# HIGH MOLECULAR-WEIGHT HEPARAN SULFATE FROM THE CELL SURFACE

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SUMMARY: Heparan sulfate fragments with molecular weight of 135,000 (as determined by equilibrium sedimentation analysis) were isolated from the trypsinate of Chinese hamster cells (line CHO) grown in culture. Evidence is presented which suggests that the intracellular heparan sulfate species with molecular weight of 10,000 to 20,000 were degradation products of the larger species. We propose that the native cell-surface heparan sulfate, in its physiological location, could serve as a nonspecific "screen" to the exposure of specific, topographically adjacent, cell-surface sites.

## INTRODUCTION

Heparan sulfate appears to be a generally occurring cell-surface molecule on cultured animal cells (1-3). We have shown previously that the cell-surface heparan sulfate of Chinese hamster cells in culture (line CHO) has an independent metabolism with respect to other cell-surface glycopeptides and is selectively released into the medium just prior to mitosis (4). We have now further characterized the cell-surface heparan sulfate and find that it is a much larger molecule than the bulk of the intracellular heparan sulfate and is, in fact, much larger than previously reported estimates of heparin and heparan sulfate from several sources.

## METHODS

Many of the materials and methods used in this work have been described previously (1,3). Bio-Gel A-0.5m and A-5m column chromatography was done with 1.2 x 130-cm columns equilibrated and eluted with 1.0 M ammonium acetate, pH ~7.0; flow rates were ~24 ml/hr, and 2-ml fractions were collected. CHO cells were prelabeled for 16 hr with 0.1 μCi/liter glucosamine-6-$^3$H (specific activity 3.6 Ci/mM, New England Nuclear). Heparan sulfate degradations with alkaline borohydride were done with 0.5 M NaOH, 1 M $NaBH_4$, room temperature, 24 hr; with 1 mg/ml Pronase, pH 7.5, 37°C, 13 hr; or with 1 mg/ml testicular hyaluronidase, pH 5.5, 37°C, 13 hr. Neutral sugar analyses were done after hydrolysis of the sample (2 N HCl, 100°C, 3 hr) and trimethylsilylation on a stainless steel column containing 15% HI-EFF 2 BP on 80/100 C-W using a Model 600-D Varian Aerograph. Equilibrium sedimentation analyses were done by the meniscus depletion method (5) using interference optics. Fractions to be analyzed were first dialyzed against 1 M NaCl. Plots of the logarithm of fringe displacement (concentration) against $r^2$ (r is the distance to the axis of rotation) were used to calculate molecular weight.

## RESULTS

Heparan sulfate was isolated and identified from three compartments of CHO cells following similar procedures to those reported previously (1). The three portions of the total heparan sulfate were designated as (a) *cell-surface heparan sulfate* (i.e., heparan sulfate recovered as acid-soluble fragments in the supernatant fluid following trypsin treatment of the cells under conditions where no irreversible cell damage occurred); (b) *cell sap heparan sulfate* (this material could be directly isolated in the acid-soluble fraction of whole cells following trypsin treatment or in the acid-soluble fraction of the supernatant of broken cells centrifuged at 105,000 x g for 1 hr); and (c) *internal membrane-associated heparan sulfate* (this was isolated as acid-soluble fragments following trypsin digestion of the acid precipitate of whole cells that had been first treated with trypsin to remove the surface heparan sulfate). Thus, each of the three heparan sulfate fractions was acid-soluble as isolated and characterized. As previously reported (1), the isolation and identification were based on characteristic elution of material from cells labeled with glucosamine-$^{3}H$ (and, in some cases, inorganic $^{35}SO_4$) from DEAE-cellulose columns, and the subsequent formation of characteristic nitrous acid degradation products was monitored by Bio-Gel P-10 chromatography.

In previous work (1), membrane-associated species had also been papain-digested; under those conditions, the elution profiles on Bio-Gel P-10 were similar for all three species and suggested that all three behaved in a manner similar to authentic bovine heparan sulfate of molecular weight 12,000 daltons. However, in the present report, papain was not used because we have found that the buffer solution alone (0.005 M cysteine-HCl, 0.001 M EDTA, 0.1 M ammonium acetate) produces random oxidation-reduction breaks in the polymer by a similar mechanism to that reported for ascorbic acid (6). We now report that the molecular weight of the tryptic fragments of heparan sulfate of the cell surface, in fact, are much larger than the other species. Figure 1 illustrates the elution profiles of the three varieties of isolated heparan sulfate run on Bio-Gel A-0.5m columns. Each variety included some material that was retained in a manner similar to that previously reported (i.e., with molecular weight similar to bovine heparan sulfate of 12,000 daltons). In addition, however, the cell-surface heparan sulfate included material excluded from Bio-Gel A-0.5m. When the excluded material was isolated and treated with nitrous acid, all radioactivity eluted from Bio-Gel P-10 as characteristic oligosaccharides of heparan sulfate. Figure 2 illustrates chromatography profiles of this high molecular-weight surface heparan sulfate on Bio-Gel A-5m. In this case, the untreated tryptic fragments (Fig. 2A control) behave as an

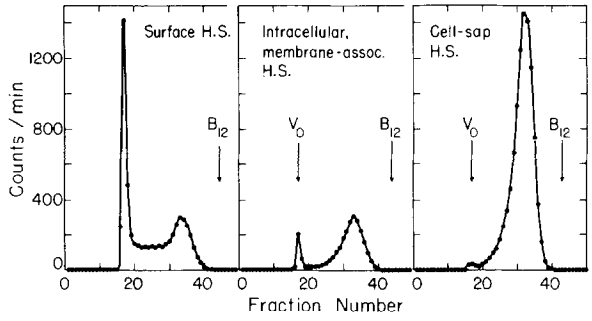


Fig. 1. Elution profiles on Bio-Gel A-0.5m of heparan sulfate isolated from various cell fractions.

extremely heterogeneous distribution that includes molecules excluded from the gel. Further treatment with either or both Pronase or alkali (Fig. 2B-D) reduced the apparent modal size of the fragments to approximately the same degree. Under these conditions and unlike results previously reported for papain-digested material, the fragments produced by Pronase or alkaline borohydride treatment were almost completely excluded from Bio-Gel P-10. This shows that the sugar chains of surface heparan sulfate, with or without a small residual peptide component, are much larger than the bulk of the intracellular heparan sulfate.

We reported earlier (7) that the Bio-Gel P-10 elution profile of cell sap heparan sulfate was unaffected by prior treatment of the material with testicular hyaluronidase (EC 3.2.1.35). Since this enzyme cleaves the β-hexosamine 1 → 4 glucuronic acid linkage, such a result was consistent with expectation. However, as illustrated in Fig. 2E, testicular hyaluronidase treatment resulted in considerable degradation, yielding fragments that eluted over a wide range. Other experiments indicated that the degree of degradation was achieved within 3 hr (1.0 mg/ml, pH 5.5, 37°C) and that further incubation or additional enzyme had no further effect. When the testicular hyaluronidase-treated material was examined by Bio-Gel P-10 chromatography, the bulk of radioactivity was still excluded. Under the same conditions, hyaluronic acid or chondroitin sulfate from these cells was completely degraded to small oligosaccharides. These findings suggest that the large tryptic fragment of the cell surface contained unusual linkages that were absent in the smaller molecules associated with internal membranes or free in the cell sap. We wonder whether these represent branch points in the larger structure.

We have been unable to detect any residual peptide in the purified cell

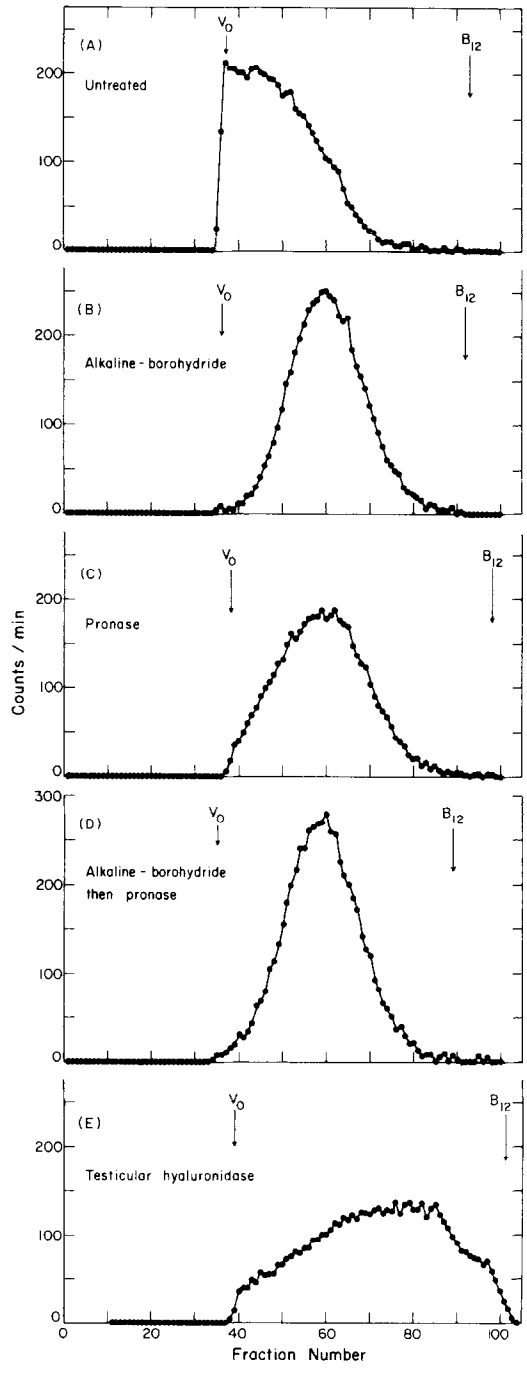


Fig. 2. Elution profiles on Bio-Gel A-5m of the tryptic fragments of cell-surface heparan sulfate, either untreated (A) or after various treatments (B-E).

sap heparan sulfate, whereas the material isolated from internal membranes was clearly a glycopeptide. Analyses for neutral sugars by gas-liquid chromatography were also distinctive: the xylose and galactose content of material from internal membranes was 0.78 and 2.22%, respectively. Under the same conditions, xylose and galactose were not detectable with cell sap heparan sulfate. If one assumes one xylose and two galactose residues per sugar chain linked to peptide and a sulfate content of 0.6 per hexosamine (7), the above values would yield an average chain length of about 40 sugars or about 18,000 daltons.

The molecular weight of trypsin-removed heparan sulfate of the cell surface appeared to range from molecules of about the same size as the major internal membrane heparan sulfate fragments to molecules that were excluded from Bio-Gel A-5m columns. To gain an estimate of the size of the molecules at the upper end of the range, the upper third of the A-5m range illustrated in Fig. 2A was isolated and studied by sedimentation equilibrium analysis. Figure 3 shows a plot of the data of the largest material obtained from the A-5m column. Analyses were done at two different concentrations and centrifuge speeds. The average molecular weight was 135,000. After alkaline borohydride treatment, the reisolated sugar chains had a molecular weight of 44,000. Comparable estimates for the middle third of the Bio-Gel A-5m size distribution were 40,000 and 27,000 daltons before and after alkaline borohydride treatment. Partial specific volumes used in the calculations were 0.47 (8) for the sugar chains isolated after alkaline borohydride treatment and 0.60 for the tryptic fragments. The latter value was based on the fact that the peptide moiety would exert an influence on the partial specific volume of the tryptic fragments. The extent of this influence is unknown, although the value used is near that others have used (9) for trypsin-derived glycoprotein fragments from the surface of mammalian cells. The molecular weight of the trypsin fragments consequently should be regarded as an approximation of the true molecular weight. Yet even using the $\bar{V}$ of heparins (0.47), the molecular weight is 94,000. This is an absolute minimum value of the molecular weight of the fragments and is five times higher than the molecular weight usually reported for heparin. We tentatively suggest that the cell-surface tryptic fragments of heparan sulfate include one to three sugar chains bound to a single polypeptide moiety.

## DISCUSSION

Our results suggest that the heparan sulfate of the cell surface of CHO cells includes surprisingly large molecules; the molecular size is large, both by comparison with other tryptic fragments removed from CHO cells as well

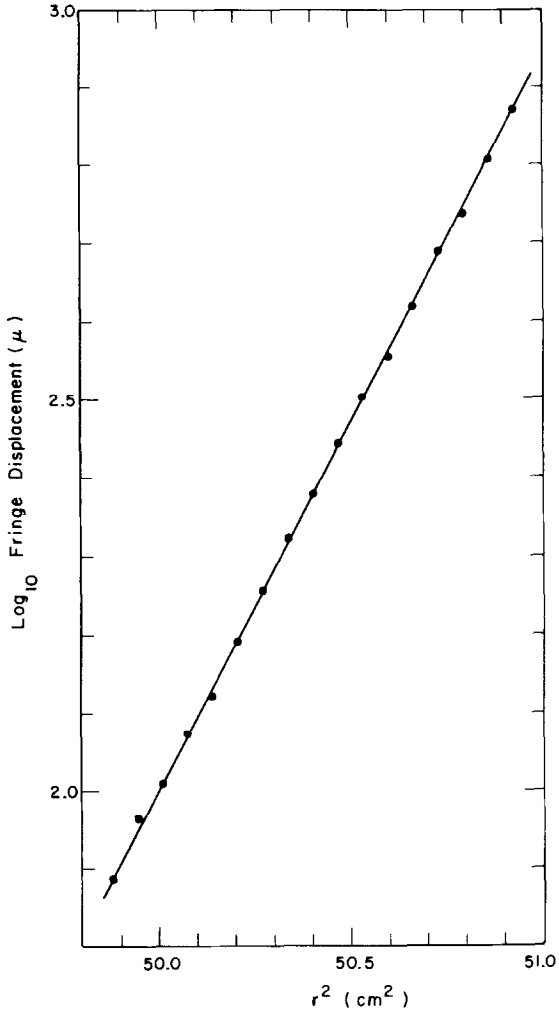


Fig. 3. Plot of sedimentation equilibrium data of cell-surface heparan sulfate in 1 M NaCl. The material used represents the first third of the isolated heparan sulfate to elute from a Bio-Gel A-5m column such as is illustrated in Fig. 2A.

as by comparison with other reports of the molecular weight of heparin and heparan sulfate. With a single exception (6), the published values range from 7,600 to 20,000 (10); by contrast, the native molecule from which we have isolated tryptic fragments of cell-surface heparan sulfate is over an order of magnitude larger and bears sugar chains that range from values similar to the published values up to several times those values.

The CHO cells studied also contained intracellular heparan sulfate species

that more closely resembled those studied by other workers. Because of the high metabolic activity of the surface variety (1) and because of the correlation of this activity with physiological events of the cell (4), it is our contention that the surface heparan sulfate is the physiologically active species at the cellular level. We also believe that all heparan sulfate species of the cell is part of a single metabolic system (1). Hence, the data presented here suggest that the bulk of intracellular heparan sulfate represents degradation products of large molecules similar to those that appear transiently on the cell surface. Under this conjecture, we would suggest that intracellular degradation of the sugar chains of membrane-bound heparan sulfate proteoglycan would release material into the cytoplasm with the characteristics of our cell sap heparan sulfate and would leave truncated proteoglycan bound to membrane with characteristics similar to the bulk of intracellular membrane-bound heparan sulfate. This notion is consistent with the fact that we have also detected a minority component of the intracellular membrane-associated heparan sulfate that is excluded from Bio-Gel A-0.5m (Fig. 1) and that labels as quickly as the surface heparan sulfate (1). We suggest that this intracellular component is the newly synthesized precursor of the other varieties.

We had speculated previously that the function of a generally occurring cell-surface molecular entity such as heparan sulfate might be related to the regulation of exposure of cell-specific entities to the immediate cell environment (4). The large size of the cell-surface heparan sulfate molecule would seem to encourage this speculation on the basis that such a large entity could effectively "overshadow" smaller, topographically adjacent structures.

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