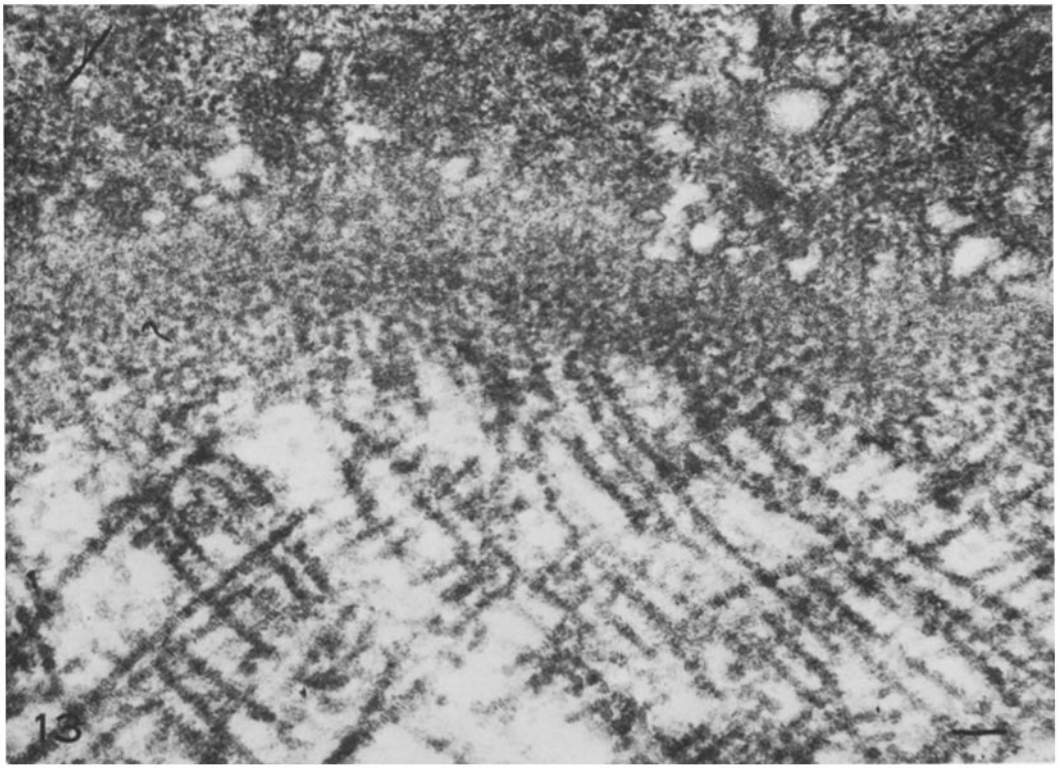


FIGURE 13 Epithelial cell fixed with lanthanum and sectioned parallel to the corneal surface. The basement membrane contains an array of electron-dense structures, each approximately 250 Å in diameter. The apparent extension of collagen fibrils into the region of the basement membrane is illustrated. × 60,750. Mark = 0.1 μm.

et al., 1972) were successful in generating two major and two minor peaks. Analysis of these peaks by acid hydrolysis with 6 N HCl and chromatography of the amino acids on an analyzer revealed some differences in the distribution of radioactivity in 4-hydroxyproline, and proline with the early eluting material containing approximately 37% of the total proline residues hydroxylated in the 4 position and the later material 45%. In all peaks, less than 2% of the total counts in hydroxyproline were in the 3 position.

Epithelial, Endothelial, and Mesenchymal Glycosaminoglycan Synthesis

The glycosaminoglycans (GAG) synthesized by the corneal epithelium, endothelium, and early mesenchyme are shown in Table I. 79% of the GAG synthesized by the epithelium of stage 29 corneas was chondroitin sulfate and 21% was enzyme resistant, presumably heparan sulfate (Meier and Hay, 1973). No hyaluronate synthesis was detected in the preparations of isolated outer epithelia, even though this is the principal GAG produced by stage 29 corneas (Toole and Trelstad, 1971). The predominant GAG synthesized by the mesenchyme plus endothelia, on the other hand, was hyaluronate. To determine whether the endothelium was capable of GAG synthesis, stage 26 corneas, which are comprised of two cell layers only, viz. epithelium and endothelium, were labeled after removal of the epithelium. These preparations made chiefly hyaluronate and no detectable chondroitin sulfate. These data clearly indicate that the two corneal epithelia are synthesizing discretely different GAG, the outer epithelium mainly chondroitin sulfate and the inner endothelium, hyaluronate.

DISCUSSION

Although morphogenesis is a complex developmental process which is unique for each tissue, this

does not preclude the existence of basic morphogenetic processes common to the development of three-dimensional form in all tissues. One such general process which is often associated with morphogenesis is the self-assembly of macromolecules (Gross, 1956; Anfinsen, 1968; Trelstad, 1973). The chick cornea presents a particularly good opportunity for the study of this aspect of morphogenesis at the tissue level since it is possible that the self-assembly of connective tissue macromolecules produced by the corneal epithelium plays an important role in the development of the optically transparent, precisely ordered corneal stroma (Trelstad and Coulombre, 1971). Many factors are instrumental in the development of the cornea and have been discussed in detail in previous publications (Hay and Revel, 1969; Conrad, 1970; Trelstad and Coulombre, 1971; Toole and Trelstad, 1971; Coulombre and Coulombre, 1972; 1974). In brief the corneal epithelium deposits an orthogonal matrix of collagen fibrils beneath its basal surface during early development. This matrix, rich also in hyaluronate, suddenly swells and serves as a scaffold for mesenchyme cells invading from the periphery. The three-dimensional organization of the matrix produced by the mesenchyme cells appears identical to that initially deposited by the epithelium, thus suggesting that the epithelial matrix is a morphogenetic template and that the genesis of the adult stromal morphology is an epithelial function (Trelstad and Coulombre, 1971). The major focus of the present study has been (*a*) to analyze the specific chemical nature of the macromolecules produced by the corneal epithelium, and (*b*) to determine ultrastructurally the distribution which they assume in space in the basement membrane which lies between the site of their excretion from the cell and their assembly into orthogonally organized arrays of collagen fibrils in the stroma.

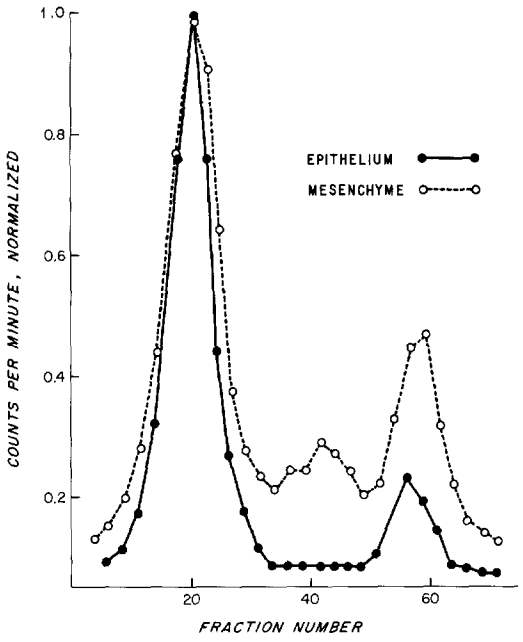


FIGURE 14 CM-cellulose chromatograms of [^{3}H]proline-labeled collagens synthesized by corneal epithelial cells and stromal mesenchyme. The epithelial profile is the average of six different but nearly identical chromatograms. The α1 chains (fractions 10–30) generally contained 10^3–10^4 cpm/fraction for both epithelium and mesenchyme. The count profiles have been normalized to facilitate comparison. Both α1 and α2 chains (fractions 50–70) cochromatographed with chick skin collagen α1 and α2 chains (optical density profile not shown). The material eluting in fractions 40–50 in the mesenchyme was not further characterized.

Epithelial and Mesenchymal Collagens

It seems likely that at least two different molecular species of collagen are being made by the

TABLE I

Synthesis of Glycosaminoglycans by Early Embryonic Corneal Epithelium, Endothelium, and Mesenchyme

	Percent hyaluronate*	Percent chondroitin sulfate*	Percent enzyme[a] resistant*	Total GAG[b] cpm per cornea
Epithelium (stage 29)	0	79	21	4,000
Mesenchyme and endothelium (stage 29)	56	26	18	20,200
Endothelium (stage 26)	75	0	25	3,300

* Expressed as percent of total counts incorporated into GAG/cornea.

[a] Enzyme-resistant GAG's are likely to be heparan sulfate and/or dermatan sulfate.

[b] Each experiment involved approximately 60 corneas.

outer epithelium: one which forms fibrils and constitutes a major constituent of the primary corneal stroma; and the other which remains associated with the epithelial cell in its basement membrane. These collagens have been isolated by neutral extraction of short-term organ cultures rendered lathyritic with β-APN. The collagen molecules have been partially characterized chemically by their chromatographic profiles on CM-cellulose and by the percent proline hydroxylation. From the CM-cellulose chromatograms it appears that the epithelium is synthesizing both α1 and α2 chains with a five- to sixfold excess of α1. One likely interpretation of these data is that equimolar amounts of an $(\alpha 1)_3$ and an $(\alpha 1)_2\alpha 2$ molecule are being synthesized.[1] The latter molecule might be type I collagen or $[\alpha 1(I)]_2\alpha 2$, which is produced by the adult corneal fibroblats as has been determined by direct chemical analysis (Trelstad and Kang, 1974) and by the stage 30–32 corneal mesenchyme as indicated by the radioisotopic profiles on CM-cellulose reported here. The $(\alpha 1)_3$ species of collagen, on the other hand, might be genetically similar to the type IV collagens which have been isolated from several different basement membranes (Kefalides, 1971).

Epithelial Glycosaminoglycans

Previous studies of the GAG synthesized in the whole cornea at early stages indicated that hyaluronate was predominant (Toole and Trelstad, 1971), and the present data demonstrate directly that this material is initially produced only by the endothelium (Table I). After mesenchymal invasion the hyaluronate is apparently synthesized by both the mesenchyme and endothelium. The epithelium, on the other hand, does not synthesize hyaluronate either before or after mesenchyme invasion, but rather sulfated GAG, chiefly chondroitin sulfate. Previous work with adult bovine corneal epithelium had demonstrated enzymes capable of synthesizing chondroitin sulfate (Smelser, 1959; Wortman, 1961), but the present report and recent studies by Meier and Hay (1973) directly demonstrate that this macromolecule is synthesized by this epithelium. In addition, Meier and Hay (1973) have identified the enzyme-resistant sulfated polysaccharide produced as heparan sulfate.

The apparent exclusive synthesis of hyaluronate by the endothelium and of sulfated GAG by the epithelium at early stages underscores the differences in function which these two epithelia have in the embryo and retain in the adult. The chondroitin sulfate produced by the embryonic epithelium probably interacts with the collagens in a noncovalent manner (Toole and Lowther, 1968) to provide a "bridging macromolecule" (Trelstad and Coulombre, 1971) influencing the precise spacing of the collagen in the fibrillar stroma and perhaps also in the basement membrane. The hyaluronate produced by the endothelium, on the other hand, may have little effect on the ordered assembly of the orthogonal primary stroma, but rather may be responsible for the timing of such events as mesenchymal cell invasion and subsequent differentiation as well as stromal dehydration and onset of transparency (Toole and Trelstad, 1971).

The possibility that epithelial GAG synthesis is restricted to specific periods of embryonic development should be explored since these materials have not been described in adult basement membranes. Recently, Bernfield and co-workers (1972) have also found that sulfated acid mucopolysaccharides are a constituent of the basement membrane of the embryonic salivary gland epithelium during morphogenesis. Both of our studies demonstrate the presence of sulfated GAG in epithelial basement membranes during morphogenetically important periods of development and it seems reasonable to postulate that these macromolecules may be in some way causally related to these events.

Spatial Order of Glycosaminoglycans

In the corneal stroma the ruthenium red-positive material is distributed at periodic intervals along the collagen fibrils equal to the collagen macroperiod of 600 Å. Similar patterns of collagen fibril staining with ruthenium red have been described in other tissues (Myers et al., 1969). Of particular interest is the new observation that the material in the basement membrane is distributed in a precise pattern with a bilaminar configuration. Although we cannot rigorously identify the ruthenium red-staining material in the basement membrane the fact that the epithelium is synthesizing chondroitin sulfate and that the ruthenium red staining is eliminated from the basement membrane with Chondroitinase ABC is strong evidence that most of the material is indeed chondroitin sulfate. Caution, however, must be exercised in interpreting the distribution of ruthenium red since

[1] For a review of collagen terminology see Trelstad (1973).

the absence of staining in particular regions may not represent the absence of sulfated GAG, but rather some form of masking such that the material does not stain. Furthermore, the staining material removed by enzyme treatment could also include a macromolecule which is dependent on chondroitin sulfate for its retention and distribution in the tissue.

Acknowledging these limitations, the present observations suggest that chondroitin sulfate is present in the basement membrane and that it is distributed in a bilaminar pattern, being located on the inner and outer faces of the dense basement lamina but not in the lamina itself. The two regions staining with ruthenium red correspond closely with the inner and outer electron-lucent layers which have been described in previous studies of basement membranes, but within which no specific structure has been recognized (Latta, 1970). The regular spacing between ruthenium red-staining sites in both inner and outer layers, as well as the bilaminar separation of about 600 Å, indicate a preferred spatial order of the chondroitin sulfate. The differences in the spacing distance revealed by the optical diffraction and manual method are unexplained. The immediate relationship between the spatial order of the GAG in the basement membrane and that present in the fibrillar stroma is an open question, as is the manner in which these packing patterns of GAG are generated. One possibility is that the spatial order results from macromolecular interactions, presumably ionic in nature (Toole and Lowther, 1968), between chondroitin sulfate-protein and the collagen of the basement membrane (probably type IV collagen) and/or that of the primary corneal stroma (probably type I collagen). Experiments designed to evaluate these various interactions (Toole and Lowther, 1968), using macromolecules produced by the epithelium, are currently in progress.

Collagen Fibrillogenesis

Previous autoradiographic studies have shown that the collagen excreted by the epithelial cells precipitates into fibrils immediately beneath the epithelium (Trelstad and Coulombre, 1971). In addition, the observation that the collagen fibrils next to the epithelium are fully formed in respect to diameter suggests that fibril growth occurs principally at the apparent end which is contiguous with the outer face of the basement membrane. In this regard the present finding of a thin unstriated fibrillar material, particularly on the outer face of the basement lamina, is of interest. This material appears to extend toward the subjacent striated collagen fibrils and may be involved with their growing ends. The manner in which these fibrils lie in an apparently overlapping or shingle-like pattern recalls the observations of Nadol et al. (1969) who have suggested that orthogonal collagen fibrillogenesis in fish dermis is dependent on the basement membrane.

These observations extend our previous study in which it was suggested that extracellular macromolecular self-assembly was a principal force in corneal stromal morphogenesis (Trelstad and Coulombre, 1971). The present data lead us to postulate that an interaction has occurred between sulfated GAG and collagen while still in the domain of the epithelial cell surface coat known as the basement membrane. This interaction could occur even before these materials are excreted from the cell, e.g., in the Golgi apparatus. The sulfation and intracellular packaging of GAG is accomplished in the Golgi apparatus (Godman and Lane, 1964) and now several lines of evidence, including the present illustration (Fig. 1) which we interpret as collagen being discharged from the corneal epithelial cell, indicate that collagen packaging and excretion also occurs in vacuoles of Golgi origin (Revel and Hay, 1963; Frank, 1970; Trelstad, 1970; Trelstad, 1971; Weinstock and Leblond, 1974). Thus the corneal epithelial cell itself by controlling the stoichiometry of synthesis, the intracellular packaging, and the timing and mode of excretion to the extracellular space, would play a very direct role in extracellular matrix morphogenesis, and the self-assembly of macromolecules in the extracellular space would occur, in part, because suitably interactive molecules were deposited at the correct morphogenetic site at the appropriate time.

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