

# Biosynthesis of Sulphated Macromolecules by Rabbit Lens Epithelium. II. Relationship to Basement Membrane Formation

J. GODFREY HEATHCOTE, ROMAINE R. BRUNS, and ROSLYN W. ORKIN  
*Developmental Biology Laboratory, Massachusetts General Hospital and Departments of Anatomy and Medicine, Harvard Medical School, Boston, Massachusetts 02114. Dr. Heathcote's present address is Department of Pathology, University of Western Ontario, London, Ontario, N6A, 5C1, Canada*

**ABSTRACT** Rabbit lens epithelial cells display a similar "cobblestone" morphology and produce the same complement of sulphated macromolecules (also see Heathcote, J. G., and R. W. Orkin, 1984, *J. Cell Biol.*, 99:852-860) whether grown on plastic or glass, dried films of gelatin or type IV collagen with laminin, or on gels of type I collagen. There was no evidence of basement membrane formation by these cells when they were grown on plastic, glass, or dried films. In contrast, cultures that had been grown on gels deposited a discrete basement membrane that followed the contours of the basal surfaces of the cells and in addition, they secreted amorphous basement membrane-like material that diffused into the interstices of the gel and associated with the collagen fibrils of the gel. A significant proportion (~70%) of the heparan sulphate proteoglycan fraction that was secreted into the culture medium (fraction MI) when the cells were grown on plastic became associated with the cell-gel layer in the gel cultures. Further, when basement membrane was isolated by detergent extraction, >90% of the <sup>35</sup>S-labeled material present was in this heparan sulphate proteoglycan.

In the preceding paper (1), we have described the sulphated macromolecules synthesized and secreted by confluent rabbit lens epithelial cells grown on a plastic (tissue culture dish) substratum. Among the secreted macromolecules is a heparan sulphate proteoglycan (designated MI) that can be precipitated from the medium by the addition of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> to 30% saturation. Although confined under these culture conditions to the medium compartment of the cultures, this component resembles the proteoglycans described in the basement membrane-like stroma of the EHS "sarcoma" (2), in the basement membrane of murine mammary epithelial cells (3), and in the basement membrane-like matrix deposited in cell culture by the murine teratocarcinoma-derived cell line, PYS-2 (4).

In this paper we show that rabbit lens epithelial cells cultured on a plastic substratum or on dried films of gelatin or laminin plus type IV collagen, do not deposit a basement membrane. In contrast, when grown on hydrated gels of type I collagen, the cells deposit basement membrane-like material that follows the contour of the basal cell surfaces. Production of this basement membrane is associated with the deposition of a heparan sulphate proteoglycan, similar to MI (see refer-

ence 1), in the cell layer. We have also compared the sulphated macromolecules produced by the epithelial cells in vitro with those synthesized by intact rabbit lenses in organ culture.

## MATERIALS AND METHODS

The materials used were generally as described in the previous paper (1). L-[5-<sup>3</sup>H]proline was purchased from New England Nuclear (Boston, MA). Gelatin (USP reagent, granular) was obtained from Eastman Kodak Co. (Rochester, NY) and laminin and type IV collagen from the EHS "sarcoma" (see references 5 and 6) were generous gifts from Dr. Hynda Kleinman, National Institute of Dental Research (Bethesda, MD). Pepsin-digested bovine dermal type I collagen (Vitrogen) was purchased from Collagen Corp. (Palo Alto, CA). Sodium deoxycholate was obtained from Sigma Chemical Co. (St. Louis, MO). NCS tissue solubilizer was purchased from Amersham Corp., Arlington Heights, IL.

**Preparation of Artificial Substrata:** To prepare films of laminin and type IV collagen, 100 μl of a solution of laminin (1 mg/ml in 0.4 M NaCl, 0.05 M Tris-HCl, pH 7.4) and 25 μl of a solution of collagen (1 mg/ml in 0.5 M acetic acid) were brought to a final volume of 500 μl with distilled water. The surface of 35-mm plastic tissue culture dishes was covered with the mixture (500 μl/dish) and allowed to dry in a laminar flow hood overnight under ultraviolet light.

Dried gelatin films were prepared by applying 1 ml of a sterile solution of 0.1% gelatin in distilled water per 35-mm tissue culture dish for 1 h at room temperature. The solution was then aspirated off and the plates were allowed to dry overnight in a laminar flow hood under ultraviolet light.

Native gels of type I collagen (0.1% final concentration) were prepared under conditions of neutral pH at 37°C. To each milliliter of a solution containing 0.43 ml Vitrogen (3 mg/ml commercial stock solution in 0.1% acetic acid) and 0.57 ml distilled water was added 0.3 ml of a 1:1 mix of 10 × Eagle's minimum essential medium (Gibco Laboratories, Grand Island, NY) and 0.14 M NaOH. All solutions were sterile and at 4°C when mixed. 2 ml of the mixed solution were added to each 35-mm culture plate and the plates incubated at 37°C for 15–30 min to allow gel formation. Gel plates were placed under ultraviolet light overnight at room temperature. Culture medium (1.5 ml per 35-mm plate) was added and the plates returned to the 37°C incubator in a humidified atmosphere containing 5% CO<sub>2</sub> for at least 1 h. At this time the culture medium was replaced with fresh medium and the gels were ready to receive cells. Electron microscopic examination of the gels revealed the presence of more banded fibrils with a 640 Å periodicity, characteristic of native type I collagen.

**Incubation of Lens Cells with Na<sub>2</sub><sup>35</sup>SO<sub>4</sub>:** Lens cells were grown to confluence in 35-mm culture dishes either directly on the plastic tissue culture surface, on dried films (see above), on carbon films evaporated onto glass coverslips, or on gels of 0.1% (wt/vol) Vitrogen in Dulbecco's modification of Eagle's medium containing 5% (vol/vol) calf serum, 5% (vol/vol) fetal bovine serum, 1% (vol/vol) antibiotic-antimycotic mixture, and 1% (vol/vol) glutamine. Cells were given fresh medium and 24 h later Na<sub>2</sub><sup>35</sup>SO<sub>4</sub> (1.0–1.25 mCi/dish) was added and the incubation continued for a further 24 h. The medium was removed and cooled on ice. For cells grown on dried films, the cells were washed two times with Hanks' balanced salt solution (HBSS)<sup>1</sup> containing protease inhibitors (see below), then harvested with a rubber policeman and processed as described previously for cells grown directly on culture dishes (see reference 1). For cells grown on collagen gels, after removal of the medium, the gels were scooped out of the culture dishes with a spatula and centrifuged at 1,500 g for 10 min to express medium contained within the gel interstices. The medium samples were combined and after the addition of 6-aminohexanoic acid (0.1 M), benzamidine hydrochloride (5 mM), and EDTA (10 mM) (see reference 7) to the concentrations indicated, centrifuged at 1,500 g for 10 min to remove cellular debris. Solid (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> was then added slowly to 30% saturation and the resulting precipitate collected by centrifugation at 80,000 g.

The cell layer-gel pellet was washed repeatedly with ice cold HBSS containing the above protease inhibitors (40–50 ml/wash) until the washings were devoid of pink color from the phenol red present in the culture medium. The pellet was stirred in 4 M guanidine hydrochloride for 24 h at 4°C and then dialyzed against the same buffer to remove unincorporated isotope. Prior to gel filtration chromatography any insoluble gel fragments were removed by centrifugation at 80,000 g for 1 h.

Aliquots of the gel washings and of the medium were dialyzed exhaustively against cold water and the <sup>35</sup>S-content of each fraction, together with an aliquot of the cell layer-gel fraction, determined.

**Purification of Lens Epithelial Cell Basement Membrane:** The basement membrane deposited by lens epithelial cells cultured on collagen gels was purified by deoxycholate extraction (8). Gels (usually 10–12 per preparation) were scooped out of the 35-mm plastic culture dishes and centrifuged as described above to express trapped culture medium. The pellet was resuspended in 200 ml of ice cold water containing 0.1 M 6-aminohexanoic acid, 5 mM benzamidine hydrochloride, and 10 mM EDTA, and stirred at 4°C for 1 h. The gel pieces were collected by low speed centrifugation and the extraction repeated twice. They were then extracted with 1 M NaCl containing the above inhibitors for 1 h at 4°C followed by 4% (wt/vol) sodium deoxycholate containing 0.1 M 6-aminohexanoic acid and 10 mM EDTA for 3 h at room temperature. After several washes with ice cold water containing protease inhibitors the gel pieces were stirred in 4 M guanidine hydrochloride for 24 h at 4°C and dialyzed against the same buffer. A parallel set of cultures were processed in the usual manner to serve as a control.

**Incubation of Intact Lenses:** Lenses were isolated from weanling (8–10 wk old) and neonatal (1–2 d) rabbits, cleaned, and incubated in Dulbecco's modification of Eagle's medium containing 10% (vol/vol) calf serum, 1% (vol/vol) antibiotic-antimycotic mixture, and 1% glutamine in an atmosphere of 95% air/5% CO<sub>2</sub>. After 1 h at 37°C, Na<sub>2</sub><sup>35</sup>SO<sub>4</sub> was added and the incubation continued for 24 h. In early experiments ~40 μCi per ml of medium was used but this was later increased by a factor of 10. Serum was included in the medium to preserve the integrity of the lenses during the incubation and it was found that the radioactivity incorporated per lens in the presence of 10% (vol/vol) calf serum was twice that incorporated in the absence of serum. The level of incorporation was the same whether lenses were incubated in 10% calf serum or a mixture of 5% (vol/vol) each of calf serum and fetal bovine serum.

After incubation the lenses were washed three times in ice cold HBSS and the capsules removed. These were rinsed briefly in fresh HBSS and then stirred for 24 h at 4°C in 2–5 ml of 4 M guanidine hydrochloride/50 mM sodium acetate, pH 5.8, containing 0.1 M 6-aminohexanoic acid, 5 mM benzamidine hydrochloride, and 10 mM EDTA. The suspension was dialyzed exhaustively against the same buffer to remove free Na<sub>2</sub><sup>35</sup>SO<sub>4</sub> and centrifuged at 105,000 g for 4–6 h at 4°C. The supernatant was clarified by filtration through a Millipore filter (type HA, 0.45-μm pore size) previously soaked in 4 M guanidine hydrochloride. To determine the distribution of radioactive sulphate the pellet was treated with 6 M guanidine hydrochloride buffer for 24 h and again centrifuged at high speed to remove insoluble material. This was washed twice with ice cold water and incubated for 36 h at 50–60°C with pronase in 50 mM NaCl/50 mM tris HCl, pH 7.4 (~1 mg/ml added every 12 h). The radioactive material released by the enzyme was separated by centrifugation at 80,000 g for 1 h and the final residue washed with water and solubilized in NCS at room temperature overnight. A small aliquot of each fraction was taken for measurement of radioactivity.

**Fractionation of Sulphated Macromolecules:** Methods used for the separation and characterization of the sulphated glycoconjugates were as described in the preceding paper (1).

**Morphology:** Lens epithelial cells plated onto carbon-coated glass coverslips were grown to confluence. The cells were fixed for 15 min in Karnovsky's fixative (9) with 2% formaldehyde and 3% glutaraldehyde, post-fixed with 1% OsO<sub>4</sub>, stained with 1% aqueous uranyl acetate for 1 h, dehydrated, and embedded in Epon. The embedded cells were separated from the glass coverslip by cleaving at the carbon-glass interface. After polymerizing additional Epon onto the base of the cell layer, thin sections were cut in a plane perpendicular to the cell layer. Lens cells grown on collagen gels in 35-mm tissue culture dishes were fixed as above. The gels with the attached cell layer were then removed from the dish, cut into small squares, and processed as one would process an ordinary piece of tissue. Thin sections were stained with alcoholic uranyl acetate and lead citrate (10).

**Assay of Collagen Biosynthesis:** Confluent cultures of lens epithelial cells on plastic tissue culture surfaces or on gelatin films were incubated with [5-<sup>3</sup>H]proline (50 μCi/ml) for periods of 20 h in the presence of increasing concentrations of ascorbic acid (0–250 μg/ml). The medium was collected, *N*-ethylmaleimide (10 mM), phenylmethanesulphonyl fluoride (2 mM) and EDTA (25 mM) were added to the final concentrations indicated, and cell debris was removed by centrifugation at 1,500 g for 10 min. To the supernatant, solid (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> was added to 30% saturation and the resulting precipitate collected by centrifugation for 1 h at 80,000 g in the cold. The cell layer was washed with HBSS containing the above inhibitors, removed from the dish with a rubber policeman, and harvested by centrifugation at 1,500 g for 10 min. Both the cell pellet and the (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> precipitate were dialyzed exhaustively against water at 4°C and hydrolyzed in 6 M HCl for 24 h at 110°C. An aliquot of each hydrolysate was taken for estimation of total tritium and the remainder was assayed for 4-hydroxy[<sup>3</sup>H]proline (11).

## RESULTS

### Deposition of Basement Membrane by Cultured Lens Cells

Confluent cultures of lens epithelial cells grown on plastic or dried films displayed a characteristic epithelioid appearance (Fig. 1*a*), but did not appear to deposit a basement membrane, as judged by biosynthetic and morphological criteria. In biosynthetic studies with Na<sub>2</sub><sup>35</sup>SO<sub>4</sub> as precursor, cultures grown on dried films of gelatin or of type IV collagen plus laminin were found to have the same sulphated components associated with the cell layer as that produced by cultures grown on plastic (see reference I, Fig. 1, components CI and CII). There was no evidence for the deposition of a heparan sulphate proteoglycan equivalent to previously described basement membrane proteoglycans (2, 3). When confluent cultures were radioactively labeled with [<sup>3</sup>H]proline to monitor collagen biosynthesis, polypeptide-bound 4-hydroxy[<sup>3</sup>H]proline was not found in the cell layer but it could be detected in that fraction of the culture medium precipitated by (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> at 30% saturation (Table I). Although the presence in the incubation medium of ascorbic acid, at concentrations up to 100

<sup>1</sup> Abbreviations used in this paper: CTAB, hexadecyltrimethylammonium; HBSS, Hanks' balanced salt solution.

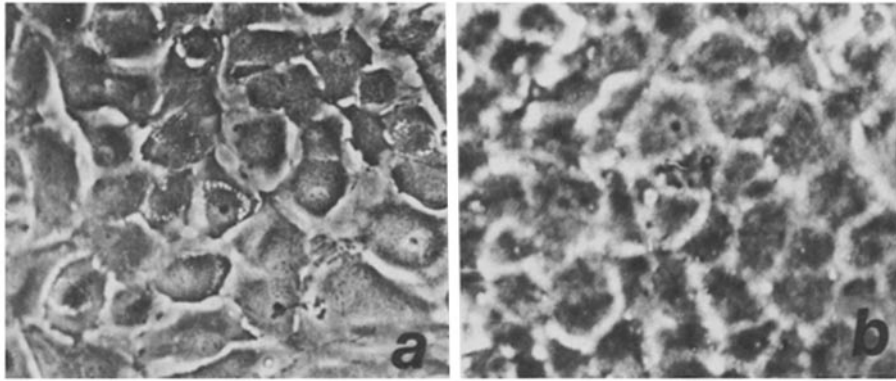


FIGURE 1 Light micrographs of rabbit lens epithelial cells cultured on plastic or on collagen gels. Rabbit lens epithelial cells grown directly on plastic tissue culture dishes (a) or on native (type I) collagen gels (b). Under both conditions the cells display a similar "cobblestone" morphology typical of epithelial cells. Cells grown on the gel tend to "burrow" slightly into the gel surface. The morphology of rabbit lens epithelial cells grown on carbon-coated coverslips and on dried films of gelatin or of type IV collagen with and without laminin is as shown in a.  $\times 260$ .

TABLE I  
4-Hydroxy[ $^3\text{H}$ ]proline Synthesis by Rabbit Lens Epithelial Cells: Effect of Ascorbic Acid

Ascorbic acid concentration $\mu\text{g/ml}$	Cell layer		Medium: $(\text{NH}_4)\text{SO}_4$ precipitate (30% saturation)		
	Total $^3\text{H}$ dpm	Hydroxy [ $^3\text{H}$ ]pro- line dpm	Total $^3\text{H}$ dpm	Hydroxy[ $^3\text{H}$ ]proline dpm	Hydroxylation of proline %
0	8,136,320	0	1,030,580	121,600	11.8
50	9,269,820	0	888,550	163,600	18.4
100	4,566,630	0	1,050,320	203,140	19.3
250	6,875,760	0	1,116,050	76,800	6.9

Confluent cultures of lens cells (8th passage,  $1.2 \times 10^6$  cells/dish) on plastic tissue culture dishes were given fresh medium containing ascorbic acid (0–250  $\mu\text{g/ml}$ ) and incubated for 24 h. Cultures were supplemented with appropriate amounts of fresh ascorbic acid, ( $^3\text{H}$ )proline (50  $\mu\text{Ci/ml}$ ) was added, and incubation continued for 20 h. The cells and a 30% saturated ammonium sulphate precipitate of the medium were dialyzed exhaustively against water at  $4^\circ\text{C}$ , hydrolyzed in 6 M HCl for 24 h at  $110^\circ\text{C}$ , and assayed for 4-hydroxy[ $^3\text{H}$ ]proline.

$\mu\text{g/ml}$ , increased the degree of hydroxylation of proline (Table I), it failed to promote deposition of basement membrane material in these cultures. Moreover, no basement membrane was detected by electron microscopy when cells were grown to confluence on carbon-coated coverslips. Rather, under these conditions cells were found to abut directly on the substratum (Fig. 2a).

Like those grown on plastic or dried films, cells grown on gels of type I collagen displayed a typical epithelioid morphology (Fig. 1b). In contrast, however, rabbit lens epithelial cells grown on type I collagen gels deposited a distinct basement membrane that followed the contours of the cells' basal surfaces (Fig. 2b). This basement membrane consisted of an electron lucid layer (lamina rara) next to the cell membrane and an electron dense layer (lamina densa) that abutted on the collagen gel. Intermittent breaks in the basement membrane did occur and in these regions, cell processes sometimes extended into the gel layer.

In addition to the basement membrane proper, lens epithelial cell cultures grown on collagen gels appeared to secrete material that diffused into the gel and formed an amorphous deposit associated with the type I collagen fibrils of the gel (Fig. 3). This material, which was present to variable extent, appeared in the gel in a gradient such that some collagen fibrils nearest the cell layer and basement membrane (within 5  $\mu\text{m}$  from the basement membrane) had associated amorphous material, whereas those collagen fibrils located deeper in the gel had a "clean" appearance (see Fig. 3, inset). Control collagen gels incubated with no cells but maintained in complete, serum-containing medium for the same period as the epithelial cultures, were found to be free of the fibril-associated amorphous material (data not shown, but as in Fig. 3, inset).

#### Incorporation of $\text{Na}_2^{35}\text{SO}_4$ by Lens Cells Grown on Collagen Gels

Following a 24-h incubation with  $\text{Na}_2^{35}\text{SO}_4$ , the cell layer plus collagen gel were extracted with 4 M guanidine hydrochloride and the extract chromatographed on Sepharose CL-4B. The elution profile (Fig. 4) resembled that observed with cells cultured on plastic (see reference 1, Fig. 1) or on dried films (data not shown), with one notable difference. A broad peak of [ $^{35}\text{S}$ ]sulphate (designated C-PG in Fig. 4), comprising ~20% of the total radioactivity in the cell layer, was eluted with a  $K_{av}$  of 0.26 (range, 0.23–0.28). This component was similar to medium component MI (see reference 1, Table II). Pronase treatment shifted the elution position of component C-PG on Sepharose CL-4B to  $K_{av} = 0.6$  (Table II), thereby establishing the proteoglycan nature of this sulphated macromolecule. The [ $^{35}\text{S}$ ]sulphate-labeled glycosaminoglycan remaining after pronase digestion of component C-PG was >90% susceptible to degradation by  $\text{HNO}_2$ , but resistant to chondroitin sulphate ABC lyase (Table II). These criteria establish component C-PG as a heparan sulphate proteoglycan.

The composition and distribution of the other sulphated macromolecules synthesized by these cultures were similar to those produced by cultures grown on dried films or on plastic (1). Thus, as had been observed for cells cultured on plastic (1), the proportion of radioactivity in the void volume (component CI) was variable. In two experiments with cells of the same strain in the 4th and 5th passages, the proportions of [ $^{35}\text{S}$ ]sulphate in CI were 18 and 31%, respectively, whereas in a third experiment with a different set of cells in the third passage, the proportion of [ $^{35}\text{S}$ ]sulphate in CI was only 6%.

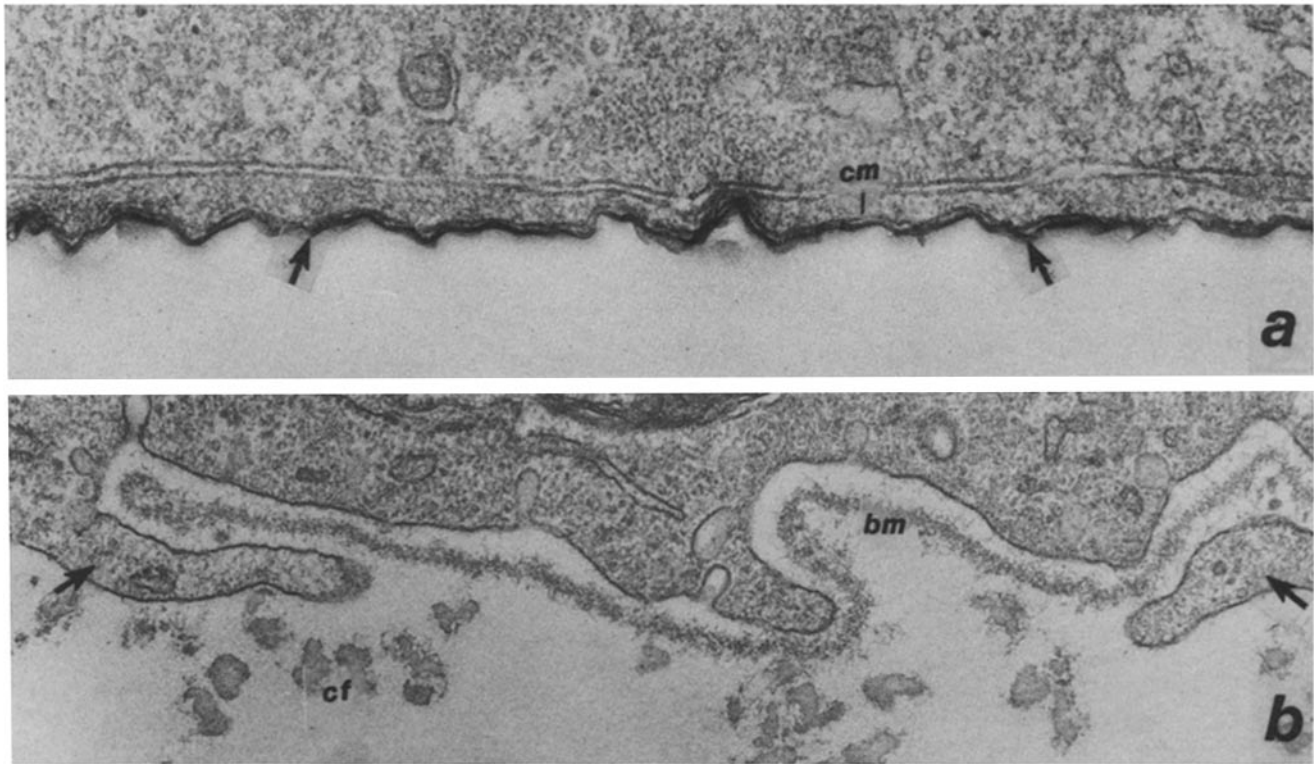


FIGURE 2 Electron micrographs of rabbit lens epithelial cells cultured on carbon-coated glass and on collagen gels. Rabbit lens epithelial cells cultured on carbon-coated glass coverslips (a) fail to deposit a basement membrane. Instead the cell membranes (cm) abut directly on the carbon film (arrows) which coats the glass. In contrast, cells grown on native (type I) collagen gels (b), deposit a well-defined basement membrane (bm) consisting of laminae rara and densa, which follows the contours of the basal surfaces of the cells. Cross-sections of collagen fibrils (cf) can be seen in the gel. At discontinuities in the basement membrane, cell processes (arrows) pass in toward the gel. (a)  $\times 87,000$ ; (b)  $\times 58,000$ .

In these three experiments however, the proportion of radioactivity in component C-PG remained constant at 21–23%. Analysis of the glycosaminoglycan content of CI and CII in the collagen gel cultures indicated that these fractions were of similar composition to that synthesized by lens cells cultured on dried films or on a plastic substratum (Table II; also see reference 1, Table II).

Sulphated macromolecules precipitated from the culture medium by 30% saturated  $(\text{NH}_4)_2\text{SO}_4$  were also analyzed by gel filtration chromatography on Sepharose CL-4B (Fig. 5a). Two components, corresponding in distribution coefficients to MI and MIII previously described (1) were observed. Analysis indicated that MI was a heparan sulphate proteoglycan and MIII largely sulphated glycoprotein (Table II). Little or no biosynthesis of components MII and MIV (reference 1, Table II) was detected in the gel cultures; however, these components were also found to variable extent in lens epithelial cultures grown on plastic (1). Examination of the sulphated glycoconjugates not precipitated by 30% saturated  $(\text{NH}_4)_2\text{SO}_4$  (Fig. 5b) revealed a marked predominance of component SII ( $K_{av} = 0.53$ ) with small amounts of SI and possible SIII (see reference 1). These macromolecules were not characterized further.

#### Relationship of Heparan Sulphate-Proteoglycan to Basement Membrane Deposition

When lens epithelial cells were grown on a plastic tissue culture surface and incubated for 24 h with  $\text{Na}_2^{35}\text{SO}_4$ , all the

newly synthesized heparan sulphate-proteoglycan (MI) was present in the culture medium (1). The corresponding experiment with cells grown on type I collagen gels revealed that 67% of the total  $^{35}\text{S}$  sulphate-labeled proteoglycan became associated with the cell-gel layer. We have designated this cell-gel associated heparan sulphate proteoglycan as C-PG.

The basement membrane deposited by the lens cells was freed of cellular contamination by sequential treatment with water, 1 M NaCl, and 4% (wt/vol) sodium deoxycholate (8). The residual insoluble basement membrane was extracted in 4 M guanidine and applied to a column of Sepharose CL-4B. Over 90% of the  $^{35}\text{S}$  sulphate in this latter isolated basement membrane fraction was present in a peak with the elution characteristics of component C-PG, the heparan sulphate proteoglycan (Fig. 6,  $K_{av} = 0.19$ ). A small proportion of component CI was detected in these preparations, but there was no evidence of the low molecular weight CII. In a control, parallel incubation, extracted and analyzed in the usual way, all three components CI, C-PG, and CII were present (data not shown, but similar to Fig. 4).

#### Incorporation of $\text{Na}_2^{35}\text{SO}_4$ by Intact Lenses

To further assess the relationships of sulphated glycoconjugates synthesized by lens epithelial cells in culture to those synthesized *in vivo*, a comparison was made with the macromolecules found in cultures of intact lenses. Most experiments on whole lenses were carried out with 10% (vol/vol) calf serum in the incubation medium but the level of incorporation of  $\text{Na}_2^{35}\text{SO}_4$  was similar when a mixture of 5% (vol/

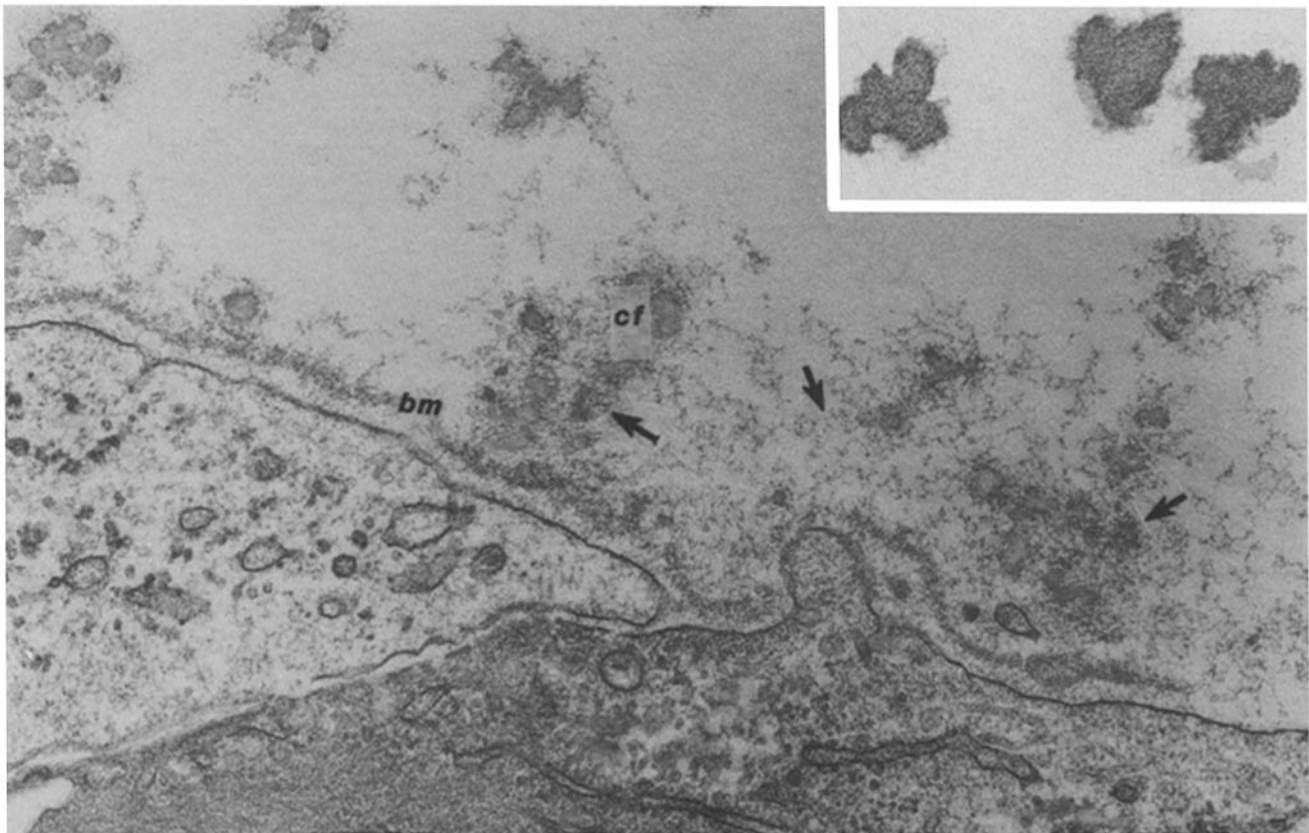


FIGURE 3 Electron micrograph of rabbit lens epithelial cells cultured on collagen gels: deposition of discrete basement membrane and of amorphous, basement membrane-like material into the gel. Rabbit lens epithelial cells grown on native (type I) collagen gels not only deposit a discrete basement membrane (*bm*), but also secrete an amorphous, basement membrane-like material (arrows) that diffuses into the interstices of the collagen gel and often associates with the collagen fibrils (*cf*) of the gel. This association is most pronounced in regions of the collagen gel abutting on the basal lamina proper. Collagen fibrils in control gels (*inset*) that were incubated only with complete, serum-containing medium but without cells, had a clean, nonfuzzy appearance. Likewise, collagen fibrils deep within the gels of lens epithelial cell-gel cultures were also free of amorphous material and appeared similar to those fibrils shown in the inset.  $\times 54,000$ ;  $\times 137,000$  (*inset*).

vol) calf serum and 5% (vol/vol) fetal bovine serum (as used for the lens epithelial cell cultures) was used (Table III). Approximately two-thirds of the nondialyzable [ $^{35}\text{S}$ ]sulphate was extracted by 4 M guanidine hydrochloride and most of the remainder was only released by protease treatment (Table III). As judged by precipitation with hexadecyltrimethylammonium bromide (CTAB), the proportion of glycosaminoglycan in each extracted fraction was different and was lower in the pronase extract than in either of the guanidine hydrochloride extracts. Although highest in the 4 M guanidine hydrochloride extract, glycosaminoglycan content was still only 60–70% indicating that a considerable proportion of the [ $^{35}\text{S}$ ]sulphate was present in glycopeptide.

The 4 M guanidine hydrochloride extract of intact lens preparations was chromatographed on a column of Sepharose CL-4B and the resulting elution profile (Fig. 7) was found to be similar to that obtained with the corresponding extract of lens cells cultured on dried films or on plastic (reference 1, Fig. 1). The peak in the void volume comprised between 8 and 19% of the total radioactivity and the remainder was largely present in a broad, included peak ( $K_{av}$ : 0.52–0.73). No distinct peak of radioactivity with a  $K_{av}$  of 0.15–0.2 (i.e., the putative heparan sulphate proteoglycan) was observed.

To determine whether lenses from younger animals had the capacity to synthesize the heparan sulphate proteoglycan, lenses were isolated from neonatal rabbits (24–36 h of age) and incubated with  $\text{Na}_2^{35}\text{SO}_4$  as described above. The elution profile of the  $^{35}\text{S}$ -labeled molecules from a column of Sepharose CL-4B differed from that obtained with older lenses. In contrast to the older lenses, neonatal lenses showed less radioactivity in the void volume (Fig. 8a). Moreover, a significant amount of radioactivity ( $\sim 10\%$ ) was present in the region of the chromatogram corresponding to the elution position of the putative basement membrane heparan sulphate proteoglycan ( $K_{av}$ : 0.15–0.2). When pooled and rechromatographed on Sepharose CL-4B, this material eluted as a broad peak of  $K_{av}$  0.16–0.24 (Fig. 8b). After digestion with pronase,  $>90\%$  of the  $^{35}\text{S}$  in this peak was precipitable with CTAB and was eluted from Sepharose CL-4B with a  $K_{av}$  of 0.55 (data not shown), thereby indicating the proteoglycan nature of the material. In addition, 76% of this S-labeled glycosaminoglycan was degraded by  $\text{HNO}_2$ . Taken together, these results suggest that intact neonatal rabbit lenses can synthesize in organ culture a heparan sulphate proteoglycan that is similar to that produced by cultured lens epithelial cells. This capacity may decline with age.

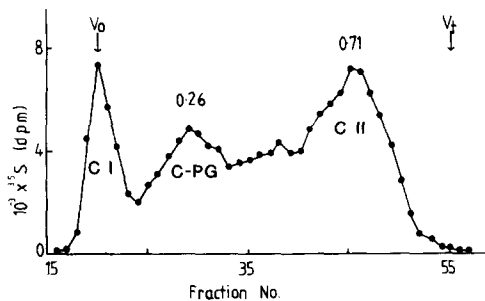


FIGURE 4 Gel filtration chromatography of sulphated macromolecules associated with the cell layer and collagen gel. Confluent cultures of lens cells (4th passage) on collagen gels in 35-mm dishes ( $n = 12$ ) were given fresh medium for 24 h.  $\text{Na}_2^{35}\text{SO}_4$  (1.25 mCi/dish) was added and the incubation continued for 24 h. The medium was removed and the cell layer and gel were washed and extracted with 4 M guanidine hydrochloride as described in the text. The extract was chromatographed on a column of Sepharose CL-4B eluted with 4 M guanidine hydrochloride at 4°C. The void volume ( $V_o$ , 54 ml) and total volume ( $V_t$ , 149 ml) of the column are indicated. The peaks of  $^{35}\text{S}$  labeled CI and CII correspond to components similarly designated in the accompanying paper.

TABLE II  
Sulphated Macromolecules Synthesized by Rabbit Lens Epithelial Cells Grown on Type I Collagen Gels

Component*	$K_{av}$ †	GAG content* %	GAG size ( $K_{av}$ )‡	GAG composition
CI	0	61	0.59	>90% HS
C-PG	0.26	100	0.61	>90% HS
CII	0.71	71		74% HS
MI	0.21	100	0.57	>90% HS
MIII	0.55	<25	ND‡	ND‡

\* Designations as described in the text and reference 1.

† Determined by chromatography on Sepharose CL-4B in 4 M guanidine hydrochloride.

‡ Percentage of  $^{35}\text{S}$  precipitable with 0.38% (wt/vol) CTAB; GAG, glycosaminoglycan.

§ Not determined.

## DISCUSSION

Although one of the first basement membranes to be studied was the lens capsule (see reference 12), surprisingly little is known of its noncollagenous constituents and their possible interspecies heterogeneity, of the manner in which component macromolecules assemble to form the lens capsule, or of the functional role of the lens capsule in vivo (but see references 13–15). Moreover, although recent studies have examined proteoglycan biosynthesis in organ cultures of normal, basement membrane-producing tissue (16–18) relatively few studies have examined the biosynthesis and deposition of sulphated basement membrane-associated proteoglycans in cell culture (3, 4, 19, 20). Of these, only two normal cell types have been examined: murine mammary epithelial cells (3) and rat parietal endoderm (20). The other studies have been done on murine teratocarcinoma-derived lines (4, 19). In this and the preceding study (1), we have sought to examine the sulphated macromolecules synthesized by rabbit lens epithelial cells in vitro, to better understand the relationships of these macromolecules to the lens capsule.

Our findings indicate that although monolayer cultures of rabbit lens epithelium display a similar "cobblestone" mor-

phology and synthesize the same complement of sulphated macromolecules whether the cells are grown on plastic or carbon-coated glass, on dried films, or on collagen gels, an identifiable basement membrane is deposited only when the cells are grown on the collagen gels. In addition to the basement membrane proper, the cells appear to secrete an amorphous material that diffuses within the interstices of the gel and that is deposited in association with the collagen fibrils of the gel. Similar observations were made by Lillie et al. (21)

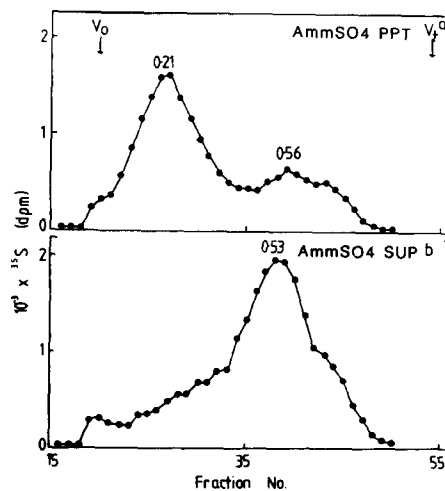


FIGURE 5 Gel filtration chromatography of sulphated macromolecules secreted into the incubation medium by lens epithelial cells grown on collagen gels. The incubation was carried out as described in the legend to Fig. 4. The medium was cooled, and after the addition of EDTA (10 mM), 6-aminohexanoic acid (100 mM), and benzamidine hydrochloride (5 mM) to the concentrations indicated, was centrifuged for 10 min at 1,500 g. Solid  $(\text{NH}_4)_2\text{SO}_4$  was added to 30% saturation and the resulting precipitate (PPT) collected by centrifugation at 80,000 g for 1 h. The precipitate was solubilized in 4 M guanidine hydrochloride and dialyzed exhaustively against the same buffer before chromatography on Sepharose CL-4B (a). An aliquot (10 ml) of the supernatant (SUP) was dialyzed against running tap water and then distilled water at 4°C before lyophilization. The lyophilized material was solubilized in 4 M guanidine hydrochloride and chromatographed on the same column of Sepharose CL-4B (b). The void volume ( $V_o$ , 54 ml) and total volume ( $V_t$ , 146 ml) of the column are indicated.

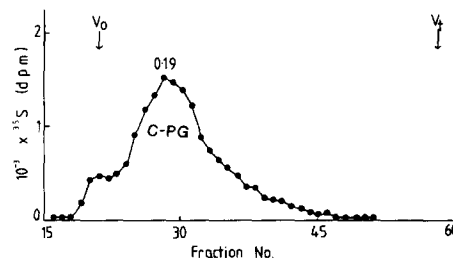


FIGURE 6 Gel filtration chromatography of sulphated macromolecules associated with the cell layer after detergent treatment. Cells grown to confluence on collagen gels (10 35-mm dishes) were incubated with  $\text{Na}_2^{35}\text{SO}_4$  (1.5 mCi/dish) for 24 h. The medium was removed and the cell layer and gel were washed successively with ice cold water, 1 M NaCl, and 4% (wt/vol) sodium deoxycholate, each containing protease inhibitors. The detergent-insoluble material was extracted with 4 M guanidine hydrochloride and chromatographed on a column of Sepharose CL-4B. The void volume ( $V_o$ , 53 ml) and total volume ( $V_t$ , 146 ml) are indicated.



TABLE III  
Extractability of [<sup>35</sup>S]Sulphated Macromolecules  
Synthesized by Intact Lenses from Weanling Rabbits

Fraction	dpm	10% calf serum		5% Calf serum/5% fetal calf serum		
		dpm	% total dpm	% GAG	dpm	% total dpm
4 M Gu-HCl xt	37,290	69	62	34,600	62	71
6 M Gu-HCl xt	5,600	10	53	5,670	10	57
Pronase xt	10,600	20	49	14,570	26	48
Final residue	350	1	ND	850	2	ND
Total dpm	53,840			55,690		
dpm/lens	6,730			6,960		

Four parallel incubations of intact lenses (8) were established, two in medium containing 10% (vol/vol) calf serum and two in medium containing a mixture of 5% each (vol/vol) calf and fetal calf serum. Na<sub>2</sub><sup>35</sup>SO<sub>4</sub> (425 μCi per incubation) was added and incubation continued for 24 h. The distribution of radioactivity in the various fractions was determined as described in the text; mean values are presented. The percentage of [<sup>35</sup>S]sulphate in glycosaminoglycan in each fraction was determined by precipitation of <sup>35</sup>S-labeled glycosaminoglycan with hexadecyltrimethylammonium bromide. *Gu-HCl*, guanidine hydrochloride; *xt*, extract; *GAG*, glycosaminoglycan.

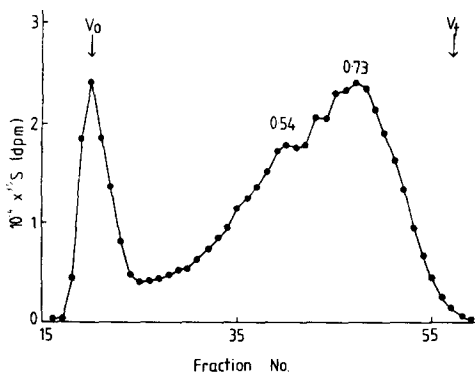


FIGURE 7 Gel filtration chromatography of <sup>35</sup>S-sulphated macromolecules synthesized by intact lenses from weanling rabbits. Intact lenses (16) were incubated with 2 mCi of Na<sub>2</sub><sup>35</sup>SO<sub>4</sub> in 50 ml of Dulbecco's modification of Eagle's medium containing 10% (vol/vol) calf serum. Capsules plus attached epithelia were isolated and extracted in 4 M guanidine hydrochloride for 24 h at 4°C. The extract, after exhaustive dialysis against the same buffer, was chromatographed on a column of Sepharose CL-4B. The void volume (V<sub>0</sub>, 23 ml) and the total volume (V<sub>t</sub>, 67 ml) of the column are marked.

in studies of the differentiation of rat lingual epithelial cells grown in collagen gel-raft cultures. The presence of the gel alone, rather than the collagen type or the presence of non-collagenous macromolecular additives appears to be essential for the rabbit lens epithelium to deposit the basement membrane in vitro. Culture dishes coated with dried films of type I collagen or of EHS "sarcoma"-derived type IV collagen mixed with laminin (see 5, 6, 22), like the uncoated culture dish surface itself all failed to provide an adequate milieu for basement membrane deposition. These ultrastructural observations were confirmed by biosynthetic studies on both the collagenous and sulphated macromolecules synthesized by cultured rabbit lens epithelial cells. More specifically, in those experiments in which we used [<sup>3</sup>H]proline as a radiolabeled probe for collagen synthesis in cells grown on plastic or dried films, the resulting macromolecular [<sup>3</sup>H]hydroxyproline was found to be present only in the medium fraction, rather than

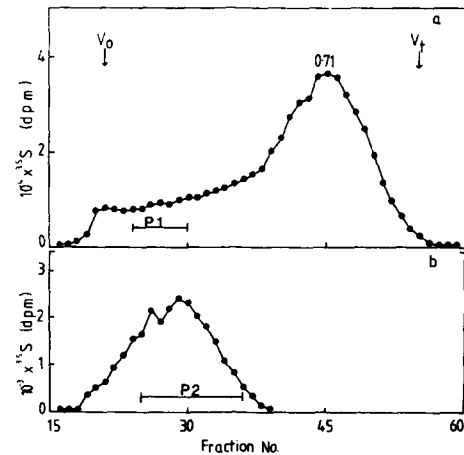


FIGURE 8 Gel filtration chromatography of the <sup>35</sup>S-sulphate-labeled macromolecules synthesized by intact neonatal rabbit lenses. Intact lenses (28) from 24–36-h old rabbits were incubated in 25 ml Dulbecco's modification of Eagle's medium containing 10% (vol/vol) calf serum for 1 h at 37°C. Na<sub>2</sub><sup>35</sup>SO<sub>4</sub> (20 mCi) was added and the incubation continued for a further 24 h. The lenses were washed four times in ice cold HBSS and the capsules stirred in 5 ml of 4 M guanidine hydrochloride containing protease inhibitors for 24 h at 4°C. The suspension was dialyzed exhaustively against the above buffer and centrifuged at 105,000 g for 4 h. The turbid supernatant was clarified by filtration through a 0.45-μm Millipore filter and an aliquot was chromatographed on a column of Sepharose CL-4B eluted with 4 M guanidine hydrochloride (a). The void volume (V<sub>0</sub>, 55 ml) and total volume (V<sub>t</sub>, 143 ml) of the column are indicated. Fractions 24–30 (P1) were pooled, concentrated over an Amicon PM10 filter, and rechromatographed on the same column (b). Fractions 25–36 (P2) were pooled for further analysis.

associated with the cell layer. Although we can not rule out the possibility that when grown on plastic or dried films that rabbit lens epithelial cells fail to synthesize a molecule(s) which is essential for deposition and assembly of component macromolecules into the basement membrane, the cells appear to synthesize and secrete the same complement of sulphated macromolecules irrespective of substratum. The notable difference that occurs in the collagen gel cultures is in the localization of a heparan sulphate proteoglycan fraction which we have designated MI (1), when it is present in the culture medium. This fraction, which is of similar size to other basement membrane-associated heparan sulphate proteoglycans (2–4, 19), was found to be secreted into the medium of the rabbit lens epithelial cells grown on plastic (1) or dried films. In contrast, when the lens epithelium was grown on collagen gels, a significant proportion (60–70%) of this heparan sulphate proteoglycan (designated C-PG) became associated with the cell-gel fraction and this was subsequently shown to be present in the isolated basement membrane fraction. The other sulphated macromolecules associated with the cell layer (CI and CII, see also reference 1) were not present in the isolated basement membrane.

It is noteworthy that the mean K<sub>av</sub> of fractions C-PG and MI described in the gel cultures is somewhat smaller than MI isolated from lens epithelial cultures grown on plastic (1) (K<sub>av</sub> ~0.2–0.25 vs. K<sub>av</sub> ~0.15, respectively). We do not know if these differences accurately reflect actual size differences in heparan sulphate proteoglycans synthesized under the two culture conditions, or if they are within the experimental error of the chromatographic analyses. If the former explanation is

correct, it suggests that in the gel cultures, the heparan sulphate proteoglycan may be processed to a somewhat lower molecular weight product. In pulse label-chase experiments, Tyree et al. (23) have reported a similar processing of the heparan sulphate proteoglycan of the EHS "sarcoma," from a higher to lower molecular weight form.

The mechanisms by which basement membrane deposition occurs have not been fully explored, although they are likely to be a function of cell type, the nature of the basement membrane constituent macromolecules, and the interactions that occur between the cell and its extracellular milieu. Observations in vitro indicate a wide range of requirements for basement membrane deposition not only among various cell types, but also for the same cell type from species to species. Although some cells appear to deposit extensive basement membranes when grown directly on a plastic tissue culture dish surface (bovine lens epithelium [24, 25] and the murine teratocarcinoma-derived PYS cells [4, 26, 27], others can, at best, form patchy deposits of basement membrane material, even when grown on collagen gels (guinea pig epidermal cells [28] and an invasive murine mammary epithelial line [29]). Among those cells that form a continuous basement membrane when grown on collagen gels are the normal rabbit lens epithelial cells described here, normal rat lingual epithelial cells (21), normal murine mammary epithelium (3, 30, 31) as well as a cell line derived from rat mammary epithelium (32). Differences in the ability to deposit basement membrane material may result from a constellation of events that may include altered rates of glycosaminoglycan degradation (3, 31, 33), production of a type IV collagenase (34), and synthesis of undersulphated heparan sulphate proteoglycans (35).

In other studies, Hay and her colleagues have examined the responses of very early embryonic chick epithelia to a variety of substrata (for reviews, see references 36 and 37). Their findings demonstrate that the presence of a collagenous substratum promotes synthesis and deposition of extracellular matrix material, including basement membrane, by cultures of neural and corneal epithelium. Although these cells were originally grown on freeze-thawed lens capsules (which may contain both collagenous and noncollagenous components), similar results were obtained by growing cells on gels of type I or type II collagen (38). Soluble collagens, laminin, or fibronectin added to the culture media also stimulate matrix production by corneal epithelial cells (37).

To determine if the biosynthetic pattern of heparan sulphate proteoglycan fractions observed in rabbit lens epithelial cell culture reflected the in vivo pattern, we also examined sulphated macromolecule synthesis in organ cultures of intact lenses. The latter experimental condition might be anticipated to more closely resemble the in vivo state than monolayer cell cultures, and organ culture has been used for the study of both lens metabolism (39-41) and the biosynthesis of lens capsule collagen (42-44). In the latter studies the newly synthesized collagenous polypeptides were found to reflect accurately the polypeptide composition of the intact lens capsule. Somewhat unexpectedly, intact lenses from weanling rabbits (the source of the lens cell cultures) failed to synthesize a discrete [<sup>35</sup>S]sulphate-labeled component with an elution position equivalent to component C-PG: only 4% of the [<sup>35</sup>S]-sulphate was found in this region of the chromatogram. However, material with the characteristics of components CI and CII was clearly identified from these intact lens cultures. In other studies of basement membrane formation in rats, Laurie and Leblond using immunoelectron microscopy, also

concluded that active synthesis of type IV collagen ceased at an early stage of cell life (45). In contrast, intact neonatal lenses could synthesize a small but significant amount (~10%) of heparan sulphate proteoglycan of molecular size equivalent to fraction C-PG, although this material also failed to elute as a discrete peak after initial molecular sieve chromatography. These findings may suggest that the biosynthesis of a basement membrane-associated (lens capsule) heparan sulphate proteoglycan is age dependent, but can be reactivated in cell culture. It also implies that the heparan sulfate proteoglycan may play a role in establishing the early structural integrity of the lens capsule.

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