Nature of sulphated macromolecules in mouse Reichert's membrane

Evidence for tyrosine *O*-sulphate in basement-membrane proteins

Mats PAULSSON,*† Marie DZIADEK,* Christian SUCHANEK,† Wieland B. HUTTNER† and Rupert TIMPL*

*Max-Planck-Institut für Biochemie and †Max-Planck-Institut für Psychiatrie, D-8033 Martinsried bei München, Federal Republic of Germany

Seven different sulphated macromolecules were detected in 6 M-guanidinium chloride extracts of metabolically [³⁵S]sulphate-labelled mouse Reichert's membrane and were partially separated. Polypeptide bands of apparent *M_r* 50000, 150000 (tentatively identified as entactin) and 170000 contained essentially tyrosine *O*-sulphate as the labelled component. Most of the radioactive sulphate was incorporated into three different proteoglycans, which could be separated by chromatography and density-gradient centrifugation before and after enzymic degradation. Enzymic analysis of glycosaminoglycans and of protein cores by immunoassays identified these components as low-density and high-density forms of heparan sulphate proteoglycan and a high-density form of chondroitin sulphate or dermatan sulphate proteoglycan.

INTRODUCTION

Sulphated macromolecules are typical constituents of basement membranes. The major sulphated components are heparan sulphate proteoglycans, which are thought to control the semipermeability properties of basement membranes by acting as charge barriers (Farquhar, 1981). Although these proteoglycans have mainly been identified in biosynthetic studies in which only trace amounts of labelled components can be characterized, some structural analyses have been done on proteoglycans isolated from the EHS (Engelbreth-Holm-Swarm) tumour (Hassell *et al.*, 1980; Fujiwara *et al.*, 1984) and glomerular basement membranes (Kanwar *et al.*, 1981, 1984; Parthasarathy & Spiro, 1984). The data demonstrate that two forms of heparan sulphate proteoglycan exist, one being a protein-poor high-density form, and the other a protein-rich low-density form. Chondroitin sulphate has also been found in basement membranes, but appears to be a minor component (Kanwar & Farquhar, 1979; Parthasarathy & Spiro, 1982; Couchman *et al.*, 1984). In addition, several sulphated glycoproteins have been identified, including entactin (Carlin *et al.*, 1981, 1983; Hogan *et al.*, 1982*a,b*) and several other polypeptide bands, which could be separated by electrophoresis of radioactively labelled glomerular basement-membrane components (Cohen *et al.*, 1981) or endothelial cell cultures (Heifetz *et al.*, 1982; Heifetz & Allen, 1982). The acceptor sites of incorporated sulphate in these proteins have not been completely identified, but they include *N*-linked oligosaccharides (Heifetz *et al.*, 1982) as well as tunicamycin-resistant acceptors (Hogan *et al.*, 1982*a,b*). Recent studies demonstrated tyrosine *O*-sulphate, which is a frequent post-translational modification in secreted proteins (Huttner, 1982, 1984; Hille *et al.*, 1984), in the extracellular-matrix protein fibronectin (Paul & Hynes, 1984; Liu & Lipmann, 1985).

Reichert's membrane is an extraembryonic basement membrane in rodent embryos that is produced by parietal endoderm cells. It reaches a thickness of several micrometres by the 14th day of gestation in mouse and rat embryos, and, because it can be cleanly isolated, it is a useful system for the study of basement-membrane structure (Hogan *et al.*, 1984). Biosynthetic studies have involved the characterization of collagen type IV, laminin, entactin, fibronectin and proteoglycans (Hogan *et al.*, 1980, 1982*a*; Cooper *et al.*, 1981; Tryggvason *et al.*, 1980; Smith & Strickland, 1981; Clark & Kefalides, 1982; Amenta *et al.*, 1983; Dziadek *et al.*, 1985), and these components have been localized at the ultrastructural level by immuno electron microscopy (Semoff *et al.*, 1982; Amenta *et al.*, 1983; Inoue *et al.*, 1983). Studies on the incorporation of radioactive sulphate demonstrated the synthesis of proteoglycans and sulphated proteins by Reichert's membrane (Hogan *et al.*, 1982*a*; Clark & Kefalides, 1982), but the nature of these molecules and the nature of the sulphate linkages have not yet been comprehensively analysed.

In the present study we have used mouse Reichert's membrane as a convenient model system to study the sulphated constituents of basement membranes. We have identified three distinct proteoglycans and several proteins that contain predominantly tyrosine *O*-sulphate.

MATERIALS AND METHODS

Tissue cultures

Reichert's membranes were dissected from 120 C3H or Balb/c mouse embryos on the 14th day of gestation, and cultured in Dulbecco's modified Eagle's medium (Flow Laboratories, Bethesda, MD, U.S.A.) containing 1% (v/v) foetal-calf serum. Radioactive labelling with carrier-free H₂³⁵SO₄ (0.5 mCi/ml, 43 Ci/mg; New Eng-

† To whom correspondence should be addressed.

Fig. 1. Separation on DEAE-cellulose of a 6 M-guanidinium chloride extract of mouse EHS tumour mixed with a [^{35}S]sulphate-labelled extract of Reichert's membrane

The column (30 cm $\times$ 2.5 cm) was equilibrated in 7 M-urea/50 mM-Tris/HCl buffer, pH 8.6, and eluted with a linear gradient (1200 ml) of 0–0.6 M-NaCl; the arrow denotes the start of the gradient. The column effluent was continuously monitored for protein absorption (A_{280} , —) and collected into 11.5 ml fractions. Portions of individual fractions were used for measurement of radioactivity (0.1 ml; ●), for determination of uronic acids by the carbazole assay (0.5 ml; A_{530} , ○) and for electrophoretic analyses (Fig. 2). The horizontal bars numbered 1 to 4 indicate pools used for further studies. Recovery of radioactivity was greater than 85%.

land Nuclear, Dreieich, W. Germany) was done for 30 h, after which tissue was separated from the medium by centrifugation and used for extraction.

Extraction

The Reichert's-membrane preparation was suspended by a few strokes in a glass homogenizer and immediately mixed with 100 ml of ice-cold 6 M-guanidinium chloride/50 mM-Tris/HCl buffer, pH 7.4, containing as proteinase inhibitors 10 mM-EDTA, 2 mM-*N*-ethylmaleimide and 2 mM-phenylmethanesulphonyl fluoride. EHS tumour (1 g dry wt.), extensively pre-extracted with neutral salt solution and dilute acetic acid (Timpl *et al.*, 1983), was also added to the extraction mixture and used as a carrier. Extraction was for 20 h at 5 °C with continuous stirring, after which the insoluble residue was removed by centrifugation at 18 000 *g*. The supernatant was extensively dialysed against the buffer used for DEAE-cellulose chromatography (see below).

Chromatography

Ion-exchange chromatography was done on a column (30 cm $\times$ 2.5 cm) of DEAE-cellulose (Whatman DE-52; W. and R. Balston, Maidstone, Kent, U.K.) eluted with a linear gradient (1200 ml) of 0–0.6 M-NaCl in 7 M-urea/50 mM-Tris/HCl buffer, pH 8.6, containing as proteinase inhibitors 2.5 mM-EDTA, 0.5 mM-*N*-ethylmaleimide and 0.5 mM-phenylmethanesulphonyl fluoride. A column (19 cm $\times$ 2.5 cm) of CM-cellulose (Whatman CM-52) was eluted with a linear gradient (1000 ml) of 0–0.15 M-NaCl in 7 M-urea/50 mM-sodium acetate buffer, pH 4.8, containing proteinase inhibitors as above. Molecular-sieve chromatography was done on a column (130 cm $\times$ 1.5 cm) of Sephacryl S-400 (Pharmacia, Uppsala, Sweden) eluted with 6 M-guanidinium chloride/50 mM-Tris/HCl buffer, pH 7.4, containing proteinase inhibitors (see above) or on a column (68 cm $\times$ 1.0 cm) of

Sephadex G-100 (Pharmacia) eluted with 0.2 M-NaCl/50 mM-Tris/HCl buffer, pH 7.4.

CsCl-density-gradient centrifugation

Proteoglycan samples in the 6 M-guanidinium chloride/50 mM-Tris/HCl buffer, pH 7.4, were adjusted to a density of 1.48 g/ml by addition of solid CsCl. The samples were then centrifuged at 40 000 rev/min (140 000 *g* at r_{av} 7.37 cm) at 18 °C for 40 h in a Beckman 50 VTi rotor. Tubes were emptied by pumping from the bottom of the tube and the contents were separated into ten 4 ml fractions.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

Electrophoresis was done on slab gels with a linear gradient of 4–15% or 3–10% acrylamide in the buffer system of Laemmli (1970). Proteins were detected by staining with Coomassie Brilliant Blue R followed by fluorography (Bonner & Laskey, 1974). M_r standards were myosin (M_r 205 000), phosphorylase *b* (M_r 94 000), albumin (M_r 67 000), ovalbumin (M_r 43 000) and carbonic anhydrase (M_r 30 000). Protein samples were reduced by treatment with 2% (v/v) 2-mercaptoethanol before electrophoresis; proteoglycan samples were run under non-reducing conditions.

Glycosaminoglycan degradations

Glycosaminoglycans were released by treatment with 0.5 M-NaOH at room temperature for 48 h. Samples were neutralized by the addition of a small volume of acetic acid. Portions containing about 10 000 c.p.m. were taken for digestion with 5 munits of heparitinase (Seikagaku Kogyo Co., Tokyo, Japan) at 37 °C for 15 h in 0.1 M-potassium phosphate buffer, pH 7.0, or with 10 munits of chondroitinase ABC (Seikagaku Kogyo Co.) at 37 °C for 18 h in the same buffer. When samples

Fig. 2. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (reducing conditions) of individual fractions separated by DEAE-cellulose chromatography (Fig. 1)

The gel (4–15% polyacrylamide) was stained with Coomassie Brilliant Blue (a) for detection of EHS-tumour proteins and was subsequently subjected to fluorography (b) for the detection of [^{35}S]sulphate-labelled Reichert's-membrane components. M_r values of calibrating proteins and positions of A- and B-chains of laminin are indicated on the left-hand side. The numbered lanes correspond to 0.15 ml portions of the fractions shown in Fig. 1. Similar analyses were done with fractions 60 to 105 (results not shown) and revealed only little stained protein but strong radioactivity at the top of the separation gel. Arrows indicate the top and the bottom of the separation gel.

of intact proteoglycan were digested, 20 mM-benzamidine hydrochloride, 5 mM-*N*-ethylmaleimide and 5 mM-phenylmethanesulphonyl fluoride were added to the buffer to avoid degradation by contaminating proteinases.

Chemical analysis

Uronic acid contents of fractions were determined by the carbazole procedure of Bitter & Muir (1962). When needed, interfering solvents were first removed by precipitating protein and proteoglycans by addition of 9 vol. of ethanol followed by incubation at 5 °C overnight.

Determination of tyrosine *O*-[^{35}S]sulphate

The determination followed a previously reported procedure (Lee & Huttner, 1983; Huttner, 1984). [^{35}S]Sulphate-labelled protein bands were identified after sodium dodecyl sulphate/polyacrylamide-gel electrophoresis by fluorography with 1 M-sodium salicylate as scintillator (Chamberlain, 1979). The corresponding regions of the dried gel were cut out and proteins were eluted by Pronase digestion. The presence of tyrosine *O*-[^{35}S]sulphate residues was determined after alkaline hydrolysis by two-dimensional thin-layer electrophoresis followed by autoradiography, with unlabelled tyrosine *O*-sulphate as standard. Recoveries were determined by

Fig. 3. Fluorographic detection of sulphate-labelled proteoglycans (HD and LD) and proteins (1 and 2) purified from Reichert's membrane

Lane 1, mixture of 170000- M_r and 150000- M_r components; lane 2, semi-purified 50000- M_r component. Proteoglycans were the high-density variant (HD), which was also examined after treatment with chondroitinase ABC (HD, C) or heparitinase (HD, H), and the low-density variant (LD). LD, ^{125}I denotes a ^{125}I -labelled low-density proteoglycan purified from the EHS tumour (Dziadek *et al.*, 1985) that was used for comparison. Protein separations (reducing conditions) were carried out on 4–15% polyacrylamide gradient gel and electrophoresis of proteoglycans (non-reducing conditions) was done on a 3–10% polyacrylamide gradient gel.

subjecting tyrosine *O*-[^{35}S]sulphate to the same hydrolysis and electrophoresis procedure. In some experiments [^{35}S]sulphate-labelled proteins were analysed for tyrosine *O*-[^{35}S]sulphate without further separation on sodium dodecyl sulphate/polyacrylamide gels.

Immunological methods

Immunoprecipitation of purified proteoglycans (500–1000 c.p.m.) was carried out in the presence of phosphate-buffered saline (0.12 M-NaCl/12 mM-sodium phosphate/4 mM-potassium phosphate buffer, pH 7.2) containing 0.04% Tween 20 and 0.02% NaN_3 . Samples were incubated for 24 h at 4 °C with affinity-purified rabbit antibodies (30 μg) directed against the high-density form of heparan sulphate proteoglycan (Dziadek *et al.*, 1985). Equivalent amounts of normal rabbit IgG or normal rabbit serum were used in the negative controls. Immune complexes were precipitated by using Tachisorb (Hoechst A.G., Frankfurt, W. Germany) and dissociated with 1 M-NaCl/2% (w/v) sodium dodecyl sulphate for liquid-scintillation counting. In other experiments antibodies against the low-density proteoglycan from the EHS tumour were used, with very similar results. It has previously been demonstrated that both sera react primarily with determinants present on the low-density proteoglycan but also with a set of determinants present on both high-density and low-density proteoglycans (Dziadek *et al.*, 1985). Radioimmunoassays for high-

density and low-density forms of heparan sulphate proteoglycan obtained from the EHS tumour were those used previously (Dziadek *et al.*, 1985).

RESULTS

Extraction and separation of ^{35}S -labelled macromolecules from Reichert's membrane

Mouse Reichert's membranes were labelled with [^{35}S]sulphate and extracted in 6 M-guanidinium chloride together with a large excess of non-labelled matrix proteins obtained from the mouse EHS tumour. By this procedure the tumour proteins were used as carriers for the trace amounts of labelled material during subsequent purification. Since the tumour contains glycoproteins and proteoglycans similar to or identical with those in Reichert's membrane, some comparisons could also be made by identifying tumour components with chemical and radioimmuno-inhibition assays. The medium fraction of the cultures contained only about 10% of the total incorporated radioactivity and was therefore discarded.

Chromatography on DEAE-cellulose in 7 M-urea separated the labelled macromolecules into two major groups, pools 1 and 2 consisting of ^{35}S -labelled proteins, and pools 3 and 4 consisting of more highly charged proteoglycans (Fig. 1). A peak containing 3% of the total radioactivity (pool 1) was eluted rather early in the gradient (0.1 M-NaCl). Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis followed by fluorography (Fig. 2) identified a major labelled polypeptide with M_r 150000 in this pool, and a minor labelled component with M_r 170000. The 150000- M_r polypeptide corresponded to a distinct protein band from the tumour carrier. However, many other tumour-derived protein bands, including the laminin B-chains, contained no or only little radioactivity. A second minor peak of radioactivity (pool 2, 1% of total label) was eluted after pool 1 (Fig. 1) and contained a major labelled polypeptide band of M_r 50000 and some label in the position of laminin A-chains (Fig. 2). The major portion of the radioactivity recovered from DEAE-cellulose was in the proteoglycan fraction (pools 3 and 4), which is described below.

Purification and characterization of ^{35}S -labelled proteins

Pools 1 and 2 were further chromatographed on CM-cellulose followed by molecular-sieve chromatography on Sephacryl S-400 (results not shown). Proteins were eluted from the latter column in positions corresponding to the apparent M_r values determined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The final products contained a mixture of the labelled 150000- M_r and 170000- M_r bands in pool 1 (Fig. 3) and corresponding protein bands from the tumour. Pool 2 contained exclusively the labelled 50000- M_r band (Fig. 3). The radioactive band in the position of laminin A-chain was only poorly recovered and not further examined. Radioimmuno-inhibition assays demonstrated large amounts of the basement-membrane protein nidogen (Timpl *et al.*, 1983) in the pool 1 material but not in pool 2 material.

The [^{35}S]sulphate-labelled 170000- M_r , 150000- M_r and 50000- M_r bands (cf. Fig. 3) were cut out, extracted and subjected to alkaline hydrolysis, with $\text{Ba}(\text{OH})_2$, followed by thin-layer electrophoresis of the alkali-stable material (Table 1). Tyrosine *O*-sulphate could be identified as the

Table 1. Presence of tyrosine *O*-sulphate in three polypeptide bands obtained from sulphate-labelled Reichert's membrane

For experimental details see the text.

Material and amount (c.p.m.)	Recovery after alkaline hydrolysis (% †)	Recovery after thin-layer electrophoresis (% ‡)
[³⁵ S]Tyrosine <i>O</i> -sulphate (2473)	85	87
170000- <i>M_r</i> band (396)	96	85
150000- <i>M_r</i> band (2704)	88	89
50000- <i>M_r</i> band (16803)	79	83

* Standard, average of two analyses.

† % of starting material in alkali-stable form.

‡ % of alkali-stable material recovered in the spot corresponding to tyrosine *O*-sulphate. **Fig. 4. Molecular-sieve chromatography of [³⁵S]sulphate-labelled samples of (b) high-density (HD) and (a) low-density (LD) proteoglycan from Reichert's membrane**

The Sephacryl S-400 column (130 cm × 1.5 cm) was equilibrated in 6 M-guanidinium chloride/50 mM-Tris/HCl buffer, pH 7.4, and the column effluent was collected into 2.5 ml fractions. Starting materials were pools 3 (LD) and 4 (HD) after DEAE-cellulose chromatography. Horizontal bars indicate pools used for further purification. Arrows denote the void volume (V_0) and total volume (V_t) of the column.

major alkali-stable product from all three bands. Recovery estimates clearly demonstrated that virtually all of the protein-bound radioactivity was due to tyrosine *O*-sulphate (Table 1), which could be further substantiated by a two-dimensional separation of the tyrosine *O*-[³⁵S]sulphate obtained from pool 1 (cf. Fig. 3). The virtually exclusive linkage of [³⁵S]sulphate to tyrosine residues in these proteins was also supported by the

observation (results not shown) that almost all [³⁵S]sulphate was hydrolysed by a short acid treatment known to hydrolyse the tyrosine *O*-sulphate ester (Lee & Huttner, 1983; Huttner, 1984).

Purification of proteoglycans

[³⁵S]Sulphate-labelled proteoglycans were separated into two main fractions by DEAE-cellulose chromatography (Fig. 1). Pool 3 contained 14% of the radioactivity and most of the carbazole-positive material and coincided in elution position with the low-density heparan sulphate proteoglycan from the EHS tumour. The second, more acidic, pool 4 contained most of the radioactivity (68%) but was low in carbazole reaction. The two pools were separately passed over Sephacryl S-400 and showed quite different profiles (Fig. 4). The major component of pool 3 was eluted close to the void volume of the column and was found to be entirely a low-density proteoglycan by CsCl-density-gradient centrifugation (Fig. 5). A few further peaks were eluted after the low-density proteoglycan from the Sephacryl S-400 column. They showed a broad uncharacteristic profile in CsCl density gradients and were not further examined.

About 90% of the radioactivity of pool 4 was eluted from Sephacryl S-400 in a distinct peak at a position indicating a smaller M_r than for the low-density proteoglycan (Fig. 4). It contained almost entirely proteoglycans of high buoyant density (Fig. 5). As shown below, this material was composed of at least two different proteoglycan species.

Characterization of the proteoglycans

Both purified proteoglycans showed broad bands after sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Fig. 3). The higher average mobility of the high-density form compared with the low-density form was compatible with their behaviour on a molecular-sieve column (Fig. 4). They were co-purified with the high-density and low-density heparan sulphate proteoglycan respectively from the mouse tumour (Fujiwara *et al.*, 1984) as demonstrated by radioimmuno-inhibition assays that are specific for the protein cores (results not shown; Dziadek *et al.*, 1985). Their correspondence to these proteoglycans was also demonstrated by direct immunoprecipitation with antisera raised against tumour proteoglycan (Table 2).

The nature of the glycosaminoglycans in the [³⁵S]-

Fig. 5. Final purification of (b) the high-density (HD) and (a) low-density (LD) proteoglycans obtained from [35 S]-sulphate-labelled Reichert's membrane by CsCl-density-gradient centrifugation

Starting materials were the respective pools after molecular-sieve chromatography (Fig. 4). ○, Density; ●, radioactivity. Horizontal bars indicate the pooled fractions (each 4 ml) used for further characterization.

sulphate-labelled proteoglycans was determined by alkaline elimination followed by enzymic degradation of the resulting side chains. The side chains of the low-density proteoglycan were resistant to digestion with chondroitinase ABC, whereas treatment with heparitinase gave quantitative degradation to fragments that were eluted late on a Sephadex G-100 column (Fig. 6), indicating these chains to be heparan sulphate. The digestion patterns obtained for the high-density proteoglycan were more complex, with portions of the glycosaminoglycans

being degraded by chondroitinase ABC as well as heparitinase (Fig. 6). This preparation was thus a mixture containing side chains both of heparan sulphate and chondroitin sulphate or dermatan sulphate.

To determine if the two types of glycosaminoglycans are present on the same or on different core proteins, intact high-density proteoglycan was digested with the two enzymes and the products were compared by electrophoresis (Fig. 3). Heparitinase degraded the upper portion and chondroitinase ABC the lower portion of the broad band of the high-density proteoglycan, indicating the presence of different macromolecules. They were then purified from appropriate digests by chromatography on the Sephacryl S-400 column. It was found that about two-thirds of the material was resistant to chondroitinase ABC (Fig. 7a) and was eluted in the same position as the original sample (cf. Fig. 4b). The resistant material therefore apparently represented a proteoglycan with only heparan sulphate side chains. After digestion with heparitinase about one-third of the sample remained as high- M_r material, which was eluted slightly later than the bulk of the starting material (Fig. 7b). As care had been taken to avoid proteolysis during digestion, this was interpreted as being a pure chondroitin sulphate or dermatan sulphate proteoglycan of somewhat smaller size than the heparan sulphate proteoglycan. By immunoprecipitation of the purified heparan sulphate and chondroitin sulphate/dermatan sulphate proteoglycans it was found that both reacted to approximately the same extent with antibodies against the tumour heparan sulphate proteoglycan (Table 2). The chondroitin sulphate/dermatan sulphate proteoglycan may therefore have a protein core that is structurally similar to that of the heparan sulphate proteoglycan.

DISCUSSION

Reichert's membrane produces seven major sulphated components, which together account for more than 90% of the incorporated [35 S]sulphate. These include polypeptide bands of M_r 170 000, 150 000, 50 000 and 440 000 (presumably laminin A-chains), which could be distinguished from typical proteoglycans by their behaviour on DEAE-cellulose and by other criteria. Determination of tyrosine *O*-sulphate in the 170 000- M_r , 150 000- M_r and 50 000- M_r bands showed the virtually exclusive linkage of labelled sulphate to tyrosine. The amount of label in the laminin A-chain was too low to allow similar determina-

Table 2. Immunoprecipitation of heparan sulphate proteoglycans (HS-PG) and chondroitin sulphate proteoglycan (CS-PG) from sulphate-labelled Reichert's membrane with antibodies against EHS-tumour-derived heparan sulphate proteoglycans

For experimental details see the text.

Material and amount (c.p.m.)	Radioactivity precipitated (%)	
	With anti-HS-PG antibodies*	With control IgG†
Low-density HS-PG (500)	65	19
High-density HS-PG (700)	36	13
High-density CS-PG (1000)	25	8

* 30 μ g of affinity-purified antibodies to high-density HS-PG.

† 38 μ g of control rabbit IgG.

Fig. 6. Nature of the glycosaminoglycan chains released from (b) high-density (HD) and (a) low-density (LD) [^{35}S]sulphate-labelled proteoglycans of Reichert's membrane

The samples were chromatographed on a Sephadex G-100 column (68 cm $\times$ 1 cm) after alkali release (control) and after a second degradation with either heparitinase or chondroitinase ABC. The arrows denote the void volume (V_0) and total volume (V_t) of the column. Fraction volume was 0.8 ml.

Fig. 7. Separation of heparan sulphate proteoglycan (HS-PG) and chondroitin sulphate proteoglycan (CS-PG) after degradation of two different samples of high-density proteoglycan with either chondroitinase ABC (a) or with heparitinase (b)

High-density proteoglycan was purified by CsCl-density-gradient centrifugation (Fig. 5) and after degradation separated on Sephacryl S-400 as described in Fig. 4. Horizontal bars indicate the pools used for immunoprecipitation. Arrows denote the void volume (V_0) and total volume (V_t) of the column.

tions to be made. Sulphation of tyrosine is a widespread post-translational modification of proteins (Huttner, 1982). The functional role of tyrosine sulphation is unknown, but, in searching for its role, three observations seem significant. First, tyrosine sulphate has so far only been found in secretory proteins (Huttner, 1984; Hille *et al.*, 1984). Secondly, the tyrosine-sulphated proteins do not share an obvious property other than being secreted. Thirdly, it is clear that not all secretory proteins are sulphated. It has therefore been suggested that tyrosine sulphation is one, but not the only, post-translational modification that may control the intracellular traffic of secretory proteins (Baeuerle & Huttner, 1984). This suggestion is consistent with the present results, where only certain basement-membrane proteins are sulphated on tyrosine though all of them are secreted. Tyrosine *O*-sulphate has also been demonstrated in another extracellular-matrix protein, fibronectin (Paul & Hynes, 1984; Liu & Lipmann, 1985), which is occasionally found in basement membranes. The presence of tyrosine *O*-sulphate appears to be restricted to certain splicing variants of the fibronectin chains (Paul & Hynes, 1984).

Sulphated proteins similar to those found in our study have been previously described in Reichert's membrane (Hogan *et al.*, 1982a). These proteins were not affected by tunicamycin, but were sensitive to HNO_2 , a treatment commonly used to identify heparan sulphate (Cifonelli & King, 1972; Shively & Conrad, 1976). Since tyrosine *O*-sulphate is very labile in acid hydrolysis conditions (Huttner, 1984), it may explain the reported sensitivity to HNO_2 . These data emphasize heparitinase treatment as a more reliable method for the identification of heparan sulphate structures.

The nature of the polypeptides in Reichert's membrane that contain tyrosine *O*-sulphate is not clear. A sulphated 150000 M_r band was originally identified in teratocarcinoma cell cultures and named entactin (Carlin *et al.*, 1981). Similar components have been found in various other cell and organ cultures (Hogan *et al.*, 1980, 1982a,b; Cooper *et al.*, 1981). Thus the 150000- M_r band of Reichert's membrane can be identified as entactin. The semi-purified product also contained substantial amounts of nidogen (Timpl *et al.*, 1983), which in its genuine form has M_r

150000 (Dziadek & Timpl, 1985). The co-chromatography of entactin and nidogen found in the present study and the immunological relationship between both components indicate that they are related proteins. However, some differences in fragment patterns have been observed (M. Dziadek, M. Paulsson & R. Timpl, unpublished work), suggesting that they are not identical. It is possible that post-translational variants exist, and further work is required to understand the precise relationship between the two proteins. The nature of the 170000- M_r and 50000- M_r bands and their possible relationship to entactin also remains to be clarified.

A better characterization exists for basement-membrane types of heparan sulphate proteoglycan, which in the EHS tumour were found as high- (M_r 130000) and low-density (M_r 450000) variants (Fujiwara *et al.*, 1984). This high-density form consists of a small protein core (M_r 5000–10000) and four heparan sulphate chains (M_r 29000); the structure of the low-density form (about 70% protein) has not yet been fully investigated. Reichert's membrane apparently contains very similar variants, as shown by their behaviour during purification, their immunological cross-reactivity and nature of the glycosaminoglycan side chains. Since recent data have shown large aberrations in values obtained for the M_r values of proteoglycans and heparan sulphate chains when determined by chromatography (Hassell *et al.*, 1980; Fujiwara *et al.*, 1984), we have avoided such calculations. However, several indirect data indicate that, as expected, the labelled high-density variant is smaller than the low-density variant.

High-density and low-density heparan sulphate proteoglycans have also been found in PYS-2 cell cultures (Tyree *et al.*, 1984), and probably exist in glomerular basement membranes (Kanwar *et al.*, 1981; Parthasarathy & Spiro, 1984). The ubiquitous occurrence of the low-density form in basement membranes was demonstrated by radioimmunoassay and other immunological assays (Dziadek *et al.*, 1985). Both the high-density and the low-density variants show some immunological cross-reactivity, but this does not necessarily indicate a close structural relationship. Pulse-chase-labelling data, however, were interpreted as indicating that the high-density form is a physiologically released fragment from the low-density form (Martin *et al.*, 1984).

The presence of chondroitin sulphate proteoglycans in basement membranes was indicated by glycosaminoglycan analyses (Kanwar & Farquhar, 1979; Parthasarathy & Spiro, 1982), by preliminary data from the EHS tumour (Hassell *et al.*, 1980; Fujiwara *et al.*, 1984) and by immunohistology with monoclonal antibodies specific for chondroitin 6-sulphate (Couchman *et al.*, 1984). Our data demonstrate production of a chondroitin sulphate proteoglycan by Reichert's membrane that is smaller in size than the high-density heparan sulphate proteoglycan. Separation of these proteoglycans into two distinct size classes indicated that chondroitin sulphate and heparin sulphate chains are most probably attached to different protein cores, as was also demonstrated for proteoglycans from the glomerular basement membrane (Kanwar *et al.*, 1981). However, cross-reaction of chondroitin sulphate proteoglycan with antibodies against heparan sulphate proteoglycan in the present study and a previous study on PYS-2 cells (Tyree *et al.*, 1984) indicates some structural similarity between the two protein cores. Rat yolk-sac carcinomas produce chondroitin sulphate

proteoglycan as the major proteoglycan component (Oldberg *et al.*, 1981), in contrast with mouse Reichert's membrane and PYS-2 cells, which produce mainly heparan sulphate proteoglycan (Hogan *et al.*, 1982a; Oohira *et al.*, 1982; Tyree *et al.*, 1984). The rat yolk-sac proteoglycan is mainly localized to the cell surface (Oldberg *et al.*, 1981), and antibodies to this proteoglycan give a completely different staining pattern from that obtained with antibodies against the EHS-tumour heparan sulphate proteoglycan on cultures of kidney cells (Hayman *et al.*, 1982). A substantial amount of chondroitin sulphate proteoglycan has also been detected in PYS-2 cultures (Höök *et al.*, 1984). Retinoic acid-stimulated F9 cells produce predominantly a chondroitin sulphate proteoglycan (Kapoor & Prehm, 1983).

The differences in the patterns of proteoglycan synthesis by these various embryonic and teratocarcinoma parietal endoderm cells may indicate that these cell types are not all equivalent to each other, and may reflect some degree of modification of synthetic profiles *in vitro*. Although the proteoglycans in these different cell systems appear to have related protein cores, as determined by immunological methods (Dziadek *et al.*, 1985), the precise structural relationships between the various heparan sulphate and chondroitin sulphate proteoglycans still needs to be established.

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