Solubilization of heparan sulfate proteoglycans from a rat liver membrane fraction was obtained by the use of the charged detergent deoxycholate or alternatively a combination of NaCl and the nonionic detergent Triton-X 100. Subsequently, proteoglycans solubilized from microsomal and plasma membrane fractions, respectively, were purified by a procedure involving gel chromatography, anion exchange chromatography, and density gradient centrifugation.

The purified heparan sulfate proteoglycan had a molecular weight of about 75,000 as determined by sedimentation equilibrium analysis or gel chromatography. Molecular weights of 17,000 to 40,000 were obtained for the ¹²⁵I-labeled core protein after removal of the heparan sulfate polysaccharide chains by different enzymatic and chemical methods. An average molecular weight of 14,000 was found for the polysaccharide chains released from the core protein by alkali treatment. The data are consistent with a proteoglycan structure containing four polysaccharide chains attached to the core protein. The amino acid composition of native and alkali-treated proteoglycan support the proposed proteoglycan model.

During the last decade a number of different cell types have been shown to contain heparan sulfate associated with their external surface (1-6). Although this finding has led to an increasing interest in this particular glycosaminoglycan, very little is known so far about the macromolecular properties of native heparan sulfate, the molecular basis for the association of heparan sulfate to cell membrane components, or the physiological significance of cell-associated heparan sulfate.

In a recent report from this laboratory we could demonstrate the presence of cell surface components which specifically bind heparin and heparan sulfate (7). Furthermore, addition of small amounts of heparin to the culture medium leads to displacement of part of the endogenous cell surface heparan sulfate from the membrane, indicating that the membrane components with affinity for heparan sulfate do in fact bind endogenous heparan sulfate to the cell surface (8, 9). In light of these discoveries, information about the macromolecular properties of cell surface heparan sulfate becomes of great importance as a multichain heparan sulfate proteoglycan could be regarded as a polyvalent ligand where each chain is capable of binding at least one "receptor."

Previous studies have demonstrated that a proteoglycan structure of heparan sulfate can be isolated from aortic tissue (10, 11) where the polysaccharide chains are linked to serine residues in a core protein via the triasaccharide galactosyl-galactose-xylose (12). Also cell-associated heparan sulfate is of proteoglycan nature (4, 13) and the isolation of a heparan sulfate proteoglycan from plasma membranes of ascites hepatoma was recently reported (14). Heparan sulfate proteoglycan has to our knowledge not before been extensively characterized. The present communication concerns the purification and characterization of a heparan sulfate proteoglycan from isolated rat liver membranes.

**EXPERIMENTAL PROCEDURES**

**Materials**

[¹²⁵I]Sulfate (carrier-free) and [¹²⁵I]iodine (carrier-free) were purchased from the Radiochemical Center, Amersham, England. H₂¹⁷O (99% purity) was obtained from Norsk Hydra Sverige AB, Stockholm, Sweden. Crystalline papain was prepared from a crude preparation (obtained from Sigma Chemical Co., St. Louis, Mo.) by the procedure of Kimmel and Smith (15). Chondroitinase ABC was obtained from Miles-Seravac, Maidenhead, England. A hepatoplasma, present in human platelets (16), was partially purified by affinity chromatography on immobilized heparan sulfate. The details of the purification procedure and the characterization of the enzyme will be reported in a separate communication. In summary, platelets lysed by repeated freezing and thawing are applied to a column of Sepharose substituted with heparan sulfate. After washing the column, the enzyme is eluted with a linear NaCl gradient from 0.04 to 0.4 M in a glycine buffer, pH 9.0. In the purified enzyme preparation no protease activity could be detected when denatured [¹²⁵I]labeled albumin was used as a substrate. Reference chondroitin sulfate polysaccharides of known molecular weight (fractions 1, 2, 3, 7, 8, 9 in Ref. 17) were kindly given by Dr. Å. Wasteson of this institute. Sprague-Dawley rats (weight 200 to 250 g) were purchased from Anticimex, Stockholm, Sweden.

**Methods**

Methods for determination of protein and uronic acid were as described (18). [¹²⁵I] Radioactivity was measured in a Nuclear Chicago model 1106 Auto-Gamma spectrometer and [³¹P] radioactivity in a Packard model 2450 liquid scintillation counter using Emulsifier E (Packard Instrument Co.) as scintillation medium.

Amino acid analyses were performed in a Beckman 121B analyzer equipped with an Autolab integrating system AA. Samples were hydrolyzed in 6 M HCl at 110°C for 24 h.

Iodination of the proteoglycan was performed with the Chloramin T method (19). Proteoglycan corresponding to 5 µg of uronic acid was isolated by passage through a column (12 x 20 mm) of Sephadex G-25 equilibrated and eluted with 0.15 M NaCl in 10 mM phosphate, pH

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1. L. Kjellén, A. Oldberg, and M. Höök, manuscript in preparation.
7.4. Labeled material eluting at the void volume of the column were further purified by anion exchange chromatography on a column (10 x 40 mm) of DEAE-cellulose eluted with a NaCl linear gradient from 0.2 to 1.0 M in 50 mM acetate buffer, pH 4.0.

Incubations of proteoglycans with chondroitinase ABC and papain were carried out as described (4). Incubations with the partially purified platelet heparitinase were performed as follows. Proteoglycan (corresponding to less than 0.1 μg of uranic acid) were incubated in 0.5 M NaOH at room temperature for 48 h. [35S]-labeled proteoglycan was incubated in 0.01 M NaOH at room temperature for 12 h. The samples were neutralized with HCl prior to analysis.

Deaminative degradation of heparan sulfate was performed according to the method of Cifonelli and King (20).

Preparation of Subcellular Fractions

Rat liver plasma membranes, labeled in vivo with [35S]sulfate, were prepared as previously described (4) except that 0.2 mM of phenylmethylsulfonyl fluoride was included in the buffer in which the livers were homogenized.

A rat liver microsomal fraction was prepared as follows. Rats (250 g) were injected intraperitoneally with 0.5 mCi Na[35S]SO4 each. Two hours later the animals were killed and the livers were taken out and prepared as previously described (4) except that 0.2 mM of phenylmethylsulfonyl fluoride was included in the buffer in which the livers were homogenized.

Table I

<table>
<thead>
<tr>
<th>Solubilizing agent</th>
<th>Heparan sulfate recovered %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 mM CaCl2, 1 mM NaHCO3, pH 7.5</td>
<td>35</td>
</tr>
<tr>
<td>+2 mM NaCl</td>
<td>65</td>
</tr>
<tr>
<td>+25 mM EDTA</td>
<td>15</td>
</tr>
<tr>
<td>+Heparin, 25 μg/ml</td>
<td>65</td>
</tr>
<tr>
<td>+1% Triton X-100</td>
<td>45</td>
</tr>
<tr>
<td>+1% Triton X-100, 2 M NaCl</td>
<td>90</td>
</tr>
<tr>
<td>0.05 M Tris-HCl, pH 8, +1% deoxycholate</td>
<td>90</td>
</tr>
</tbody>
</table>

The capacity of different agents to solubilize the heparan sulfate macromolecules in the membrane fraction was investigated. The results show (Table I) that a significant amount of labeled material was released by treatment of the membrane fraction with buffer alone. Addition of the chelating agent EDTA to the solubilizing medium did not result in any increase of solubilized heparan sulfate. Sixty-five percent of the labeled molecules was released by treatment of the membrane fraction with heparin or with NaCl at high concentration, whereas the nonionic detergent Triton X-100 caused the solubilization of somewhat less heparan sulfate. The most efficient solubilization releasing more than 90% of the labeled material was obtained with the charged detergent deoxycholate or a combination of NaCl and Triton X-100. Deoxycholate was used in the further work.

Purification of Heparan Sulfate Proteoglycans from Isolated Plasma Membranes

A purification procedure, involving gel chromatography, anion exchange chromatography, and density gradient cen-
Cell-Surface Heparan Sulfate

Effluent fractions containing $^{35}$S radioactivity were pooled as indicated in Fig. 1A and applied without prior concentration to a column of DEAE-cellulose that was subsequently eluted with a linear gradient of NaCl. The labeled material emerged as a distinct, single peak, well after the bulk of the protein (Fig. 2). The purification step resulted in a 4-fold increase in specific activity.

After concentration by ultrafiltration the pooled material was subjected to density gradient centrifugation in CsCl (Fig. 3A). Most of the labeled material was recovered in the bottom of the tube and material in fractions with a buoyant density of more than 1.55 was pooled, concentrated, and dialyzed against water in a Sartorius membrane filter apparatus. The yield of heparan sulfate proteoglycan obtained by this purification technique corresponded to 15 μg of uronic acid from 10 rats representing a 30% recovery of $^{35}$S radioactivity from the plasma membrane fraction.

Fig. 2. DEAE-ion exchange chromatography of material obtained from plasma membranes after Sepharose 6B chromatography. (The elution pattern for $^{35}$S radioactivity is virtually the same for material obtained from microsomes.) The run was performed at 4°C. A column of DEAE-Sepharose (50 ml) equilibrated with 0.1 M NaCl, 0.05 M Tris-HCl, pH 8.0, was loaded with the pooled fractions from the Sepharose 6B chromatography (see Fig. 1). The deoxycholate was washed off the column by eluting with 200 ml of 0.2 M NaCl, 0.01 M Tris-HCl, 0.1% Tween 80, pH 8.0. The $^{35}$S-labeled material was eluted with a linear gradient (500 ml) 0.2 to 1.0 M NaCl, 0.01 M Tris-HCl, 0.01% Tween 80, pH 8. Each 5-ml fraction was analyzed for $^{35}$S radioactivity (○) and $A_{291}$ (△). Fractions were pooled as indicated.

Fig. 3. Density gradient centrifugation in CsCl of material derived from plasma membranes (A) and microsomes (B). The centrifugation was performed in the presence of 4 M guanidine HCl, 0.02 M Tris-HCl, pH 8.0, after addition of solid CsCl to give a starting density of 1.50 g/ml. The centrifuge was run for 72 h at 100,000 × g and 20°C. The bottom of the centrifuge tube was punctured and 1-ml fractions were collected and analyzed for $^{35}$S radioactivity (○) and density (△).

Effluent volume (ml)

Fig. 4. Separation of microsomal heparan sulfate proteoglycan and nucleic acid on DEAE-Sepharose. Material obtained from the density gradient (Fig. 3B) was dialyzed against water and applied to a column (1 × 6 cm) of DEAE-Sepharose equilibrated with 0.2 M NaCl, 0.05 M acetate buffer, pH 4.0. The column was eluted at 18°C with a linear gradient (150 ml) 0.2 to 1.0 M NaCl in 0.05 M acetate buffer, pH 4.0. Fractions of 2.8 ml were collected and analyzed for $^{35}$S radioactivity (○) and $A_{291}$ (△). Heparan sulfate proteoglycan was pooled as indicated.

Fig. 5. Gel chromatography on Sepharose 6B of heparan [$^{35}$S]sulfate proteoglycan before (○) and after alkali (△) and papain (▲) treatment. The column (1 × 120 cm) was eluted at 18°C with 0.1% sodium lauryl sulfate, 0.05 M Tris-HCl, pH 8.0, at a flow rate of 3 ml/h. Fractions of 1.5 ml were collected and analyzed for radioactivity. Fig. 5 represents material purified from the plasma membrane fraction. Essentially identical results were obtained with proteoglycans derived from microsomal membranes (not shown).
FIG. 6. Sedimentation equilibrium centrifugation of heparan sulfate proteoglycan at different concentrations (0.44 mg/ml, C₁; 0.16 mg/ml, C₂; 0.08 mg/ml, C₃; 0.013 mg/ml, C₄). In Log fringe displacement plotted versus r² for proteoglycan in 1 M NaCl. The vertical line indicates the cell bottom. Inserted is a plot of Mᵥ versus the solute concentration. For further details, see the text.

TABLE II
Amino acid composition (expressed as residues/1000) of heparan sulfate proteoglycans isolated from rat liver microsomes

<table>
<thead>
<tr>
<th></th>
<th>Untreated</th>
<th>Alkali-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lys</td>
<td>41</td>
<td>39</td>
</tr>
<tr>
<td>His</td>
<td>12</td>
<td>13</td>
</tr>
<tr>
<td>Arg</td>
<td>21</td>
<td>31</td>
</tr>
<tr>
<td>Asp</td>
<td>195</td>
<td>119</td>
</tr>
<tr>
<td>Thr</td>
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<td>45</td>
</tr>
<tr>
<td>Ser</td>
<td>142</td>
<td>117</td>
</tr>
<tr>
<td>Glx</td>
<td>161</td>
<td>167</td>
</tr>
<tr>
<td>Pro</td>
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<td>66</td>
</tr>
<tr>
<td>Gly</td>
<td>100</td>
<td>152</td>
</tr>
<tr>
<td>Ala</td>
<td>57</td>
<td>53</td>
</tr>
<tr>
<td>Cys</td>
<td>nil</td>
<td>nil</td>
</tr>
<tr>
<td>Val</td>
<td>33</td>
<td>33</td>
</tr>
<tr>
<td>Met</td>
<td>15</td>
<td>13</td>
</tr>
<tr>
<td>Ile</td>
<td>34</td>
<td>36</td>
</tr>
<tr>
<td>Leu</td>
<td>68</td>
<td>72</td>
</tr>
<tr>
<td>Tyr</td>
<td>19</td>
<td>24</td>
</tr>
<tr>
<td>Phe</td>
<td>16</td>
<td>20</td>
</tr>
<tr>
<td>GlcNH₂</td>
<td>870</td>
<td>986</td>
</tr>
<tr>
<td>GalNH₂</td>
<td>trace</td>
<td>trace</td>
</tr>
</tbody>
</table>

*The number of glucosamine residues are expressed per 1000 amino acid residues, without correction for destruction during hydrolysis.

Purification of Heparan Sulfate Proteoglycan from a Microsomal Fraction

As only small amounts of purified heparan sulfate proteoglycan were obtained from the isolated plasma membrane fraction, a microsomal fraction was used to prepare enough of purified proteoglycan for a characterization of the macromolecule. To isolate heparan sulfate proteoglycan from the microsomal fraction the purification procedure had to be slightly modified.

The microsomal fraction obtained from 10 animals previously injected with [35S]sulfate was resuspended in 100 ml of 0.2 M NaCl, 20 mM Tris-HCl, pH 8, containing 10 g of deoxycholate and treated as described above.

The solubilized material was applied to a column of Sepharose 6B which was eluted as described. In addition to a peak of [35S]labeled material eluting at the same Kb, as the heparan sulfate proteoglycan from the plasma membrane fraction, a second peak of radioactivity was observed (Fig. 1B). The second peak which contained material of low molecular weight was not further analyzed.

The labeled material of high molecular weight was subjected to ionic exchange chromatography on DEAE-cellulose followed by density gradient centrifugation in CsCl (Fig. 3B) as described above. [35S]-labeled material was recovered at densities above 1.55 g/ml and also at the top of the tube. Such material of low buoyant density was occasionally found also in material derived from plasma membranes and was not further analyzed.

The high density heparan sulfate fraction, dialyzed as above, was finally separated from a contaminant (presumably nucleic acid) having an absorption maximum of about 260 nm by chromatography on DEAE-cellulose as shown in Fig. 4. The final yield of heparan sulfate proteoglycan using the microsomal fraction from 10 rats corresponded to 150 μg of uronic acid.

Characterization of Heparan Sulfate Proteoglycans

The purified [35S]-labeled heparan sulfate macromolecules derived from plasma membrane and microsomal fractions, respectively, were both degraded by treatment with papain or alkali (Fig. 5) indicating a proteoglycan nature of the heparan sulfate macromolecules.

The amino acid composition of the heparan sulfate proteoglycan isolated from the microsomal fraction was analyzed before and after treatment with alkali. As shown in Table II serine, glycine, glutamine (glutamic acid), and asparagine (aspartic acid) are the dominating amino acids, whereas cysteine residues are lacking. Alkaline treatment of the proteoglycan molecules results in reduction of the serine content by about 15%, but also a marked reduction of the threonine content is observed. Amino acid analyses of the proteoglycan derived from the plasma membrane fraction gave similar results (not shown).

*Gel chromatography of rat liver heparan sulfate proteoglycans previously reported by us (Fig. 5, Ref. 4) indicates a much higher molecular weight than that found in the present communication (cf. Fig. 5). The reason for this discrepancy is not known although one could speculate that extraction with guanidinium hydrochloride used in the former study might not break all hydrophobic interactions in membrane resulting in the extraction of heparan sulfate proteoglycans to which membrane components are associated. In support of this explanation solubilization by sodium lauryl sulfate of [35S]-labeled material from a freshly prepared microsomal fraction yielded no [35S]-labeled material larger in size than the isolated proteoglycan as shown by gel chromatography on a column of Sepharose 6B eluted with 0.1% sodium lauryl sulfate in 10 mM Tris, pH 8.0.
major labeled component with approximate $M$, of 30,000 (Fig. 7). When the heparan sulfate polysaccharide chains were removed by use of nitrous acid deamination, a $M$, of about 40,000 was found for the remaining 125I-labeled protein core which eluted as a homogenous peak on gel chromatography (not shown). Alkali treatment of the 125I-heparan sulfate proteoglycan yielded several labeled components; the major peak corresponded to a protein with a $M$, of 17,000 (Fig. 7).

**DISCUSSION**

Solubilization of heparan sulfate from the rat liver plasma membranes could not be obtained by incubation of the membranes with the cation chelator EDTA. This finding is inconsistent with the idea that heparan sulfate is bound to the liver cell surface by EDTA-susceptible calcium bridges, which has been suggested for fibroblastic cell systems (25). Incubation of the plasma membrane fraction with 2 M sodium chloride or heparin, treatments which are expected to break heparan sulfate-receptor interactions, led to the liberation of only 65% of the membrane-associated heparan sulfate. The remaining portion of the heparan sulfate requires detergent solubilization of the membranes to be released, which might support the suggestion of a heparan sulfate proteoglycan species that has its protein core anchored in the hydrophobic region of the membrane (26).

A procedure involving gel chromatography, ion exchange chromatography, and density gradient centrifugation was used in the purification of heparan sulfate proteoglycans from rat liver membranes. The product appeared to be reasonably pure as (a) apparent molecular weight homogeneity was observed on sedimentation equilibrium centrifugation and (b) 125I iodination of the material yielded a labeled component that was quantitatively degraded by procedures that specifically cleaves N-sulfated glycosaminoglycans. Amino sugar analysis indicated lack of galactosaminoglycans.

The data presented here suggest that cell surface heparan sulfate occurs mainly as a proteoglycan with a molecular weight of approximately 75,000. The molecular weight of the core protein determined after removal of the heparan sulfate carbohydrate is in the order of 17 to 40 x 10^3. The higher value is an upper limit and was obtained in experiments where the polysaccharide chains were degraded by nitrous acid deamination, a degradation procedure in which glucosamine residues carrying sulfamingo groups are attacked and cleaved (20). As a consequence of the specificity of this degradation method, remnants of the polysaccharide chains are left covalently attached to the core protein. These remnants which are composed of the linkage trisaccharide galactosyl-galactosyl-xylose and additional uronic acid N-acetylglucosamine disaccharide units can therefore significantly contribute to the observed molecular weight of 40,000. Also, digestion of the proteoglycan with heparitinase leaves polysaccharide remnants attached to the core protein and, hence, the observed molecular weight of 30,000 is probably too high.

The lower value was obtained in experiments where the polysaccharide was removed from the core protein by alkali treatment, a procedure in which the carbohydrate chains are

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**FIG. 7.** Gel chromatography on Sepharose 6B of 125I-labeled heparan sulfate proteoglycan before and after removal of polysaccharide chains. Samples of 125I-labeled heparan sulfate proteoglycan before (A: $\Delta$) and after heparitinase (B; $\bigcirc$) and alkali (C; $\bullet$) treatment were applied to a column (1 x 92 cm) of Sepharose 6B eluted with 4 M guanidine hydrochloride at 18°C and a flow rate of 1.5 ml/h. Fractions of 0.75 ml were collected and analyzed for radioactivity. The column was calibrated with the following proteins: IgG heavy chain (I: $M$, = 50,000), ovalbumin (II: $M$, = 43,000), IgG light chain (III: $M$, = 23,000), and lysozyme (IV: $M$, = 14,300). Blue dextran and dinitrophenylalanine were used as markers for the void volume ($V_0$) and the total volume ($V_t$) of the column, respectively. Inserted is a plot of the log $M$, versus $K_r$ for samples and calibration proteins.
Thus, the observed value of 17,000 for the polypeptide does represent a lower limit for the molecular weight of the core protein. A proteoglycan structure of $M_r$ 75,000 with a core protein of $M_r$ 17 to 40 $\times$ 10^3 can, in addition, contain a maximum of four polysaccharide chains with the observed average $M_r$ of 14,000.

Support for the proposed model of heparan sulfate proteoglycans is provided by the amino acid analyses. Assuming a molecular weight of 17,000 to 25,000 for the core protein, 4 to 5 serine residues are destroyed per molecule during alkali treatment of the proteoglycan suggesting that the hydroxyl groups of these residues are substituted with carbohydrate (i.e. heparan sulfate chains) (28). It should be noted that some threonine residues also were destroyed during alkali treatment which might indicate that also this amino acid carries carbohydrate.

The size of the cell surface heparan sulfate proteoglycan distinctly differs from that of a heparin proteoglycan which was recently reported from this laboratory (29). The heparin macromolecule had a $M_r$ of 900,000 and was composed of 10 to 15 polysaccharide chains, each with a $M_r$ of about 80,000, attached to a polypeptide largely consisting of alternating serine and glycine residues. Further work will hopefully reveal whether the difference in size between the heparin and heparan sulfate proteoglycans also is accompanied by a structural difference in the polypeptide core.

In the speculations about the physiological function of cell surface heparan sulfate, its proteoglycan properties must be taken in account. A heparan sulfate proteoglycan containing four polysaccharide chains attached to a core protein could presumably act as a polyanion ligand to the heparan sulfate receptors in the cell membrane, each polysaccharide chain binding one receptor. The receptor molecules would thus become organized within the membrane in a pattern which is determined by the shape of the proteoglycan molecule. Such a clustering of membrane receptor proteins by a polyvalent ligand has been suggested to affect the organization of intracellular actomyosin-containing filaments (30, 31).

Acknowledgments—The excellent technical assistance of Miss I. Pettersson is gratefully acknowledged. We are indebted to Drs. H. Bennich and H. Pertoft for help with the amino acid and ultracentrifugation analyses, respectively.

REFERENCES

Cell-surface heparan sulfate. Isolation and characterization of a proteoglycan from rat liver membranes.
A Oldberg, L Kjellén and M Höök


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