Structure and Affinity for Antithrombin of Heparan Sulfate Chains Derived from Basement Membrane Proteoglycans*

(Received for publication, October 9, 1986)

Gunnar Pejler, Gudrun Backström, and Ulf Lindahl

From the Department of Veterinary Medical Chemistry, Swedish University of Agricultural Sciences, The Biomedical Center, S-751 23 Uppsala, Sweden

Mats Paulsson‡, Marie Dziadek§, Sakuhei Fujiwaraf, and Rupert Timpl

From the Max-Planck-Institut für Biochemie, D-8033, Martinsried/Munich, Federal Republic of Germany

Metabolically 35S- or 3H-labeled heparan sulfate was isolated from murine Reichert’s membrane, an extracellular basement membrane produced by pavietal endothelial cells, and from the basement membrane-producing Engelbreth-Holm-Swarm mouse tumor. The polysaccharides were subjected to structural analysis involving identification of products formed on deamination of the polysaccharides with nitrous acid. The polysaccharide from Reichert’s membrane contained N- and O-sulfate groups in approximately equal proportions. It bound almost quantitatively and with high affinity to antithrombin. A high proportion of antithrombin-binding sequence was also indicated by the finding that 3-O-sulfated glucosamine residues accounted for about 10% of the total O-sulfate groups. In contrast, at least 80% of the sulfate residues in the heparan sulfate isolated from the mouse tumor were N-substituents. Only a minor proportion of this polysaccharide bound with high affinity to antithrombin, and no 3-O-sulfated glucosamine residues were detected. These results are discussed in relation to the possible functional role of heparan sulfate in basement membranes.

Heparin and heparan sulfate are both synthesized as proteoglycans, heparin by connective-tissue-type mast cells and heparan sulfate by a variety of cells (Rodén, 1980). Both types of polysaccharides are composed of alternating hexuronic (D-glucuronic or L-iduronic) acid and D-glucosamine units, the latter residues being either N-sulfated or N-acetylated. In addition, the sugar residues carry O-sulfate groups in certain positions (Lindahl and Höök, 1978; Rodén, 1980; Bienkowski and Conrad, 1985; Gallagher et al., 1986; Lindahl and Kjellén, 1987). Whereas there is thus no apparent qualitative difference between the two types of polysaccharide, the sugar composition and sulfation pattern generally provide a quantitative distinction, heparin containing more sulfate and iduronic acid but less N-acetylglucosamine than heparan sulfate (Gallagher and Walker, 1985). The blood anticoagulant activity of heparin is due to the presence of a specific pentasaccharide sequence (Lindahl et al., 1984; Atha et al., 1985; see Fig. 1), the distinguishing feature of which is a 3-O-sulfated glucosamine residue (Lindahl et al., 1980). Polysaccharide chains possessing this sequence bind with high affinity to antithrombin and thus drastically increase the rate by which this proteinase inhibitor inactivates enzymes involved in the coagulation process (Bjork and Lindahl, 1982). Recent findings suggest that the same antithrombin-binding region may occur also in heparan sulfate (Marcam and Rosenberg, 1986; Lane et al., 1986; Marcum et al., 1986).

The proteoglycan forms of the two polysaccharides appear to be more clearly distinct. In the heparin proteoglycan, 10–15 polysaccharide chains are attached to a polypeptide core composed essentially of alternating serine and glycine residues (Robinson et al., 1978). At least 2 out of 3 serine residues carry a polysaccharide substituent, and the macromolecule is thus characteristically resistant to digestion by proteolytic enzymes such as Pronase. Heparan sulfates are generally assembled into more conventional proteoglycan structures, with polypeptide cores of complex composition and relatively sparsely distributed polysaccharide chains. The occurrence of immunologically distinct core proteins has been reported (Dziadek et al., 1985; see also Gallagher et al., 1986).

Basement membranes contain, in addition to other macromolecules, proteoglycans, in particular heparan sulfate proteoglycans. It has been proposed that these proteoglycans control the permeability properties of basement membranes by functioning as a charge barrier (Farquhar, 1981). Structural studies of heparan sulfate proteoglycans from the basement membrane-producing Engelbreth-Holm-Swarm tumor (EHS)1 have demonstrated a high- and a low-density form of the proteoglycan (Fujiiwar et al., 1984; Hassell et al., 1985).

* This work was supported by Grant 13X-2309 from the Swedish Medical Research Council, Grant 600/80/D98/2 from the Swedish Council for Forestry and Agricultural Research; the Faculty of Veterinary Medicine, Swedish University of Agricultural Sciences; KabiVitrum AB, Stockholm; and Grant Ti 95/6-2 from the Deutsche Forschungsgemeinschaft. A preliminary account of part of this work has appeared (Paulsson et al., 1986). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Supported by fellowships from the European Molecular Biology Organization and the Alexander von Humboldt Foundation. Present address: Dept. of Biophysical Chemistry, Biocenter, University of Basel, Basel, Switzerland.

‡ Supported by fellowships from the European Molecular Biology Organization and the Alexander von Humboldt Foundation. Present address: The Murdoch Institute, Royal Children’s Hospital, Parkville, Victoria 3052, Australia.

§ Present address: Dept. of Dermatology, Medical College of Oita, Oita, Japan.

1 The abbreviations used are: EHS, Engelbreth-Holm-Swarm tumor; HexA, unspecified hexuronic acid; GlcA, D-glucuronic acid; IdoA, L-iduronic acid; GcNAc, 2-deoxy-2-acetamido-2-glucose (N-acetyl-D-glucosamine); αManα, 2,5-anhydro-b-mannitol formed by reduction of terminal 2,5-anhydro-b-mannose residue with NaBH₄; NSO₃, N-sulfate group; OSO₃, O-sulfate group. (The locations of O-sulfate groups are indicated in parentheses.)


5036
The high-density form (M, $\sim$130,000) contains a small protein core (M, $\sim$10,000) to which four 30-nm-long heparan sulfate chains (M, $\sim$25,000) are attached (Fujinawa et al., 1984). The low-density proteoglycan (M, $\sim$600,000) carries three 90-nm-long heparan sulfate chains (M, $\sim$43,000) connected to a large core protein (M, $\sim$400,000) (Paulsson et al., 1986). High- and low-density heparan sulfate proteoglycans have also been isolated from Reichert's membrane, an extraembryonic basement membrane produced by parietal endoderm cells in the pregnant rodent uterus (Paulsson et al., 1983). Contrary to EHS material, Reichert's membrane is a product of normal cells, and since it can be cleanly isolated, it is a useful system for the study of basement membrane structure. In analogy with the findings for EHS proteoglycans, gel chromatography indicated a larger molecular size for the low-density proteoglycan from Reichert's membrane than for the high-density proteoglycan from Reichert's membrane than for the high-density proteoglycan from Reichert's membrane (Paulsson et al., 1985). Immunological characterization showed that high- and low-density EHS heparan sulfate proteoglycans shared common epitopes with each other and with the proteoglycans obtained from Reichert's membrane, but not with the heparan sulfate proteoglycan isolated from liver plasma membrane (Dziadek et al., 1985; Paulsson et al., 1985). It thus may be concluded that the heparan sulfate proteoglycans in EHS and in Reichert's membrane represent a unique class of proteoglycans with preferential localization to basement membranes.

In the present study, heparan sulfate proteoglycans isolated from EHS and from Reichert's membrane have been characterized with regard to polysaccharide structure. The heparan sulfate from Reichert's membrane showed a higher O/N-sulfate ratio than did the corresponding tumor material. Furthermore, it bound with high affinity to antithrombin and contained large amounts of the 3-O-sulfated glucosamine residue previously implicated as a unique component of the antithrombin-binding region of heparin.

**EXPERIMENTAL PROCEDURES**

**Materials.—** Reference samples of unlabeled glycosaminoglycans (hyaluronic acid, chondroitin sulfate, heparin) were as described (Enderback et al., 1985). HexA-[1-3H] instrs disaccharides, with or without O-sulfate groups in various positions, were prepared initially by a procedure involving paper chromatography as the last step (Jacobsson et al., 1979a) and later by high-performance ion-exchange chromatography, following HNO$_3$ (pH 1.5)-NaB$_3$H$_4$ treatment (Thunberg et al., 1982) of heparan or heparin sulfate. The two tetrasccharides, IdoA-GlcNAc(6-OSO$_3$)-GlcA-[1-3H]Man$_3$(3,6-di-OSO$_3$) and IdoA-GlcNAc(6-OSO$_3$)-GlcA-[1-3H]Man$_3$(3,6-di-OSO$_3$) were obtained as described (components a and b, respectively, in Fig. 7A in Thunberg et al., 1982).

The $^3$H-labeled disaccharides, GlcA-[1-3H]Man$_3$(3-OSO$_3$) and GlcA-[1-3H]Man$_3$(3,6-di-OSO$_3$) were obtained through N-deacetylation of the corresponding tetrasaccharides by hydrazinolysis, followed by cleavage of the products with HNO$_3$ at pH 3.9 (Thunberg et al., 1982). The O-sulfated monosaccharides, [1-3H]Man$_6$(6-OSO$_3$) and [1-3H]Man$_3$(6-6-di-OSO$_3$), were obtained by digestion of the appropriate disaccharides with bovine liver $\beta$-d-glucuronidase (Jacobsson et al., 1979a).

**Fig. 1. Structure of antithrombin-binding region in heparin.** Structural variants are indicated by $R'$, -SO$_3$ or -H, $R''$, -SO$_3$, or -COCH$_3$ or the alternative hexuronic acid residue indicated at unit 7. The pentasaccharide sequence composed of units 2-6 constitutes the actual binding region (within brackets). The 3-O-sulfate group (marked by an asterisk) at glucosamine unit 4 is unique to the antithrombin-binding region and is essential to the interaction of the polysaccharide with antithrombin, together with the 6-O-sulfate residue at unit 2 and the N-sulfate groups of units 4 and 6. For additional information, see Lindahl et al. (1984) and Atha et al. (1985).
yalated by treatment with hydrazine/hydrazine sulfate at 100 °C for 2 h (Thunberg et al., 1982). The products were desalted by passage through a column (1 × 64 cm) of Sephadex G-15, equilibrated with 10% ethanol.

Reduction of saccharides with nitrous acid at pH 1.5 (deamination of N-sulfated glucosamine residues; 10 min at room temperature) or at pH 3.9 (deamination of N-unsubstituted glucosamine residues; 10 min at room temperature) was carried out as described (Thunberg et al., 1982), with some modification. In these reactions, the glucosamine target residues are converted into 2,5-anhydro-D-mannose units, with cleavage of the corresponding glucosaminidic linkages (see Shively and Conrad, 1976). Reduction of these units to terminal anhydromannitol residues was achieved by adding, per each 200-μl portion of pH 1.5 reaction mixture, either 75 μl of 1 m Na₂CO₃ containing 13 mg of unlabeled NaBH₄/ml or 75 μl of 1 m NaOH, followed by 5 ml of NaB³H₄ (specific activity, 2.5-7.5 Ci/mmol) in 200 μl of 0.01 m NaOH. The products obtained after deamination at pH 3.9 (600-μl reaction mixture) were neutralized with 75 μl of 2 m Na₂CO₃ containing 13 mg of unlabeled NaBH₄/ml. After reduction of deamination products at room temperature for ~15 h, the pH was lowered to ~4 with glacial acetic acid (‘H-containing samples in a fume hood) and was then adjusted to pH 7 with 4 m NaOH.

Labeled di- and tetrasaccharides were separated by high-performance ion-exchange chromatography on a Partisil-10 SAX column (Whatman), eluted at a rate of 1 ml/min using step gradients with increasing concentrations of aqueous K₂HPO₄ (Bienkowski and Conrad, 1986). The column was connected to a model Flo-One HS radioactivity-flow detectors (Radiomatic Instruments & Chemical Co., Inc., Tampa, FL), equipped with a 2.5-ml cell, using Flo-Scint III (Radiomatic) as scintillation medium (Radiomatic) as scintillation medium. The radioactivity was integrated continuously and recorded on a strip chart. The retention times of labeled components were related to those of di- and tetrasaccharide standards. The identity of ³²S-labeled sample components was ascertained by use of ³²P-labeled internal standards, by rerunning samples after digestion with β-glucuronidase, or, in the case of tetrasaccharides, by identifying saccharides formed by deacetylation, followed by deaminative cleavage (pH 3.9 reaction) of the tetrasaccharide.

Ion-exchange chromatography of intact glycosaminoglycans was performed on columns (1 × 3 cm) of DEAE-cellulose (Whatman DE52), eluted at a rate of ~6 ml/h with a gradient extending from 0.05 to 1.5 m LiCl in 0.05 m acetate buffer, pH 4.0. Gel chromatography was carried out on columns of Sephadex G-15 (1 × 150 cm, eluted with 0.2 m NH₄HCO₃ at ~6 ml/h), G-25 (1 × 190 cm, eluted with 0.2 m NH₄HCO₃ at ~6 ml/h), or Sepharose CL-4B (1 × 90 cm, eluted with 50 mM Tris·HCl, 0.15 m NaCl, 0.1% sodium dodecyl sulfate, pH 8.0, at ~3 ml/h). Affinity chromatography on antithrombin-Sepharose was carried out as described (Thunberg et al., 1982) using a 3 ml-column of the affinity matrix. Generally, heparin (1 mg) was added to radiolabeled samples as an internal standard and was detected by the carbazole reaction. Paper chromatography was performed using Whatman No. 3MM paper, developed with ethyl acetate/acetate acid/water (3/1/1). High-voltage electrophoresis was conducted on Whatman No. 3MM paper in 0.083 m pyridine, 0.050 m acetic acid (pH 5.3, 80 V/cm).

Hexuronic acid was detected by the carbazole reaction (Bitter and Muir, 1962), and radioactivity was detected either by the flow detector described above or by liquid scintillation spectrometry. Labeled samples separated by paper chromatography or paper electrophoresis were quantified by cutting the paper strips in 1-cm pieces which were then soaked with 1 ml of water in the counting vial before the addition of scintillation medium (4 ml of Packard Emulsifier Scint 299).

RESULTS
Characterization of Intact Polysaccharide

Macromolecular Properties—Gel chromatography on Sepharose 4B of high-density, ³²S-labeled heparan sulfate proteoglycan from Reichert’s membrane showed a major peak (Kᵥ 0.34) and, in addition, a shoulder corresponding to smaller components (Fig. 2A). Alkaline treatment of this material resulted in a pronounced shift of the elution profile to a position (Kᵥ 0.65), indicating single polysaccharide chains. In contrast, the same material was only marginally affected by digestion with Pronase (Fig. 2A). Similar digestion of heparan sulfate proteoglycan derived from cultured rat hepatocytes yielded single polysaccharide chains only, as indicated by the nearly identical elution patterns obtained with alkaline-treated and with Pronase-digested material (Fig. 2B). These results suggest that the heparan sulfate proteoglycan from Reichert’s membrane is relatively resistant to proteolytic degradation. The product obtained on digesting the corresponding EHS proteoglycan with Pronase showed a peak elution position intermediate to those of the intact proteoglycan and the single heparan sulfate chains (not shown).

Ion-exchange chromatography of polysaccharide chains, released from proteoglycans by alkaline treatment, yielded essentially symmetrical peaks that emerged somewhat retarded compared to standard chondroitin sulfate, yet clearly before heparin (Fig. 3). The elution position of heparan sulfate isolated from Reichert’s membrane (Fig. 3A) did not differ significantly from that of EHS polysaccharide (Fig. 3B). The polysaccharides derived from the low-density proteoglycans (not shown) appeared slightly before those of the high-density proteoglycans (in Fig. 3).

Affinity for Antithrombin—³²S-Labeled heparan sulfate proteoglycans from Reichert’s membrane and from EHS tissue were treated with alkaline to release heparan sulfate chains. The labeled chains were mixed with unlabeled heparin and were then subjected to affinity chromatography on a column of antithrombin-Sepharose that was eluted with a linear salt gradient. The heparin preparation was separated into approximately one-third of high-affinity and two-thirds of low-affinity components (Fig. 4), as is commonly seen with commercially available heparin preparations (see Björk and Lindahl, 1982). In contrast, about 80% of the polysaccharide chains from the high-density heparan sulfate proteoglycan of Reichert’s membrane (Fig. 4A) and ~70% of the chains from the corresponding low-density proteoglycan (not shown) emerged
isolated from Reichert’s membrane
glycans from Reichert’s membrane
affinity for antithrombin, the elution
alkali treatment of the high-density proteoglycan, the elution
on the other hand, showed largely low affinity for the immo-
chains.
the high-density heparan sulfate proteoglycan which were eluted with a salt gradient as described under “Methods.” Fractions of 3 ml were collected and analyzed for radioactivity (O) and for hexuronic acid (carbazole reaction; — —), LiCl concentration.

as retarded as or even later than the high-affinity component of the heparan standard. The heparan sulfate from EHS tissue, on the other hand, showed largely low affinity for the immobilized antithrombin, although ~30% of the material appeared at an elution position intermediate to the high-affinity and low-affinity heparin components (Fig. 4B; chromatogram of polysaccharide from high-density proteoglycan, the elution profile of polysaccharide from the corresponding low-density proteoglycan being essentially similar (not shown). The un-retarded peak in Fig. 4B probably represents at least partly galactosaminoglycan since this sample had not been digested with chondroitinase.

**Composition Analysis**

Heparan Sulfate from Reichert’s Membrane— Biosynthetically [35S]-labeled heparan sulfate proteoglycan, digested with chondroitinase ABC, was depolymerized by treatment with nitrous acid (pH 1.5 reaction), and the products were reduced with NaBH4. Chromatography of the deamination products obtained from the high-density proteoglycan on Sephadex G-25 (Fig. 5A) showed a major fraction (~60% of the total label) corresponding to disaccharides and inorganic sulfate, but also appreciable amounts of tetrasaccharides (~30%) and larger oligosaccharides (~10%). The corresponding pattern relating to the low-density proteoglycan (not shown) was essentially similar, with fractions of disaccharides (including inorganic sulfate), tetrasaccharides, and larger oligosaccharides constituting ~50, ~30, and ~20%, respectively, of the total [35S] label.

**Fig. 3. Anion-exchange chromatography of heparan sulfate chains.** Free [35S]-labeled polysaccharide chains were obtained by alkaline β-elimination of [35S]-labeled high-density heparan sulfate proteoglycan isolated from Reichert’s membrane (A) or from EHS (B). Samples (~20,000 cpm) were mixed with unlabeled standard hyaluronan (HA, 1 mg), chondroitin sulfate (CS, 2 mg), and heparin (Hep, 3 mg) and applied to columns (1.2 cm × 10 cm) of DEAE-cellulose (Whatman DE52) which were eluted with a salt gradient as described under “Methods.” Fractions of 3 ml were collected and analyzed for radioactivity (O) and for hexuronic acid (carbazole reaction; — —), LiCl concentration.

**Fig. 4. Affinity chromatography of heparan sulfate on antithrombin-Sepharose.** Heparan sulfate chains (~20,000 cpm), obtained by alkaline β-elimination of [35S]-labeled high-density proteoglycans from Reichert’s membrane (A) and EHS (B), were mixed with 1 mg of standard pig mucosal heparin and fractionated on a column (3 ml) of antithrombin-Sepharose as described (Thunberg et al., 1982). Effluent fractions of 2 ml were collected and analyzed for radioactivity (O) and for hexuronic acid (carbazole reaction; — —). The heparin standard is separated into fractions of low and high affinity for antithrombin, as indicated. — —, NaCl concentration.

**Fig. 5. Gel chromatography on Sephadex G-25 of products obtained on deaminative cleavage of heparan sulfate isolated from Reichert’s membrane (A) and EHS tissue (B and C).** Samples of [35S] sulfate-labeled (A and B; ~500,000 cpm; preparative runs) or [3H]glucosamine-labeled (C; ~15,000 cpm; analytical run) chondroitinase ABC-digested high-density proteoglycans were treated with nitrous acid at pH 1.5, and the products were reduced with NaBH4 and subjected to gel chromatography (see “Methods”). Effluent fractions of ~2 ml were collected, analyzed for radioactivity, and combined into pools corresponding to disaccharides (also including inorganic sulfate) and tetrasaccharides as indicated by the horizontal bars. Of the two peaks in the disaccharide/inorganic sulfate fraction in A, the most retarded one corresponds largely to inorganic sulfate and mono-O-sulfated disaccharides, whereas the less retarded peak is due to di-O-sulfated disaccharides. The inorganic [35S] sulfate accounted for 74% of the total label in this fraction as determined by high-performance ion-exchange chromatography. Elution patterns highly similar to those in A–C were obtained with polysaccharides isolated from the corresponding low-density proteoglycans (not shown). Disaccharides corresponding to the most retarded peak in C were isolated by preparative gel chromatography on Sephadex G-15 (not shown); the pooled fractions were lyophilized before further analysis.
The label recovered in di- and oligosaccharides represented the total incorporated O-[^35]S sulfate groups, whereas the released inorganic[^35]S sulfate derived from N[^35]S sulfate groups in the intact polysaccharide (Shively and Conrad, 1976). Further separation of the most low-molecular-weight fraction by ion-exchange chromatography (see below) allowed an assessment of the amount of inorganic[^35]S sulfate relative to total[^35]S sulfate incorporated and thus of the N/O-sulfate ratio of the intact polysaccharide. Such analyses showed that N-sulfate constituted ~50 and 40% of the total labeled sulfate groups of the high- and low-density heparan sulfate proteoglycans, respectively.

The di- and tetrasaccharide fractions were analyzed further by high-performance ion-exchange chromatography. The labeled Hexa-a-Mans disaccharides thus identified would correspond to various O[^35]S sulfated -GlcNSO_3-HexA-GlcNSO_3 sequences in the parent polysaccharide; whereas tetrasaccharides of the general structure, HexA-GlcNAc-GlcA-aMans, would represent -GlcNSO_3-HexA-GlcNAc-GlcA-GlcNSO_3; sequences (Shively and Conrad, 1976; Thunberg et al., 1982; Bienkowski and Conrad, 1985). The larger oligosaccharides should contain 2 or more consecutive N-acetylated disaccharide units, the internal units presumably lacking O[^35]S sulfate groups (Jacobsson and Lindahl, 1980; Gallagher et al., 1986). It should be noted that tetrasaccharides and larger oligosaccharides may be formed in the deamination reaction not only due to the presence of resistant, N-acetylated glucosamine residues, but also due to an aberrant ring contraction reaction which converts initially N-sulfated glucosamine residues into 3-aldehydo-pentose units, without cleavage of the corresponding glycosidic linkage (Shively and Conrad, 1976; Thunberg et al., 1982; Bienkowski and Conrad, 1985). Finally, it should be emphasized that the analytical procedure employed relates exclusively to incorporated[^35]S sulfate groups and thus will provide no information regarding nonsulfated saccharide sequences.

Ion-exchange chromatography showed the di- and sulfated species, IdOA(2-OSO_3)-aMans(6-OSO_3), to be the predominant disaccharide component obtained on deamination of the high-density (Fig. 6A) as well as the low-density (not in Fig. 6; see Table I) heparan sulfate proteoglycans from Reichert’s membrane; it accounted for almost half of the total O[^35]S sulfated disaccharide. In contrast, the disaccharide isomer, GlcA-aMans(3,6-di-OSO_3), was obtained in significant amounts from the high-density proteoglycan (Fig. 6A) but not from the low-density proteoglycan (Table I). Of the mono-[^35]S-sulfated disaccharide structures, IdOA(2-OSO_3)-aMans was the most abundant component, followed by GlcA-aMans(6-OSO_3) and IdOA-aMans(6-OSO_3), again with close agreement in composition between the two different proteoglycan fractions (Table I).

The tetrasaccharide fractions derived from the high- and low-density heparan sulfate proteoglycans contained appreciable amounts of mono-, di-, and tri-[^35]S-sulfated species (Fig. 6B and Table I). Major[^35]S-labeled peaks emerged together with H-labeled standards of the two tetrasaccharides, IdOA-GlcNAc(6-OSO_3)-GlcA-aMans(3-OSO_3) and IdOA-GlcNAc(6-OSO_3)-GlcA-aMans(3,6-di-OSO_3), previously identified as markers for the antithrombin-binding region of heparin (Thunberg et al., 1982). The structures of these components, in particular the occurrence of the unique 3-O-sulfated terminal 2,5-anhydromannitol residue, were ascertained in the following manner. The tetrasaccharides (derived from the high-density proteoglycan only) were N-deacetylated by hydrazinolysis as described under "Methods" and were then cleaved by deamination with nitrous acid at pH 3.9. The resulting[^35]S-labeled disaccharide products included two components with the same retention times as standard GlcA-aMans(3-OSO_3) and GlcA-aMans(3,6-di-OSO_3), respectively (Fig. 6C). The amounts of these disaccharides were in fair agreement with the predicted yields, based on the contents of the alleged parent compounds in the tetrasaccharide fraction (Table I). Both disaccharides were abolished by digestion with bovine liver β-glucuronidase, which instead yielded mono-[^35]O-sulfated and 3,6-di-[^35]O-sulfated 2,5-anhydro-
would not separate anhydromannitol (3-O- and 6-O-sulfate) in Tetrasaccharide from the appropriate amounts (Table I).

### Table I

| Source                                | Disaccharides | Tetrasaccharides |
|---------------------------------------|---------------|------------------|
|                                       | aManα-(3-OSO₂)-aManα- | IdoA-GlcNAc(6-OSO₂)-GlcA-aManβ- | Other disulfated tetrasaccharides | IdoA-GlcNAc(3-OSO₂)-GlcA-aManα(5,6-di-OSO₂) | Other trisulfated tetrasaccharides |
| High-density proteoglycan             | 16/6.7/24/ND  | 32/18/26/12       | 46/7.0                              |                                             |
| Low-density proteoglycan              | 18/8.4/27/ND  | 45/21/14/11       | 47/ND                               |                                             |
| Tetrascarbohydrate from high-density proteoglycan | (60)/27(22)/ND (18)/ND | (18)/ND            | ND/ND                                | ND/ND                                 |

- Applicable only after digestion of disaccharides with β-D-glucuronidase.
- Molar composition calculated with regard to the number of O-[35S]sulfate groups in each component.
- ND, none detected. Under the conditions of the analysis, the limit of detection for a single component corresponded to 0.3% of the radioactivity applied.
- The entire tetrascarbohydrate fraction was N-deacetylated by hydrazinolysis and was then cleaved by treatment with HNO₂ at pH 3.9 as described under "Methods." The resulting disaccharides were subjected to ion-exchange chromatography either before or after digestion with β-D-glucuronidase (digested samples are indicated in parentheses). The somewhat lower amounts of IdoA-GlcNAc(6-OSO₂) in the digested sample might be due to contaminating α-L-iduronidase.

Previous studies have identified the 3-O-sulfated glucosamine unit as a unique component of the antithrombin-binding region of heparin, present only in heparin molecules with high affinity for the proteinase inhibitor (Björk and Lindahl, 1982). One of the glucosamine residues (unit 2 in Fig. 1) of the pentasaccharide-binding region may be either N-acetylated or N-sulfated, whereas the two remaining glucosamine residues (units 4 and 6) are invariably N-sulfated (Lindahl et al., 1984). Deaminative cleavage of this sequence converts the unique 3-O-sulfated (or 3,6-di-O-sulfated) glucosamine unit 4 into a terminal 2,5-anhydromannose residue, which is recovered in a disaccharide when unit 2 is N-sulfated but in a tetrascarbohydrate when unit 2 is N-acetylated. Clearly, the tetrascarbohydrate variety predominated during the degradation products of heparan sulfate from Reichert's membrane (Table I); only a minor proportion (~10%) of the antithrombin-binding regions in the high-density proteoglycan yielded the disaccharide and thus appear to have been exclusively N-sulfated. The total amounts of 3-O-sulfate groups in the heparan sulfates were estimated from the overall yields of 3-mono-O-sulfated and 3,6-di-O-sulfated anhydromannose residues, as calculated from the gel chromatographic distribution of the degradation products and the compositional analysis of the resulting fractions. Assuming that no 3-O-sulfate groups occurred in the mono-O-sulfated tetrascarbohydrate fraction or in oligosaccharides larger than tetrascarbohydrates, the 3-O-[35S] sulfate groups would account for ~5% of the total incorporated sulfate groups or ~10% of the O-sulfate groups. These values would essentially apply to both the high- and low-density heparan sulfate proteoglycans. Due to the lack of information regarding nonsulfated regions, it is not possible to calculate the number of antithrombin-binding regions per polysaccharide chain. However, assuming a minimum average of approximately one sulfate group/disaccharide unit, which seems reasonable in view of the ion-exchange chromatography properties of the intact polysaccharide chains (Fig. 3A), we estimate that the heparan sulfate from Reichert's membrane contains a 3-O-sulfate group, and hence an antithrombin-binding site, for each 10-20 disaccharide units. The antithrombin-binding capacity of this polysaccharide thus matches or exceeds that of conventional heparin with high affinity for antithrombin.

Heparan Sulfate from EHS—The sulfation pattern of the tumor heparan sulfate differed drastically from that of the Reichert's membrane polysaccharide. Biosynthetically 35S-labeled tumor heparan sulfate, freed from chondroitin sulfate by digestion with chondroitinase ABC, was treated with nitrous acid at pH 1.5; and the products were separated by gel chromatography on Sephadex G-25. Contrary to the heparan sulfate from Reichert's membrane which yielded O-[35S]sulfated oligosaccharides and inorganic [35S]sulfate (derived from N-sulfate groups) in about equal amounts (Fig. 5A), the chromatograms relating to EHS heparan sulfate were dominated by peaks of inorganic [35S]sulfate, with only minimal amounts of labeled di- or oligosaccharides (Fig. 5B). Calculations of N/O-[35S]sulfate ratios, following more accurate determination of inorganic [35S]sulfate by high-performance ion-exchange chromatography (not shown) of the retarded gel chromatography fraction, indicated that at least 80% of the total sulfate groups in the high- and low-density tumor heparan sulfate proteoglycans were N-substituents. These N-sulfate groups accounted for ~50% of the total N-substituents, as calculated (Jacobsson et al., 1979b) from the size distribution of oligosaccharides obtained by deaminative cleavage of heparan sulfate biosynthetically labeled with [3H]glucosamine (Fig. 5C). Di- and tetrascarbohydrates each accounted for ~25% of the total labeled products, the remainder being hexascarbohydrides or larger fragments containing 2 or more consecutive N-acetylated disaccharide units.
The disaccharides isolated after deamination of the \[^{3}H\] glucosamine-labeled heparan sulfate were analyzed further by high-voltage paper electrophoresis at pH 5.3 (Fig. 7). About 70% of the disaccharides obtained from either high- or low-density proteoglycans were nonsulfated, 20–25% were mono-O-sulfated, and only ~5% were di-O-sulfated. The amounts of radioactivity incorporated into EHS heparan sulfate by the biosynthetic labeling procedures employed were too low to permit a more alternative characterization of the deamination products. Therefore, an alternative route was exploited, involving deamination of unlabeled polysaccharide at pH 1.5, followed by reduction of the products with NaBH\(_4\) (see "Methods"). The labeled disaccharides obtained showed the same separation into nonsulfated, monosulfated, and disulfated species on paper electrophoresis (not shown) as was observed for the \[^{3}H\] glucosamine-labeled sample. The nonsulfated components were isolated by preparative paper electrophoresis and were then separated further by paper chromatography along with standards (Lindahl et al., 1984) of \[^{14}C\] GlcA-aMan\(_{8}\) and IdoA-[^3]H]aMan\(_{9}\) (not shown). Quantification of the separated glucuronic acid- and iduronic acid-containing disaccharides gave molar ratios of 1.4/1 and 1.6/1 for samples derived from high- and low-density heparan sulfate proteoglycans, respectively. These ratios thus are representative for disaccharide units that lack O-sulfate substituents but are located within N-sulfated block regions of the polysaccharide chains. The O-sulfated disaccharides were quantified by high-performance ion-exchange chromatography, which showed GlcA[^3]H]aMan\(_{6}\)-(6-OSO\(_{3}\)), IdoA[^3]H]aMan\(_{6}\)-(6-OSO\(_{3}\)), IdoA(2-OSO\(_{3}\))-[^3]H]aMan\(_{6}\), and IdoA(2-OSO\(_{3}\))-[^3]H]aMan\(_{6}\)-(6-OSO\(_{3}\)) but no GlcA[^3]H]aMan\(_{3}\)-(3-OSO\(_{3}\)) or GlcA[^3]H]aMan\(_{6}\)-(3,6-di-OSO\(_{3}\)) (not shown). Similar results, albeit based on lower levels of radioactivity, were obtained with \[^{33}S\] labeled disaccharides (isolation shown in Fig. 5B). These observations indicate a ratio of glucuronic acid to iduronic acid of ~0.3/1 for the O-sulfated disaccharide units, again with no significant difference between high- and low-density proteoglycans. Finally, similar chromatography of \[^{3}H\] labeled tetrasccharides showed these components to be largely nonsulfated, with trace amounts of mono-, di-, and tri-O-sulfated species (data not shown).

![Fig. 7. High-voltage paper electrophoresis of disaccharides isolated from EHS heparan sulfate.](image)

**DISCUSSION**

The immunochemical distinction between various heparan sulfate proteoglycans reflects the structural diversity of the core proteins. The basement membrane proteoglycans isolated from EHS tissue and from Reichert's membrane share antigenic determinants that are lacking in similar species produced by other types of cells, e.g. hepatocytes (Dziadek et al., 1985; Paulsson et al., 1985). A difference in core protein structure is further indicated by the finding that the basement membrane heparan sulfate proteoglycans partially resisted digestion by Pronase, whereas the hepatocyte proteoglycan was completely degraded to single polysaccharide chains. Resistance toward proteolysis appears to be a characteristic of proteoglycans in which the protein core is densely substituted by polysaccharide chains (Robinson et al., 1978; Bourdon et al., 1985). In fact, the high-density heparan sulfate proteoglycan from Reichert's membrane appeared almost unaffected by Pronase (Fig. 2A) and thus mimicked the heparin proteoglycan from mast cells (Robinson et al., 1978).

The similarity between the heparan sulfate proteoglycan from Reichert's membrane and the heparin proteoglycan includes also the presence of the antithrombin-binding sequence in the polysaccharide chains. The occurrence of this highly specific structure in heparan sulfate from certain tissues was recently reported (Marcum and Rosenberg, 1985; Lane et al., 1986; Marcum et al., 1986) and was found to be associated with anticoagulant activity. However, in all of these preparations, the fraction with high affinity for antithrombin amounted to at most a few percent of the total heparan sulfate molecules available. For instance, ~1% of the heparan sulfate produced by cloned bovine aortic endothelial cells binds to antithrombin, yet accounts for most of the anticoagulant activity expressed by the unfractionated starting material (Marcum et al., 1986). Even in commercially available heparin preparations, only about one-third of the molecules bind with high affinity to antithrombin (Byrk and Lindahl, 1982). The heparan sulfate produced by Reichert's membrane is thus exceptional in that practically all of the material has high affinity for antithrombin. Moreover, the 3-O-sulfated glucosamine residue, which is a marker for the antithrombin-binding region, is at least as abundant in this unfractionated heparan sulfate as in the fraction of heparin molecules with high affinity for antithrombin. These findings applied to both the high- and low-density forms of the proteoglycan; in fact, the high-density heparan sulfate proteoglycan was completely degraded to single polysaccharide chains by Pronase, whereas the hepatocyte proteoglycan was completely degraded to single polysaccharide chains. Resistance toward proteolysis appears to be a characteristic of proteoglycans in which the protein core is densely substituted by polysaccharide chains (Robinson et al., 1978; Bourdon et al., 1985). In fact, the high-density heparan sulfate proteoglycan from Reichert's membrane appeared almost unaffected by Pronase (Fig. 2A) and thus mimicked the heparin proteoglycan from mast cells (Robinson et al., 1978).

The functional role of antithrombin binding is unclear. The

| Source of polysaccharide | N-Sulfate/total N-substituents | O-Sulfate/total sulfate groups | Glucosamine 3-O-sulfate/total O-sulfate groups | Fraction with high affinity for antithrombin |
|-------------------------|-------------------------------|--------------------------------|-----------------------------------------------|---------------------------------------------|
| Reichert's membrane     | ~50                           | ~10                            | >70                                           |                                             |
| EHS                     | 50                            | <20                            | ND                                            | <30                                         |

*The results apply to polysaccharides from both high- and low-density proteoglycans.*

*—, not determined; ND, none detected.*
active heparan sulfate proteoglycan on the vascular endothelium has been attributed important functions in hemostasis, and it has been suggested that it is required to provide a nonthrombogenic surface at the vessel wall (Marcum et al., 1986). It is possible that heparan sulfate in at least some types of basement membrane fulfills a similar function, preventing the deposition of fibrin in filtration processes. Indeed, whereas Reichert’s membrane is normally covered by cells, the trophoblast layer in later stages of development becomes discontinuous such that the membrane is in direct contact with the blood (Jollie, 1968). On the other hand, the functional role of the antithrombin-binding saccharide sequence is not necessarily restricted to regulation of the major hemostatic mechanism. The interaction of antithrombin with mast-cell heparin thus has been implicated in the control of inflammatory reactions (Lindahl et al., 1982). Moreover, certain marine molluscs produce a heparin-like polysaccharide which has high anticoagulant activity (Dietrich et al., 1985), high affinity for antithrombin, and a high content of 3-O-sulfated glucosamine residues (Jordan and Marcum, 1986); yet these animals do not have any blood coagulation mechanism related to that of mammals. It will be important to establish whether this high affinity for antithrombin is characteristic of heparan sulfates from basement membranes in general or a specific feature of the polysaccharide produced by Reichert’s membrane. Preliminary studies suggest that heparan sulfate synthesized by mouse mammary epithelial cells and deposited in basement membrane also contains an unusually large proportion of molecules with high affinity for antithrombin.

The heparan sulfate isolated from EHS proteoglycans differed considerably from the Reichert’s membrane polysaccharide (Table II). Whereas the N-sulfate contents were well within the range typical for heparan sulfates (cf. Gallagher and Walker, 1985), O-sulfate groups were scarce, and the amounts of glucosamine 3-O-sulfate residues, if any, fell below the limit of detection. In accord with these findings, only a minor fraction of the polysaccharide was capable of binding with high affinity to antithrombin. Again, it is possible that these features are an expression of tissue specificity and reflect the biosynthetic capacity of the basement membrane-producing cells before their neoplastic transformation. On the other hand, there are numerous examples of cell transformation resulting in the formation of a low-sulfated heparan sulfate that is no longer representative of the parent cell (see Gallagher et al., 1986, for references). Such an effect was indeed noted in a recent study comparing a basement membrane heparan sulfate produced by mammary epithelial cells with that synthesized by the corresponding transformed cells.

REFERENCES

Atha, D. H., Lormeau, J.-C., Pettou, M., Rosenberg, R. D., and Choay, J. (1985) Biochemistry 24, 6723-6729

2 G. Pejler, A. Danielsson, I. Björk, U. Lindahl, and C. P. Dietrich, unpublished data.

3 G. Pejler and G. David, unpublished data.

Bienkowski, M. J., and Conrad, H. E. (1985) J. Biol. Chem. 260, 356-365

Bittner, T., and Muir, H. M. (1962) Anal. Biochem. 4, 330-334

Björk, I., and Lindahl, U. (1982) Mol. Cell. Biochem. 48, 161-182

Bourdon, M. A., Oldberg, A., Pierchbacher, M., and Rusoizahi, E. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 1321-1325

Dietrich, C. P., dePaiva, J. F., Moraes, C. T., Taaheshi, H. K., Porcinoatto, M. A., and Nader, H. B. (1985) Biochim. Biophys. Acta 843, 1-7

Dziadek, M., Fujiiwara, S., Paulsson, M., and Timpl, R. (1985) EMBO J. 4, 905-912

Enerback, L., Kolset, S. O., Kusche, M., Hjerpe, A., and Lindahl, U. (1985) Biochem. J. 227, 661-668

Farquhar, M. G. (1981) in Cell Biology of Extracellular Matrix (Has, E. D., ed.) pp. 355-375, Plenum Press, New York

Fujiiwara, S., Wiedemann, H., Timpl, R., Luniig, A., and Engel, J. (1984) Eur. J. Biochem. 143, 145-157

Gallagher, J. T., and Walker, A. (1985) Biochem. J. 230, 665-674

Gallagher, J. T., Lyon, M., and Steward, W. P. (1986) Biochem. J. 236, 315-325

Hassell, J. R., Leyshon, W. C., Ledbetter, S. R., Tyree, B., Suzuki, S., Kato, M., Kimata, K., and Kleinman, H. K. (1985) J. Biol. Chem. 260, 8098-8105

Hook, M., Björk, I., Hopwood, J., and Lindahl, U. (1976) FEBS Lett. 66, 90-93

Jacobsson, I., and Lindahl, U. (1980) J. Biol. Chem. 255, 5084-5100

Jacobsson, I., Höök, M., Pettersson, I., Lindahl, U., Larn, O., Wiren, E., and von Figure, K. (1979a) Biochem. J. 179, 77-87

Jacobsson, I., Backström, G., Höök, M., Lindahl, U., Feingold, D. S., Malmström, A., and Roden, L. (1979b) J. Biol. Chem. 254, 2975-2982

Jollie, W. P. (1968) Am. J. Anat. 122, 513-532

Jordan, R. E., and Marcum, J. A. (1986) Arch. Biochem. Biophys. 245, 690-695

Lau, D. A., Pejler, G., Flynn, A. M., Thompson, E. A., and Lindahl, U. (1986) J. Biol. Chem. 261, 3980-3986

Lindahl, U., and Höök, M. (1978) Annu. Rev. Biochem. 47, 385-417

Lindahl, U., and Kjellman, N. (1987) in The Biology of the Extracellular Matrix: Proteoglycans (Wight, T. N., and Mecham, R., eds.) pp. 59-104, Academic Press, New York, in press

Lindahl, U., Backstrom, G., Thunberg, L., and Leder, I. G. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 6551-6555

Lindahl, U., Kolset, S. O., Bøgwald, J., Østervid, B., and Seljeld, R. (1982) Biochem. J. 206, 231-237

Lindahl, U., Thunberg, L., Backström, G., Rinnfeld, J., Nordling, K., and Björk, I. (1984) J. Biol. Chem. 259, 12368-12376

Marcum, J. A., and Rosenberg, R. D. (1985) Biochem. Biophys. Res. Commun. 126, 365-372

Marcum, J. A., Atha, D. H., Fritze, L. M. S., Naworth, P., Stern, D., and Rosenberg, R. D. (1986) J. Biol. Chem. 261, 7007-7017

Oldberg, Å., Kjellman, L., and Höök, M. (1979) J. Biol. Chem. 254, 8505-8510

Paulsson, M., Dziadek, M., Suchaneck, C., Hüttnner, W. B., and Timpl, R. (1986) Biochem. J. 231, 571-579

Paulsson, M., Fujiwara, S., Dziadek, M., Timpl, R., Pejler, G., Backstrom, G., Lindahl, U., and Engel, J. (1986) Ciba Found. Symp. 145, 383-391

Robinson, H. C., Horner, A. A., Höök, M., Ögren, S., and Lindahl, U. (1978) J. Biol. Chem. 253, 6687-6693

Roësel, J. (1980) in The Biochemistry of Glycoproteins and Proteoglycans (Lennarz, W. J., ed.) pp. 267-371, Plenum Press, New York

Shivey, J. E., and Conrad, H. E. (1976) Biochemistry 15, 3932-3942

Thunberg, L., Backström, G., and Lindahl, U. (1982) Carbohydr. Res. 100, 393-410

Timpl, R., Paulsson, M., Dziadek, M., and Fujiwara, S. (1987) Methods Enzymol., in press.