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 [^{35}S]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 ^{35}S-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 $(NH_4)_2SO_4$ and eluted from Sepharose CL-4B columns at approximately the same position (K_{av} = 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-

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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 sulphated 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 sulphated 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 lb body weight (8–10-wk old) were obtained through the animal farm of the Massachusetts General Hospital. $Na_2{}^{35}SO_4$ (~1,000 mCi/mmol) in water was purchased from New England Nuclear (Boston, MA). Dulbecco's modification of Eagle's medium (DME), Hanks' balanced salt solution (HBSS[1]), L-glutamine, antibiotic-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. (Logan, UT). Sepharose CL-2B, CL-4B, Sephacryl S-300, and Sephadex G-50 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 sulphate (mixed isomers, grade II), hexadecyltrimethylammonium bromide (CTAB), pronase (protease 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-aminohexanoic 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 CsCl (biological grade) was from Becton Dickinson Immunodiagnostics, 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 (Amersham Corp., Arlington Heights, IL) which were counted in 20 ml of 2,5-diphenyloxazole (5 g/l) and 1,4-bis-(5-phenyloxazol-2-yl)benzene (0.1 g/l) dissolved in toluene.

Culture of Lens Epithelial Cells: After sacrifice of the rabbits by intravenous injection of sodium pentabarbitol, 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 Separation, Inc., Clifton, NJ) to remove adherent fragments of iris and suspensory ligament. Lenses were then washed in sterile HBSS and capsules were isolated with iris forceps and a Ziegler 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% CO_2 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 transferred to 35-mm dishes. Fresh medium was supplied three times per week and cells were subcultured when they formed typical epithelioid sheets with a cobblestone appearance (see Fig. 1 of accompanying paper). Confluent cultures (~2×10^5 cells per 35-mm dish) in the 13th–15th passage were generally used for isotopic labeling, but the same pattern of [^{35}S]sulphate incorporation was seen with cells of lower passage number.

Incorporation of $Na_2{}^{35}SO_4$ by Cultured Lens Cells: Confluent dishes (35 mm) of lens cells were given fresh medium for 24 h and after the addition of $Na_2{}^{35}SO_4$ (100 μCi–1 mCi per 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-aminohexanoic acid, 5 mM benzamidine hydrochloride, and 10 mM EDTA (see reference 32). The cells were harvested with a rubber policeman, suspended in 1–2 ml of 4 M guanidine hydrochloride/50 mM sodium acetate, pH 5.8 at 4°C, containing the above protease inhibitors (hereafter referred to simply as 4 M guanidine hydrochloride), and stirred for 24 h at 4°C. After exhaustive dialysis against the same buffer, the extract was centrifuged at 105,000 *g* for 50–60 h at 4°C and the supernatant removed: an aliquot was taken for determination of radioactivity. When the supernatant was turbid, it was clarified by filtration through a Millipore filter (type HA, 0.45-μm pore size) that had been soaked in 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 50–60°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 sulphated macromolecules from the culture medium by precipitation with cold ethanolic potassium acetate were unsuccessful and in later experiments precipitation with $(NH_4)_2SO_4$ was adopted (also see reference 22). The medium was cooled on ice and 6-aminohexanoic acid (0.1 M), benzamidine hydrochloride (5 mM), and EDTA (10 mM) were added to the concentrations indicated. After removal of cellular debris by centrifugation at 1,500 *g* for 10 min, solid $(NH_4)_2SO_4$ 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 $Na_2{}^{35}SO_4$ and $(NH_4)_2SO_4$. The final solution was turbid and, after an aliquot was removed for determination of radioactivity, was clarified by filtration as described above.

An aliquot (10 ml) of the $(NH_4)_2SO_4$ 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 sulphated macromolecules in the $(NH_4)_2SO_4$ 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; 4:3, 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 [^{35}S]sulphate was lost in the organic phase.

Fractionation of Sulphated Macromolecules: Initial characterization of the sulphated 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 × 90 cm (flow rate ~3 ml/h, fraction volume 1.1–1.3 ml) or 1.5 × 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 recovery of [^{35}S]sulphate from 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 (V_o) of each column and ^3H_2O was used to determine the total volume (V_t).

Density gradient centrifugation was carried out in 4 M guanidine hydrochloride to which CsCl had been added (0.55 g/g 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 [^{35}S]sulphate-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-aminohexanoic 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 × 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 ^3H_2O 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; β-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. D. J. S. Hulmes (University of Manchester, England) and ovalbumin (mol wt 45,000).

[1] *Abbreviations used in this paper:* CTAB, hexadecyltrimethylammonium bromide; HBSS, Hanks' balanced salt solution.

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, [^{35}S]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 × 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 $Na_2{}^{35}SO_4$ by Lens Epithelial Cells

When confluent cultures of lens epithelial cells were incubated with $Na_2{}^{35}SO_4$ 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 $(NH_4)_2SO_4$ 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) (K_{av}) of 0.70 (range 0.69–0.73) (Fig. 1). The distribution of radioactivity between

TABLE I

Distribution of Nondialyzable Radioactive Sulphate in Lens Epithelial Cultures

	Exp. 1		Exp. 2	
	dpm	*% of total dpm*	*dpm*	*% of total dpm*
Cell layer				
4 M guanidine-HCl extract	771,240		1,890,000	
Pronase extract	267,210	62.2	270,250	37.1
Final residue	15,890		12,340	
Medium				
$(NH_4)_2SO_4$ precipitate	294,580	17.4	748,390	12.8
$(NH_4)_2SO_4$ supernatant	346,800	20.4	2,939,660	50.1

Confluent cultures of lens cells of comparable passage number (exp. 1, 13th; exp. 2, 15th) were labeled with $Na_2{}^{35}SO_4$ (500 μCi/ml) for 24 h at 37°C. The medium was cooled to 4°C and spun to remove cellular debris, and solid $(NH_4)_2SO_4$ 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 supernatant was dialyzed exhaustively against water to remove free $Na_2{}^{35}SO_4$, 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.

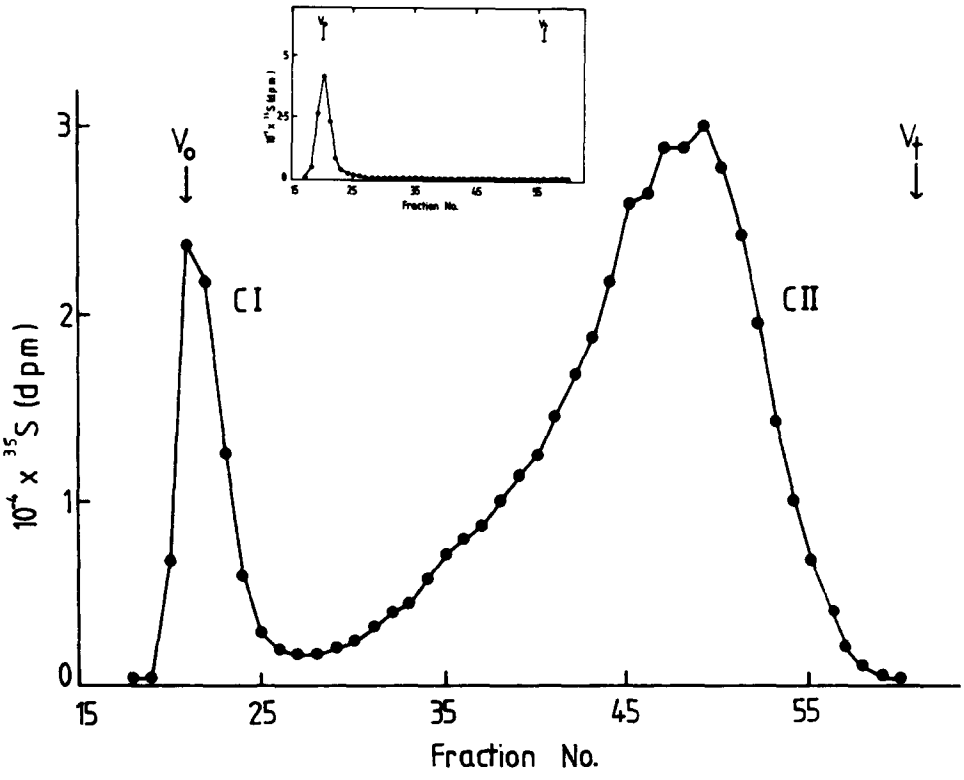

FIGURE 1 Gel filtration chromatography on Sepharose CL-4B and CL-2B of 4 M guanidine hydrochloride extract of cell layer. Lens cell cultures were incubated with $Na_2{}^{35}SO_4$ (125 μCi/ml) in Dulbecco's modification of Eagle's medium containing 5% (vol/vol) calf serum and 5% (vol/vol) fetal calf serum 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-aminohexanoic acid, 10 mM EDTA, and 5 mM benzamidine hydrochloride for 24 h at 4°C, dialyzed against the same buffer to remove unincorporated $Na_2{}^{35}SO_4$ and, after centrifugation, chromatographed on a column of Sepharose CL-4B (0.9 × 90 cm), eluted with the above buffer at 4°C. The void volume (V_O) was 27 ml and the total volume (V_t) 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 (V_o) was determined by measuring the absorbance at 590 nm and was 23 ml; the total volume (V_t) 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. 2*a*).

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 [^{35}S]sulphated components of CI were precipitable with CTAB and a slightly higher proportion (~60%) of the [^{35}S]sulphate was eluted in the void volume after molecular sieve chromatography on G-50 (Fig. 3). These findings indicate that ~50–60% of the [^{35}S]sulphate labeled macromolecules in fraction CI are in glycosaminoglycans. Upon treatment of this material with HNO_2, 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 HNO_2 or testicular hyaluronidase, it was presumed to consist of sulphated glycopeptides.

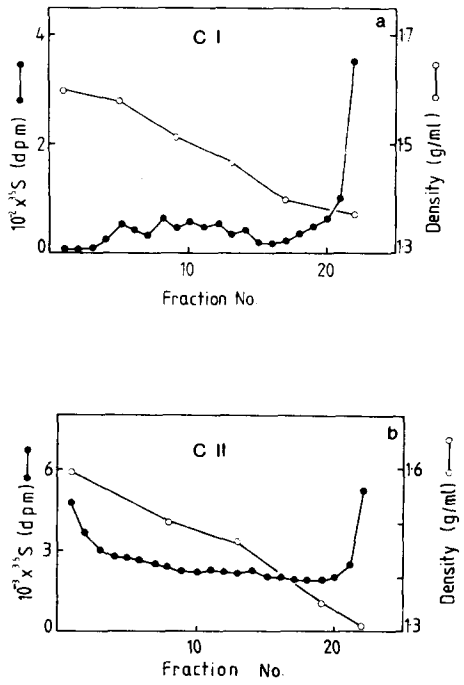

FIGURE 2 Cesium chloride density gradient centrifugation of sulphated components of epithelial cell layers. Components CI and CII were isolated as shown in Fig. 1. Solid CsCl (0.55 g/g) was added to each component and the mixtures were centrifuged at 105,000 *g* for 48–72 h at 8°C. Fractions (0.5 ml) of CI (*a*) and CII (*b*) were collected from the bottom of the centrifuge tube and aliquots were taken for determination of radioactivity (●) and density (○).

Separation of Components of CII

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 [^{35}S]sulphate was excluded from a column of Sephadex G-50 (Fig. 4*a*) and could be precipitated with CTAB, indicative of glycosaminoglycan. The radioactivity included by Sephadex G-50 was not

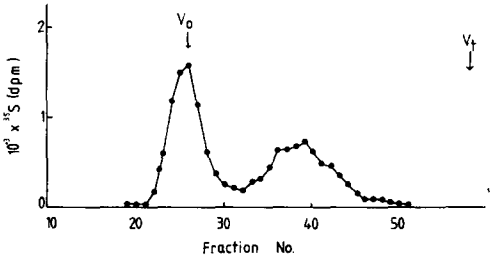

FIGURE 3 Chromatographic separation of ^{35}S-glycosaminoglycans and ^{35}S-glycopeptides from CI on Sephadex G-50. Component CI isolated by chromatography on Sepharose CL-4B was dialyzed exhaustively to remove guanidine hydrochloride and digested with pronase for 18 h at 60°C. After boiling the sample was lyophilized, resuspended in 0.1 M ammonium acetate in 20% (vol/vol) ethanol, and chromatographed on Sephadex G-50. Fractions of ~1.3 ml were collected and 100-μl aliquots taken for determination of radioactivity.

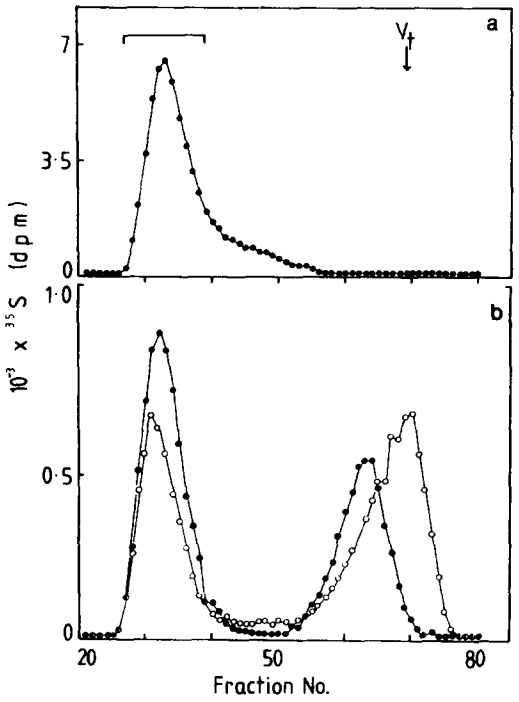

FIGURE 4 Chromatographic analysis of ^{35}S-glycosaminoglycans from CII on Sephadex G-50. Component CII isolated by chromatography on Sepharose CL-4B was digested with pronase and chromatographed on Sephadex G-50 (*a*), as described in the text. Fractions of ~0.9 ml were collected and 200-μl aliquots were taken for determination of radioactivity. The remainder of fractions 27–39 were pooled, divided in half, and lyophilized. One portion was treated with HNO_2 for 2 h and the other with chondroitin ABC lyase for 24 h, both at room temperature. The reaction mixtures were lyophilized, suspended in 0.1 M ammonium acetate in 20% (vol/vol) ethanol, and rechromatographed on Sephadex G-50 (*b*). ○, products of nitrous acid degradation; ●, products of chondroitin ABC lyase digestion.

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 HNO_2 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. 4*b*, 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. 2*b*). The material sedimenting 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 [^{35}S]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 K_{av} 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 $(NH_4)_2$ SO_4

The $(NH_4)_2SO_4$ precipitate was solubilized in 4 M guanidine hydrochloride and chromatographed on Sepharose CL-4B (Fig. 6). The major [^{35}S]sulphate-labeled component, accounting for over 50% of the radioactivity, was a broad peak with a K_{av} of 0.15 (0.11–0.19) (Fig. 6, *MI*). After treatment with pronase the [^{35}S]sulphate was largely (>80%) precipitable with CTAB and was eluted from Sepharose CL-4B with a K_{av} 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. 8*a*). The glycosaminoglycan portion of MI was analyzed by treatment with HNO_2 and chondroitin ABC lyase and found to be >95% heparan sulphate (Fig. 9).

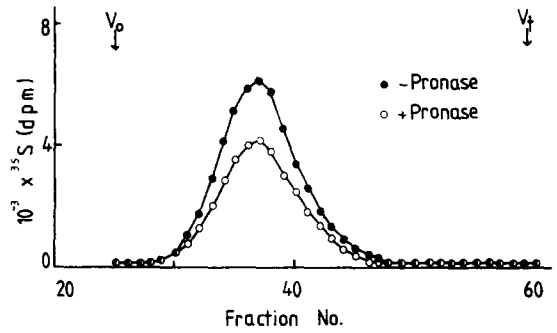

FIGURE 5 Gel filtration chromatography on Sephacryl S-300 of ^{35}S-glycosaminoglycan from CII. CII was isolated by gel filtration chromatography on Sepharose CL-4B. After density gradient centrifugation under dissociative conditions, the bottom four fractions (buoyant density >1.60 g/ml) were pooled and split into two portions. One was chromatographed directly on Sephacryl S-300 in 4 M guanidine hydrochloride buffer (●). The other was dialyzed to remove guanidine hydrochloride, digested with an excess of pronase at 60°C for 18 h, and then chromatographed on Sephacryl S-300 (○). The void volume (V_o) of the column was 27 ml and the total volume (V_t) was 65 ml.

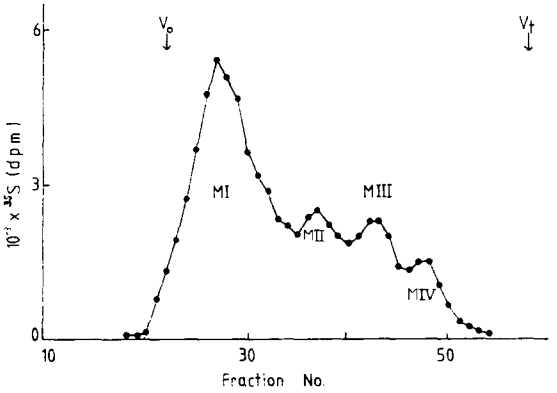

FIGURE 6 Gel filtration chromatography of sulphated macromolecules precipitated from the culture medium by $(NH_4)_2SO_4$ at 30% saturation. Lens cell cultures were incubated with $Na_2{}^{35}SO_4$ (500 μCi/ml) for 24 h at 37°C. The medium was removed, cooled to 4°C, and spun to remove cellular debris. Benzamidine hydrochloride (5 mM), 6-aminohexanoic acid (0.1 M), and EDTA (10 mM) were added to the concentrations indicated and $(NH_4)_2SO_4$ (176 mg/ml) was added slowly, with stirring. The resulting precipitate was collected by centrifugation at 80,000 *g* for 1 h at 4°C, resuspended in 4 M guanidine hydrochloride/50 mM sodium acetate, pH 5.8 containing the above inhibitors and dialyzed exhaustively against the same buffer at 4°C. The suspension was clarified by filtration and an aliquot chromatographed on a column of Sepharose CL-4B (1.5 × 90 cm) in 4 M guanidine hydrochloride buffer at 4°C. The void volume (V_o) was 57 ml and the total volume (V_t) was 151 ml. For reference purposes the peaks of radioactivity have been designated *MI–MIV*.

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 (K_{av} 0.41) and MIV (K_{av} 0.69) were present from preparation to preparation and were not characterized further; however, MIII (K_{av} 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 $(NH_4)_2$ SO_4

This fraction, comprising 20–50% of the total sulphated macromolecules 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 Sepharose CL-4B in the same buffer (Fig. 10). Approximately 10% (5.7–15.1%) of the [^{35}S]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 $(NH_4)_2SO_4$ to precipitate it were not due to the presence of lipid.

Most of the radioactivity in the $(NH_4)_2SO_4$ supernatant of

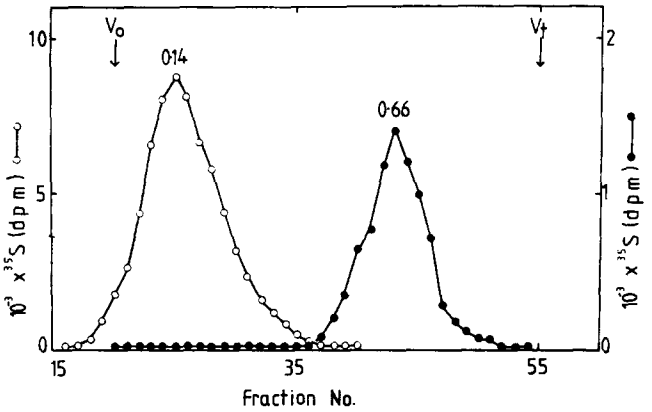

FIGURE 7 Sepharose CL-4B chromatography of component MI before and after pronase digestion. Component MI was purified by rechromatography on Sepharose CL-4B (○), dialyzed exhaustively against distilled water, and lyophilized. After treatment with pronase as described in the text, the digest (●) was reapplied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

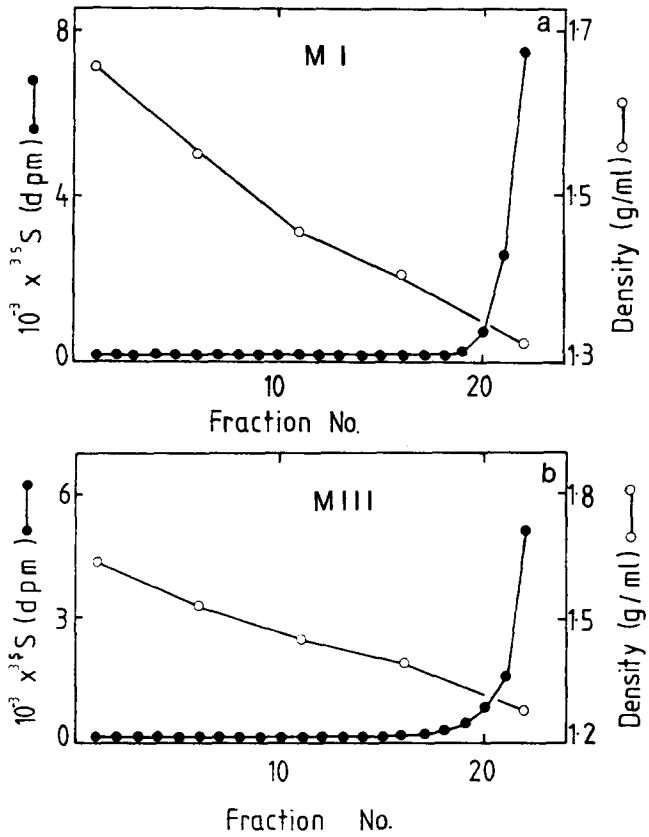

FIGURE 8 Cesium chloride density gradient centrifugation of the major sulphated components in 30% saturated $(NH_4)_2SO_4$ precipitate of lens epithelial cell medium. Components MI and MIII from Fig. 6 were rechromatographed on Sepharose CL-4B. To each was added solid CsCl (0.55 g/g) before centrifugation 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 (○).

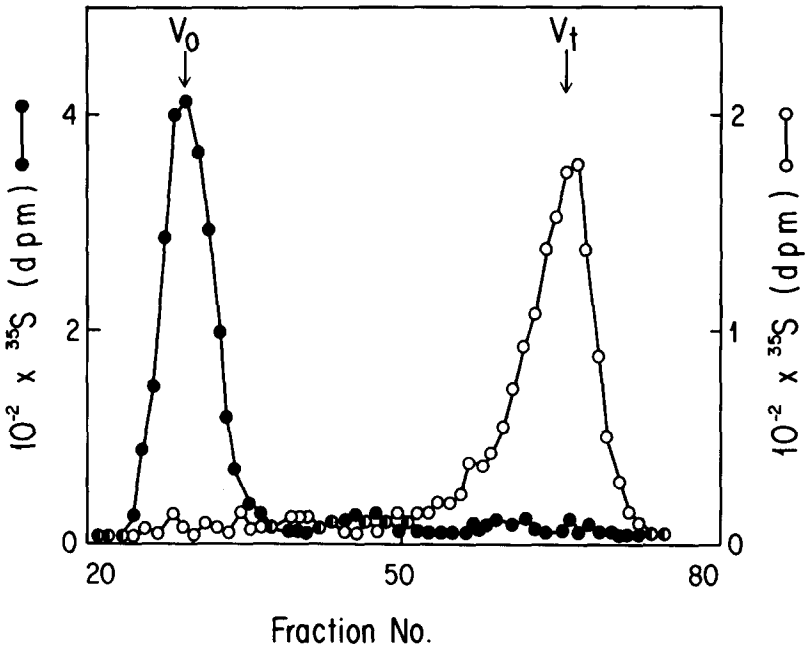

FIGURE 9 Chromatographic analysis of ^{35}S-glycosaminoglycans from MI on Sephadex G-50. The glycosaminoglycan component of MI (Fig. 7, K_{av} = 0.66) was dialyzed exhaustively against water to remove 4 M guanidine hydrochloride, split into two, and lyophilized. One portion was treated with nitrous acid (○) 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 (V_o) of the column was 28 ml and the total volume (V_t) was 63 ml.

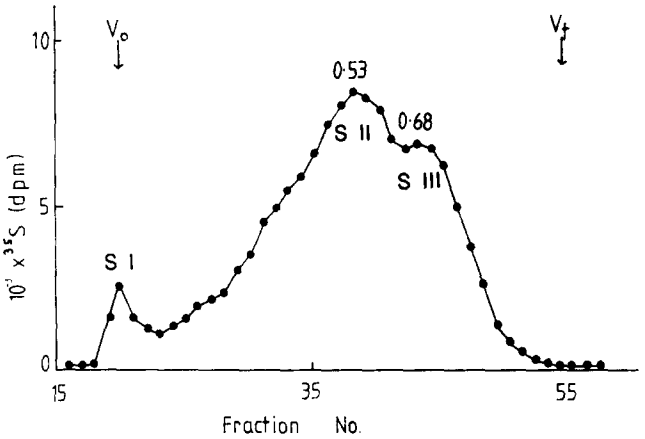

FIGURE 10 Gel filtration chromatography of sulphated macromolecules in lens cell medium that are not precipitated by 30% saturated $(NH_4)_2SO_4$. The experimental conditions were as described in Fig. 6. After centrifugation of the 30% saturated $(NH_4)_2SO_4$ precipitate the supernatant was dialyzed exhaustively against cold water to remove all unincorporated isotope and then lyophilized. The lyophilized material was redissolved in 4 M guanidine hydrochloride and an aliquot was chromatographed on Sepharose CL-4B in the same buffer. The void volume (V_o) was 55 ml and the total volume (V_t) was 149 ml. For reference purposes the peaks of radioactivity have been designated *SI–SIII*.

the medium was present in two barely separated components of K_{av} 0.53 (range 0.51–0.53) and 0.66 (range 0.61–0.70), designated *SII* and *SIII*, respectively (Fig. 10). Although components SI–SIII were not individually analyzed by CsCl density gradient centrifugation, samples of the unfractionated $(NH_4)_2SO_4$ 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 SIII 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 components 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 described in basement membranes of developing murine salivary gland (38), cultured murine mammary epithelium (39), and rat glomerulus (40). In addition, chondroitin sulphate appears to be present in the rat glomerular basement membrane (19). Early reports concentrated on the characterization of these glycosaminoglycans but more recently the proteoglycans from which they are derived have been investigated (19–22, 24, 41). At present no published reports exist on the sulphated proteoglycan(s) present in lens capsule. In addition to the heparan sulphate proteoglycan, three glycoproteins of high molecular weight have been identified in basement membranes: fibronectin, laminin, and entactin (see references 11 and 12) and the last two may be sulphated (9, 42–44). The content and distribution of these glycoconjugates in basement membranes may depend upon the tissue and species of origin and in general, little is known of their functions. However, 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 components 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 sulphated 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 (*SI*, Fig. 10). Although these cell-associated and secreted components may not be identical, they share certain characteristics. For example, fractions CI and SI eluted in the void volume of both Sepharose CL-4B and CL-2B (Figs. 1 and 10) indicating a molecular weight greater than 1×10^6. Despite its large size, however, SI was not precipitated from the culture medium by 30% saturated $(NH_4)_2SO_4$. Biosynthesis of high molecular weight lipid-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 CI was not possible due to the limited quantities available, density gradient centrifugation in 4 M guanidine hydrochloride failed to dissociate CI into smaller components (Fig. 2*a*). 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 sulphated 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 (*MI*) synthesized by the lens epithelial cells was secreted into the culture medium from which it could be recovered by precipitation with 30% $(NH_4)_2SO_4$ (Fig. 6). The results indicate that MI consists almost exclusively of a heparan sulphate proteoglycan, although when centrifuged on a CsCl density gradient

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} ~0.15 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} ~0.45; 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 MIII, 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 (>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 (~60%) of the sulphated macromolecules were cell-associated (Table I, exp. 1) and in the other ~60% was present in the medium (Table I, exp. 2). In the first of these, at least 98% of the cell-associated [^{35}S]-sulphate was present in CII, and in the medium, equivalent amounts were found in the $(NH_4)_2SO_4$ precipitate and supernatant fractions. However, when the secreted macromolecules predominated, the levels of CI rose to ~10% of the total cell-associated [^{35}S]sulphate. In addition, the glycoproteins SII and SIII (not precipitated by $(NH_4)_2SO_4$) formed ~80% of the secreted [^{35}S]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 unexpected 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).

TABLE II

Principal Sulphated Macromolecules Synthesized by Rabbit Lens Epithelial Cells Cultured on a Plastic Substratum

Component	K_{av}*	Composition	Buoyant density‡
			(g/ml)
CI	0	Heparan sulphate/glycoprotein	1.31–1.37
CII	0.70	Glycosaminoglycan (78%) (72% heparan sulphate)	1.60–1.68
		Glycoprotein	1.27–1.34
MI	0.15	Heparan sulphate proteoglycan	1.32
MII	0.43	Glycoprotein, glycosaminoglycan	ND§
MIII	0.54	Glycoprotein	1.29
MIV	0.69	Glycoprotein	ND
SI	0	Heparan sulphate/glycoprotein	ND
SII	0.53	Glycoprotein	ND
SIII	0.66	Glycoprotein	ND

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

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