Biosynthesis of Sulphated Macromolecules by Rabbit Lens Epithelium. I. Identification of the Major Macromolecules Synthesized by Lens Epithelial Cells In Vitro

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ABSTRACT Rabbit lens epithelial cells synthesize and secrete a variety of [35S]sulphate-labeled glycoconjugates in vitro. Associated with the cell layer, and with the medium, was a high molecular weight glycoconjugate(s) that contained heparan sulphate which was apparently covalently linked to sulphated glycoprotein. This component(s) was eluted in the void volume of a Sepharose CL-2B column and could not be fractionated by detergent treatment or extraction with lipid solvents. The cell layer also contained glycosaminoglycans (72% heparan sulphate, 28% chondroitin sulphate), as well as a small proportion of a low molecular weight sulphated glycoprotein. The major 35S-labeled species secreted into the medium were sulphated glycoproteins with approximate molecular weights of 120,000 and 35,000 together with a heparan sulphate proteoglycan. This proteoglycan could be precipitated from the culture medium with 30% saturated (NH₄)₂SO₄ and eluted from Sepharose CL-4B columns at approximately the same position (Kₒᵥ = 0.15) as heparan sulphate proteoglycans described in the basement membrane of the EHS "sarcoma" (Hassell, J. R., P. G. Robey, H. J. Barrach, J. Wilczek, S. I. Rennard, and G. R. Martin, 1980, Proc. Natl. Acad. Sci. USA, 77:4494-4498) and of the mouse mammary epithelium (David, G., and M. Bernfield, 1981, J. Cell Biol., 91:281-286). Its presence in the culture medium was unanticipated but may be explained by the inability of these cultures to deposit a basement membrane when grown on a plastic surface. The relationship of this heparan sulphate proteoglycan to the lens epithelial basement membrane is the subject of the following paper.

The anterior subcapsular epithelium of the lens is responsible for the formation of the anterior lens capsule and also differentiates into the fiber cells that form the body of the lens (1, 2). The anterior capsule is a thick basement membrane consisting principally of cross-linked aggregates of a procollagen-like molecule (3-6), together with other macromolecular components such as heparan sulphate (see below) and the basement membrane glycoprotein laminin (7, 8). To date there is no conclusive evidence for the presence of entactin (9) or fibronectin (10) in lens capsule, but since each basement membrane may contain tissue- and species-specific components (for reviews, see references 11 and 12) lens capsule may possess its own characteristic glycoconjugates.

The initial identification of the glycosaminoglycan heparan sulphate in rabbit and bovine lens capsule by Dische (13, 14) has since been confirmed in the bovine by others (15, 16). Moczar et al. (17) have also shown that cultured bovine lens epithelial cells can synthesize heparan sulphate and other sulphated glycosaminoglycans. At present, for the lens capsule no information is available on the nature of the proteoglycans from which these glycosaminoglycans may be derived, although heparan sulphate proteoglycans have been described in the basement membranes of the rat glomerulus (18-20), murine mammary epithelium (21), and PYS-2 teratocarcinoma cells (22), and the basement membrane-like stroma of a murine tumor (EHS "sarcoma" [23, 24]). In addition, heparan sulphate proteoglycans have also been identified at the surface of a number of cell types (see references 25 and 26) where they may serve to anchor cells to the pericellular matrix (27-29). Possible relationships between plasma mem-
brane- and basement membrane-associated heparan sulphate proteoglycans are not yet known. As part of a study of the formation of basement membranes in which we have used specific ocular basement membranes as experimental models, we have examined the nature of the sulfated macromolecules synthesized by normal rabbit lens epithelial cells in vitro. The results clearly indicate that in culture, the lens epithelium can produce heparan sulphate proteoglycan(s) together with sulfated glycosaminoglycans and glycoproteins. In this paper, we describe the biochemical characterization of these macromolecules. Their relationship to the basement membrane deposited by lens epithelial cells in culture is discussed in the accompanying paper (30).

MATERIALS AND METHODS

Materials: New Zealand white rabbits of either sex and of 4.5-5.5 lb body weight (8-10 wk old) were obtained through the animal farm of the Massachusetts General Hospital. Na235SO4 (~1,000 mCi/mmol) in water was purchased from New England Nuclear (Boston, MA). Dulbecco's modification of Eagle's medium (DME), Hanks' balanced saline solution (HBSS), l-glutamine, antibacterial-antimycotic mixture, and trypsin-EDTA (0.05 g trypsin and 0.2 g EDTA per liter of calcium- and magnesium-free HBSS) were obtained from Gibco Laboratories (Grand Island, NY) and sera were supplied by Sterile Systems, Inc. (Denver, CO). Sepharose CL-2B, CL-4B, Sephacryl S-300, and Sephadex G-25 were bought from Pharmacia (Piscataway, NJ), and Bio-Gel A-5m was from Bio-Rad Laboratories (Richmond, CA). Guanidine hydrochloride (practical grade) was obtained from Sigma Chemical Co., (St. Louis, MO) and stock solutions were treated with activated charcoal before use. Heparin (grade III), hyaluronic acid (grade III-S), chondroitin sulfate (mixed isomers, grade II), hexadecyltrimethylammonium bromide (CTAB), pronase (pro tease types VI and XIV) were also obtained from Sigma Chemical Co. Chondroitin ABC lyase (E.C. 4.2.2.4) was purchased from Miles Laboratories (Elkhart, IN); n-butyl nitrite, benzamidine hydrochloride, and 6-amino-hexanoic acid was from Eastman Kodak (Rochester, NY); sodium dodecyl sulphate (SDS) was from Pierce Chemical Co., (Rockford, IL); N-dodecyl-N,N-dimethyl-3-ammonio-1-propane-sulphonate (Zwittergent 3-12) was from Calbiochem-Behring Corp. (La Jolla, CA) and Cee (biological grade) was from Becton Dickinson Immunodiagnostic, Schwarz/Mann Div. (Spring Valley, NY). Other reagents were of "analytical" grade and obtained wherever they were available. Radioactivity was determined in Hydrofluor (National Diagnostics, Somerville, NJ) except for samples solubilized in NCS (Amer sham Corp., Arlington Heights, IL) which were counted in 20 ml of 2,5-diphenyloxazole (5 g/l) and 1,4-his-(5-phenyloxazol-3-yl)amine (5 ml) dissolved in toluene.

Culture of Lens Epithelial Cells: After sacrifice of the rabbits by intravenous injection of sodium pentobarbitone, lenses were removed from the eyes, cleaned of vitreous and iris by trimming with fine scissors, and rolled along their equators on Whatman no. 3 filter paper (Whatman Chemical Co., London). Lenses were then washed in sterile HBSS and capsules were isolated with iris forceps and a Zieger iridectomy knife. No attempt was made to separate the anterior and posterior capsules, but any visible clumps of fiber cells were teased away. The capsules were rinsed in culture medium consisting of Dulbecco's modification of Eagle's medium (with glutamine), containing 5% (vol/vol) calf serum, 5% (vol/vol) fetal bovine serum, and 1% (vol/vol) antibiotic-antimycotic mixture (31), cut into fragments with scissors and applied to 60-mm plastic tissue culture dishes in a drop of medium. After incubation for 1 h at 37°C in an atmosphere of 95% air/5% CO2 culture medium (3 ml) was carefully added and the dishes were returned to the incubator. Over a period of 2-6 d, epithelioid cells grew out from the explants and after ~2 wk the dishes were treated with trypsin-EDTA and the cells subcultured. Growth was slow on 60-mm dishes but accelerated when the cells were subcultured when they formed typical epithelioid sheets with a cobblestone appearance (Fig. 1 of accompanying paper). Confluent cultures (~2 × 106 cells per 35-mm dish) in the 13th-15th passage were generally used for isotopic labeling, but the same pattern of 35S sulfate incorporation was seen with cells of lower passage number.

Incorporation of Na235SO4 by Cultured Lens Cells: Cell-fluent dishes (35 mm) of lens cells were given fresh medium for 24 h and after the addition of Na235SO4 (100 μCi in 1 ml) incubation was continued for 24 h. The medium was removed and the cell layer briefly washed (twice) with ice-cold HBSS containing 0.1 M 6-amino-hexanoic acid, 5 mM benzamidine hydrochloride, and 10 mM EDTA (see reference 32). The cells were harvested with a rubber policeman, suspended in 1 ml of 4 M guanidine hydrochloride/m Ma HCl pH 7.5, centrifuged at 1,500 g, supernatant was discarded, and the remaining pellet was resuspended in 1 ml of 4 M guanidine hydrochloride, and a second aliquot taken for determination of radioactivity. In some experiments the high speed pellet was washed twice with ice-cold water, resuspended in 1 ml of 50 mM NaCl/50 mM Tris-HCl, pH 7.5 at 4°C, and incubated for 36 h at 60-60°C with three additions of pronase (1 mg each). The mixture was boiled for 5 min and the pronase extract separated by centrifugation at 80,000 g for 90 min. The final residue was washed once with water and solubilized in NCS for determination of radioactivity.

Initial attempts to isolate sulfated macromolecules from the culture medium by precipitation with cold ethanolic potassium acetate were unsuccessful and in later experiments precipitation with (NH4)2SO4 was adopted (also see reference 22). The medium was cooled on ice and 6-amino-hexanoic acid (0.1 M), benzamidine hydrochloride (5 mM), and EDTA (10 mM) were added to the preparations indicated. After removal of cellular debris by centrifugation at 1,500 g for 10 min, solid (NH4)2SO4 was added to 30% saturation and the resulting precipitate collected by centrifugation at 80,000 g for 1 h at 4°C. The precipitate was resuspended in 4 M guanidine hydrochloride and dialyzed against the same buffer to remove free Na235SO4 and (NH4)2SO4. The final solution was turbid and, after an aliquot was removed for determination of radioactivity, was clarified by centrifugation.

An aliquot (10 ml) of the (NH4)2SO4 supernatant was dialyzed exhaustively against running tap water followed by distilled water at 4°C, and freeze-dried. The lyophilized material was suspended in 4 M guanidine hydrochloride and its radioactivity was determined. To examine the effect of lipid extraction upon the sulfated macromolecules in the (NH4)2SO4 supernatant, lyophilized material was treated with the detergent Zwittergent 3-12 (4% wt/vol in 4 M guanidine hydrochloride) prior to chromatography on Sepharose CL-4B in a buffer system of 4 M guanidine hydrochloride containing 0.5% (wt/vol) Zwittergent 3-12 (33). Alternatively the lyophilized material was dissolved in HBSS (3 ml) and treated with ethyl acetate/acetone (7 ml; 43. vol/vol) at 65°C for 30 min (34). The aqueous and organic phases were separated by low speed centrifugation at room temperature and the aqueous phase lyophilized. Less than 1% of the 35S sulfate was lost in this phase.

Fractionation of Sulfated Macromolecules: Initial characterization of the sulfated macromolecules was carried out by gel filtration chromatography on columns of Sepharose CL-4B in 4 M guanidine hydrochloride at 4°C. Columns of 0.9 x 90 cm (flow rate ~ 3 ml/h, fraction volume 1.1-1.3 ml) or 1.5 x 90 cm (flow rate ~ 9 ml/h, fraction volume 2.6-2.7 ml) were eluted with buffer under gravity and a 0.2-ml aliquot of each fraction was taken for liquid scintillation spectrometry. Columns of Sepharose CL-2B and Sephacryl S-300 were run under similar conditions. The void volume of both Sepharose CL-4B and Sephacryl S-300 was ~80%, but from Sepharose CL-2B it was lower and more variable (34-68%). Blue Dextran 2000 was used to determine the void volume (V0) of each column and H2O was used to determine the total volume (Vt).

Density gradient centrifugation was carried out in 4 M guanidine hydrochloride to which CsCl had been added 65% of solution. The starting density was 1.47 g/ml. Samples were centrifuged in polyallomer tubes at 105,000 g for 48-72 h at 8°C and 0.5-ml fractions were collected from the bottom of the gradient. Aliquots of each fraction were taken for determination of radioactivity and density.

To obtain an estimate of the molecular size of the 35S sulfate-labeled glycoproteins synthesized by the lens epithelial cells (see below), appropriate fractions from the Sepharose CL-4B column were dialyzed exhaustively against water containing 10 mM EDTA and 10 mM 6-amino-hexanoic acid at 4°C and lyophilized. The material was denatured in 2% (wt/vol) SDS, reduced and alkylated, and chromatographed on a column of 6% agarose (Bio-Gel A-5m, 1.5 x 90 cm) as previously described (6). Fractions of ~2 ml were collected and an aliquot of each taken for measurement of radioactivity. Blue Dextran was used to determine the void volume and H2O was used to determine the total column volume. For molecular weight (mol wt) estimation, the column was calibrated with rat tail tendon collagen (γ components, mol wt 294,000; α-components, mol wt 196,000; and α chains, mol wt 98,000), chick tendon pro-α chains (mol wt 150,000), which was a gift of Dr. J. S. Hulmes (University of Manchester, England) and ovalbumin (mol wt 45,000).

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Analysis of Glycosaminoglycans: Samples in 4 M guanidine hydrochloride were dialyzed exhaustively against 50 mM NaCl/50 mM Tris HCl, pH 7.5 at 4°C and incubated for 18 h at 50-60°C with pronase (100 µg/ml). After boiling for 5 min, [35S]sulphate-labeled glycosaminoglycans were precipitated by the addition of 2% (wt/vol) CTAB in water to a final concentration of 0.32% in the presence of carrier glycosaminoglycans (0.5% (wt/vol) chondroitin sulphate and 0.5% (wt/vol) hyaluronic acid). The precipitated glycosaminoglycans were pelleted by centrifugation (1,500 g, 10 min, room temperature), washed twice in 0.05 M NaCl/0.05% (wt/vol) CTAB, and solubilized in 1 ml of methanol. Radioactivity in the supernatant and precipitate was measured. Alternatively, the pronase digest was chromatographed on a column of Sephadex G-50 (medium grade, 0.9 x 100 cm) eluted with 0.1 M ammonium acetate, pH 7.5, containing 20% (vol/vol) ethanol as a bacteriostatic agent (35). Fractions of 0.9-1.0 ml were collected at a flow rate of 2.9 ml/h.

Radioactively labeled glycosaminoglycans were identified by assessing their susceptibility to treatment with chondroitin ABC lyase and nitrous acid as described by Hart (35). As a control, samples of nonradioactive chondroitin sulphate (2 mg) were incubated with and without enzyme. Likewise, two samples of nonradioactive heparin (2 mg) were treated identically with the radioactive material except that for one of these absolute ethanol was substituted for 20% (vol/vol) butyl nitrite in the reaction mixture (35). Controls were assayed by the addition of CTAB as described above and in each case undegraded glycosaminoglycan produced a turbid precipitate whereas turbidity was minimal in the degraded samples.

RESULTS

Incorporation of Na₂³⁵SO₄ by Lens Epithelial Cells

When confluent cultures of lens epithelial cells were incubated with Na₂³⁵SO₄ for 24 h the nondialyzable radioactivity was distributed among the various fractions as shown in Table I which gives data from two representative, but not duplicate, experiments. Two patterns of distribution were seen: in one, the radioactivity was predominantly associated with the cell layer (exp. 1, Table I) and in the other with the medium, particularly that fraction not precipitated by (NH₄)₂SO₄ at 30% saturation (exp. 2, Table I). Most of the radioactivity in the cell layer (63-84%) was extractable in 4 M guanidine hydrochloride and an additional 12-32% was released by digestion with pronase.

The 4 M guanidine hydrochloride extract of the cell layer was fractionated on a column of Sepharose CL-4B into two components, one in the void volume (CI) and the other (CII) eluted with a distribution coefficient (36) of 0.70 (range 0.69-0.73) (Fig. 1). The distribution of radioactivity between

### Table I

<table>
<thead>
<tr>
<th>Component</th>
<th>Exp. 1</th>
<th>Exp. 2</th>
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<tbody>
<tr>
<td>Cell layer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 M guanidine-HCl</td>
<td>771,240</td>
<td>1,890,000</td>
</tr>
<tr>
<td>Pronase extract</td>
<td>267,210</td>
<td>270,250</td>
</tr>
<tr>
<td>Final residue</td>
<td>15,890</td>
<td>12,340</td>
</tr>
<tr>
<td>Medium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(NH₄)₂SO₄ precipitate</td>
<td>294,580</td>
<td>748,390</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ supernatant</td>
<td>346,800</td>
<td>2,939,660</td>
</tr>
</tbody>
</table>

Confluent cultures of lens cells of comparable passage number (exp. 1, 13th; exp. 2, 15th) were labeled with Na₂³⁵SO₄ (500 µCi/ml) for 24 h at 37°C. The medium was cooled to 4°C and spun to remove cellular debris, and solid (NH₄)₂SO₄ was added to 30% saturation. The precipitate was spun down, resuspended in 4 M guanidine hydrochloride buffer, and dialyzed exhaustively against the same buffer at 4°C. A portion of the supematant was dialyzed exhaustively against water to remove free Na₂³⁵SO₄ lyophilized, and suspended in 4 M guanidine hydrochloride buffer. The cell layer was washed in HBSS and extracted as described in the text. Aliquots of each fraction were taken for determination of radioactivity.

![Figure 1: Gel filtration chromatography on Sepharose CL-4B and CL-2B of 4 M guanidine hydrochloride extract of cell layer. Lens cell cultures were incubated with Na₂³⁵SO₄ (125 µCi/ml) in Dulbecco's modification of Eagle's medium containing 5% (vol/vol) calf serum and 5% (vol/vol) fetal calf serum for 24 h at 37°C. The cell layer was extracted in 4 M guanidine hydrochloride/50 mM sodium acetate, pH 5.8 containing 0.1 M 6-amino-2-hexanoic acid, 10 mM EDTA, and 5 mM benzamidine hydrochloride for 24 h at 4°C, dialyzed against the same buffer to remove unincorporated Na₂³⁵SO₄ and, after centrifugation, chromatographed on a column of Sepharose CL-4B (0.9 x 90 cm), eluted with the above buffer at 4°C. The void volume (V₀) was 27 ml and the total volume (Vₜ) was 79 ml. For reference purposes the peaks of radioactivity have been designated CI and CII and pooled fractions used for further analysis. The inset shows the elution profile of component CI on Sepharose CL-2B. CI was concentrated, mixed with Blue Dextran 2000, and applied to a column of Sepharose CL-2B eluted with 4 M guanidine hydrochloride at 4°C. The void volume (V₀) was determined by measuring the absorbance at 590 nm and was 23 ml; the total volume (Vₜ) was 64 ml. |
these two peaks varied and bore no obvious relationship to the passage number (no. 2-15) of the cultured cells, to the interval between subculture and incubation with isotope (7-47 d), to the presence of ascorbate in the medium, or to the presence of conditioned or fresh medium during the incubation period.

Preliminary Characterization of CI

CI appears to be a single component of large molecular size. When chromatographed on Sepharose CL-2B in 4 M guanidine hydrochloride, radioactivity was recovered only in the void volume (Fig. 1, inset). CsCl density gradient centrifugation in 4 M guanidine hydrochloride also failed to fractionate CI: most of the radioactivity recovered from the gradient banded at the top of the gradient with a buoyant density of 1.31-1.37 (Fig. 2a).

To examine its glycosaminoglycan content, CI was digested with pronase followed by precipitation with CTAB or chromatography on Sephadex G-50. Approximately 50% of the [35S]sulphated components of CI were precipitable with CTAB and a slightly higher proportion (~60%) of the [35S]sulphate was eluted in the void volume after molecular sieve chromatography on G-50 (Fig. 3). These findings indicate that ~50-60% of the [35S]sulphate labeled macromolecules in fraction CI are in glycosaminoglycans. Upon treatment of this material with HNO2, 95% was degraded to small fragments indicating that it was largely heparan sulphate. Since material included by Sephadex G-50 was neither precipitable with CTAB nor degraded by HNO2 or testicular hyaluronidase, it was presumed to consist of sulphated glycopeptides.

Separation of Components of CI

The broad and asymmetrical shape of the CII peak (Fig. 1) suggests the presence of more than one component. After pronase digestion 78% (SE 2.59) of the [35S]sulphate was excluded from a column of Sephadex G-50 (Fig. 4a) and could be precipitated with CTAB, indicative of glycosaminoglycan. The radioactivity included by Sephadex G-50 was not...
precipitable with CTAB and taken to represent sulphated glycopeptides. Of the glycosaminoglycans present in fraction CII, most (mean = 72%, SE 2.72) was degraded by HNO₂ and was therefore likely to be heparan sulphate. The remainder was susceptible to chondroitin ABC lyase indicating the presence of a substantial amount of chondroitin and/or dermatan sulphates. In the specific experiment illustrated in Fig. 4b, heparan sulphate accounted for 60% of the glycosaminoglycans present in fraction CII. From these results it appeared probable that CII consisted principally (>80%) of one or more glycosaminoglycan(s) (or proteoglycans, but see below) together with a variable but relatively small amount of sulphated glycoprotein.

An attempt to separate the glycosaminoglycans in CII from glycoprotein by gel filtration chromatography on Sephacryl S-300 was not successful (data not shown) but separation was affected by CsCl density gradient centrifugation in 4 M guanidine hydrochloride (Fig. 2b). The material settling at the bottom of the gradient with a buoyant density of 1.60–1.68 g/ml was collected and digested with pronase after removal of guanidine hydrochloride by dialysis. Over 80% of the [³⁵S]sulphate was precipitable by CTAB indicating its presence in glycosaminoglycan. In contrast, <10% of the radioactivity at the top of the gradient (buoyant density 1.27–1.34 g/ml) was precipitable with CTAB after pronase digestion. Radioactive material of intermediate density was largely glycosaminoglycan since most of the label was precipitable with CTAB and on recentrifugation it sedimented to the bottom of the gradient with a buoyant density >1.60 g/ml. The distribution of radioactivity within the gradient was such that when CII was thought, on the basis of precipitability with CTAB, to contain little glycoprotein, only 2% of the label at most was found at the top of the gradient. This value was increased, but never to more than 17%, when CII appeared to contain more glycoprotein.

To determine whether the dense sulphated species was glycosaminoglycan or proteoglycan it was collected from the gradient and split into two portions, one of which was digested with pronase. When chromatographed on Sephacryl S-300 in 4 M guanidine hydrochloride the radioactivity in both the digested and untreated samples was eluted with a Kᵥ of 0.35 (Fig. 5) clearly establishing its presence in glycosaminoglycan rather than proteoglycan.

CII glycoprotein material was collected from the top of the CsCl density gradient and rechromatographed on Sepharose CL-4B where it was found to elute with a distribution coefficient of 0.64 (data not shown). Further characterization of the CII sulphated glycoprotein has yet to be undertaken because of the small quantities obtained.

Medium Macromolecules Precipitated by 30% Saturated (NH₄)₂SO₄

The (NH₄)₂SO₄ precipitate was solubilized in 4 M guanidine hydrochloride and chromatographed on Sepharose CL-4B (Fig. 6). The major [³⁵S]sulphate-labeled component, accounting for over 50% of the radioactivity, was a broad peak with a Kᵥ of 0.15 (0.11–0.19) (Fig. 6, MI). After treatment with pronase the [³⁵S]sulphate was largely (>80%) precipitable with CTAB and was eluted from Sepharose CL-4B with a Kᵥ of 0.63 (0.60–0.66) (Fig. 7), clearly indicating the proteoglycan nature of MI. Nevertheless, MI floated to the top of a CsCl density gradient with a buoyant density of 1.32 g/ml (Fig. 8a). The glycosaminoglycan portion of MI was analyzed by treatment with HNO₂ and chondroitin ABC lyase and found to be >95% heparan sulphate (Fig. 9).

In contrast to MI, components MII, MIII, and MIV appeared to consist principally of sulphated glycoprotein(s), judging by their low precipitability with CTAB after pronase treatment (31, 24, and 9%, respectively). Variable amounts of MII (Kᵥ 0.41) and MIV (Kᵥ 0.69) were present from preparation to preparation and were not characterized further; however, MIII (Kᵥ 0.54, range 0.51–0.58) was a more con-
sistent feature of the chromatogram. When centrifuged under
dissociative conditions in a CsCl density gradient, MIII was
found to have a low buoyant density (1.29 g/ml; Fig. 8 b).

Medium Macromolecules Not Precipitated by
30% Saturated (NH₄)₂SO₄

This fraction, comprising 20-50% of the total sulphated
carbohydrates synthesized by the lens epithelial cell cultures
(Table I), was analyzed after exhaustive dialysis against cold
water and lyophilization. The dry material was solubilized in
4 M guanidine hydrochloride and chromatographed on Sepha-
rose CL-4B in the same buffer (Fig. 10). Approximately 10%
(5.7-15.1%) of the [³⁵S]sulphate was eluted in the void volume
designated SI and this, like its counterpart in the cell layer
(CI), was ~50% glycosaminoglycan (>95% heparan sulphate).
The elution profile on Sepharose CL-4B was not changed by
prior treatment of the lyophilized material with either the
detergent Zwittergent 3-12 or a mixture of ethyl acetate and
acetone, suggesting that the large size of SI and the inability
of 30% saturated (NH₄)₂SO₄ to precipitate it were not due to
the presence of lipid.

Most of the radioactivity in the (NH₄)₂SO₄ supernatant of

![Figure 7](https://via.placeholder.com/150)

**Figure 7** Sepharose CL-4B chromatography of component MI before and after pronase digestion. Component MI was purified by rechromatography on Sepharose CL-4B (O), dialyzed exhaustively against distilled water, and lyophilized. After treatment with pronase as described in the text, the digest (●) was reapplied to the column of Sepharose CL-4B. The void volume (V₀) of the column was 54 ml and the total volume (V₁) was 149 ml.

![Figure 8](https://via.placeholder.com/150)

**Figure 8** Cesium chloride density gradient centrifugation of the major sulphated components in 30% saturated (NH₄)₂SO₄ precipitate of lens epithelial cell medium. Components MI and MIII from Fig. 6 were rechromatographed on Sepharose CL-4B. To each was added solid CsCl (0.55 g/g) before centrifugation at 105,000 g for 48-72 h at 8°C. Fractions (500 µl) of MI (a) and MIII (b) were collected from the bottom of the centrifuge tube and aliquots were taken for determination of radioactivity (●) and density (O).

![Figure 9](https://via.placeholder.com/150)

**Figure 9** Chromatographic analysis of ³⁵S-glycos-
aminoglycans from MI on Sephadex G-50. The gly-
cosaminoglycan component of MI (Fig. 7, Kᵥ = 0.66)
dialyzed exhaustively against water to remove 4 M guanidine hydrochloride, split into two, and lyophi-
lized. One portion was treated with nitrous acid (O) and the other with chondroitin ABC lyase (●) as described in the text. Each was then chromatographed on a column of Sephadex G-50 eluted with 0.1 M ammonium acetate in 20% (vol/vol) ethanol. Fractions of 0.95 ml were collected and the radioactivity determined. The void volume (V₀) of the column was 28 ml and the total volume (V₁) was 63 ml.
scribed in basement membranes of developing murine sali-
glycosaminoglycan although hyaluronic acid has been de-

In recent years increasing attention has been paid to the
component with an apparent tool wt of ~35,000 (each value
representing the mean of two determinations; data not

Although not dramatic, these differences do suggest a possible
association of [35S]sulphated glycosaminoglycan(s) with SII
the medium was present in two barely separated components
of $K_v$, 0.53 (range 0.51–0.53) and 0.66 (range 0.61–0.70),
designated SII and SIII, respectively (Fig. 10). Although
components SII-SIII were not individually analyzed by CsCl
density gradient centrifugation, samples of the unfraccionated
(NH$_4$)$_2$SO$_4$ supernatant of the medium were centrifuged under
dissociative conditions. Under these conditions all of the
radioactivity was recovered at the top of the gradient with a
buoyant density of 1.28 g/ml (data not shown). On the basis
of their distribution coefficients on Sepharose CL-4B and
their low precipitability with CTAB, SII, and SIII appeared to
be similar to MIII and MIV described above. However, the
glycosaminoglycan content of the S fractions appeared to be
somewhat higher than that of the corresponding M fractions.
Thus, after further purification by rechromatography, 41% of
the $[^{35}S]$sulphate in SII was precipitated with CTAB, as
compared with 24% of the radioactivity in MIII. For SII and
MIV the corresponding values were 16 and 9%, respectively.
Although not dramatic, these differences do suggest a possible
association of $[^{35}S]$sulphated glycosaminoglycan(s) with SII
and SIII.

Chromatography of partially purified SII and SIII compo-
ents on a column of agarose A-5m in 0.1% (wt/vol) SDS
resolved SII into two components of apparent mol wt
~120,000 and (by extrapolation) ~35,000. SIII gave a single
component with an apparent mol wt of ~35,000 (each value
representing the mean of two determinations; data not
shown.)

DISCUSSION

In recent years increasing attention has been paid to the
contributions of noncollagenous glycoconjugates to the
structure and function of a variety of basement membranes (see
11, 12, 37). Typically, heparan sulphate is the predominant
glycosaminoglycan although hyaluronic acid has been de-
scribed in basement membranes of developing murine sali-

the heparan sulphate proteoglycan appears to provide the
main charge barrier in the glomerular basement membrane
(37) and the glycoproteins may be involved in the adhesion of
cells to the basement membrane (see reference 11).

As part of an investigation of the macromolecular compo-
nents of the lens capsule, we have shown that rabbit lens
epithelial cells, when grown to confluence on a plastic (tissue
culture) substratum, can synthesize and secrete various sul-
phated glycoconjugates.

The largest sulphated macromolecule(s) synthesized by the
lens epithelial cells was found to be present in both the cell
layer (CI, Fig. 1) and the culture medium (SII, Fig. 10).
Although these cell-associated and secreted components may
not be identical, they share certain characteristics. For example,
fractons CI and SII eluted in the void volume of both
Sepharose CL-4B and CL-2B (Figs. 1 and 10) indicating a
molecular weight greater than $1 \times 10^6$. Despite its large size,
however, SII was not precipitated from the culture medium by
30% saturated (NH$_4$)$_2$SO$_4$. Biosynthesis of high molecular
weight lipido-associated heparan sulphate proteoglycans by
cells in culture has been described (28, 29, 45), but treatment
of SI with detergent or organic solvents had no obvious effect.
Although similar treatment of fraction Cl was not possible
due to the limited quantities available, density gradient cen-
trifugation in 4 M guanidine hydrochloride failed to dissociate
Cl into smaller components (Fig. 2a). Only protease digestion
separated the complex into its component heparan sulphate
glycosaminoglycan and sulphated glycopeptide moieties (Fig.
3). Although we have been unable to determine whether in the
native macromolecules, the heparan sulphate is part of a proteoglycan,
the findings to date do suggest that such a proteoglycan might be covalently linked with a sulphated glycoprotein to constitute a high molecular weight complex. Recently a number of high molecular weight glycoprotein-

proteoglycan complexes has been described. These include a
high molecular weight glycoprotein-chondroitin sulphate
proteoglycan complex on the surface of M21 human melanoma
cells (46), a sulphated glycoconjugate fraction from rat liver
plasma membranes containing heparan sulphate and a sul-
phated glycopeptide (47), and a complex of heparan sulphate
and lactosaminoglycans covalently bound to fibronectin in
differentiated F9 mouse teratocarcinoma cells (48).

The second major sulphated macromolecule (M1) synthe-
sized by the lens epithelial cells was secreted into the culture
medium from which it could be recovered by precipitation
with 30% (NH$_4$)$_2$SO$_4$ (Fig. 6). The results indicate that M1
consists almost exclusively of a heparan sulphate proteogly-
can, although when centrifuged on a CsCl density gradient

FIGURE 10 Gel filtration chromatography of sulphated macromolecules in lens cell medium that are not precipitated by 30%
saturated (NH$_4$)$_2$SO$_4$. The experimental conditions were as
described in Fig. 6. After centrifugation of the 30% saturated
(NH$_4$)$_2$SO$_4$ precipitate the supernatant was dialyzed exhaustively
against cold water to remove all unincorporated isotope and then
lyophilized. The lyophilized material was redissolved in 4 M gua-
nidine hydrochloride and an aliquot was chromatographed on
Sepharose CL-4B in the same buffer. The void volume ($V_v$) was 55
ml and the total volume ($V_t$) was 149 ml. For reference purposes
the peaks of radioactivity have been designated SI-SIII.
under dissociative conditions it floated to the top (Fig. 8 a). This may reflect a high protein : glycosaminoglycan ratio and/or a low sulphate content. The molecular size of this proteoglycan \( (K_{av} \approx 0.15 \text{ on Sepharose CL-4B} ) \) is similar to that of the heparan sulphate proteoglycan deposited by the EHS "sarcoma" (24), by murine mammary epithelium (21) and to at least one of the heparan sulphate proteoglycans recently reported to be synthesized by a murine embryonal carcinoma-derived cell line (41). However, fraction MI is larger than the heparan sulphate proteoglycan of the glomerular basement membrane \( (K_{av} \approx 0.45; \text{ reference 19}) \). It seems likely that MI represents a basement membrane proteoglycan. Its occurrence in the medium rather than the cell layer was unanticipated, especially since lens epithelial cells are known to be responsible for the production of the lens capsule in vivo (1). This finding may be explained however by the inability of rabbit lens epithelial cells cultured under the conditions described here to deposit a basement membrane (see accompanying paper).

A third category of sulphated macromolecule synthesized by the lens epithelial cells consisted of components of lower molecular weight. This group was eluted from Sepharose CL-4B with distribution coefficients in the approximate range of 0.5–0.7 and included cell fraction CII (Fig. 1) and medium fractions MRI, MIV (Fig. 6), and SII, SIII (Fig. 10). Whereas CII was predominantly heparan sulphate glycosaminoglycan (with some chondroitin sulphate and/or dermatan sulphate), the medium components consisted almost entirely of sulphated glycoproteins.

Sulphated glycoproteins represented an unexpectedly high percentage (\( \geq 50\% \)) of the total sulphated macromolecules synthesized by these cultures. Most were of relatively low apparent molecular weight as judged by SDS/agarose gel filtration chromatography and were too small to be related to the sulphated glycoproteins previously described in basement membranes, i.e., entactin and laminin. They could conceivably represent degradation products of these high molecular weight glycoproteins but it should be noted that whenever possible, experimental procedures were carried out at 4°C in the presence of protease inhibitors. Perhaps more likely is a relationship to the low molecular weight (24,000–60,000) sulphated glycoproteins on the surface of vascular endothelial cells (49).

Although the sulphated macromolecules synthesized by the lens epithelial cells were qualitatively similar in all experiments, quantitative differences were observed. Two biosynthetic patterns emerged: in one, most (\( \approx 60\% \)) of the sulphated macromolecules were cell-associated (Table I, exp. 1) and in the other \( \approx 60\% \) was present in the medium (Table I, exp. 2). In the first of these, at least 98% of the cell-associated \( [35S] \) sulphate was present in CII, and in the medium, equivalent amounts were found in the \( (\text{NH}_4)_2\text{SO}_4 \) precipitate and supernatant fractions. However, when the secreted macromolecules predominated, the levels of CI rose to \( \approx 10\% \) of the total cell-associated \( [35S] \) sulphate. In addition, the glycoproteins SII and SIII (not precipitated by \( (\text{NH}_4)_2\text{SO}_4 \)) formed \( \approx 80\% \) of the secreted \( [35S] \) sulphate labeled macromolecules. No discernible variables in the culture conditions appeared responsible for these differences although one possible inference is that low molecular weight sulphated glycoproteins may be essential constituents of CI.

The results presented indicate that lens epithelial cells can synthesize an array of sulphated macromolecules of unex-pected complexity (Table II). These have been partially characterized but before a definite statement about their interrelationships can be made it will be necessary to examine their biosynthesis with other radioactive precursors such as amino acids and sugars. Among the newly synthesized macromolecules is a heparan sulphate proteoglycan (MI) similar to previously described basement membrane proteoglycans, and the relationship of this to basement membrane deposition by lens epithelial cells is considered in the following paper (30).

This work was supported by National Institutes of Health grant no. EYO3810. J. G. Heathcote is the recipient of a Career Development Award of the Juvenile Diabetes Foundation. We would like to thank Drs. Jerome Gross and Bryan Tooze for many helpful discussions during this study, and Brian Gliniak for expert technical assistance.

This is publication no. 956 of the Robert W. Lovett Memorial Group for the Study of Diseases Causing Deformities, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts.

Received for publication 28 December 1983, and in revised form 21 May 1984.

TABLE II

<table>
<thead>
<tr>
<th>Component</th>
<th>( K_{av}^* )</th>
<th>Composition</th>
<th>Buoyant density ( (g/ml) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>CI</td>
<td>0</td>
<td>Heparan sulphate/glycoprotein</td>
<td>1.31–1.37</td>
</tr>
<tr>
<td>CII</td>
<td>0.70</td>
<td>Glycosaminoglycan (78%) (72% heparan sulphate)</td>
<td>1.60–1.68</td>
</tr>
<tr>
<td>MII</td>
<td>0.54</td>
<td>Glycoprotein</td>
<td>1.27–1.34</td>
</tr>
<tr>
<td>MI</td>
<td>0.15</td>
<td>Heparan sulphate proteoglycan</td>
<td>1.32</td>
</tr>
<tr>
<td>SII</td>
<td>0.53</td>
<td>Glycoprotein</td>
<td>ND</td>
</tr>
<tr>
<td>SIII</td>
<td>0.66</td>
<td>Glycoprotein</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Sepharose CL-4B in 4 M guanidine hydrochloride.
† 
Cl density gradient in 4 M guanidine hydrochloride.
‡ Not determined.

REFERENCES


