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Endothelial Heparan Sulphate: Compositional Analysis and Comparison of Chains from Different Proteoglycan Populations

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From cultures of human umbilical vein endothelial cells incubated with ^{3}H-glucosamine or ^{35}S-sulphate, we have purified three heparan sulphate proteoglycans: 1) a low density (1.31 g/ml) proteoglycan from the cell extract, 2) a low density proteoglycan from the medium, and 3) a high density (>1.4 g/ml) proteoglycan from the medium. The disaccharide composition of heparan sulphate chains from the low density proteoglycan of the medium was examined, using specific chemical and enzymic degradations followed by gel chromatography and strong anion exchange HPLC. Chains released from each of the different proteoglycan populations were then compared by gel chromatography and gradient polyacrylamide gel electrophoresis before and after various specific degradations. The results indicate that heparan sulphate from human endothelial cells are large polymers (MW >50,000) of low overall sulphation (32-35% *N*-sulphated glucosamine and an N/O-linked sulphate ratio of 2.0) with rare and solitary heparin-like disaccharides. Heparan sulphate from the different proteoglycan populations appeared to have similar structure except that chains from the high density fraction were larger polymers.

Heparan sulphate proteoglycans (HSPG) consist of a protein core to which polyanionic polysaccharide chains are covalently attached. Several different core proteins have been described, with M_r ranging from 35 kDa [1] to approximately 400 kDa [2]. The proteoglycans

Abbreviations: HSPG, heparan sulphate proteoglycan; DSPG, dermatan sulphate proteoglycan; GlcNAc(6S), *N*-acetylglucosamine 6-sulphate; GlcNAc6R, glucosamine with either -OH or -OSO_3 at C-6; GlcNR, glucosamine with either -SO_3 or -$COCH_3$ as N-substituent; $GlcNSO_3$, *N*-sulphated glucosamine; $GlcNSO_3$(3S), *N*-sulphated glucosamine 3-sulphate; GlcA, D-glucuronic acid; IdoA, L-iduronic acid; IdoA(2S), iduronic acid 2-sulphate; HexA, hexuronic acid; DHexA, hexuronic acid with a 4,5-double bond; Xyl, xylose; SAX, strong anion exchange; d.p., degree of polymerization (a disaccharide has d.p.=1 etc); AUFS, absorbance units full scale.

The codes used for proteoglycans denote in turn : C 2, low-density (1.35-1.28 g/ml) HSPG from the cell extract; M 1a, high density (>1.4 g/ml) HSPG fraction from the spent medium; M 2a, low-density (1.31 g/ml) HSPG from the spent medium [6].

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are present in most tissues and located either in the extracellular matrix/basement membrane or in the plasma membrane. The polysaccharide chains consist of alternating uronic acid and glucosamine residues, the latter being either *N*-acetylated or *N*-sulphated. Heparan sulphate is structurally related to heparin, which has the same carbohydrate structure but is more sulphated and is synthesized exclusively by mast cells. The chains are linked to serine residues in the core protein *via* a specific fragment identified as GlcA-Gal-Gal-Xyl-Ser [3].

Both heparin and heparan sulphate chains are initially formed as polymers of GlcAβ1-4GlcNAcα1-4 [4]. Specific glycosyltransferases in the Golgi apparatus catalyse the addition of the constituent monosaccharides from UDP-sugars to the non-reducing end of the growing chain. Subsequently, the polymer undergoes a series of modification reactions starting with *N*-deacetylation and *N*-sulphation of *N*-acetylated glucosamines, followed by 5'-epimerization of glucuronate residues (to iduronic acid) and formation of ester-linked sulphate groups at various sites (on C-6 or C-3 of glucosamine and on C-2 of the uronic acid). The different modifying enzymes recognize structural features formed by preceding modifications [4]. For example, 5'-epimerization of D-glucuronic acid to form L-iduronic acid requires the presence of an *N*-sulphated glucosamine linked to C-4 of the uronic acid. The reactions are not 'complete', i.e. not all potential sites in a chain are modified, and especially not in heparan sulphate. In contrast to heparin, heparan sulphate has about 50% *N*-acetylated glucosamine residues and a higher number of *N*-sulphamido groups than ester-linked sulphate. Thus, in heparan sulphate segments of the native, unsulphated form of the polysaccharide alternate with domains that are more modified. This segregation of charge density within the chains is characteristic of heparan sulphate [5] and leaves room for structural variation.

Earlier studies have indicated that heparan sulphate from different cells and tissues display different functional and structural properties (see [5] and references cited therein). However, most of these studies have dealt with heparan sulphate isolated by proteolytic digestion of tissues containing an unknown variety of HSPG's, perhaps carrying distinctively different chains. Human endothelial cells synthesize two major types of proteoglycans [6], a large (M_r 600-800 kDa) HSPG and a smaller DSPG (M_r 100-200 kDa). The HSPG has a relatively low buoyant density (1.31 g/ml in CsCl/4 M guanidinium chloride) and a protein core with M_r 350 kDa. It is the major proteoglycan of the cell layer (C 2), but is also secreted into the medium (M 2a), where an additional core protein of M_r 250 kDa is seen, perhaps due to proteolytic processing. In the medium a small amount of HSPG is also found in a high density fraction (M 1a) and with a protein core similar to that of the low density HSPG in the medium. The higher density of this proteoglycan fraction may therefore be caused by a different polysaccharide content.

Here, we have examined the heparan sulphate chains from each of these proteoglycans. The sizes of the polysaccharides were estimated by gel chromatography. Chains from the low density HSPG of the medium were degraded using specific enzymic and chemical methods. From analyses of the degradation products by gel chromatography and strong anion exchange HPLC, the approximate disaccharide composition of the chains was inferred. Chains from the three different proteoglycan populations were then degraded by the same methods and the oligosaccharide patterns compared by polyacrylamide gel electrophoresis.

Materials and Methods

Materials

Human umbilical veins were collected in sterile buffer immediately after delivery, and endothelial cells were prepared by collagenase digestion and cultured on fibronectin-coated dishes as described by Lindblom *et al.* [6]. Cells from the first two passages (split ratio 1:3) were used in this study. Serum from healthy donors was purchased from the Blood Bank, Malmö General Hospital. Oligosaccharide standards were non-sulphated (DHexA1-4GlcNAc) and monosulphated (DHexA1-4GlcNR6R) disaccharide obtained by heparitinase digestion of a low sulphated heparan sulphate (bovine lung), and trisulphated disaccharide [DHexA(2S)1-4GlcNSO$_3$(6S)] obtained by heparinase digestion of heparin. The standards were purified by gel chromatography on Bio-Gel P-2.

The following materials were obtained from the sources indicated: $Na_2{}^{35}SO_4$ (25-40 Ci/mg), D-[6-^{3}H]glucosamine hydrochloride (20-40 Ci/mmol), Amplify™ spray and Hyperfilm™ MP from The Radiochemical Centre, Amersham, UK; acrylamide and *N,N'*-methylene bisacrylamide (2x) from Serva, Heidelberg, West Germany; Sepharose CL-6B from Pharmacia-LKB, Uppsala, Sweden; a semi-dry electroblotter B from Ancos A/S, Vig, Denmark; Bio-Gel P-2 and P-6, and Zetaprobe nylon from Bio-Rad, Richmond, CA, USA; chromatography paper 3 MM from Whatman, Maidstone, UK; heparitinase (heparan sulphate lyase, EC 4.2.2.8), heparinase (heparin lyase, EC 4.2.2.7), chondroitinase ABC (chondroitin ABC lyase, EC 4.2.2.4) from Seikagaku Kogyo Inc., Tokyo, Japan; heparinase II (heparin lyase II from *Flavobacterium heparinum*) and Pronase (EC 3.4.24.4; type VI) from Sigma, St. Louis, MO, USA; dialysis membranes Spectrapor 1 (m.w.c.o. 6000-8000) and Spectrapor 3 (m.w.c.o. 3500) from Spectrum, Los Angeles, CA, USA. Other materials were from sources listed previously [6].

Isolation of Proteoglycans and Polysaccharides

Heparan sulphate proteoglycans, metabolically labelled with either ^{3}H-glucosamine or $^{35}SO_4^{2-}$, were purified by isopycnic density-gradient centrifugation, gel chromatography and ion-exchange FPLC as earlier described [6]. Heparan sulphate chains were released by treating the purified proteoglycans with 0.5 M NaOH, 0.1 M $NaBH_4$ at 20°C overnight. After neutralization with 0.1 M acetic acid and dilution (1:10) in 6 M urea, 0.1% (by vol) Triton X-100, 10 mM Tris-HCl buffer, pH 8.0, the samples were applied on columns of DEAE-cellulose DE-53 (0.5 ml) equilibrated in the same buffer. The columns were washed with equilibrating buffer, followed by 5 ml 6 M urea, 0.1% (v/v) Triton X-100, 0.2 M sodium acetate buffer, pH 5.8 and 5 ml 6 M urea/0.1% Triton X-100, 0.5 M sodium acetate buffer, pH 5.8. The heparan sulphate was finally eluted with 5 x 0.25 ml 4 M guanidinium chloride, 50 mM sodium acetate buffer, pH 5.8, containing 5 mg/ml ovalbumin, supplemented with 200 µg heparan sulphate (bovine lung) and dialyzed against 2 x 1000 vol. of water. Finally, aliquots were evaporated, *in vacuo*, in Eppendorf tubes (1 ml), using a Speed-Vac centrifuge (Heto, Denmark), and stored at -60°C.

For quantitative experiments purified heparan sulphate from the low density HSPG of the medium (containing approx. 200 µg carrier) was treated with 10 mIU chondroitinase ABC in 10 mM Tris-acetate buffer, pH 7.0 for 2 h at 37°C, heated for 1 min (100°C), and further

treated with 10 μg Pronase for 2 h at 37°C. After treatment, the heparan sulphate was recovered by stepwise elution from DEAE-cellulose, dialysed against water and freeze-dried (as above).

Degradative Methods

For compositional analysis, lyophilized aliquots of isolated heparan sulphate from the low density HSPG of the medium (containing 10-50 μg heparan sulphate carrier) were redissolved in 100 μl 3 mM calcium acetate, 0.1% Triton X-100, 10 mM Hepes-NaOH buffer, pH 7.0 and digested with heparitinase (2 mIU) for 16 h at 43°C, or with heparinase (1 mIU) for 16 h at 30°C. Digestions were terminated by heating for 1 min in a boiling water bath. The amounts of heparitinase and heparinase used was primarily based on those required for complete digestion of standard heparan sulphate (heparitinase) and heparin (heparinase) preparations, as measured by absorbance at 232 nm. To ascertain complete digestion of the radioactive samples equivalent and higher amounts of enzyme were then tested and the effect was monitored by gel chromatography. For example, with heparitinase a slight increase in number of cleaved linkages (from 74 to approx. 77%) was obtained when the amount of enzyme was increased from 1 to 2 mIU (further amounts had no significant effect). For oligosaccharide mapping of chains from the different HSPG's, aliquots containing the same amount of ^{3}H dpm and heparan sulphate carrier (10 μg) were dissolved in 50 μl 3 mM $CaAc_2$, 0.1% Triton X-100, 10 mM Hepes-NaOH buffer, pH 7.0, and digested for 2 h at 43°C with 0.5 mIU of heparitinase or for 2 h at 30°C with 0.5 mIU heparinase. All experiments were repeated at least twice.

For combined digestion the sample was dissolved in a total of 100 μl 3 mM calcium acetate, 10 mM Hepes-NaOH buffer, pH 7.0. Enzymes were then added (1 mIU each of heparitinase, heparinase and heparinase II) and the mixture incubated at 37°C. The degradation was monitored by drawing aliquots (100,000 ^{3}H dpm) for chromatography on Bio-Gel P-2 after 16, 40 and 64 h of incubation. On each of these occasions, a further addition (1 mIU) of each enzyme was made. At 69 h, the enzymes were inactivated by heating (100°C) for 1 min. No significant change in chromatographic pattern was observed between 40 and 69 h.

For deaminative cleavage, samples were dissolved in 10 μl 0.5 M HNO_2 [7] on a shaker at room temperature for 10 min, and subsequently neutralized by the addition of 1.3 μl 2 M Na_2CO_3. Samples intended for electrophoresis were lyophilized in a Speed-Vac centrifuge and redissolved in 20 μl electrophoresis buffer.

Gel Chromatography

For gel filtration of oligosaccharides, we used columns (1.5 x 100 cm) of Bio-Gel P-6 and Bio-Gel P-2, equilibrated in 0.5 M NH_4HCO_3 and eluted at 8 ml/h. Samples of 1 ml were applied and fractions of 1.8 ml collected.

A column (1 x 100 cm) of Sepharose CL 6B was equilibrated in 0.3 M NaCl, 10 mM Tris-HCl buffer, pH 7.5, and eluted at 4 ml/h. Void volume (28 ml) and total volume (70 ml) were determined with blue dextran and free $^{35}SO_4^{2-}$, respectively. Samples of 500 μl were applied and fractions of 1 ml collected.

Strong Anion Exchange (SAX) HPLC

HPLC was performed on a Varian model 5560 system. Disaccharides were separated on a Partisil 10/25 SAX (Whatman) fitted with a 5 cm x 4.5 mm guard column (Whatman). The columns were equilibrated in water. Samples (200-1000 µl) diluted to less than 50 mM salt were injected and the column was eluted with water for 10 min, followed by a linear gradient from 0-0.6 M KH_2PO_4, pH 4.2, for 60 min. The column was then rinsed for 10 min with 1 M KH_2PO_4 (the nominal gradient used is outlined in Fig. 2b). Flow was 1 ml/min and 1 ml fractions were collected. In each run, disaccharide standards were used and their elution monitored by absorbance at 232 nm.

Fractions from ion-exchange and gel chromatography were analyzed for ^{3}H and/or ^{35}S radioactivity in a scintillation counter (LKB-Wallach) using Ready-Safe (Beckman) as a scintillator.

Polyacrylamide Gel Electrophoresis and Electroblotting

Polyacrylamide gel electrophoresis was performed on linear gradient gels (20-30% T/0.5-3.6%C) as described by Fransson *et al.* [8]. For electroblotting we slightly modified the protocol of Kyhse-Andersen [9]. The trans-unit, starting from the anodic side, consisted of a) six layers of filter paper soaked in 0.3 M Tris, pH 10.3 (anode buffer No.1), b) three layers of filter paper in 25 mM Tris, pH 10.4 (anode buffer No.2), c) the nylon-membrane wetted in water, d) the resolving gel, which had been equilibrated for 15 min in anode buffer No.2 and finally, e) three filter papers soaked in 26 mM Tris, 40 mM 6-amino-*n*-hexanoic acid, pH 9.4 (cathode buffer). Trapped air bubbles between the membrane and the gel were eliminated by gently rolling a glass test tube on the gel resting on the membrane. Transfer to the nylon membrane was effected at 0.8 mA/cm^2 for 40 min. No dye or radioactive material was detectable in the second nylon membrane when a double layer was used, nor was any dye left in the gel after the transfer. After blotting, the nylon membranes were first air-dried for 10 min, then 'baked' in a Napco model 5831 vacuum oven at 80°C for 1 h. The dried membranes were sprayed with Amplify™, briefly dried in a fume hood and exposed to a film at -60°C for 7-10 days.

Results and Discussion

Compositional Analysis. 1: Specific Degradations

For a quantitative assessment of the composition of endothelial heparan sulphate, we chose to study the most abundant fraction, the low density HSPG from the medium (M 2a). Chains liberated from this proteoglycan (see the Methods section) eluted as a unimodal peak both on ion-exchange chromatography (gradient elution from a Mono-Q column; not shown) and gel chromatography (see below). After brief digestion with chondroitinase ABC and Pronase to eliminate any remaining contamination, the chains were subjected to exhaustive degradation with either heparinase, heparitinase or nitrous acid, followed by gel chromatography on Bio-Gel P-6. The relative amount of linkages susceptible to the various degradations was calculated by using the formula $\Sigma A_n/n$, where A_n was defined as the relative

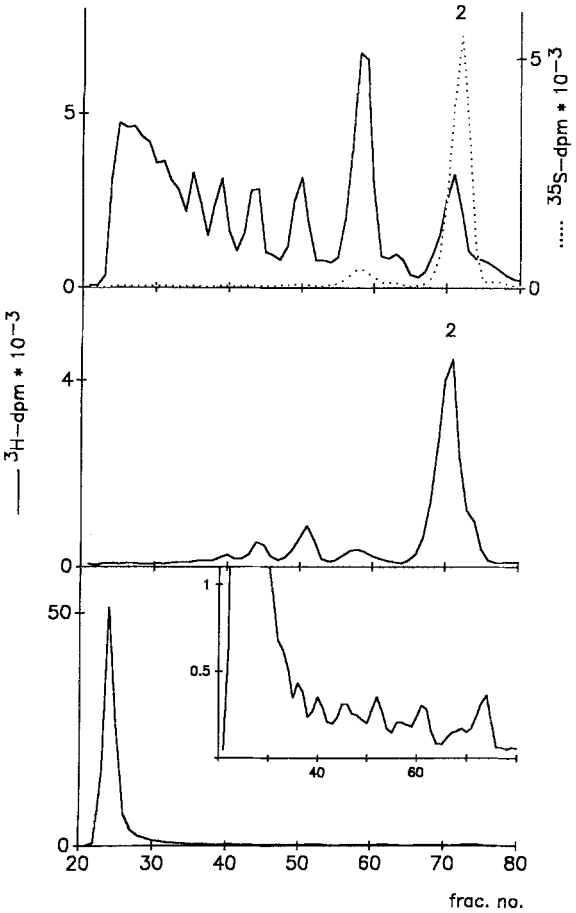

Figure 1. Gel chromatography on Bio-Gel P-6 of endothelial heparan sulphate chains after degradation with a) nitrous acid, b) heparitinase and c) heparinase.

Heparan sulphate was released from the purified low density HSPG of the spent culture medium by alkaline cleavage and further purified by digestion with chondroitinase ABC and Pronase as described in the Methods section. In a), the profiles of ^{3}H-glucosamine- and $^{35}SO_4^{2-}$-labelled heparan sulphate are derived from separate runs. In the inset in c) the y-axis scale has been magnified to demonstrate the small but significant amounts of smaller oligosaccharides. In a) and b) the most retarded peaks (indicated by the number 2 and representing disaccharides) were pooled and further analyzed by SAX HPLC (see Fig. 2).

amount of ^{3}H-glucosamine radioactivity within a chromatographic peak of a certain oligosaccharide size (d.p.) and n is d.p. [10-12]. By comparing the amounts of di-, tetra- and larger oligosaccharides in the chromatograms it is also possible to determine the spacing of susceptible linkages.

Table 1. Relative amount and distribution of various linkages in heparan sulphate derived from low density HSPG of the medium based on results by specific degradations followed by gel chromatography (Fig. 1).

Method	Linkage[a]	Relative occurrence (% of all all linkages)	Distribution		
			Contiguous	Alternate	Spaced
Low pH HNO_2	GlcNS(6R)α1-4HexA	35	38	32	30
Heparitinase	GlcNR(6R)α1-4GlcA	78	87	5	8
Heparinase	GlcNS(6R)α1-4IdoA2S	6.5	26	12	62

[a] Based on the results of Shively and Conrad [7] and Linhardt *et al.* [16].

Oligosaccharides covering a broad range of sizes were generated when the chains were treated with nitrous acid (Fig. 1a). Low pH deamination selectively cleaves the hexosaminidic bond in $GlcNSO_3$6Rα1-4HexA [7], and the result indicated that 32-35% of all disaccharides bear N-linked sulphate. By integration of the disaccharide peak it was calculated that 38% of these occur in clusters (Table 1). Free $^{35}SO_4^{2-}$ co-eluted with the disaccharides and 82% of the ^{35}S label was present in this peak (denoted 2 in Fig. 1a), whereas 17% was present in mostly tetra- and hexasaccharides. The latter represent predominantly O-linked sulphate groups on *N*-acetylated disaccharides [13]. The fractions containing disaccharides and free SO_4^{2-} (peak 2, Fig. 1a) was pooled and further analyzed by SAX HPLC (Fig. 2a). Only a portion of the non-sulphated HexA-AnMan generated by the HNO_2 treatment was retained by the column. Free $^{35}SO_4^{2-}$ was incompletely separated from monosulphated disaccharide, but by comparing the relative amounts of ^{3}H-glucosamine and $^{35}SO_4^{2-}$ in the mono- and disulphated disaccharide peaks, it was estimated that free $^{35}SO_4^{2-}$ contributed approximately 82% of the ^{35}S label in the large retarded peak from Bio-Gel P-6 (Fig. 1a), whereas 10 and 8% were present in mono- and disulphated disaccharides, respectively (Fig. 2a). These calculations indicate that *O*-sulphate represents 33% of total sulphate, implying an N/O-sulphate ratio of approximately 2.0:1.

Scission of the endothelial heparan sulphate with heparitinase, which cleaves the hexosaminidic linkage in GlcNR6Rα1-4GlcA [14-16], resulted in extensive degradation. From the chromatographic profile in Fig. 1b it was calculated that 76-80% of all linkages were cleaved (see Table 1). The large amount (68% of total radioactivity) of disaccharides indicates that 87% of the glucuronate containing disaccharides occur repetitively in clusters, which thus comprise 59% of total chain length. Disaccharide separation on SAX HPLC (Fig. 4b) showed that 24% of these disaccharides are monosulphated, the rest being unsulphated. By treating the disaccharides with nitrous acid prior to SAX HPLC it is possible to evaluate their contents of sulphamido groups [12], and the major part (approx. 70%) of the monosulphated disaccharides generated by heparitinase (see the second peak in Fig. 2b) were desulphated by HNO_2 (result not shown).

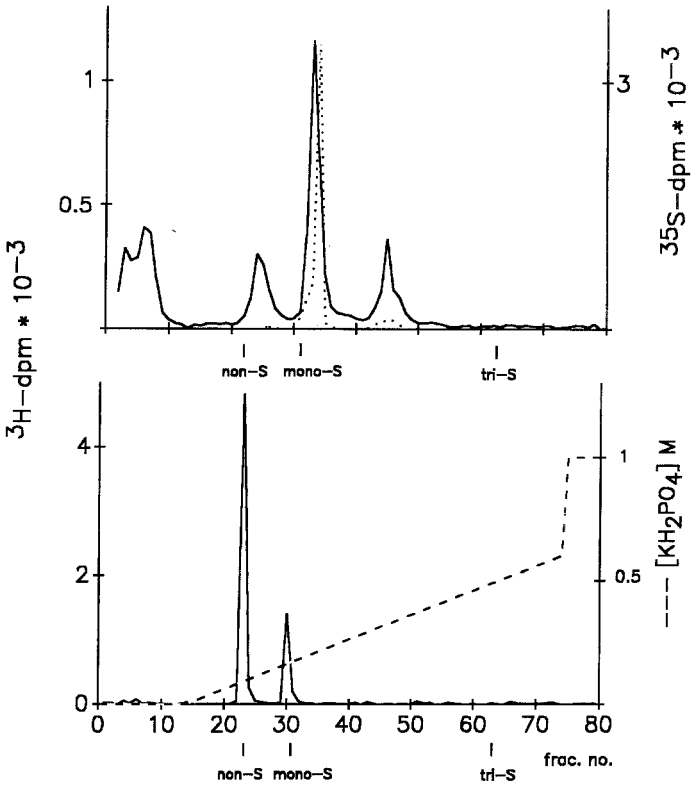

Figure 2. SAX HPLC of disaccharides obtained by a) treatment with HNO_2, and b) heparitinase digestion of endothelial heparan sulphate.

Disaccharides were pooled after gel chromatography on Bio-Gel P-6 (see Fig. 1), lyophilized, redissolved in water and injected into the column. The elution positions of standards (as monitored by A_{232}, 0.2 AUFS) are indicated, and the nominal gradient used is outlined in b. Non-S, non-sulphated disaccharide; mono-S, monosulphated disaccharide; tri-S, trisulphated disaccharide.

Digestion with heparinase, with specificity for the hexosaminidic bonds in $GlcNSO_36R\alpha1$-4IdoA(2S) [14-16], yielded very few small oligosaccharides (Fig. 1c with inset). However, by chromatography on Sepharose CL 6B the digested chains eluted at K_{av} approximately 0.6 (not shown), indicating an average M_r of 15,000-20,000 Da, whereas the intact chains had an M_r of approximately 60,000 (see below). From these results it was estimated that only approximately 6.5% of all linkages were cleaved by the enzyme. This indicates a low content of IdoA2S [16] and it can also be seen that most of the susceptible linkages occur solitarily (Table 1). The tiny amount of disaccharides generated by the enzyme did not allow further analysis on SAX HPLC.

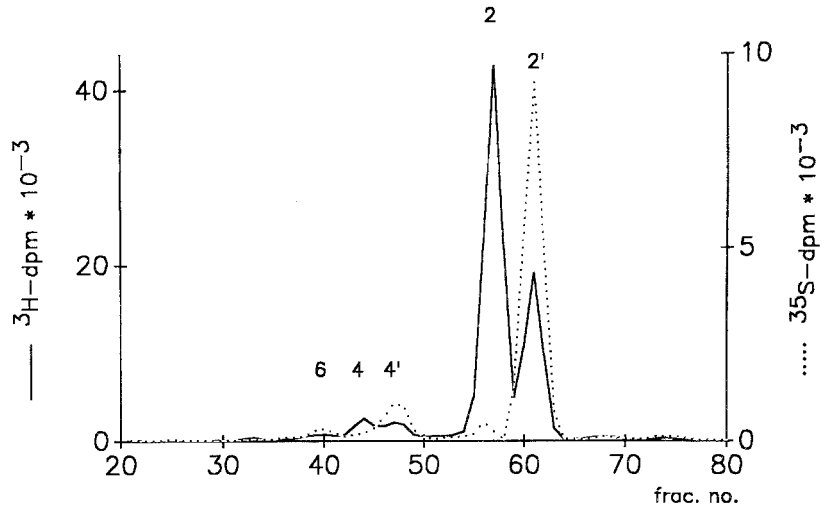

Figure 3. Gel chromatography on Bio-Gel P-2 after digestion of heparan sulphate chains with heparinase, heparitinase and heparinase II.

Samples of ^{3}H-glucosamine (10^6 dpm) and $^{35}SO_4$-labelled (2.5×10^5 dpm) heparan sulphate chains derived from purified low density proteoglycan of the medium were pooled and dissolved in 100 µl 3 mM $CaAc_2$, 10 mM Hepes-NaOH buffer, pH 7.0. Digestion was performed as described in the Methods section. Fractions from the marked peaks (6, hexasaccharide; 4, low sulphated tetrasaccharide; 4', high sulphated tetrasaccharide; 2, *N*-acetylated disaccharides; 2, *N*-sulphated disaccharides) were pooled separately for further analysis by treatment with nitrous acid and/or SAX HPLC.

Compositional Analysis. 2: Combined Digestion

Use of heparinase II in combination with heparinase and heparitinase has been shown to result in extensive degradation with a high yield of disaccharides [12]. The former enzyme has a broad substrate specificity [15, 16] and, in contrast to both heparinase and heparitinase, it also cleaves the hexosaminidic linkage between *N*-sulphated glucosamine and non-sulphated iduronic acid. We degraded endothelial heparan sulphate by this method and the resulting oligosaccharide profile is shown in Fig. 3. As described by Turnbull and Gallagher [12], Bio-Gel P-2 separates disaccharides into two populations, tentatively identified as *N*-acetylated and *N*-sulphated disaccharides by their sensitivity to treatment with HNO_2. Here, 60% of the total ^{3}H-glucosamine radioactivity was recovered in the early disaccharide peak (*N*-acetylated, denoted 2) and 27% in the later (*N*-sulphated, denoted 2'). Both peaks were pooled and applied to SAX HPLC for determination of disaccharide sulphation (Fig. 4). Disaccharides in peak 2 (*N*-acetylated) eluted mostly (91%) as non-sulphated species, the rest being monosulphated (Fig. 4a). Peak 2' (*N*-sulphated disaccharides) contained predominantly (72%) monosulphated disaccharides, a small amount of non-sulphated

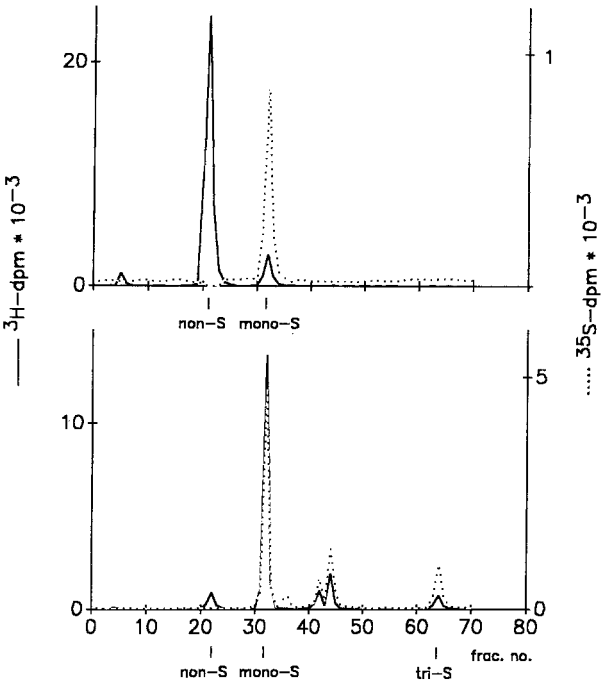

Figure 4. SAX HPLC of disaccharides from a) peak 2, and b) peak 2', obtained from Bio-Gel P-2 after combined digestion (Fig. 3) with heparinase, heparinase II and heparitinase.

Fractions comprising peak 2 and 2' in Fig. 3 were pooled, lyophilized, redissolved in water and injected into the column. Gradients were run as in Fig. 2b. The elution positions of standards are indicated.

species (6.3%), the rest being disulphated (17%) and trisulphated (5.8%) disaccharides (Fig. 4b). The split elution profile of the disulphated disaccharide suggests the presence of at least two different species, most likely DHexA1-4GlcNSO$_3$(6S) and DHexA(2S)1-4GlcNSO$_3$. To fit the data from heparinase digestion with the amount of disulphated disaccharides, Ido(2S)α1-4GlcNSO$_3$ should be the major disulphated disaccharide in the parent chain.

The small $^{35}SO_4^{2-}$ peak between the mono- and disulphated disaccharide peaks in Fig. 4b, probably represents free SO_4^{2-} liberated by a sulphatase contaminant in some of the enzyme preparations. The amount constitutes approximately 3% of the total ^{35}S radioactivity (to compare with the 40% of $^{35}SO_4^{2-}$ present in monosulphated disaccharides), and may have caused an underestimation (1-2%) of the amount of sulphated disaccharides. As seen in Fig. 3, the combined digestion also generated small but significant amounts of larger oligosaccharides (1.8% and 8.6% of the ^{3}H radioactivity in hexasaccharide and tetrasaccharide peaks, respectively). These saccharides may contain linkages absolutely or relatively

resistant to the enzymes used, as some low M_r oligosaccharides are known to require very high enzyme concentrations to be cleaved [14, 16, 17]. The tetrasaccharides were partly separated into one with a low and one with a higher sulphate content. The hexasaccharides and the two tetrasaccharide populations were pooled separately as indicated in Fig.3 (peaks designated 6, 4 and 4', respectively), and treated with nitrous acid followed by gel chromatography on Bio-Gel P-6 (results not shown). The tetrasaccharide material in 4' showed a marked sensitivity to deaminative cleavage (77% of the ^3H radioactivity was eluted as disaccharide after the treatment) and the resulting disaccharides were further analyzed by chromatography on SAX HPLC (results not shown). The oligosaccharides in peaks 6 and 4 showed a more limited sensitivity to HNO_2 (50 and 37%, respectively). The results indicate that the hexa- and tetrasaccharides persisting after combined digestion are heterogeneous populations.

The data from SAX HPLC of disaccharides and nitrous acid treatment of tetra- and hexasaccharides are summarized in Table 2. Including the analysis of hexa- and tetrasaccharides by deaminative cleavage, the results account for 90% of disaccharides (Table 2). In total, 34-36% of these were found to carry at least one sulphate group, somewhat less than expected from the estimated amount of *N*-sulphated residues and the fact that the chains also contained *O*-sulphate groups at C-6 of *N*-acetylated glucosamine residues. The deficit, approximately 5-7% as estimated from the amount of $^{35}SO_4^{2-}$ label present in tetra- and higher oligosaccharides after deaminative cleavage (Fig. 1a), may be explained by either the sulphatase activity revealed by the very small free $^{35}SO_4^{2-}$ peak in Fig. 4b, or the presence of *O*-sulphate groups in some of the HNO_2-resistant hexa- and tetrasaccharides in Fig. 3 (see Table 2).

From the results of the selective and combined degradations, the disaccharide composition of endothelial heparan sulphate was inferred as explained in Table 3. Some *N*-acetylated disaccharides (of the form HexA1-4GlcNAc) in heparin/heparan sulphate bear *O*-sulphate groups [5]. The amount of such disaccharides can be estimated from the amount of $^{35}SO_4^{2-}$ label present in tetra- and higher oligosaccharides after deaminative cleavage. In a detailed study of nitrous acid resistant tetrasaccharides in heparan sulphate from bovine lung, Sanderson *et al.* [13] showed that sulphate groups on *N*-acetylated disaccharides are attached exclusively to C-6 of glucosamine. The uronic acid marking the non-reducing end of these tetrasaccharides was never sulphated, but was identified as non-sulphated L-iduronic acid and D-glucuronic acid in approximately equal amounts. A content of approximately 8% monosulphated *N*-acetylated disaccharides in endothelial heparan sulphate was estimated from the amount of $^{35}SO_4$ label in tetrasaccharides after deaminative cleavage (Fig. 1a). This can be compared to the 6% of total ^3H radioactivity present in *N*-acetylated, monosulphated disaccharides after combined digestion (see Fig. 4a and Table 2).

The amount of linkages susceptible to heparinase (Table 1) was very close to the total amount of di- and trisulphated disaccharides generated by combined digestion (and nitrous acid treatment of resistant oligosaccharides), indicating that nearly all of these contain IdoA(2S). However, the split elution profile of disulphated disaccharides in Fig. 4b suggests the presence of a minor different species, probably IdoAα1-4GlcNSO$_3$(6S) [but also possibly GlcAβ1-4GlcNSO$_3$(6S)]. Thus there is little, if any, room for di- or trisulphated disaccharides containing glucuronic acid (listed in Table 3).

Table 2. Disaccharide sulphation in the various peaks resolved by Bio-Gel P-2 after combined digestion of endothelial heparan sulphate with heparitinase, heparinase II and heparinase (see the Methods section).

Disaccharides (peak 2 and 2' in Fig. 3) were analyzed directly by SAX HPLC. Tetra- and hexasaccharides (peak 4, 4' and 6 in Fig. 3) were treated with HNO_2 followed by chromatography on Bio-Gel P-6 to evaluate their contents of *N*-sulphated glucosamine. The disaccharides obtained by deaminative cleavage of tetrasaccharide 4' were also analyzed by SAX HPLC. Numbers within parentheses are estimates solely from nitrous acid treatment/gel chromatography, whereas other numbers are derived by integration of peaks on the SAX HPLC chromatograms.

Analyzed peak[a]	Relative amount (^{3}H dpm)	Disaccharide sulphation[b]			
		Non-S	Mono-S	Di-S	Tri-S
6	1.8	(0.9)[c]	(0.9)[d]	-[d]	-[d]
4	4.8	(3.2)[c]	(1.6)[d]	-[d]	-[d]
4'	3.8	(0.8)[c]	1.5	0.8	0.4
2	60.3	54.6	5.7		
2'	27.1	1.7	19.4	4.6	1.4
Subtotal	93.8	56.3	27.5	5.4	1.8

[a] Denotes peak in Fig. 3.
[b] Non-S, non-sulphated disaccharide; mono-S, monosulphated disaccharide; di-S, disulphated disaccharide; tri-S, trisulphated disaccharide.
[c] The material resistant to HNO_2 may contain some O-linked sulphate groups.
[d] Degree of sulphation not determined by SAX-HPLC.

Comparison of Heparan Sulphate Chains from Different Proteoglycan Populations

Polysaccharide chains isolated from the three major endothelial HSPG's were compared by chromatography on Sepharose CL-6B (Fig. 5). The heparan sulphate chains originating from the low density fractions of the cell layer (Fig. 5a) and medium (Fig. 5c) produced elution profiles with peak maximum at K_{av} 0.33 and 0.28, respectively. HS derived from the high density fraction of the medium eluted at an earlier position, K_{av} 0.24 (Fig. 5b). All values are outside the calibration range of Wasteson [19], indicating M_rs above 50,000 Da.

The three heparan sulphate populations were also analyzed by gradient polyacrylamide gel electrophoresis before and after various specific degradations. After deaminative cleavage, the chains from the low density HSPG's (C 2 and M 2a) generated a wide range of oligosaccharides from disaccharides (migrating in the front) up to approximately 20 disaccharides (see Fig. 6d and f). Heparitinase gave rise to a complex pattern of small oligosaccharides of d.p. 1-≈6 (Fig. 6g and i). When subjected to heparinase, it could be seen that most of the chains had been cleaved since the degradation products (Fig. 6k-m) moved further into the gel than the intact chains (Fig. 6a-c). However, the products comprised large oligosaccharides, not fully resolved by the gel, and only barely detectable amounts of material migrating in the front. The heparan sulphate chains from the high density HSPG (M 1a) yielded degradation patterns slightly different from those of the low density HSPG's. After treatment with nitrous acid (Fig. 6e), smaller amounts of long fragments were produced compared to the other heparan sulphates, and conversely, digestion with heparitinase generated more of the longer fragments (Fig. 6h). Heparinase, however, yielded fragments similar to those of C 2 and M 2a (Fig. 6k-m). Heparinase fragments from the three fractions also eluted at the same position on gel chromatography (results not shown).

General Discussion

The development of cell culture techniques and adequate methods for proteoglycan purification provides an interesting opportunity to study the regulation of heparan sulphate biosynthesis. Earlier reports have indicated that some heparan sulphate modifications are more common in certain tissues. For example, the 3-*O*-SO_3 group on glucosamine in the antithrombin III-binding region was shown to be highly expressed in a HSPG from murine Reichert's membrane [20] and in rat brain heparan sulphate [21]. Heparan sulphate from endothelial cells has attracted special interest because of its possible interaction with circulating antithrombin III and other factors of the coagulation system. Studies on bovine aortic endothelial cells [22, 23] have indicated that these cells can synthesize distinct subspecies of heparan sulphate. Marcum *et al.* [22] reported the presence of a endothelial HSPG population carrying chains with the same anticoagulant activity as in commercial heparin preparations. The subpopulation was also distinguished by its hydrophobic core protein and was proposed to be located in the plasma membrane. However, only less than 1% of the hydrophobic proteoglycans showed affinity for antithrombin III, diminishing the possibility of further characterization.

In a previous study, we characterized the major proteoglycans and core proteins synthesized by human umbilical vein endothelial cells [6]. The proteoglycans were purified to homogeneity as judged by SDS-polyacrylamide gel electrophoresis. In the present work we have analyzed the composition of the heparan sulphate chains attached to the major HSPG of this cell type. The method we used for compositional analysis is similar to that presented by Turnbull and Gallagher [12] in a recent study of fibroblast heparan sulphate. An alternative method using chemical degradation for the compositional analysis of heparin/heparan sulphate, was recently described by Guo and Conrad [24]. In comparison to the report from fibroblasts [12], endothelial heparan sulphate chains have a somewhat higher M_r but a distinctively lower content of both N- and O-linked sulphate (see Table 3). Correspondingly, the size of the *N*-acetylated segments, of up to approximately 20 disaccharides or more (Fig. 1a and Fig.

Table 3. Composition of endothelial heparan sulphate as estimated by selective and combined degradations.

In the Table are listed all disaccharides hitherto identified in heparin and heparan sulphate [18]. The amounts of disaccharides containing *N*-sulphated/*N*-acetylated glucosamines and glucuronic/iduronic acids were inferred from degradations using nitrous acid and heparitinase, respectively (Table 1). Content of IdoA(2S) was as estimated from heparinase digestion.

Disaccharide	Sulphation	*N*-acetylated (65-68%)		*N*-sulphated (32-35%)	
GlcA-containing-disaccharides (78%)	Non-S	52-56[a]	GlcAβ1-4GlcNAc		
	Mono-S	5-9[b]	GlcAβ1-4GlcNAc(6S)	15-20[c]	GlcAβ1-4GlcNSO$_3$
	Di-S			0	GlcAβ1-4GlcNSO$_3$(6S) GlcAβ1-4GlcNSO$_3$(3S) GlcA(2S)β1-4GlcNSO$_3$
	Tri-S			0	GlcAβ1-4GlcNSO$_3$(3,6-diS) GlcA(2S)β1-4GlcNSO$_3$(6S)
IdoA-containing-disaccharides (22%)	Non-S	0-4[d]	IdoAα1-4GlcNAc		
	Mono-S	0-4[e]	IdoAα1-4GlcNAc6S (IdoA(2S)α1-4GlcNAc)[g]	7-15[f]	IdoAα1-4GlcNSO$_3$
	Di-S	0	(IdoA(2S)α1-4GlcNAc6S)[g]	2[h] 5	IdoAα1-4GlcNSO$_3$(6S) IdoA(2S)α1-4GlcNSO$_3$
	Tri-S			2	IdoA(2S)α1-4GlcNSO$_3$(6S)

[a] The minimum amount of this disaccharide was given by the analysis of disaccharides after heparitinase digestion (Fig. 2b).

[b] 5% is a minimum based on experiments where disaccharides generated by heparitinase digestion (Fig. 1b) were analyzed on SAX HPLC after treatment with HNO_2 (not shown). 7% of the disaccharides retained their sulphation after this treatment.

[c] The amount was reached by satisfying the total amount of glucuronate-containing disaccharides (as estimated by heparitinase digestion of the chains). SAX HPLC of disaccharides after heparitinase digestion (Fig. 2b) suggested that there were no di- and trisulphated disaccharides containing glucuronic acid.

[d] This is the only alternative to GlcAβ1-4GlcNAc for non-sulphated disaccharide in heparan sulphate, and it has been identified in heparan sulphate from bovine lung [13]. 4% represents a maximum reached by subtracting the amount of non-sulphated disaccharides in the heparitinase digest (Fig. 2b) from the amount these disaccharides after combined digestion (Table 2).

[e] This disaccharide was identified in heparan sulphate from bovine lung [13]. 4% represents a maximum reached by subtracting the minimum amount of GlcAβ1-4GlcNAc(6S) from the estimated total amount of monosulphated *N*-acetylated disaccharides (see the Results and Discussion section).

[f] Reached by satisfying the total amount of iduronate containing disaccharides.

[g] Disaccharides within parentheses have only been identified in heparin [18] and are probably absent from heparan sulphate [13].

[h] The split peak of disulphated disaccharide in Fig. 4b suggested two different species. To fit results from heparinase digestion the major component had to be IdoA2Sα1-4GlcNSO$_3$.

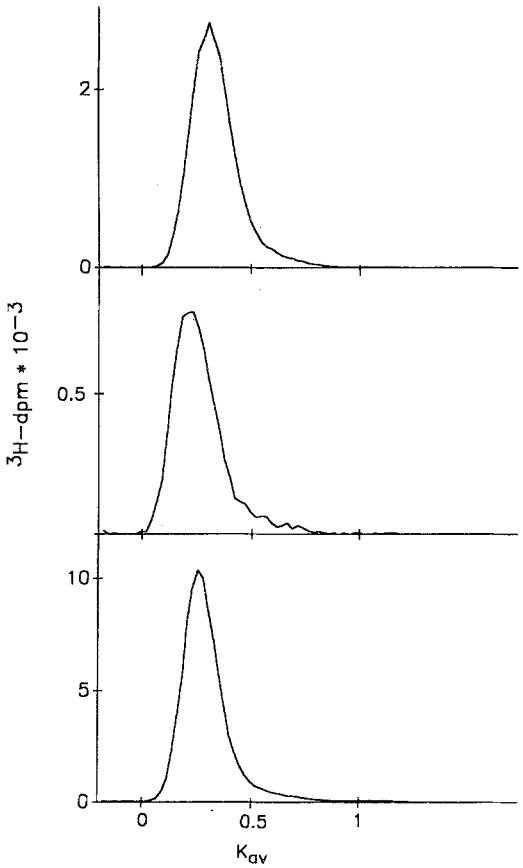

Figure 5. Gel chromatography on Sepharose CL 6B of ^{3}H-glucosamine-labelled polysaccharides of purified proteoglycans.

Polysaccharide chains were obtained from purified proteoglycan fractions by alkaline elimination as described in the Methods section. The Figure shows heparan sulphate chains from a) low density HSPG in cell extract (C 2), b) high density HSPG in spent culture medium (M 1a), c) low density HSPG in spent culture medium (M 2a). Void volume ($K_{av} = 0$) and total volume ($K_{av} = 1$) were determined by the elution positions of blue dextran and free $^{35}SO_4^{2-}$

6d-f), seems larger in endothelial cells. The N/O-sulphate ratio for endothelial heparan sulphate and that reported for fibroblasts [12], 2.0 vs. 1.8, are similar and also the relations of iduronate (as estimated from the amount of heparitinase resistant saccharides) to *N*-sulphated glucosamine residues. The size of *N*-sulphated segments cannot be directly assessed from the present data, but as iduronate residues are only found attached to C-1 of $GlcNSO_3$, a minimum size is given by the heparitinase resistant fragments in Fig. 1b and Fig. 6g-i. These fragments seem significantly smaller in endothelial cells than in fibroblasts [12]. Furthermore, the content of disulphated disaccharides is unexpectedly low in endothelial heparan sulphate, and one might guess that these two phenomena are related.

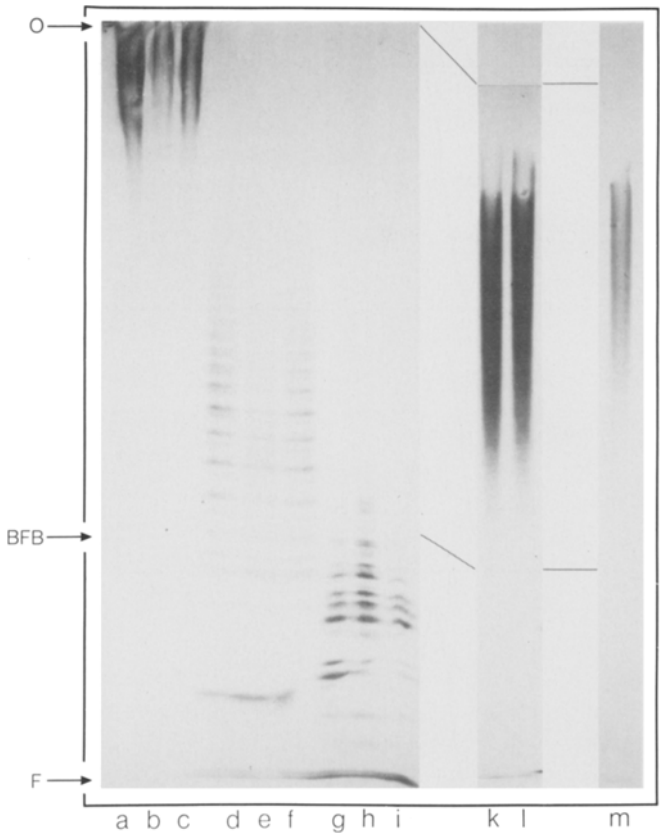

Figure 6. Gradient polyacrylamide gel electrophoresis of intact and degraded ^{3}H-glucosamine-labelled heparan sulphate chains obtained from low density proteoglycan fraction of cell extract (a, d, g, k), the high density proteoglycan (b, e, h, m) of spent culture medium or the low density proteoglycan (c, f, i, l) of the spent culture medium.

Isolated heparan sulphate chains were run either intact (a, b, c), after degradation with nitrous acid (d, e, f), or after digestion with heparitinase (g, h, i) or heparinase (k, l, m), as described in the Methods section. Lanes a-i, k and l, and m were run on separate but similar (20-30%) gels. The d.p. of individual bands after treatment with HNO_2 were estimated by counting the number of 'doublet' bands (each doublet roughly representing one d.p.) above the band of bromophenol blue, which has been shown to migrate just ahead of heparan sulphate dodecasaccharides [27]. O, origin of separating gel; BFB, bromophenol blue; F, migration front.

These findings confirm the concept of a domain structure of alternating segments of sulphated and non-sulphated disaccharides [5]. Furthermore, the preferentially wide spacing of sulphated iduronate residues, as indicated by results from heparinase digestion (Fig. 1c, Fig. 6k-m, Table 1) indicates that this modification occurs both internally as well as towards the non-reducing end of the chains. The long heparin-like segments found by Nader *et al.* [25] in a HSPG from rabbit endothelial cells, are clearly not present in the heparan sulphate studied here.

In an effort to search for subspecies of endothelial heparan sulphate we have compared three different proteoglycan populations. Comparison of chain sizes was performed on Sepharose CL 6B. All the endothelial heparan sulphate chains are unusually large and in this respect reminiscent of the chains of the heparin proteoglycan in mouse mastocytoma [26], with an estimated M_r of 60,000-100,000. The small amount of high density HSPG found have chains that appear to have somewhat larger average M_r (Fig. 5b) than those of the low-density HSPG's. The larger chains may explain the higher density of their parent proteoglycan molecules. For oligosaccharide mapping we used polyacrylamide gel electrophoresis, which has been shown to have a resolution superior to gel chromatography [27]. The presence of domains with different sulphation patterns in a subgroup of heparan sulphate chains should give rise to unique oligosaccharide bands after specific degradations. The electrophoretic oligosaccharide patterns after enzymic or chemical degradations of chains from high- and low density proteoglycans show a significant amount of identical bands, though the intensity of each band may vary between the different populations (Fig. 6). The observed differences may at least partly be explained by the fact that the longer chains (derived from the high density HSPG) contain a proportionately lower amount of the extended *N*-acetylated sequences seen in the polysaccharide-protein linkage region [28]. Taken together the results do not indicate the presence of distinct subspecies of heparan sulphate in any of the examined proteoglycans.

From what is known of the biosynthesis of heparin and heparan sulphate, it is not clear what determines the degree and distribution of secondary modifications within the polysaccharide chain. The recent report by Horner [21] shows that heparan sulphates of different tissue origins vary approximately fourfold in the amount of chains showing high affinity for antithrombin III. Our study shows that heparan sulphate from human endothelial cells has properties distinct to those recently reported from human fibroblast cells [12]. Identification of distinct sub-species of heparan sulphate may provide clues to the possibly diverse functions of this polysaccharide.

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