One of the major sulphated proteins secreted by rat hepatocytes contains low-sulphated chondroitin sulphate

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When isolated hepatocytes are incubated with $^{35}\text{SO}_4^{2-}$, a specific set of secretory proteins is labelled. One of these proteins is electrophoretically heterogeneous, with an apparent molecular mass of 35–45 kDa [Marcks von Würtemberg & Fries (1989) *Biochemistry* 28, 4088–4093]. Here we report that treatment with chondroitinase ABC converted the broad electrophoretic band of this protein, with a 50–60% loss of radioactivity, into a relatively homogeneous band with a molecular mass of 28 kDa. Size determination by gel chromatography of the protein's oligosaccharide chain (released by alkali treatment) indicated that it contained about 40 hexose units. Similar analysis of the enzyme-resistant oligosaccharide chain remaining linked to the protein after chondroitinase ABC treatment indicated a size of between six and eight hexose units. These observations suggest that the protein's oligosaccharide chain carries only three or four sulphate groups, of which one or two are located close to the polypeptide chain. Consistent with this hypothesis, the free oligosaccharide behaved like a low-sulphated glycosaminoglycan upon ion-exchange chromatography.

INTRODUCTION

Sulphation is a ubiquitous modification of secretory proteins which has attracted much attention (Huttner, 1988; Roux *et al.*, 1988). The sulphated proteins secreted by a human hepatoma cell line (HepG2) have been particularly well studied (Liu *et al.*, 1985; Hortin *et al.*, 1986). Analysis by SDS/PAGE of the medium of these cells after labelling with $^{35}\text{SO}_4^{2-}$ showed that the major part of the incorporated radioactivity was in proteoglycans, which appeared as a smear in the upper part of the gels. We have recently described the secretory proteins that are labelled in cultures of rat hepatocytes (Marcks von Würtemberg & Fries, 1989). In this system, one of the proteins that incorporated most sulphate had a molecular mass of 35–45 kDa. Here we show that this protein carries a chondroitin sulphate chain with an atypical sulphation pattern.

EXPERIMENTAL

Materials

Culture media were purchased from Labassco, Stockholm, Sweden; saponin (white pure) from Merck, Darmstadt, Germany; phenylmethanesulphonyl fluoride, *N*-ethylmaleimide and pepstatin from Sigma, St. Louis, MO, U.S.A.; antibodies to rat serum proteins from Dakopatts, Glostrup, Denmark; Blue Dextran 2000 from Pharmacia, Uppsala, Sweden; $\text{Na}_2^{35}\text{SO}_4$ from Dupont Scandinavia AB, Stockholm, Sweden; ^{14}C -methylated molecular mass standards from Amersham International, Amersham, Bucks., U.K.; heparinase (heparin lyase; EC 4.2.2.7), heparitinase, chondroitinase ABC (chondroitin ABC lyase; EC 4.2.2.4) and chondroitinase AC II (chondroitin AC II lyase; EC 4.2.2.5) from Seikagaku Kogyo Co., Tokyo, Japan. Radio-labelled chondroitin sulphate disaccharides were kindly given by Dr. M. Kusche and monosulphated chondroitin sulphate from bovine nasal cartilage and heparin by Professor U. Lindahl (both at the Swedish University of Agricultural Sciences, Uppsala, Sweden). [^3H]Hyaluronan oligosaccharides (containing

20, 30 and 50 hexose units) were given by Dr. T. Laurent of this department, and glutaraldehyde-fixed *Staphylococcus aureus* was given by Dr. N.-P. Nilsson, Pharmacia, Uppsala, Sweden.

Labelling with [^{35}S]sulphate

Cultures of rat hepatocytes, prepared as described (Persson *et al.*, 1988), were grown for 20–24 h. Each dish (35 mm diam.) containing 1×10^6 cells was rinsed twice with 1.5 ml of Dulbecco's $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free phosphate-buffered saline. Then, 1.0 ml of Eagle's minimum essential medium lacking MgSO_4 and NaHCO_3 supplemented with MgCl_2 and Hepes/HCl (pH 7.4) and containing one-tenth of the normal concentrations of methionine and cystine was added. This medium was replaced after 10 min by 1.0 ml of the above medium containing 0.25 mCi of $^{35}\text{SO}_4^{2-}$ (1200–1400 Ci/mmol) and the cells were further incubated for 1 or 2 h. In some experiments the medium also contained 50 μM unlabelled sulphate. At the end of the labelling period the medium was collected, cell debris was removed by centrifugation (5 min at 14000 g) and *N*-ethylmaleimide, phenylmethanesulphonyl fluoride and pepstatin were added (final concentrations 0.1 mM, 0.2 mM and 1 $\mu\text{g}/\text{ml}$ respectively). The cells were rinsed twice with 1.5 ml of an ice-cold buffer containing 137 mM-NaCl, 5 mM-KCl, 1.2 mM- CaCl_2 , 0.7 mM- MgSO_4 and 10 mM-Hepes/HCl (pH 7.4). The soluble proteins in the cells were released by the addition of 1.0 ml of a solution containing 1 mg of saponin/ml, 0.15 M-NaCl, 5 mM-EDTA and 50 mM-Tris/HCl, pH 8.0, supplemented with *N*-ethylmaleimide, phenylmethanesulphonyl fluoride and pepstatin as described above. The labelled media and cell extracts were frozen in liquid N_2 and stored at -70°C .

Immunoprecipitation

Rabbit antibodies to rat serum proteins were added in excess to portions of media and cell extracts. After 3–4 h on ice, the immune complexes were recovered using *S. aureus* particles as described (Persson *et al.*, 1988).

Abbreviation used: UTI, urinary trypsin inhibitor.

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Acid precipitation

RNA (250 µg) and trichloroacetic acid (final concentration 120 g/l) were added to the samples. After a 30 min incubation on ice, the acid-insoluble material was pelleted by centrifugation and washed once with trichloroacetic acid (100 g/l) and once with ice-cold 95% (v/v) ethanol.

SDS/PAGE

Slab gels (200 mm × 200 mm × 0.75 mm) with a 10–15% polyacrylamide gradient were made as described by Maizel (1971). After electrophoresis was completed the gels were prepared for fluorography according to Skinner & Griswold (1983). Finally, the gels were put on preflashed X-ray films and kept at –70 °C, typically for 7 days.

Isolation of $^{35}\text{SO}_4^{2-}$ -labelled 35–45 kDa protein

The medium (1.0 ml) from a culture of rat hepatocytes (1×10^6 cells), which had been labelled with $^{35}\text{SO}_4^{2-}$ for 2 h, was subjected to chromatography on an Ultrogel AcA 34 column eluted with buffer containing 50 mM-Tris/HCl and 0.15 M-NaCl (pH 8.0). The radioactivity in each fraction was determined by liquid scintillation counting. Protein was precipitated with trichloroacetic acid and analysed by PAGE. The remainder of the fractions containing the 35–45 kDa protein were pooled, frozen in liquid N_2 and stored at –70 °C.

Alkali treatment (β -elimination)

To release the polysaccharide chains, the isolated 35–45 kDa protein was treated with 0.5 M-NaOH and 1 M-NaBH₄ for 24 h at 45 °C (Rosenberg *et al.*, 1985). The sample was then neutralized by the addition of acetic acid and chromatographed in 50 mM-Tris/HCl (pH 8.0)/0.15-M NaCl on a Sephadex G-25 column (1 cm × 56 cm). Fractions (1.0 ml) were collected and their radioactivity was measured.

Enzymic digestions

Immune complexes attached to *S. aureus* particles were washed by centrifugation in 300 µl of a buffer containing 50 mM-Tris/HCl, 50 mM-sodium acetate, 50 µg of BSA/ml, 1 mM-EDTA, 0.1 mM-N-ethylmaleimide, 0.2 mM-phenylmethanesulphonyl fluoride and 1 µg of pepstatin/ml (final pH 7.0). Then, 50 µl of the same buffer containing SDS (3 mg/ml) was added and the bacteria were suspended. After a 3 min incubation at 95 °C, the bacteria were removed by centrifugation, and chondroitinase ABC (25 munits) was added in 50 µl of the above Tris/acetate buffer supplemented with Triton X-100 (100 g/l). As a control, heparinase (4 units) or heparitinase (0.5 units) in 50 µl of 0.1 M-sodium acetate buffer containing 3 mM- CaCl_2 , 0.2 M-phenylmethanesulphonyl fluoride, 0.1 mM-N-ethylmaleimide and 1 µg of pepstatin/ml (pH 5.5) was added to identical samples. After a 4 h incubation at 37 °C the proteins were recovered by acid precipitation and analysed by SDS/PAGE.

Samples from gel chromatography, containing protein or free oligosaccharide chains, were mixed with chondroitinase ABC (25 munits) in a total volume of 600 µl containing 50 mM-Tris/HCl, 50 mM-sodium acetate, 0.15 M-NaCl, 60 µg of BSA/ml, 1 mg of monosulphated chondroitin sulphate/ml, 1 mM-EDTA, 0.1 mM-N-ethylmaleimide, 0.2 mM-phenylmethanesulphonyl fluoride and 1 µg of pepstatin/ml (final pH 8.0) and incubated for 20 h at 37 °C. Alternatively, samples containing free oligosaccharide chains were mixed with either 25 munits of chondroitinase ABC or 50 munits of chondroitinase AC II in the above buffer (also containing 1 mM-NaF) and incubated for 2 h

at 37 °C. The samples from these digestions were then analysed by gel chromatography.

Paper electrophoresis

Charged components released by chondroitinase treatment of the intact protein or of the free oligosaccharide chains were separated by high-voltage paper electrophoresis essentially as described by Kusche *et al.* (1988). The appropriate fractions from gel chromatography were freeze-dried, dissolved in 100 µl of distilled water and applied in one spot in 5 µl portions to a strip of Whatman 3 MM paper. The electrophoresis was performed in 1.6 M-formic acid (pH 1.7) at 36 V/cm for 80 min. ^3H -labelled mono- and di-sulphated disaccharides (Kusche *et al.*, 1988) and free $^{35}\text{SO}_4^{2-}$ were used as standards. After electrophoresis, the paper was dried, sprayed with 2,5-diphenyloxazole (200 g/l) in acetic acid, put on a pre-exposed X-ray film and kept at –70 °C for about 7 days. The amounts of radioactivity in the different spots along each lane were determined by densitometric scanning.

RESULTS

When hepatocytes are incubated with $^{35}\text{SO}_4^{2-}$, most of the label that is incorporated into acid-insoluble material is in high-molecular-mass proteoglycans. Upon gel electrophoresis, these components appear as a smear in the upper part of the gel, which obscures many protein bands. With the use of antibodies against rat serum proteins, however, most of the labelled proteins can be recovered with little co-precipitation of the interfering proteoglycans (Marcks von Würtemberg & Fries, 1989). Furthermore, the major hepatic proteoglycan, which contains heparan sulphate, occurs predominantly in a membrane-bound form (Kjellén *et al.*, 1981) and can therefore be eliminated if a saponin rather than a detergent extract of the cells is used. The left-hand lane of Fig. 1 shows sulphate-labelled cellular proteins obtained by saponin extraction followed by immunoprecipitation. The second

Fig. 1. Sensitivity to chondroitinase ABC of $^{35}\text{SO}_4^{2-}$ -labelled secretory proteins from rat hepatocytes

Monolayer hepatocyte cultures were incubated for 1 h with $^{35}\text{SO}_4^{2-}$. Antibodies against rat serum proteins were then added to portions of the medium (m) or to saponin extracts of the cells (c). The immunoprecipitates were collected, dissociated and incubated with (+) or without (–) chondroitinase ABC. The proteins were finally analysed by SDS/PAGE. A fluorogram of the dried gel is shown. The positions of the chondroitinase-sensitive protein in the gel before and after enzyme treatment are indicated by a and b respectively. The positions of ^{14}C -methylated standard proteins are indicated on the left.

Fig. 2. Gel chromatography of ³⁵SO₄²⁻-labelled secretory proteins

Medium from rat hepatocytes labelled for 2 h with ³⁵SO₄²⁻ was chromatographed on an Ultrogel AcA 34 column (1 cm × 85 cm) and the radioactivity in each fraction (1.3 ml) was determined (a). The elution position of Blue Dextran 2000 is indicated by V₀ (31 ml). The total volume (72 ml, not shown) was determined using ³H₂O. The acid-insoluble material in the indicated fractions was analysed by SDS/PAGE followed by fluorography (b).

lane shows the ³⁵SO₄²⁻-labelled proteins secreted into the medium obtained by immunoprecipitation. The most apparent difference between the two patterns is the occurrence in the medium sample of a broad diffuse band in the 35–45 kDa region (a). This component can also be seen in the cell sample, but here it is much weaker.

The third and the fourth lanes in Fig. 1 show the effects of treating cell and medium samples with chondroitinase ABC, an

Fig. 3. Size determination of the oligosaccharide chain released from the 35–45 kDa protein

The ³⁵SO₄²⁻-labelled protein was isolated as described in the legend to Fig. 2. The oligosaccharide was released by β-elimination and purified by gel filtration as described in the Experimental section. The chain was then chromatographed on a Sephadex G-75 column (1 cm × 91 cm) and the radioactivity in the fractions was determined. HA₅₀, HA₃₀ and HA₂₀ denote the elution positions of hyaluronans consisting of 50, 30 and 20 hexose residues respectively.

enzyme which degrades chondroitin and dermatan sulphate chains to disaccharides. The most dramatic effect of the enzyme is seen in the medium sample, where the diffuse 35–45 kDa band disappears and a new 28 kDa double band (band b) appears. In the cell samples the same conversion can be detected, although the intensities of the corresponding bands are much lower. Densitometric scanning of the fluorogram indicated that band b contained 40–50% of the radioactivity of band a. Treatment with heparinase and heparitinase did not result in any changes in the electrophoretic pattern (results not shown). (It is unlikely that these enzymes were inactivated by the SDS present in the reaction mixture because it also contained a 3-fold excess of Triton X-100.)

For further analysis of the chondroitinase-sensitive protein, it was separated from the other major labelled proteins by gel chromatography. Fig. 2(a) shows the radioactivity profile obtained when medium from ³⁵SO₄²⁻-labelled cells was chromatographed on Ultrogel AcA 34. Three peaks were obtained, with K_{av} values of 0.1, 0.3 and 0.45. Calibration of the column indicated that the latter two peaks contained proteins of about 200 and 110 kDa respectively. Fig. 2(b) shows the fluorogram of an electrophoresis gel used for the analysis of the acid-precipitated

Fig. 4. Gel chromatography of chondroitinase ABC- or AC II-treated oligosaccharides released from the 35–45 kDa protein

The oligosaccharide chain released from the 35–45 kDa protein was described in Fig. 3, and portions of this material were treated with chondroitinase ABC for 2 h (a) or 20 h (b) or with chondroitinase AC II for 2 h (c). The digests were then chromatographed on a Sephadex G-25 column (1 cm × 50 cm) and the radioactivity in the fractions was determined. The asterisks indicate the fractions subsequently analysed by paper electrophoresis.

Fig. 5. Gel chromatography of oligosaccharides released from protein after chondroitinase ABC treatment

The protein was prepared as described in the legend to Fig. 2 and treated with chondroitinase ABC and low-molecular-mass components were removed by chromatography on a short Sephadex G-25 column (1.5 cm $\times$ 5 cm). The oligosaccharide bound to the protein was then released by β -elimination and the reaction mixture was applied to a Sephadex G-75 column (1 cm $\times$ 91 cm). The radioactivity in each fraction was determined. HA₂₀ denotes the elution position of hyaluronan consisting of 20 hexose residues.

proteins in the fractions. This analysis showed that the material eluting close to the void volume ($K_{av} = 0.1$) hardly entered the separating gel. The adjacent peak ($K_{av} = 0.3$) contained three of the major sulphated proteins, with apparent molecular masses of 190, 150 and 130 kDa. We have recently found that the 190 kDa protein is α_1 -inhibitor₃ and that the 150 kDa band is formed from this protein through autolytic cleavage during sample preparation (E. M. Sjöberg & E. Fries, unpublished work). The next peak ($K_{av} = 0.45$) contained the 35–45 kDa protein as the major component; the contribution of other radioactive proteins was less than 5%.

To determine the size of the chondroitinase-sensitive carbohydrate, the 35–45 kDa protein was treated with alkali to release glycosaminoglycans by β -elimination. Low-molecular-mass components were removed by chromatography on a short Sephadex G-25 column (results not shown). Subsequent analysis of the main radioactive product, which constituted about 90% of the total radioactivity, on a Sephadex G-75 column yielded one symmetrical peak (Fig. 3), indicating that all sulphate groups were linked to one type of oligosaccharide chain. Comparison with hyaluronans of known size (as indicated) suggested that the sulphated chain contained 37–40 (non-sulphated) hexose units, corresponding to a molecular mass of 7.5–8.0 kDa.

To verify that the effect of the chondroitinase ABC treatment was specific, i.e. that disaccharides were formed, the isolated oligosaccharide chain was treated with the enzyme for 2 h and the products were analysed by chromatography on Sephadex G-25. Fig. 4(a) shows that some of the radioactive material was excluded from the gel and some was eluted with a K_{av} of about 0.6, the value also observed for isolated monosulphated disaccharide (results not shown). However, free sulphate ions were not separated from the disaccharide on this column. To determine the relative amounts of these two components, the fraction indicated with an asterisk in Fig. 4(a) was analysed by paper electrophoresis, which showed that 60% of the radioactivity migrated as a monosulphated disaccharide and the remainder as free sulphate. The radioactivity profile in Fig. 4(a) also indicated the presence of an intermediate size component ($K_{av} = 0.2$). If the enzymic reaction was prolonged, the amount of excluded radioactive material decreased (Fig. 4b). A distinct peak with a

Fig. 6. Ion-exchange chromatography of the oligosaccharide chain

A sample containing the $^{35}\text{SO}_4^{2-}$ -labelled oligosaccharide chain (isolated as described in the legend to Fig. 3), and ^3H -labelled hyaluronan (10 kDa), chondroitin sulphate (1.3 mg) and heparin (1.0 mg) was applied to a DEAE-Sephadex CL-6B column (1.5 cm $\times$ 2.5 cm) in a buffer containing 0.05 M-sodium acetate and 0.05 M-LiCl (pH 4.0). Elution was with a linear gradient of 160 ml of 0.05–1.5 M-LiCl in 0.05 M-sodium acetate buffer (pH 4.0). The fractions (2.7 ml) were analysed for uronic acid (Bitter & Muir, 1962) and radioactivity. The peak fractions of hyaluronan (HA), chondroitin sulphate (CS) and heparin (HN) are indicated.

K_{av} of 0.2 became apparent, corresponding to an oligosaccharide of between four and eight hexose units (calibration not shown). Under these conditions, however, most of the low-molecular-mass material was in the form of sulphate.

Chondroitinase ABC degrades both chondroitin sulphate and dermatan sulphate chains into disaccharides, whereas chondroitinase AC does not degrade the latter type (Hiyama & Okada, 1975). To see if the oligosaccharide chain contained dermatan sulphate, we treated it with chondroitinase AC II and separated the reaction products on a Sephadex G-25 column (Fig. 4c). Clearly, most of the oligosaccharide was degraded by this enzyme. Subsequent analysis of the low-molecular-mass material by paper electrophoresis showed that 95% of the radioactivity migrated as a monosulphated disaccharide and the remaining 5% as free sulphate. Thus the oligosaccharide chain seems to be of the chondroitin sulphate type.

It has been shown that, upon treatment of chondroitin sulphate chains with chondroitinase ABC, the six hexose units linking the chain to the protein remain intact (Hascall *et al.*, 1972). To see if the radioactive material eluting with a K_{av} of 0.2 (Fig. 4b) might be identical with this segment of the oligosaccharide, the 35–45 kDa protein was treated with this enzyme. Low-molecular-mass material was then removed by gel filtration and the remaining oligosaccharide was released by β -elimination. Analysis on a Sephadex G-25 column yielded a K_{av} of 0.2 for the main component (results not shown). To obtain an additional estimate of the size of the oligosaccharide, we also chromatographed it on a Sephadex G-75 column (Fig. 5), which showed that the oligosaccharide was slightly smaller than a hyaluronan containing 8 hexose units. Part of the radioactivity was eluted with the total volume, indicating that some of the sulphate groups were released from the oligosaccharide during the β -elimination.

Ion-exchange chromatography may be used to assess the degree of sulphation of glycosaminoglycans; for a given chain length, the ionic strength required for elution will depend on the degree of sulphation (Humphries *et al.*, 1986). To exploit this fact, we applied the free $^{35}\text{SO}_4^{2-}$ -labelled chondroitin sulphate chain to a DEAE-Sepharose column together with a hyaluronan of 10 kDa, a monosulphated chondroitin sulphate of 8 kDa and

heparin (see Fig. 6). Upon elution with a LiCl gradient, the hyaluronan appeared first, closely followed by the $^{35}\text{SO}_4^{2-}$ -labelled chondroitin sulphate. The chondroitin sulphate and the heparan sulphate chains were eluted at higher salt concentrations.

DISCUSSION

In this paper we show that one of the major sulphated proteins secreted by rat hepatocytes contains chondroitin sulphate. Earlier studies have shown that, when isolated rat hepatocytes are incubated with $^{35}\text{SO}_4^{2-}$, most of the label is incorporated in heparan sulphate (Prinz *et al.*, 1980; Ninomiya *et al.*, 1981). In agreement with those studies, we found that when samples of detergent-solubilized cells were analysed by gel electrophoresis, most of the radioactive material hardly entered the separating gel (Marcks von Würtemberg & Fries, 1989), a property characteristic of the heparan sulphate proteoglycan of hepatocytes. However, we also found that little of this proteoglycan was released into the medium. This finding is in agreement with earlier observations, both *in vitro* and *in vivo*, that (low sulphated) chondroitin sulphate is the major glycosaminoglycan released from rat hepatocytes (Hata & Nagai, 1978; Ninomiya *et al.*, 1981).

The synthesis of chondroitin sulphate in rat hepatocytes seems to be under developmental control; it decreases dramatically during and shortly after birth (Gressner & Vasel, 1985) and is greater in transformed than in normal hepatocytes (Kojima *et al.*, 1982). We therefore considered the possibility that the synthesis of the chondroitin sulphate proteoglycan in isolated rat hepatocytes was induced by the isolation of the cells. To determine the pattern of proteins labelled in intact liver, $^{35}\text{SO}_4^{2-}$ -containing medium was circulated for 20 min immediately after the liver was removed from the animal; essentially the same protein pattern was obtained as with the isolated hepatocytes (E. M. Sjöberg & E. Fries, unpublished work).

The cell suspension obtained from rat liver after collagenase perfusion contains different types of hepatic cells (reviewed by Pertoft & Smedsrød, 1987). We isolated the parenchymal cells (hepatocytes) by differential centrifugation followed by density gradient centrifugation in colloidal silica (Percoll). Although this procedure yields parenchymal cells with little contamination by other cell types (Pertoft & Smedsrød, 1987), the possibility could not be excluded that specific proteins were produced exclusively by a small number of contaminating non-parenchymal cells. In fact, it has recently been shown that the so-called stellate cells, or lipocytes, produce 6-fold more glycosaminoglycans per cell in culture than do hepatocytes (Arenson *et al.*, 1988). Therefore we were concerned that the sulphated proteins we observed might have originated from these cells. To test this possibility, we analysed the incorporation of $^{35}\text{SO}_4^{2-}$ into proteins by cells in fractions enriched with the lipocytes; these cells can be partially separated from the parenchymal cells on account of their lower density (Pertoft & Smedsrød, 1987). We found that the incorporation of $^{35}\text{SO}_4^{2-}$ by the lighter cells was far less than by the same number of hepatocytes (E. M. Sjöberg & E. Fries unpublished work).

Chondroitinase ABC degrades chondroitin sulphate chains to disaccharides but leaves the innermost six sugar residues (Xyl-Gal-Gal-GluA-GalNAc-GluA) intact. Of these, the GalNAc and possibly one of the Gal residues may be sulphated (Sugahara *et al.*, 1988). Therefore hydrolysis of a $^{35}\text{SO}_4^{2-}$ -labelled chondroitin sulphate chain by chondroitinase ABC will yield both radioactive disaccharides and a radioactive hexasaccharide (Hascall *et al.*, 1972). Our analysis of the protein described in this paper indicated that these compounds were formed upon enzyme treatment. Furthermore, we found that the resistant oligosaccharide con-

tained about one-third of the total radioactivity and that the whole chondroitin sulphate chain contained about 40 hexose residues. These findings imply that most of the disaccharide units were unsulphated. This conclusion was supported by the observation that, upon ion-exchange chromatography, the chondroitin sulphate chain behaved like an unsulphated hyaluronan of similar size (Fig. 6). To our knowledge such a distribution of sulphate groups has not been documented previously.

Hascall *et al.* (1972) have shown that chondroitinase AC I degrades chondroitin sulphate chains, including the innermost GalNAc-GluA repeat. When the $^{35}\text{SO}_4^{2-}$ -labelled chondroitin sulphate chain was treated chondroitinase AC II, whose effect on the innermost residues has not been studied, little if any radioactive oligosaccharide was formed. This finding indicates that the latter enzyme also removes the innermost disaccharide repeat and that this segment of the chain carries a substantial part of the sulphate groups.

The amount of radioactive sulphate incorporated into hepatocytes was found to decrease rapidly with increasing sulphate concentration in the medium (E. M. Sjöberg & E. Fries, unpublished work). For practical reasons we therefore labelled the cells in media with a low sulphate concentration (50 μM) or no sulphate. Studies with cartilage tissue and aortic endothelial cells have shown that, in the absence of sulphate, otherwise fully sulphated chondroitin sulphate may be almost unsulphated (Ito *et al.*, 1982; Humphries *et al.*, 1986). In the presence of $\geq 40 \mu\text{M}$ sulphate, however, a major part of the $^{35}\text{SO}_4^{2-}$ -labelled chondroitin sulphate in aortic endothelial cells was observed to behave as fully sulphated chondroitin sulphate (Humphries *et al.*, 1986). To see if the sulphation pattern of the 35–45 kDa protein was affected in a similar fashion, we labelled the cells in media containing no or 50 μM sulphate. The chondroitin sulphate chain was then isolated and analysed by ion-exchange chromatography. In both cases the radioactivity eluted as one peak, slightly after the hyaluronan standard. The results from the enzyme digestion of chains isolated from cells labelled under the two different conditions also seemed to be identical.

A urinary trypsin inhibitor (UTI), isolated from human urine, has properties very similar to those of the protein described in this paper. UTI has a molecular mass of 40–45 kDa (Tanaka *et al.*, 1982), which is decreased to 26 kDa upon chondroitinase ABC treatment (Balduyck *et al.*, 1986). It contains two sulphate groups and apparently about 20 GluA-GalNAc repeats (Balduyck *et al.*, 1986; Selloum *et al.*, 1987). No murine equivalent to UTI has been isolated, and antibodies against the human UTI do not precipitate a sulphated protein of the expected molecular mass from the medium of isolated rat hepatocytes (E. M. Sjöberg & E. Fries, unpublished work). Sequencing of clones from a human liver cDNA library (Kaumeyer *et al.*, 1986) has shown that UTI is linked via two arginine residues to α_1 -microglobulin, a 25 kDa plasma protein. Based on kinetic studies, we have previously suggested that an intracellular sulphated protein of 60–80 kDa might be the precursor of the 35–45 kDa protein (Marcks von Würtemberg & Fries, 1989). Consistent with this idea, we have found that antibodies against rat α_1 -microglobulin precipitate this larger protein (E. M. Sjöberg & E. Fries, unpublished work). Apparently this is the fully glycosylated precursor of α_1 -microglobulin and rat UTI, which is cleaved shortly before externalization.

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