SECRETION OF COLLAGEN BY CORNEAL EPITHELIUM

I. Morphology of the Collagenous Products Produced by Isolated Epithelia Grown on Frozen-Killed Lens

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ABSTRACT

Corneal epithelium from 5–7-day old chick embryos was isolated with EDTA and grown in culture on frozen-killed lens as a substratum. Autoradiographs showed that in the presence of [³H]proline, the corneal epithelium synthesized and secreted onto the lens substratum, radioactive materials resistant to extraction by sodium hydroxide. The radioactive label was associated with newly formed striated collagen fibrils, large "sheets" of collagen, and basal lamina. The repeat period and interband pattern of the abundant new collagen sheets and fibrils was typical of "native" or so-called "mesenchymal" collagen. Collagen-like materials were observed in secretory (Golgi) vacuoles within the corneal cells and collagen fibrils within the intercellular canals (lateral interfaces) of the epithelium, as well as at the base of the cells. Both the granular endoplasmic reticulum and Golgi complexes were highly developed in the corneal epithelium. In the discussion, the role of cytoplasmic organelles in collagen secretion, the origin and structure of the basal lamina, and variations in collagen polymerization patterns in vitro are reviewed and evaluated. The morphogenetic significance of the synthesis and secretion of collagen by embryonic epithelium is appraised and the production of true native-striated collagen by epithelium is stressed.

INTRODUCTION

In 1963, we observed that the epidermis of salamander larvae secretes a proline-rich product into the dermis (Hay and Revel, 1963) in exactly the same sequence as chondrocytes delivered labeled product into the cartilaginous matrix (Revel and Hay, 1963). Glutaraldehyde, which seemingly binds free amino acids, was not used as a fixative and the product withstood washing and dehydration procedures. Since the amphibian larval dermis is almost entirely collagen (Edds and Sweeney, 1961), we concluded that the proline-rich protein secreted by the epidermis was, in all likelihood, collagen. Kallman and Grobstein (1965) challenged the idea in autoradiographic studies of salivary gland epithelium and Bernfield (1970), studying the same system, has concluded that mesenchyme is the "major source of epithelial collagen." Subsequently, Hay and Revel (1969) described good circumstantial evidence for the secretion of collagen by corneal epithelium in early chick embryos and corneal epithelium was shown to produce a protein containing hydroxyproline (Revel, 1965; Goodfellow et al., 1969; Conrad, 1970 b), as were certain other non-fibroblastic cells (Green and Goldberg, 1965). In this report, we present definitive experiments

with isolated corneal epithelium grown in culture that establish the fact that epithelium can indeed produce abundant quantities of collagen, not only of the basement lamina variety, but also of the "native" striated fibril type which heretofore has been thought to originate only from cells of mesenchymal origin.

One of us has shown that frozen-killed connective tissue can efficiently substitute for the living mesenchyme in supporting development of isolated epidermis and formation of basement membrane, as observed with the light microscope (Dodson, 1963; 1967). Therefore, we used frozen-killed substrata, such as lens (this report) and corneal stroma (Dodson and Hay, 1972)[1] on which to grow epithelium isolated by EDTA (ethylenediaminetetraacetic acid). A preliminary note has been published (Dodson and Hay, 1971). The most dramatic secretion of morphologically recognizable collagen by the isolated corneal epithelium occurred when frozen lens was the substratum and, interestingly, frozen lens subsequently proved to be an excellent substratum for the demonstration of collagen secretion by other embryonic epithelia, such as neural tube (Cohen and Hay, 1971). Millipore filter and collagen gels were poor substrata in this regard (Dodson and Hay, 1971). We report here our observations on the corneal epithelium-frozen lens system; we will compare the various substrata in detail in another communication (Dodson and Hay, 1972).[1]

MATERIALS AND METHODS

Separation of Epithelium

Corneas were dissected from white Leghorn (*Gallus gallus*) embryos after 5–14 days of incubation at 37°C. Whole corneas were soaked in three changes of 0.04% EDTA (disodium salt) in calcium- and magnesium-free Hanks' solution, pH 7-7.4 for a total of 40–60 min. The epithelium was then carefully peeled from the stroma using fine forceps. As it separated, the epithelial sheet tended to curl up, with the basal surface on the outside.

15 isolated epithelia from nine different experiments were very carefully examined by both light and electron microscopy to verify the purity of the epithelial preparation. In no case did fibroblasts or fibrillar corneal stroma ever adhere to the epi-

[1] Dodson, J. W., and E. D. Hay. 1972. Secretion of collagen by corneal epithelium. II. Effect of the underlying substratum on secretion and polymerization of epithelial products. In preparation.

thelium. In one case, the basement lamina of the corneal stroma did adhere to the epithelium (see Results).

Preparation of Lens Substratum

Lenses from 5–7-day old embryos were separated from iris by peeling iris and supporting ligament away with forceps. Each lens was passed back and forth through the forceps several times to remove vitreous. Sometimes, the ciliary body-lens supporting ligament contaminated the meridian of a lens. This material consists of beaded microfibrils of the same appearance and size (100–120 Å in diameter) as the elastic microfibrils described by Ross and Bornstein (1970). The observations reported here will be restricted to the microfibril-free front and back surfaces of the lens.

Lenses were frozen at −18°C and then thawed, the freezing and thawing being repeated three times (Dodson, 1967). To determine whether the lens cells were killed, frozen-thawed lenses were cultivated for 5 days on medium containing [³H]proline and then examined autoradiographically. There was no incorporation of label and no viable cells as judged by microscopic appearance in any of the explants. Control explants of lens substratum without epithelium were cultivated and processed exactly as explants bearing epithelium in all experiments.

Cultivation

The corneal epithelium was placed over a substratum immediately after separation, before it had time to curl tightly, and the combined explants were cultivated in Falcon culture dishes (Falcon Plastics, Division of B-D Laboratories, Inc., Los Angeles, Calif.) containing 0.3–0.5 ml of medium. The dishes were incubated at 37°C with air as the gas phase and the medium was changed every second day (Fig. 1).

The medium was 30% embryo extract in Leibovitz medium L-15 (Grand Island Biological Co., Grand Island, N. Y.). It was chosen after trying simpler balanced salt solutions, varying concentrations of embryo extract, and the addition of fetal calf serum; a clot of plasma and embryo extract was also tried, but the cells contained many fat droplets. The embryo extract was prepared by centrifuging a mixture of equal parts of medium L-15 and the finely minced pulp of 13-day old chicken embryo; antibiotics were added to the extract to give a concentration in the final medium of 50 U of penicillin and 50 μg of streptomycin per milliliter.

Fixation, Embedding, Microscopy

Explants were fixed in Karnovsky's formaldehyde-glutaraldehyde fixative, postosmicated, and
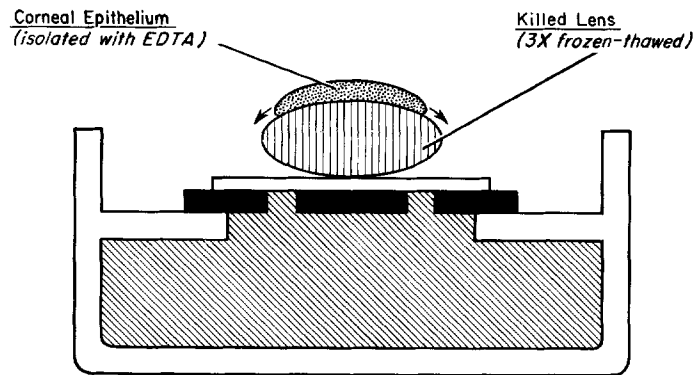
FIGURE 1 Diagram of the culture system. The isolated corneal epithelium is grown on a frozen-killed lens placed on rayon cloth on top of a stainless steel mesh. The culture medium below the tissue is changed every 2 days. A film of medium covers the tissue (not shown). Within a day, the epithelium begins to spread over the lens capsule (arrows). By 3–5 days it completely encloses the lens.

stained in uranyl acetate before dehydration and embedding in Araldite. Thin sections (0.5–1 µm) were stained with toluidine blue for light microscopy. Ultrathin sections (~0.1 µm; silver) on uncoated grids, were stained with lead citrate for examination in an ordinary transmission electron microscope (Siemens Elmiskop I). The procedures and solutions used are described in the appendix to Hay and Revel's monograph (1969).

Sections for high voltage electron microscopy were 1 µm thick, mounted on copper grids without a supporting film. The material had been stained en bloc with uranyl acetate; lead citrate was applied to one surface of the section. The lead citrate did not penetrate the section very deeply, but the superficial density gave the micrographs a three-dimensional appearance which can be appreciated even without stereo-optics (Fig. 13). Sections were viewed in the RCA high voltage electron microscope located at the University of Virginia (see acknowledgment).

Autoradiography

For autoradiography, 50 µCi of L-proline, 2, 3, ^{3}H (New England Nuclear Corp., Boston, Mass.) was added to the medium in each culture dish; explants were grown in the presence of the isotope for 2–9 days, then cultivated on "cold" medium, lacking the isotope but containing proline, for a further 24 h before fixation. Isotope was thus added routinely in most experiments.

For light microscopy, slides bearing sections were dipped in Ilford K5 or L4 emulsion diluted to 25%; after exposure in the dark for 2, 4, or 8 wk they were developed in 33% Dektol for 3–5 min and then fixed: some were stained with toluidine blue. For electron microscopy, sections on grids were coated

with Ilford L4 emulsion, diluted to 65% or 50%, using the loop technique; after exposure in the dark for 3–4 wk, they were developed in D-19 for 3 min and then fixed.

Since silver grains in the autoradiographs could be due to any of several sources, we prepared autoradiographs of control sections of tissues treated in various ways. (a) Sections of tissue treated or not treated with uranyl acetate showed that the uranium did not impart detectable radioactivity; (b) Control substrata, lacking epithelium, demonstrated that free [^{3}H]proline, adsorbed or otherwise bound to the tissue, contributed very little to the autoradiographs, and then only after 8–12 wk of exposure of the autoradiographs to the tissue; (c) The remnants of cells in frozen-killed substrata did not incorporate [^{3}H]proline; (d) Heavy labeling, present after only 2 wk or 4 wk of exposure, was only found when epithelial cells were present; the source, then, is tritium in molecules that were insoluble after fixation, dehydration, and that were produced by epithelium.

In order to remove noncollagenous proteins before autoradiography, some unfixed blocks of tissue were extracted for 24–48 h at 5°C in two to three changes of 100 vol of 0.1 M sodium hydroxide, as suggested to us by Dr. Jerome Gross (see Cohen and Hay, 1971).

Remark on Terminology: "Substrate" or "Substratum"; "Basal Lamina" or "Basement Membrane"

For the physical support on which cells rest, or are placed, the term "substratum" is used here. A substrate is "a substance acted upon (as by an enzyme)" and a substratum is "something that... underlies and supports or forms a base for something

else; an underlying structure, layer or part" (Gove, 1961). Where the role of a substance is mainly metabolic (active, reactive) then the term substrate may be more suitable; where the role is, or appears to be, mainly physical, the term substratum seems more appropriate.

The term "basement membrane" has been used for decades by light microscopists to refer to the periodic acid Schiff (PAS)-positive layer under epithelia or around muscle fibers. This layer is composed ultrastructurally of a juxtacellular filamentous sheet (basal or basement lamina) and an associated layer of collagen fibers (reticular lamina). Only in the renal glomerulus and lens capsule is the reticular lamina absent, so that only in these two cases is it correct to refer to the basal lamina as a basement membrane. Even there, the term "basal lamina" is to be preferred and we will in this paper use that term to refer to the filamentous sheet next to an epithelium or muscle fiber.

RESULTS

Fine Structure of Corneal Epithelium and Lens in Vivo

The corneal epithelium at the time of isolation from the embryo consists of an inner layer of tall basal cells rich in granular endoplasmic reticulum and an outer layer of flattened epithelial cells referred to as the periderm. Cells of the periderm have dense cytoplasm and possess microvilli on their free surface. Golgi complexes are very well developed in the tall inner cells and often occupy a basal position in the cytoplasm between the nucleus and basal plasmalemma (Hay and Revel, 1969; Trelstad, 1970). The granular endoplasmic reticulum is hypertrophied and the basal cells have large nuclei with prominent nucleoli. Under the basal plasmalemma, there is a basement lamina 0.1 μm in width composed of fine filaments (Hay and Revel, 1969). The underlying first formed or primary corneal stroma is rich in collagen fibrils, which we will show in this report are unquestionably of epithelial origin at this time The collagen fibrils are 250 Å in diameter with a repeating major period of 550–600 Å and they are arranged in orthogonal layers.[2] Through 5 days of development, there are no fibroblasts

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[2] After glutaraldehyde and osmium fixation, collagen fibrils, including those in adult avian tendon, show a repeat period of 550–600 Å rather than the 640 Å average repeat reported (Gross, 1956) in nonfixed collagen fibrils.

present in the stroma. At 6–7 days of incubation, mesenchymal cells migrate into the corneal stroma between the orthogonal layers of collagen fibrils (Hay and Revel, 1969; Trelstad and Coulombre, 1971) and subsequently the cornea increases dramatically in thickness due to the deposition of additional collagen by the invading mesenchymal cells. The corneal epithelium continues to possess well-developed secretory organelles through hatching and undoubtedly continues to contribute collagen and extracellular matrix to the juxtaepithelial stroma throughout development (Hay and Revel, 1969; Trelstad, 1970). We chose 5–7-day old epithelium for most of our experiments because at that time the corneal epithelium seems to be producing most of the corneal connective tissue stroma.

The 5–7-day old lens used in the experiments reported here consisted before freezing of an anterior epithelium and an elongated posterior epithelium which is differentiating into lens fibers. The lens capsule, a basement membrane composed of several layers of basement lamina, survives the freezing and serves as a very suitable insoluble collagenous substratum. It is thinner anteriorly than posteriorly, a feature which permits identification of the surfaces in sections (Fig. 2). The cornea is separated from the lens by the anterior chamber at 5 days of development and thus both are readily isolated without contamination of one by the other (Fig. 4–4, Hay and Revel, 1969).

Appearance of Isolated Corneal Epithelium, Lens, and Control Cultures

As in the case of corneal epithelium isolated with trypsin or collagenase (Figs. 3–10, 3–12, Hay and Revel, 1969), corneal epithelium isolated by EDTA treatment shows tremendous blebbing of its naked basal surface (Fig. 3). Extension of such naked pseudopodia or blebs seems to be characteristic, indeed, to be diagnostic of removal of basal lamina from basal surface of an epithelium (see Cohen and Hay, 1971). The response of a cell to loss of its glycoprotein basal surface coat, then, is to send out extrusions of membrane-bounded cytoplasm. The apical and lateral surfaces may show mild blebbing after enzyme treatment (Cohen and Hay, 1971), but the free surface and intercellular surfaces seem little affected by the mild EDTA treatment used

here. The intercellular junctions described by Hay and Revel (1969) in corneal epithelium are preserved during the EDTA treatment used here and the tissue can be handled with forceps because it remains an intact sheet. Intracellular organelles also seem little affected by EDTA in the concentration used here.

15 isolated epithelia were examined immediately after isolation for contamination by extracellular matrix. 14 of these epithelia showed blebs on the basal surface when examined by light microscopy; electron microscopy confirmed the conclusion that the basal lamina had been completely removed from the basal surface of the epithelium (Fig. 3). In one case, the basal surface of an EDTA-isolated epithelium was smooth (not blebbed) as examined in the light microscope. In this case, electron microscope examination revealed that the basal lamina adhered to some parts of the epithelium when it was pulled off of the EDTA-treated stroma. The epithelium was from a 9 day old stroma in this case; possibly the adherence of epithelial basal plasmalemma to basal lamina is more secure at 9 days than at the earlier 5–7 day period used for the majority of the experiments. We estimate the effective rate for isolation of perfectly clean (sans basal lamina) epithelium by EDTA treatment to be about 90%, whereas enzyme treatment is probably 100% effective in removing basal lamina (Cohen and Hay, 1971). In no case were any contaminating collagen fibrils or fibroblasts present in association with isolated epithelia.

Isolated lenses were also examined carefully in the electron microscope. The collagen of the lens capsule is insoluble under the conditions used here (Kefalides, 1970). The lens capsule both in controls lacking epithelia and in the experimental series remained distinct and intact throughout the experiment, with no significant alteration in fine structure. Lens cells often lysed and slipped out through tears in the capsule, leaving a few dead cytoplasmic remnants within the capsule (Fig. 2).

There were never any striated collagen fibrils contaminating the lens preparation.

Control lenses lacking epithelia were grown on the same rafts as the lenses with epithelia in each experiment. Seven lenses were examined carefully in the electron microscope and by autoradiography. They never showed any significant radioactivity, nor did the numerous other control substrata that were also employed (Dodson and Hay, 1972).[1] The lysed lens cells within the lens capsule were definitely dead, then, as judged by lack of ability to incorporate radioactive isotopes as well as by their microscopic appearance (Fig. 2). Additional evidence that the dead lens makes no contribution to the corneal stroma that appears in culture comes from controls employing other types of epithelia. Back epidermis and apical limb epithelium do not produce striated fibrils when grown on lens (Dodson and Hay, 1972)[1]; neural epithelium produces only a few fibrils (Cohen and Hay, 1971).
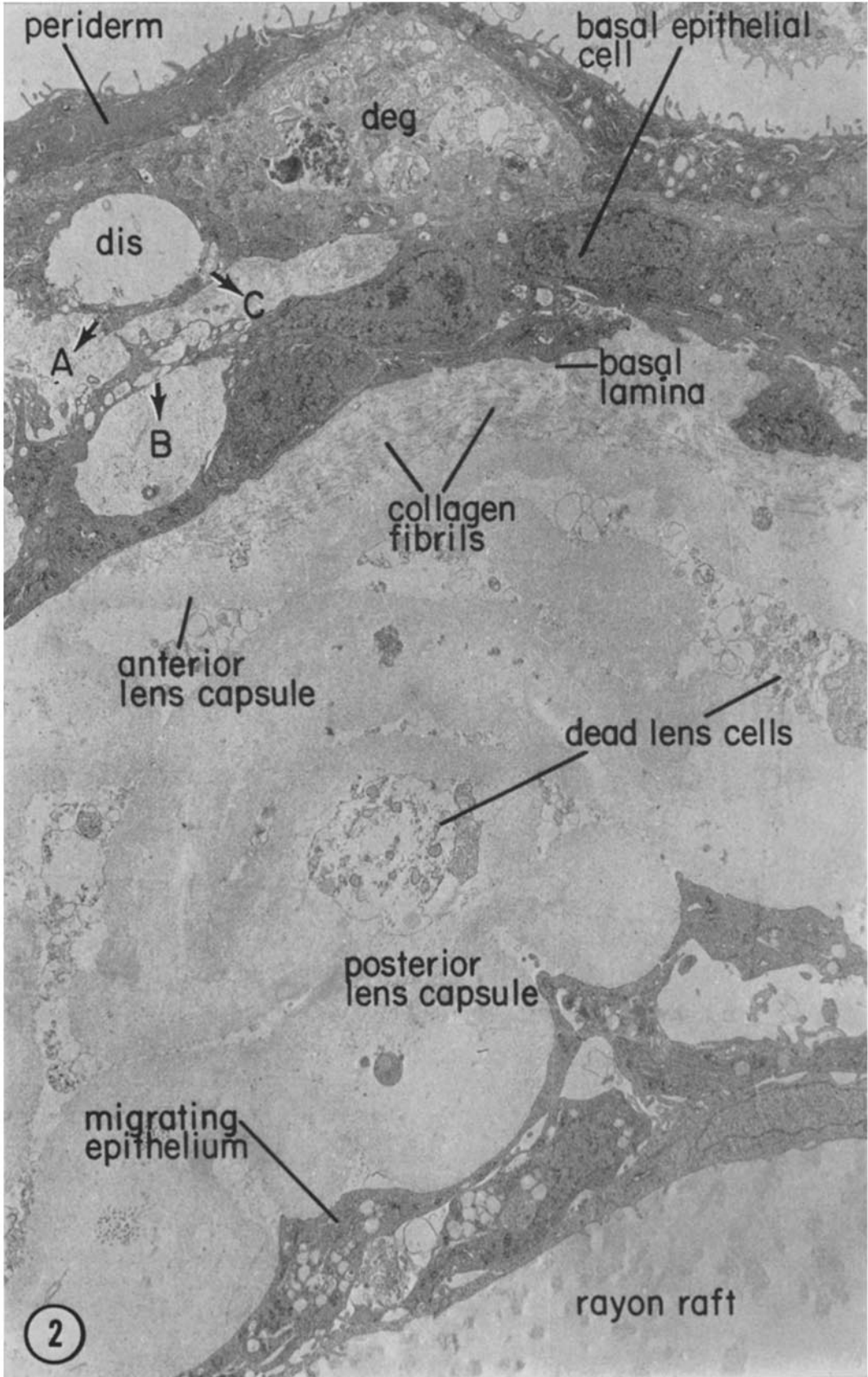
Fine Structure of Epithelium Grown on Killed Lens

The EDTA-isolated epithelium was generally placed on the anterior surface of the frozen-killed lens where, as we have noted, the capsule is relatively thin (anterior lens capsule, Fig. 2). After 3–5 days in culture, the growing epithelium spreads completely around the lens substratum, covering its posterior surface (posterior lens capsule, Fig. 2). In order to do this, the migrating epithelial cells treat the supporting raft (rayon raft, Fig. 2) as a free surface, preferring the underlying lens capsule as a substratum upon which to migrate. Under other conditions, with less desirable substrata, epithelium will migrate away randomly along the threads of the rayon raft (Dodson and Hay, 1972).[1]

The cultured corneal epithelium after 3 days in vitro is generally two layers thick and actively proliferating, as in vivo at an equivalent time

FIGURE 2   After the isolated corneal epithelium has grown on the killed lens in organ culture for 3 days, it has spread completely around the substratum as shown here. The oldest part of the epithelium, on top of the substratum, shows some degeneration (*deg*) and distended intercellular clefts (*dis*). Other intercellular clefts (*A, B, C*) are full of newly manufactured collagen. Clefts *A* and *B* are shown at higher magnification in a serial section in Fig. 10. Newly formed collagen is abundant under the older epithelium at the top. The migrating cells at the bottom have just joined up to enclose the substratum; they have not yet made any detectable collagen and rest directly on the lens capsule. The epithelium shown here and in Fig. 4 was isolated from a 7 day old chick embryo. × 3,000.

periderm

basal epithelial cell

deg

dis

C

A

B

basal lamina

collagen fibrils

anterior lens capsule

dead lens cells

posterior lens capsule

migrating epithelium

rayon raft

2

(8–9 days, Hay and Revel, 1969). The outer layer of the epithelium (periderm, Fig. 2), like the equivalent layer in vivo, consists of flattened cells with rather dense cytoplasm linked by zonulae occludentes and desmosomes. Peridermal cells in vitro increase the number of microvilli on their free surface, as they would have done in vivo in this period. Microvilli on the free surface of the periderm are more numerous in the original area of the explant, where the cells presumably are more differentiated (top, Fig. 2). The migrating cells on the lower lens surface are younger in appearance in other respects as well; their cytoplasm is not as dense and nuclei are more euchromatic (chromatin more dispersed) than those of peridermal cells at the top of the explant.

The basal cells of the cultured corneal epithelium tend to be rounded or cuboidal in the most differentiated areas of the explant (top, Fig. 2), and flattened in areas where they have recently been migrating (bottom, Fig. 2). They rarely assume a columnar shape reminiscent of that seen in vivo, even though a basal lamina is present. Tonofilaments and microtubules are not as well developed as in normal corneal epithelium (Hay and Revel, 1969). Nuclei are euchromatic in basal cells and nucleoli are prominent, as in vivo. Mitochondria are generally normal in appearance and often contain a finely filamentous material (Fig. 4). Lipid droplets are more numerous than in normal cytoplasm and focal areas of cell degeneration (deg, Fig. 2) are not uncommon in the cultured epithelium. The intercellular clefts are more often distended than is the case in vivo. They contain either liquid or nonstructured materials (dis, Fig. 2) or fibrillar material (A, B, C, Figs. 2 and 10). Desmosomes, hemidesmosomes, and other intercellular connections are present (Fig. 9). In the original area of the explant (top, Fig. 2), the basal surface of the epithelium tends to be smooth due to the presence of newly secreted basal lamina and collagenous stroma. On the lower side of the lens capsule, where at 3 days the edges of the migrating epithelial sheet are just meeting, the basal surface of the cells follows the contour of the lens capsule upon which the cells are migrating. There is not as yet any newly secreted extracellular matrix on this surface (Fig. 2).

As in the intact embryo, the basal cells of the cultured corneal epithelium are very rich in granular endoplasmic reticulum and each contains a prominent Golgi complex usually located lateral to the nucleus (Fig. 4). The endoplasmic reticulum extends into the Golgi complex and may connect with smooth-surfaced lamellae. The Golgi complex consists of the usual flattened lamellae, numerous small coated and uncoated vesicles, and various sized vacuoles (Hay and Revel, 1969). Golgi vacuoles may appear empty, especially those located near or in the faces of the stacked Golgi lamellae (vac 4, Fig. 4). A second type of vacuole (vacs 5 and 6, Fig. 4), seemingly derived from the first type, contains finely filamentous material of the same density as the basal lamina. The filamentous material, which we believe is likely to be collagen, has the same fine structure as that in the cavity of the endoplasmic reticulum (er, Fig. 4). Vacuoles in the cultured cells containing the basement lamina-like material are the same size (vacs 5 and 6, Fig. 4) as in vivo (Figs. 3–5, Hay and Revel, 1969) or they may be considerably larger (vacs 1 and 7, Fig. 4), presumably due either to overproduction of the filamentous material or to overaccumulation due to impaired excretion in culture.

The idea that the filamentous material in the Golgi vacuoles is collagen and that its progress out of the cell is impaired in vitro is supported by the fact that it polymerizes into striated fibrils within intraepithelial clefts (vac 2, Figs. 4 and 5),

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FIGURE 3   The basal surface of the freshly isolated corneal epithelium shows numerous blebs as a result of separation from the basal lamina and underlying stroma after EDTA treatment. × 30,000.

FIGURE 4   A higher magnification electron micrograph of part of a corneal epithelial cell in a 3 day culture shows the secretory organelles to good advantage. The granular endoplasmic reticulum (er) extends into the Golgi apparatus where it becomes smooth surfaced and sometimes connects to Golgi lamellae (short arrows). Secretory vacuoles with a content similar to that in the endoplasmic reticulum (vacs 1, 5, 6) seem to derive from the Golgi complex. They may become quite large (vac 7). Vac 2, which seems to connect to vac 1 (long arrow), contains a striated fibril (Fig. 5). It is likely that this vacuole is, in fact, an extracellular cleft. The basal or basement lamina is newly formed; indeed, isolated epithelia grown on substrata other than lens do not make a lamina. × 30,000.
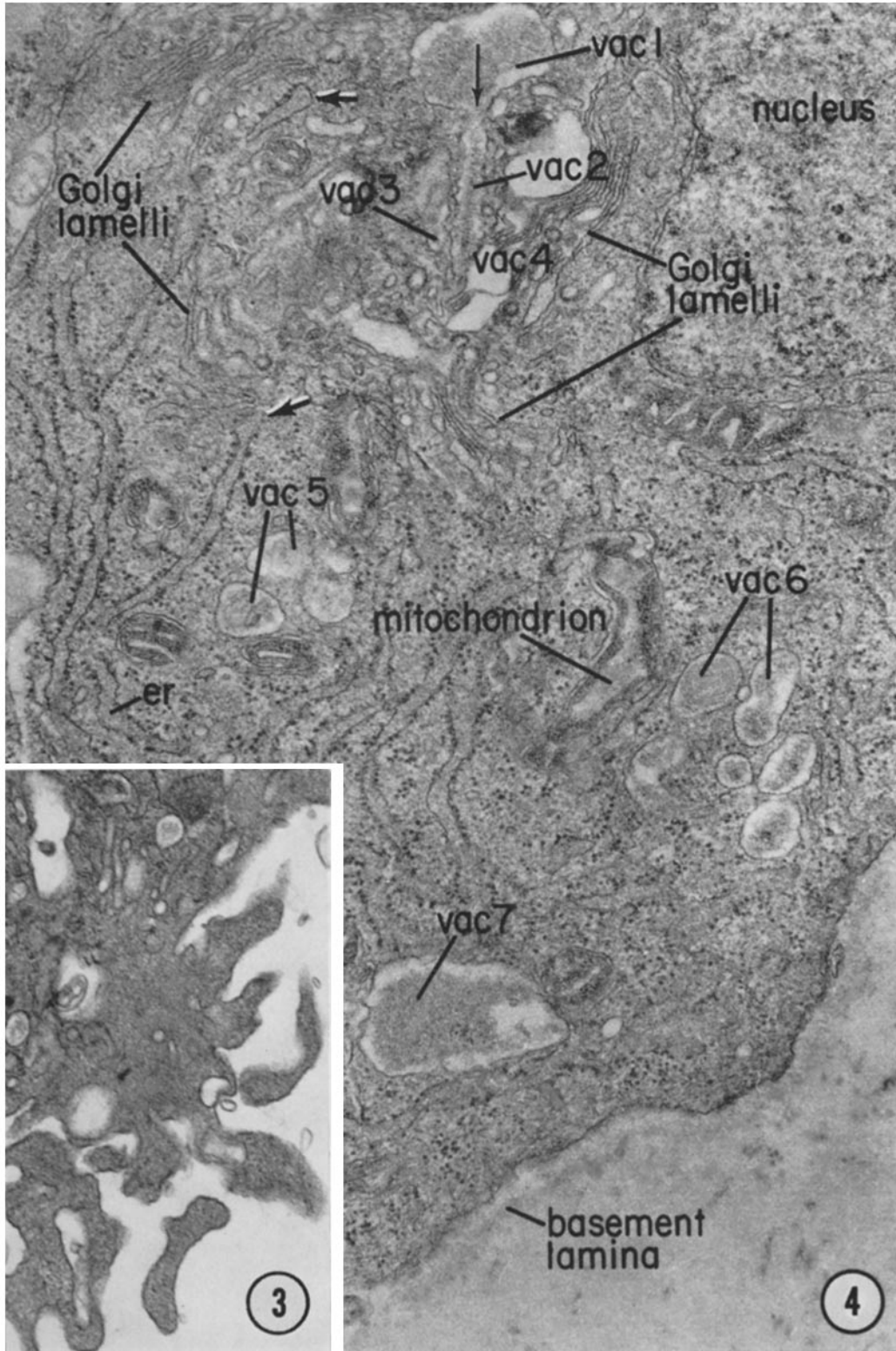
that abut onto the Golgi vacuoles (vac 1, Figs. 4 and 5). Such clefts are not often observed in vivo in corneal epithelium. Oblong or rodlike vacuoles containing fibrillar, but not overtly striated, material are seen in vivo and in vitro (vac 3, Figs. 4 and 5; vac 4, Fig. 10). Some of the round profiles (vac 1, Fig. 10) may be cross sections of rodlike vacuoles. We have not observed vacuoles containing filamentous material directly connected to the cell surface, although profiles resembling emptied vacuoles are common next to the lateral and basal plasmalemmas (vac 3, Fig. 10). It is likely that the rare clefts or vacuoles in the Golgi complex containing fibrils that are actually striated (vac 2, Fig. 5) connect to the cell surface, even though the bounding membrane seems to be like that of other Golgi vacuoles (vac 1, Fig. 5). We do not have serial sections near Fig. 5. Re-examination of serial sections prepared for this purpose, however, revealed intracellular clefts (Fig. 6) that connect to the lateral cell surface (Fig. 7); the fibrils they contain are the same size or larger than that in vac 2 (Fig. 5). Certain vacuoles, which may or may not be oblong in shape, depending on the plane of section, have a denser content (vac 2, Fig. 10) and may correspond to the very dense vacuoles thought to contain collagen in the process of condensation before secretion (Trelstad, 1971). These observations, which lend weight to the Golgi theory of collagen secretion (Revel and Hay, 1963), will be considered further in the Discussion.

Fine Structure of the Collagen Secreted by Isolated Corneal Epithelium

By 3 days in culture, in 12 of 18 cultures studied with the electron microscope, the corneal epi-

thelium produced a basement lamina over part, but by no means all, of its basal surface (Figs. 4, 9, and 10). Usually located on the anterior (top) surface where the epithelium is the thickest, the lamina consists of a moderately dense filamentous sheet several hundred angstroms thick located 200–300 Å from the basal plasmalemma, to which it is seemingly connected by small filaments 20 Å in diameter. As in vivo, small filaments and amorphous strands on the stromal side of the basal lamina seem to link it to the underlying collagenous stroma. The density of the central sheet of the lamina (basement lamina, Fig. 9) is almost exactly the same as that of nearby collagen fibrils cut in cross section (f 2, Fig. 9).
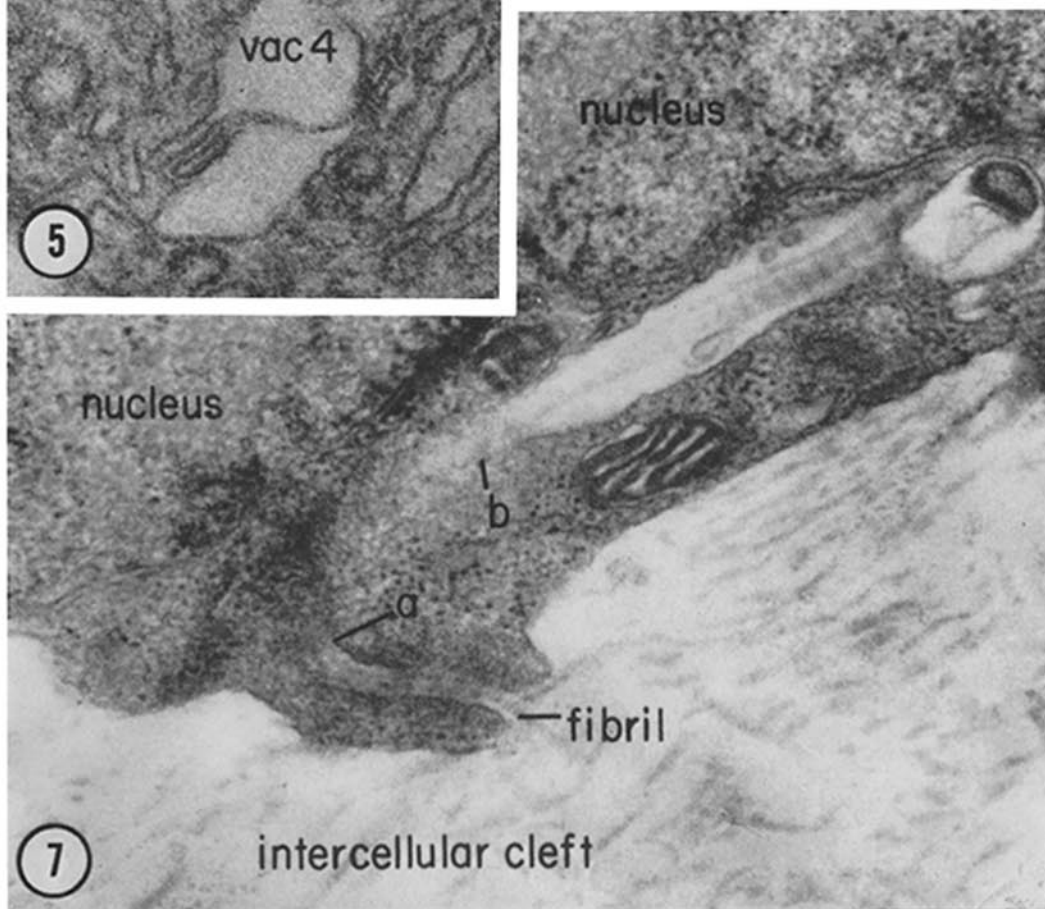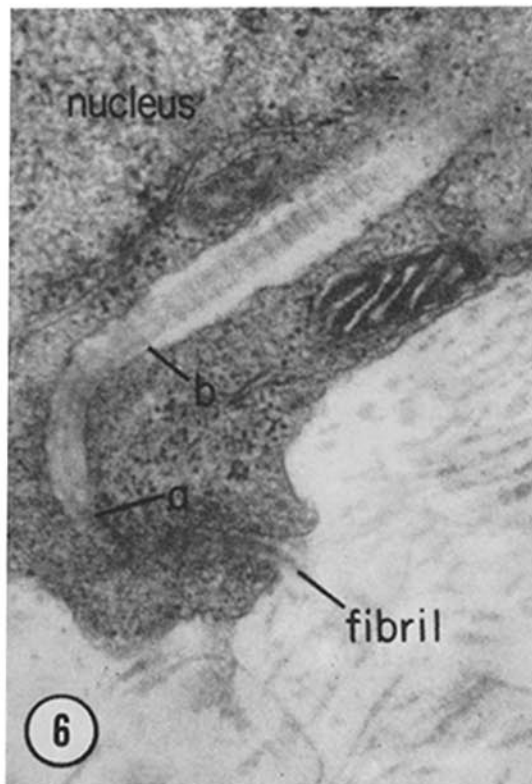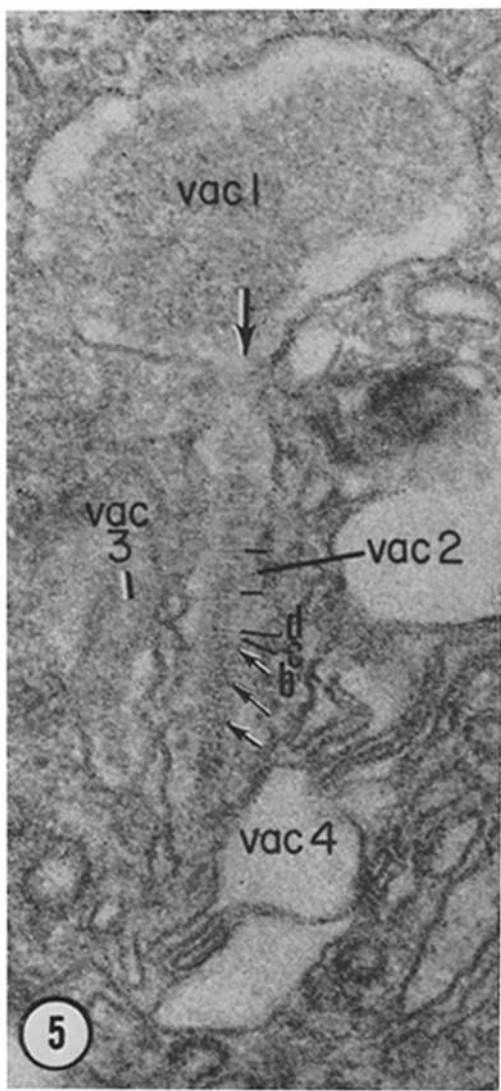
The second type of newly formed collagen which polymerizes under the epithelium of origin is striated and takes the form both of fibrils (collagen fibrils, Figs. 4, 9, 10), and of sheets (Figs. 10–13). The fibrils are seemingly almost identical in fine structure to those seen in the corneal stroma in vivo. They average 250 Å in diameter with a typical repeating major period (550–600 Å intervals) and the usual minor interbands (a, b, c, d, e, Fig. 8). Some of the fibrils are smaller in diameter than 250 Å, however, and some may even be juxtaposed to form sheets, as will be described below. The fibrils tend to be arranged at right angles to each other (e.g., f 4 and 5, Fig. 9), as in vivo, but their general distribution is much more random than in the normal stroma.

The polymerization of newly secreted collagen into striated sheets was an unexpected, but interesting finding. The banding is identical to native collagen (Fig. 8). Rarely cut in perfect longitudinal section, such sheets appear to consist of dense (S 1, S 3, Fig. 11) and less dense (S 2,

Figure 5 The striated fibril in the cleft labeled vac 2 (Fig. 4) is shown here at high magnification. The repeat period (500 Å) is the same as native collagen. The dashes mark d bands and the small arrows mark b bands. Vac 2 either connects with or is closely apposed to vac 1 (arrow). Vac 1 contains the unpolymerized filamentous (basal lamina-like) material we believe to be collagen precursor. Vac 3 contains material similar to that in vac 2, but striations are not apparent. Vac 4, which is an expanded saccule forming from a Golgi lamella, seems empty but perhaps contains mucopolysaccharide. × 100,000.

Figures 6 and 7 Shown here are two sections less than half a micron apart showing an "intracellular" vacuole containing striated collagen. The regions labeled a and b correspond to each other in the two sections. The so-called vacuole is clearly a tortuous intracellular cleft which connects to the lateral cell surface at the point labeled "fibril." It is likely that vac 2 (Fig. 5) also connects to the lateral cell surface, because intracellular clefts containing striated collagen with a repeat period (500 Å) of native collagen are fairly numerous in the cultured corneal epithelium. × 60,000.

Figure 5. vac 1, vac 3, vac 2, vac 4, d, e, b

Figure 6. nucleus, b, a, fibril

Figure 7. nucleus, nucleus, b, a, fibril, intercellular cleft

S 4, Fig. 11) areas that are in register. High voltage electron microscopy reveals the dense edges (*S 1*, *S 3*, Fig. 13) to be located on cut edges of the section. They are seemingly more intensely stained by the lead citrate applied to the cut edge. Deeper parts of the sheet (*S 2*, *S 4*, Fig. 13) are less dense probably because they were stained only by the uranyl acetate used en bloc. The fact that striations extend across from less dense to dense areas of the sectioned sheets (*S 1–S 4*, Figs. 11 and 13) attests to the integrity of the sheets. At the ends of the sheets individual fibrils seem to splay out (*f*, Figs. 10 and 13). High voltage electron microscopy of thick sections (Fig. 13) shows that the sheets twist and turn and also take on a scalloped shape. In thin sections, the shape of each longitudinally cut sheet is also seen to be highly irregular (Figs. 11 and 12).

There are many profiles in the thin sections which we interpret to be cross sections of the scalloped, interwound sheets (sheets, Fig. 10). The density in cross section is identical to that of the cross-sectioned fibrils, even to the presence of the punctate staining, stippled areas (*p*, inset, Fig. 10). Oblique sections show continuity of the striated longitudinal profiles and the stippled profiles (*obl*, Figs. 11 and 12). The cross-sectioned profiles suggest that some of the sheets are branched (*br*, Figs. 10 and 11). The alternative to the interpretation of such profiles as cross sections of striated sheets is that they are curled fibers of lamina-like collagen. They do not, however, closely resemble the lamina (compare sheets and basal lamina, Fig. 10). Random sampling could explain the predominance of cross sections (Fig. 10).

Another interesting finding with no in vivo parallel was the polymerization of striated col-

lagen fibrils within the intercellular clefts of the epithelium (Fig. 10), as well as within cytoplasmic vacuoles and clefts as we already noted (Figs. 5–7). In addition to fibrils, sheets similar to those in the subepithelial compartment were very commonly seen in such clefts (Fig. 10). That these clefts were not oblique sections through the basal surface is shown by the lack of penetration of basal lamina into the cleft (lower right, Fig. 10) and by the location of collagen-filled clefts next to the periderm. Moreover, we ascertained by serial sections the fact that the plane of section illustrated in Fig. 2 was perpendicular to the base of the epithelium. Fig. 10 is a micrograph of a section near that shown in Fig. 2. The intraepithelial pockets labeled *A* and *B* can be identified in both micrographs. They are connected to the basal surface by a very narrow intercellular cleft (Fig. 10). Smaller "intracellular" clefts containing collagen, as at *a–b* in Figs. 6 and 7, were observed to open into dilated interfacial canals that also contained collagen.
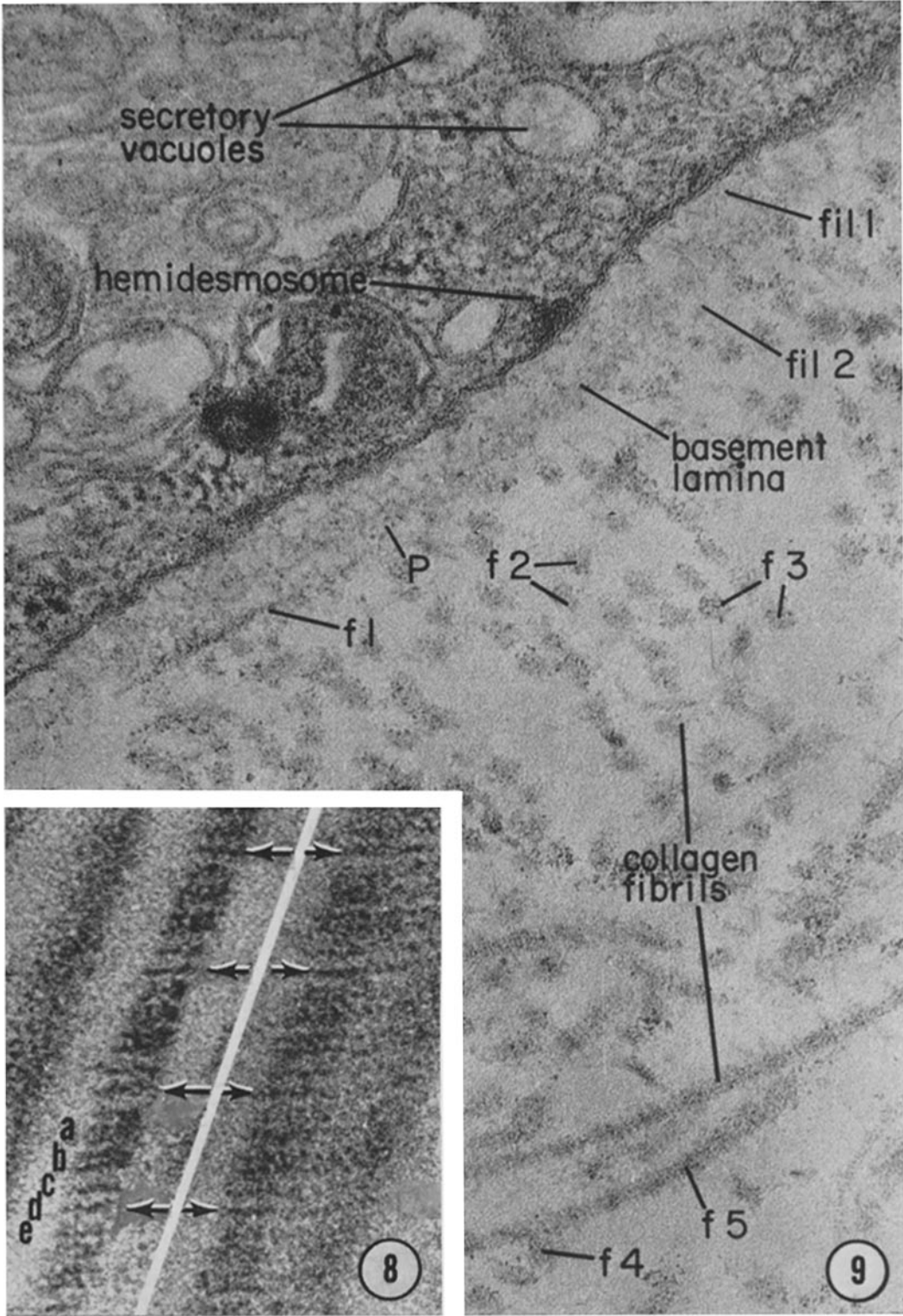
Autoradiographic Studies

Autoradiographic studies of cultures exposed to [³H]proline showed that intensely labeled, insoluble products were concentrated in areas rich in striated fibrils and sheets as, for example, within the intercellular canals and epithelial clefts (Fig. 14) described above (Figs. 6, 10). Basal lamina (Figs. 14, 15) and striated fibrils under the epithelium were also radioactive. Silver grains occur over the lens capsule (silver grain, Fig. 14) in the experimental series but not in the controls, suggesting that some of the newly secreted proline-rich material diffuses into the lens without polymerizing into the recognizable collagenous products described above. Diffuse

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FIGURE 8   At higher magnification, the fibrils (200–250 Å in diameter), labeled "collagen fibrils" in previous figures, can be seen to contain the native collagen repeating period (left); the collagenous sheets have a similar period (right). × 400,000.

FIGURE 9   The newly formed basal lamina (like the lamina in vivo) seems to be connected to the basal plasmalemma by small filaments or amorphous strands (*fil 1*). It connects to underlying collagen fibrils (*f 1*) by similar small strands (*fil 2*). The distance from *fil 2* to the cell membrane is 0.1 μm, the usual width of the lamina. Cross sections of the newly formed collagen fibrils are similar to the lamina in density (*f 2*) and may contain punctate structures (*f 3*) like those in the lamina (*p*). The fibril labeled *f 5* is orthogonal to the cross-sectioned fibril labeled *f 4*. × 150,000.

secretory
vacuoles

fil 1

hemidesmosome

fil 2

basement
lamina

P

f 2

f 3

f l

collagen
fibrils

a
b
c
d
e

f 5

f 4

8

9

label over nuclei and cytoplasm indicated incorporation of proline into structural components of the growing cells, as well as into collagen. The intense radioactivity of the basal lamina, striated fibrils, and sheets supports the conclusion reached above that these are new products made by the corneal epithelium, the only living cells present in the cultures.

We carried out an additional test to show that the [³H]proline labeled material under the epithelium was collagen. The fact that it withstood fixation and washing procedures that removed free proline from control lens already indicated the label was a large molecule. We extracted the control and experimental cultures with 0.1 N NaOH to remove noncollagenous protein. The intensely radioactive material on the surface of lenses cultivated with corneal epithelium withstood this procedure well and is likely to be collagen. It is unlikely to be alkali-insoluble membrane protein because epithelial cells were removed by the NaOH extraction and electron microscopy revealed little or no membrane residue in the extracellular materials remaining after NaOH extraction. For further discussion of this extraction technique, the reader is referred to Cohen and Hay (1971).

DISCUSSION

Our evidence for the synthesis and secretion of collagen by corneal epithelium can be summarized as follows: (a) basal lamina and abundant native striated collagen are laid down under and within the isolated corneal epithelium in culture; (b) basal lamina and collagen fibrils are intensely labeled when [³H]proline is added to the medium and the [³H]proline label over the extracellular fibrils resists NaOH extraction, a procedure designed to demonstrate radioactivity in collagen;

(c) possible sources of nonradioactive basal lamina and striated fibrils, such as repolymerization of lens capsule protein, were ruled out by appropriate controls; (d) no living cells other than the corneal epithelium are present in the culture; lens cells in the substratum are dead as judged both by their morphology and their inability to metabolize [³H]proline; EDTA isolated epithelium is completely free of contaminating cells and collagen fibrils, and is rarely contaminated by basal lamina; (e) the corneal epithelial cells are rich in secretory organelles, vacuoles, and intercellular clefts containing radioactive product; thus, they are synthetically active.

The production by isolated epithelium of both basal lamina and striated collagen fibrils is the best evidence to date that corneal epithelium can produce both "epithelial type collagen" (basal lamina) and "fibroblast type collagen" (striated fibrils). These morphological data are considerably more significant than the chemical data previously available, because the morphological fingerprint of the native collagen fibril is so unmistakable. Chemical studies have shown only that isolated corneal epithelium produces a protein containing hydroxyproline (Revel, 1965; Goodfellow, et al., 1969; Conrad, 1970 b). Together with a concurrent study of neural epithelium (Cohen and Hay, 1971), the work makes it safe to conclude that production of fibrous collagen is not "the exclusive function of the fibrocyte family of cells" (Porter, 1964, page 28), as was heretofore so widely believed. Some of the biological implications of this statement will be considered at the end of the discussion. Let us first consider variations in collagen polymerization patterns in vitro, the origin and structure of the basal lamina, and the role of cytoplasmic organelles in collagen synthesis, since our results also bear on these topics.

FIGURE 10 The intercellular clefts A and B shown in Fig. 4 are shown here at higher magnification in an adjacent section. Cleft B connects to the basal surface via a narrow intercellular cleft which has a desmosome-like junction on its interface. The collagen fibrils in the intercellular clefts and basal epithelial compartment are produced by the epithelium. They are radioactive when [³H]proline is added to the medium (Figs. 14, 15); no other living cells are present in the culture (Fig. 2). Several vacuoles containing collagen-like matrix precursor are labeled (vacs 1, 2, 4), one of which is rod-shaped (vac 4). Vac 3 connects to the cell surface where it may have recently discharged its contents. Large fibrils or sheets with the native collagen repeat period occur in the intercellular canals (arrow, main figure and inset). They connect to fibrils (f). In cross section, the sheets appear to curl and branch (br, inset). They contain punctate structures (p, inset) like those in collagen fibrils and basal lamina (Fig. 9). × 33,000. Inset, × 150,000.

A

collagen
fibrils

f

B

sheets

intercellular
cleft

basal
lamina

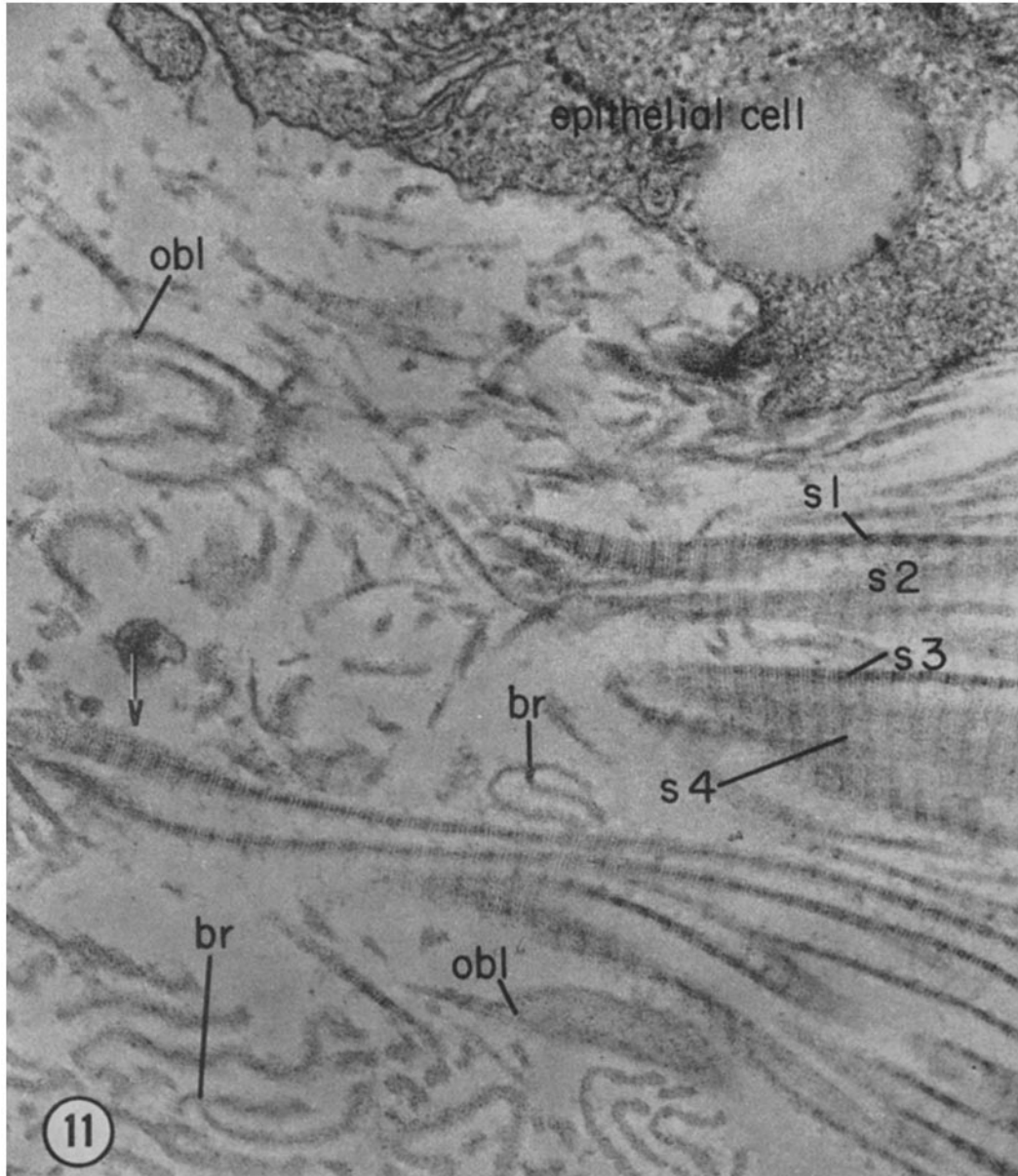vac 2

vac 4

vac 3

br

br

vac 1

P

(10) collagen
fibrils
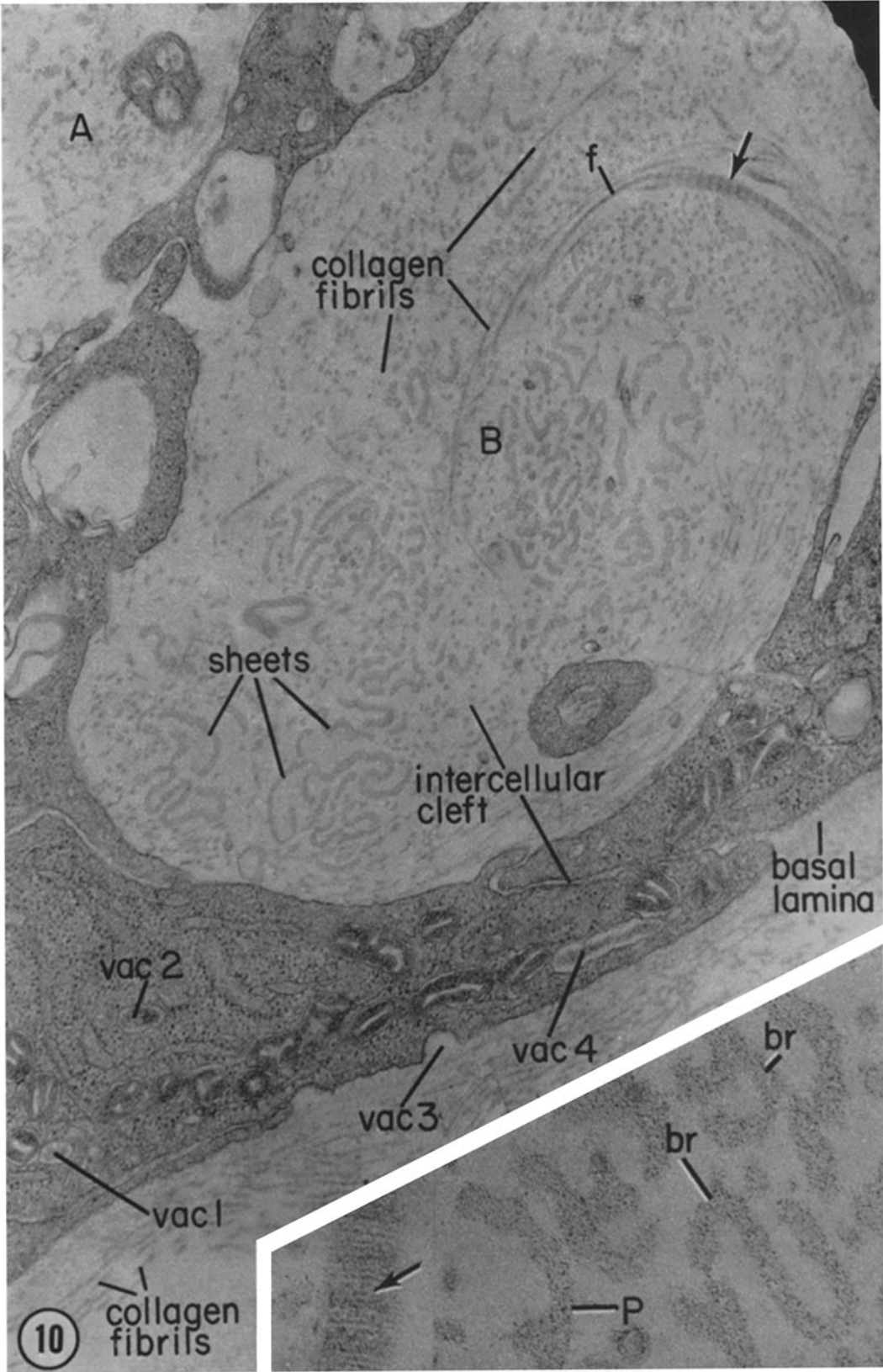
FIGURE 11 Another view of the collagenous sheets formed in the cultures shows that dense edges (*s 1, s 3*) connect to less dense portions (*s 2, s 4*). The dense edges are located near the surface of the section and stain more intensely with lead citrate than the deeper portions of the sheets (Fig. 13). In oblique sections, striated portions of the sheets connect to the unstriated cross-sectioned face (*obl*). Cross sections reveal branching within the sheets (*br*). A vesicle-like structure of unknown significance is present (*v*). × 70,000.

Polymerization of Collagen into Striated Sheets

In addition to basal lamina and the native "640 Å" striated fibrils[2] of the small diameter (250 Å) typical of corneal stroma, tortuous sheets of collagen with the period characteristic of the smaller fibrils also polymerized in vitro. The sheets were particularly common in intraepithelial pockets, but were also seen under the epithelium.
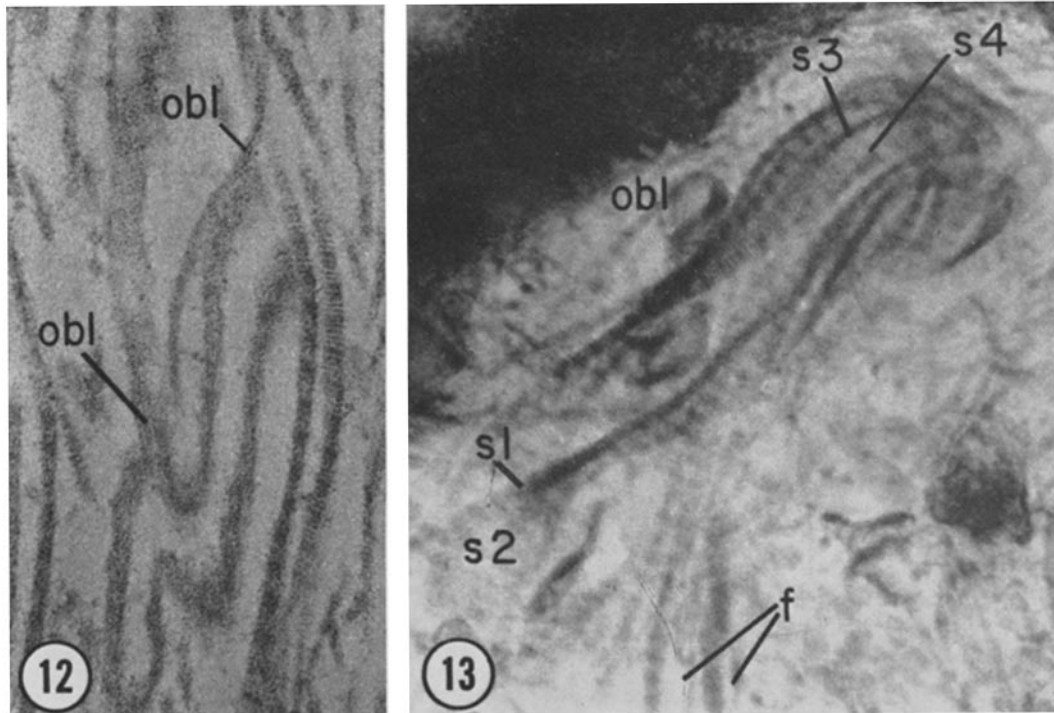
FIGURE 12 Another view of a thin section shows to good advantage the twisting of collagen sheets. Oblique planes of section show the connection of the striated long faces of the sheets to the unstriated cross faces (*obl*). × 85,000.

FIGURE 13 A high voltage electron micrograph of a 1 μm thick section shows the twisting and interconnections of the striated collagenous sheets. Compare the obliquely sectioned profile (*obl*) to those shown in Fig. 12 in a thin section. Near the surface of the section, the sheets are stained more intensely (*s 1*, *s 3*) than deeper in the section (*s 2*, *s 4*). The sheets connect to fibrils (*f*). × 50,000.

Gross (1956, 1961) reported reprecipitation of fibroblast-derived collagen into fibrous long-spaced sheets and also into sheets with a native 640 Å period similar to those reported here. Reports of striated collagen occurring in vivo in sheet-like array are uncommon, however. Concentric sheets or large fibers of striated collagen have been noted in the fins of fishes (Fitton-Jackson, 1968) and very wide fibrils are present in pre-dentine (Slavkin, 1971). Broad aggregates of native striated fibrils up to 2,000 Å in diameter also occur in the cartilage matrix of a chondrodystrophic mutant mouse (Seegmiller et al., 1971) and these large fibrils or sheets anastomose with one another, much as in the present case.

The matrix of the chondrodystrophic mutant seems to have less than the normal amount of acid polysaccharide. Seegmiller et al. (1971) have suggested that glycosaminoglycan keeps the collagen fibrils small in diameter (100–250 Å) in

cartilage by binding to cross-linking sites on the collagen molecules; in the mutant, extra sites might be available for binding of collagen to collagen. The normal corneal stroma resembles the cartilage matrix in its high content of acid mucopolysaccharides and, interestingly, also contains very small collagen fibrils. We have no quantitative data on acid mucopolysaccharide secretion by corneal epithelium in vitro as compared to in vivo. The alcian blue reaction is weak at this early stage (Conrad, 1970 a), and thus it is difficult to estimate a difference between in vivo and in vitro stromas by histochemistry. If acid mucopolysaccharide could be shown to be low in the areas where sheets were seen, we might entertain the same hypothesis for abnormal corneal fibrillogenesis here as was suggested by Seegmiller et al. (1971) for the chondrodystrophic mutant.

Other abnormalities that were noted in vitro were incompleteness of the basal lamina on the

undersurface of the epithelium and the lack of strict orthoganality in the arrangement of the layers of collagen fibrils themselves. Factors other than the epithelial undersurface (Edds and Sweeney, 1961; Berliner, 1969) and basal lamina (Nadol et al., 1969) must operate in vivo to order the orthogonal layers in such a precise gridwork (Trelstad and Coulombre, 1971). We have not determined whether or not the basal lamina would eventually have become complete or the collagen fibrils more regular in long-term cultures. We did examine the effect of living mesenchyme on the production of basement lamina by corneal epithelium and found the added tissue had little effect (Dodson and Hay, 1972).[1] It is possible that 5-7-day old corneal epithelium in vivo is already deficient (before isolation) in synthesis of basement lamina materials due to its advanced "age"; a comparison with younger neural epithelium in this regard will be made in the next section of the discussion.

Origin and Structure of the Basal Lamina

Unlike the striated collagen fibril, the basal lamina has often been considered to be a product of the adjacent epithelial cell (Porter, 1964; Fawcett, 1966). Direct evidence, however, for its epithelial origin such as presented here, and obtained from cultures of isolated neuroepithelium (Cohen and Hay, 1971), has not been available heretofore. In the kidney, the endoplasmic reticulum of glomerular epithelial cells contains a material antigenically (Andres et al., 1962) and ultrastructurally (Farquhar, 1964) similar to the basal lamina or so-called basement membrane. There are many situations in vivo where a basal lamina forms under an epithelium, Schwann cell, or muscle cell with few if any fibroblasts being nearby, but it can be argued that the collagen was produced elsewhere and merely polymerized next to the cell in question (Kurtz and Feldman, 1962; Nathaniel and Pease, 1963; Dische, 1964; Thomas, 1964; Pierce, 1966, 1970; Fawcett,

1966; Hay and Revel, 1969; Jensen and Mottet, 1970; Briggaman et al., 1971). In the case of developing amphibian epidermis, we suggested that both underlying fibroblasts and epidermis contributed to the basal lamina and striated collagen fibers of the basement membrane (basal lamella), because [3H]proline labeled products of both origins accumulated under the epithelium in vivo (Hay and Revel, 1963). The most direct evidence previously available for a nonfibroblastic origin of the epithelial basement lamina is the case of synthesis of basement membrane-like material by presumably pure lines of carcinoma cells in vitro (Pierce et al., 1962). In addition to corneal epithelium and neuroepithelium (Dodson and Hay, 1971; Cohen and Hay, 1971), isolated smooth muscle has now been shown to be capable of reconstructing its basal lamina in culture (Ross, 1971).

The fact that the basal lamina contains a true collagen has not yet been generally appreciated. In a recent morphogenetic theory, for example, Bernfield et al., (1972, p. 675) suggest that disruption of the basal lamina by collagenase means that the "treatment affects materials other than collagen;" they draw conclusions about the morphogenetic role of basal lamina that erroneously take into account only the polysaccharide component. It is now generally agreed that the hydroxyproline-rich component of the basal lamina is collagen. This molecule has a helical structure and can form SLS type aggregates with ATP (Kefalides, 1970), but it contains only one kind of alpha chain (Kefalides, 1971), which can be distinguished from the $\alpha 1$ of both cartilage and skin.

It remains to be learned from biochemical studies whether the basal lamina and striated collagen fibrils produced by epithelia are composed of two different kinds of collagen molecules or the same kind of collagen molecule. The extent to which epithelial products polymerize into one or the other of these morphological forms of collagen does differ significantly, however, among various

FIGURES 14 and 15   Autoradiographs of cultured epithelium grown for 3 days in the presence of [3H]proline reveal intense incorporation of proline into the intercellular and extracellular collagen fibrils and sheets. Label also appears over the basal lamina (basement lamina) and lens capsule (silver grain). Resistance of label in the extracellular compartment to extraction with NaOH indicates that it is largely in newly formed collagen secreted by the isolated epithelial cells. The epithelium shown here was taken from a 7 day old chick embryo. × 25,000.

fibrils

fibrils

intercellular cleft

silver grain

basement lamina

(14)

fibrils

basement lamina

(15)
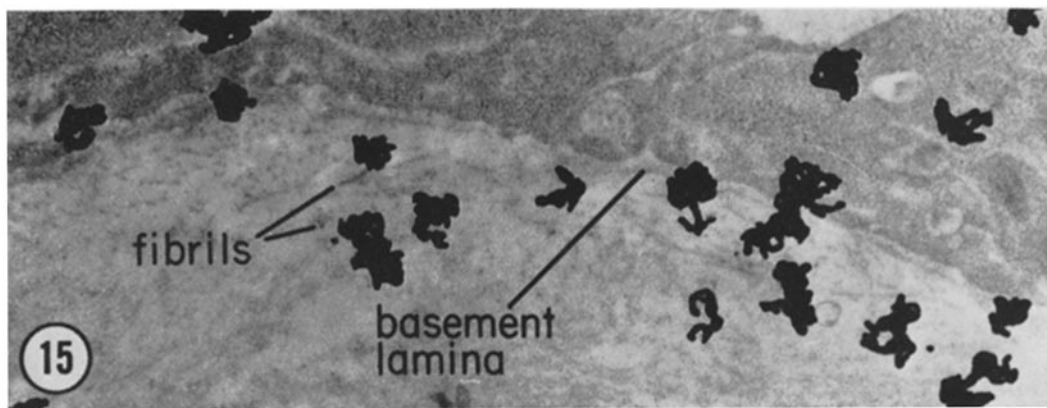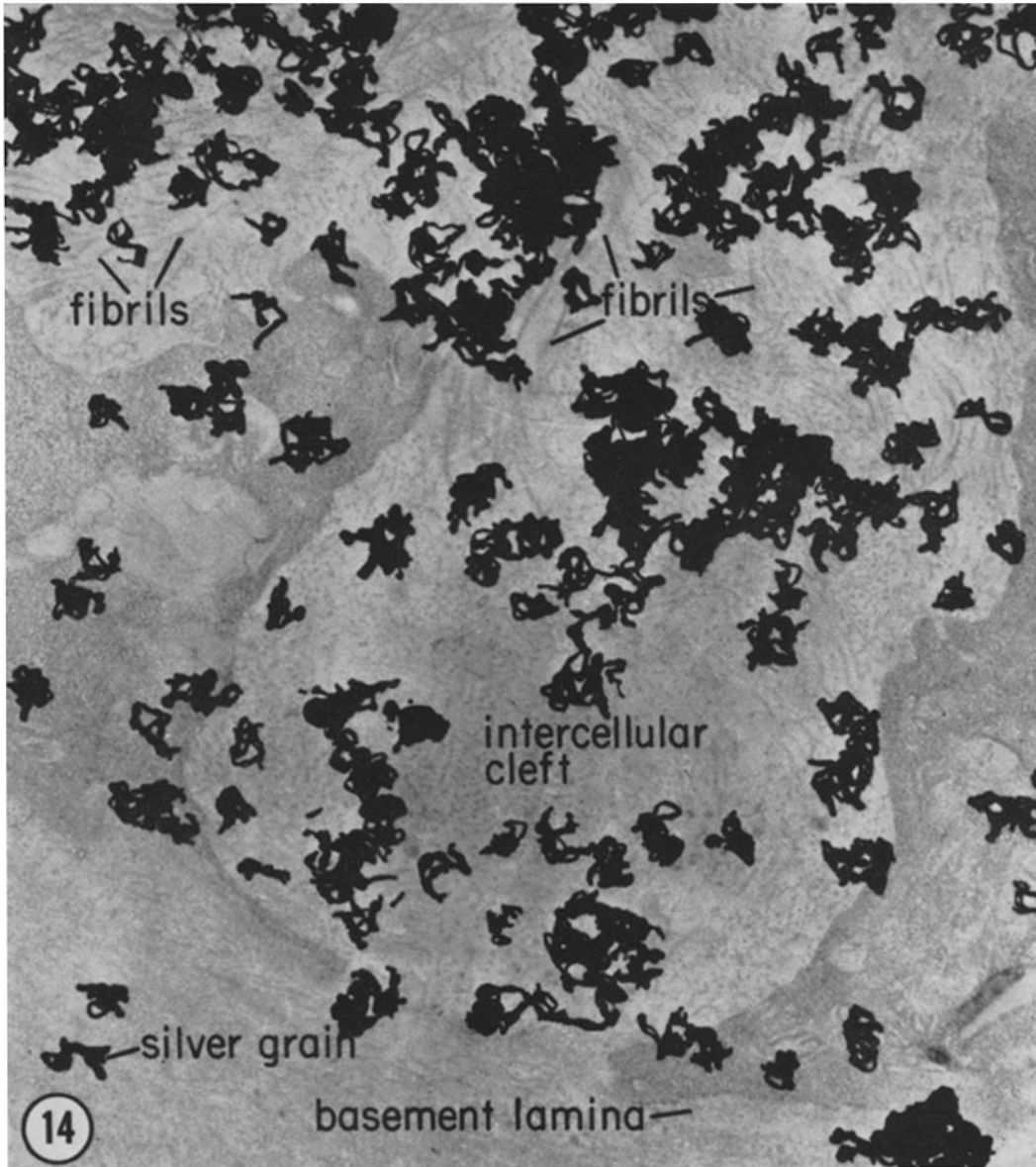
tissues in vivo and in vitro. Trypsin-isolated embryonic neural tube produces extensive basal lamina and few striated collagen fibrils in culture (Cohen and Hay, 1971). EDTA-isolated corneal epithelium produces many more collagen fibrils than does neuroepithelium, but the amount of basal lamina is less. The difference is unlikely to be the mode of isolation, since the same difference in amount of striated collagen underlying these two epithelia is observed in the intact embryo and, moreover, the cells had ample time in culture to recover from the isolation procedure. The difference in the amount of basal lamina produced could be due to a difference in either the age or state of differentiation of the two epithelia, or both. The younger (2 day old) neuroepithelium may have been producing more basal lamina type collagen than the older (5–7-day old) corneal epithelium at the time of isolation. Along these lines, it is pertinent to mention that our unpublished studies of embryonic chick back and limb epidermis grown on killed lens indicate that, here again, few if any striated fibrils are produced, although basal lamina is formed. These several epithelial cells seem to differ, then, in their ability to produce basal lamina and striated collagen fibrils at the time of their isolation from the embryo. These quantitative and qualitative differences among the cells' ability to produce collagen may describe an important parameter of the state of differentiation of the cells.

Cell Organelles Involved in Collagen Synthesis

Since collagen is an extracellular protein analogous in many ways to the proteinaceous products secreted by gland cells onto their luminal surface, it was tempting to explain the abundant granular endoplasmic reticulum in fibroblasts and chondroblasts with the suggestion that the same secretory organelle was involved in the synthesis of connective tissue extracellular matrix (Hay, 1962) as in the synthesis of zymogen (Palade et al., 1961). Autoradiographic studies of cartilage cells and fibroblasts showed that a proline-rich product likely to be largely collagen appears within the cisternae of the endoplasmic reticulum shortly after injection of [³H]proline (Revel and Hay, 1963; Ross and Benditt, 1965), a finding which has been confirmed for odontoblasts (Frank, 1970). Cooper and Prockop (1968) were unable to obtain similar resolution in autoradiographs of cartilage

and Salpeter (1968) was unable to localize [³H]proline in secretory organelles of chondroblasts in growing regeneration blastemas. In the present study of embryonic cornea and in autoradiographs of growing neural epithelium (Cohen and Hay, 1971), we observed as did Salpeter (1968) that [³H]proline entered the background cytoplasm and nucleus as well as the secretory organelles. Proline is present in nucleoproteins and structural proteins of the cell. Therefore, nongrowing cells that are making primarily collagen have to be studied in order to follow collagen synthesis by autoradiography. Biochemical studies of microsomes (Udenfriend, 1966) and the accumulated autoradiographic data on nongrowing cells, make the conclusion that collagen is manufactured by the granular endoplasmic reticulum seem a safe one, in spite of recent statements to the contrary (Cooper and Prockop, 1968; Salpeter, 1968). Indeed we were encouraged to test the now established hypothesis that corneal epithelium makes collagen by the large amount of granular endoplasmic reticulum observed in corneal epithelial cells at a time when a collagenous stroma appears beneath them.

The central question which remains to be resolved about the intracellular secretory pathway of collagen is the degree to which the Golgi complex is involved. We noted transposition of most, but not all, of the proline-rich product from the endoplasmic reticulum to the Golgi vacuoles of chondrocytes and we described in the Golgi vacuoles themselves nonstriated fibrils of the same size and staining characteristics as the fibrils of the extracellular matrix (Revel and Hay, 1963), observations that have been extended to odontoblasts and osteoblasts (Frank, 1970; Weinstock, 1972). Ross and Benditt (1965) noted transposition of [³H]proline labeled material to Golgi zones in fibroblasts, but they concluded from careful quantitative studies that most of the collagen-containing vesicles go directly to the cell surface from the endoplasmic reticulum, without passing through the Golgi zone as defined by its characteristic lamellae. Since Golgi lamellae are not significantly labeled by [³H]proline (Revel and Hay, 1963), the disagreement may be one of defining the Golgi complex. Fibroblasts do not contain the large Golgi vacuoles that characterize cartilage cells, so that secretory vesicles that originate from the Golgi zones might not be recognized as such in fibroblasts. However, even in odontoblasts, more label appears in the endoplasmic reticulum than subsequently moves

to the well-localized Golgi zones of these cells. The question is not trivial. To put it another way, do proteins synthesized into and transported by the endoplasmic reticulum have to be complexed to material (e.g. glycosaminoglycan) produced in the Golgi zone before they can be secreted; is it absolutely necessary for a vesicle (vacuole) to acquire cell surface type membrane from the Golgi complex (Whaley et al., 1972) before it can be transported to and fuse with the surface of the cell?

The observations on corneal epithelial cells presented in this paper and elsewhere (Hay and Revel, 1969; Trelstad, 1970, 1971; Coulombre and Coulombre, 1972) support the Golgi theory of collagen secretion, for the following reasons (a) the Golgi complex shifts to the basal cytoplasm (stromal side) of the corneal epithelial cells during the period of most active collagen secretion (Hay and Revel, 1969; Trelstad, 1970); (b) direct connections and intermingling of contents can be observed between cisternae of endoplasmic reticulum and Golgi saccules (see especially Figs. 3–6 and 4–10, Hay and Revel, 1969); (c) intermediate stages can be observed between the Golgi saccules and the final secretory granules; the Golgi saccules and lamellae probably synthesize the mucopolysaccharide of the extracellular matrix (Revel, 1970; Weinstock and Leblond, 1971); (d) the Golgi complex decreases in size and content when corneal collagen synthesis is prevented (Coulombre and Coulombre, 1972); (e) the possibility that the content of the dense Golgi vacuoles is collagen is supported by its resemblance to basal lamina material; indeed, in the cultured corneal cells, vacuoles full of lamina-like material become quite large.

In a case described here a vacuole containing the amorphous "precursor" (lamina-like) material seemed to be connected to a vacuole containing a striated fibril, while in another case (Trelstad, 1971), a corneal epithelial vacuole was seen that contained both the amorphous material and striated collagen fibrils. A point that requires further discussion is the possible continuity with the cell surface of intracellular "vacuoles" that contain truly striated collagen (Sheldon and Kimball, 1962; Trelstad, 1971; Weinstock, 1972; Seegmiller et al., 1972; Carlson, 1972). We suspect that these vacuoles connect to the cell surface because very careful reexamination of serial sections with this point in mind revealed such connections in our material. The so-called intracellular vacuole containing prominent striated fibrils is

perhaps better described as an intracellular cleft or canaliculus, then. Published illustrations of serial sections would be necessary to prove that "secretory vacuoles," as well as "ingestion vacuoles" (Usuku and Gross, 1965; Welsh and Meyer, 1967; Azar, 1969; Norman and Schmidt, 1967) containing native striated collagen are not, in fact, merely clefts in the cell surface. Along these lines, we might note that there is no evidence of collagen resorption in our system; the collagen described in the intracellular clefts is radioactive and most likely newly secreted.

It is of some interest to note that the vacuoles or intracellular clefts containing striated collagen fibrils open into the lateral cell compartment and the lateral cell compartment itself is often packed with striated collagen. Autoradiographic studies suggested that amphibian epidermal cells secrete collagen onto their lateral as well as basal surfaces (Hay and Revel, 1963; Hay, 1964). No recognizable striated collagen or basal lamina actually appeared in the interfacial canals of the epidermis and most of the proline-labeled product quickly moved to the basal extracellular compartment. No striated collagen is seen in the intercellular canals of corneal epithelium in vivo. It seems likely, however, that soluble collagen on its way to the underlying stroma may routinely enter the lateral as well as the basal compartment.

Biological Significance of Collagen Secretion by Epithelium

In addition to neural epithelium and corneal epithelium discussed here, a number of other embryonic epithelia probably produce extracellular matrix. Cardiac muscle is an epithelium whose basal surface faces an extracellular matrix, the cardiac jelly that supports the endocardium. Myocardium secretes sulfate-rich materials into the cardiac jelly (Manasek, 1970) and possibly produces collagen as well. Amphibian and fish epidermis secrete proline- and glucose-rich products into the underlying acellular dermis, the so-called basal lamella (Hay and Revel, 1963; Berliner, 1969; Nadol and Gibbons, 1970). We mentioned earlier in the discussion and have reviewed elsewhere (Hay, 1964; Hay and Revel, 1969) indirect evidence that a variety of epithelia make their own basal laminas or so-called basement membranes, even in the adult. The importance of extracellular matrix to the biology of the mature individual has received considerable review in the

past; its function in supporting the tissues and in mediating intercellular transport is widely recognized (Porter, 1964; Fitton-Jackson, 1968). The role of connective tissue in the embryo, particularly that of the extracellular matrices produced by epithelia, is only now receiving the attention it deserves. Yet, the extracellular matrices produced by embryonic epithelia are the principal supporting materials of the early embryonic body.

It is a natural consequence of the timing of development that these scaffoldings of epithelial origin should serve to guide the migration of the mesenchymal cells when the latter make their appearance. In the case of the chick cornea, for example, the primary (epithelial) stroma serves as a substratum for the migration, on the fourth day of development, of the presumptive endothelium. On the fifth day, stroma begins to swell, presumably due to hydration (Hay and Revel, 1969) related to increased concentration of hyaluronate (Toole and Trelstad, 1972). Mesenchymal cells then invade the stroma, using the component collagenous layers as a substratum (Hay and Revel, 1969; Trelstad and Coulombre, 1971). Collagen both supports the migration physically and may also serve in "contact guidance" (Hay, 1968). The collagen of the amphibian basal lamella seems to play a similar role at metamorphosis when it is invaded by presumptive fibroblasts (Kemp, 1961; Usuku and Gross, 1965). The cardiac jelly supports and may guide the fibroblasts that invade the myocardium (Manasek, 1970) and the basal laminas on the basal surface of epiblast and hypoblast probably guide the migration of mesoblast (Hay, 1968).

Another morphogenetic function of epithelial matrices in the embryo could be to "induce" differentiation of cells of the fibrocyte family. In the case of the chick cornea, the mesenchymal cells that first invade the stroma have relatively small amounts of endoplasmic reticulum and other secretory organelles compared to fully differentiated fibroblasts. When the invasion of the stroma is completed, endoplasmic reticulum and Golgi complexes become very prominent in the cells and then abundant collagen of fibroblast origin is laid down on the original stromal collagen. The idea that an epithelium stabilizes or promotes the early differentiation of fibroblasts through the intermediate of an extracellular matrix is compatible with current ideas about the inductive effect (later in embryogenesis) of mesenchymal collagen and mucopolysaccharide on epithelial differentiation

(Konigsberg and Hauschka, 1965; Bernfield et al., 1972).

Certainly, the time has come for a closer look at the connective tissue matrices which epithelia create in the embryo and even in adult vertebrates. Connective tissue cells of mesenchymal origin are so numerous in the bird and mammal, that we have fallen into the habit of defining a "connective tissue cell" as a mesenchymal cell that secretes collagen. The mesenchymal connective cell differs from the epithelial connective tissue cell in that it eventually becomes trapped within the matrix it secretes, whereas the epithelial cell surmounts its connective tissue products. That the collagenous products of the connective tissue cells, whether epithelial or mesenchymal, can be morphologically the same is established by the present study; possible similarities and differences in their biological function and chemistry in the embryo and the adult deserve further study.

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