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ENTITLED

Use of Base-Catalyzed $\beta$-Elimination in Structural Studies on Chondroitin Sulfate

IS APPROVED BY ME AS FULFILLING THIS PART OF THE REQUIREMENTS FOR THE

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by

Tommy Wah Chu

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Table of Contents

Acknowledgements 1
List of Figures iv
List of Tables v
List of Abbreviations vi

1. Introduction 1
1.1 Chondroitin sulfate structure 1
1.2 Possible significance of 6- or 4- sulfation 7
1.3 Preparation of chondroitin sulfates for structural study 8
1.4 Previous structural work on chondroitin sulfate 11
1.5 Determination of regions of 4- and 6- sulfation in the chondroitin sulfate polymer 12

2. Experimental Procedures 14
2.1 Materials 14

2.2 Methods 15
2.2.1 Preparation of chondroitin-sulfate proteoblycan 15
2.2.2 Enzymatic degradation with chondroitinases 15
2.2.3 Microdialysis 16
2.2.4 Reduction with NaB3H4 16
2.2.5 β-Elimination 17
2.2.6 Paper chromatography and paper electrophoresis 17
2.2.7 Assay for xylose content 19
2.2.8 Gel filtration chromatography 19
2.2.9 Hyaluronidase/β-glucuronidase digestion 20
2.2.10 Analytical polyacrylamide gel electrophoresis 20
2.2.11 Miscellaneous procedures 21

3. Results 24
3.1 Initial characterization of proteoglycan 24

3.2 β-Elimination assay 24
3.2.1 Xylose released by total hydrolysis 25
3.2.2 Xylitol formed from β-elimination 28
3.2.3 Time course 32

3.3 Generation of linkage region fragments 32
<table>
<thead>
<tr>
<th>Figure</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Structure of Chondroitin Sulfate</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>Proteoglycan Aggregate Structure</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>Mechanisms of Base-Catalyzed β-Elimination</td>
<td>10</td>
</tr>
<tr>
<td>4</td>
<td>Total Acid Hydrolysis of Chondroitin Sulfate</td>
<td>27</td>
</tr>
<tr>
<td>5</td>
<td>β-Elimination of Chondroitin Sulfate</td>
<td>31</td>
</tr>
<tr>
<td>6</td>
<td>Percentage of Elimination Incurred Over Time</td>
<td>35</td>
</tr>
<tr>
<td>7</td>
<td>Preparative Gel Filtration of Chondroitinase ABC and ACII Digests</td>
<td>38</td>
</tr>
<tr>
<td>8</td>
<td>Analytical Gel Filtration of I and II</td>
<td>40</td>
</tr>
<tr>
<td>9</td>
<td>Radioelectrophoresis of I and II</td>
<td>43</td>
</tr>
<tr>
<td>10</td>
<td>Chromatography of I and II in System III</td>
<td>46</td>
</tr>
<tr>
<td>11</td>
<td>Monosaccharide Content of II</td>
<td>49</td>
</tr>
<tr>
<td>12</td>
<td>Effects of Bacterial Δ4,5-β-Glucuronidase on II</td>
<td>52</td>
</tr>
<tr>
<td>13</td>
<td>Polyacrylamide Gel Electrophoresis of I</td>
<td>54</td>
</tr>
<tr>
<td>14</td>
<td>PAGE of Larger Oligosaccharides Containing Linkage Region Sugars</td>
<td>56</td>
</tr>
<tr>
<td>15</td>
<td>PAGE of C4S Digested at pH 6 with Hyaluronidase and β-Glucuronidase</td>
<td>61</td>
</tr>
<tr>
<td>16</td>
<td>Gel Filtration Profile of pH 6 Hyaluronidase/β-Glucuronidase Digest of Commercial C4S</td>
<td>63</td>
</tr>
<tr>
<td>17</td>
<td>PAGE of Size Classes</td>
<td>65</td>
</tr>
<tr>
<td>18</td>
<td>Paper Electrophoresis of Size Classes</td>
<td>68</td>
</tr>
<tr>
<td>19</td>
<td>PAGE of Hyaluronidase/β-Glucuronidase Digestions of C4S Performed for Various Amounts of Time.</td>
<td>71</td>
</tr>
</tbody>
</table>
## List of Tables

<p>| I. | Xylose Assay | 29 |
| II. | β-Elimination Time Course | 33 |
| III. | Summary of Characteristics of I and II | 57 |
| IV. | Summary of Gel Filtration Profile of Hyaluronidase/β-Glucuronidase of C4S | 66 |</p>
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>chondroitinase ABC</td>
<td>chondroitin ABC lyase; E.C. No. 4.2.2.4</td>
</tr>
<tr>
<td>chondroitinase AC II</td>
<td>chondroitin AC lyase; E. C. No. 4.2.2.5</td>
</tr>
<tr>
<td>chondro-4-sulfatase</td>
<td>2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-4-O-sulfo-D-galactose-4-sulfohydrolase; E. C. No. 3.1.6.9</td>
</tr>
<tr>
<td>C4S</td>
<td>chondroitin-4-sulfate</td>
</tr>
<tr>
<td>C6S</td>
<td>chondroitin-6 sulfate</td>
</tr>
<tr>
<td>d</td>
<td>dalton</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>gal</td>
<td>D-galactose</td>
</tr>
<tr>
<td>GalNAc</td>
<td>2-acetamido-2-deoxy-D-galactose</td>
</tr>
<tr>
<td>GloA</td>
<td>D-glucuronic acid</td>
</tr>
<tr>
<td>β-glucuronidase</td>
<td>β-D-glucuronide glucuronohydrolase; E. C. No. 3.2.1.31</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>hyaluronidase</td>
<td>hyaluronate lyase; E. C. No. 3.2.1.35</td>
</tr>
<tr>
<td>IIB</td>
<td>4-O-(2-O-sulfo-α-L-idopyranosyluronic acid)-6-O-sulfo-2,5-anhydro-D-mannitol</td>
</tr>
<tr>
<td>mA</td>
<td>milliamperes</td>
</tr>
<tr>
<td>mCi</td>
<td>milliCuries</td>
</tr>
<tr>
<td>MW</td>
<td>molecular weight</td>
</tr>
</tbody>
</table>
nmoles  nanomoles
PAGE  polyacrylamide gel electrophoresis
Rg  Ratio of glycitol migration relative to distance traveled by $^{14}$C-D-glucoitol
R-glo  Ratio of glycitol migration relative to distance traveled by $^{14}$C-D-glucose
Tris  tris-(hydroxymethyl)aminomethane
Xyl  D-xylose
µl  microliter
µM  micromolar
1 Introduction

1.1 Chondroitin Sulfate Structure

Chondroitin sulfate, a straight chain carbohydrate polymer approximately 20,000 daltons in molecular weight, is composed of the repeating 1-3 linked disaccharide D-glucuronic acid (GlcA) : 1-4 linked to 2-acetamido-2-deoxy-D-galactosamine (GalNAc). The GalNAc residue may be found sulfated at the 4-carbon, giving chondroitin 4-sulfate (C4S), or at the 6-carbon to give chondroitin 6-sulfate (C6S). Approximately 10% of the N-acetyl galactosamine (GalNAc) residues remain unsulfated [1]. Previously it had been believed that chondroitin sulfate existed as a homopolymer with respect to the position of sulfation on the GalNAc moiety within a given chain. However, recent evidence has accumulated (see 1.4 below) suggesting that hybrid polymers exist containing both 4- and 6-sulfated GalNAc residues in a single polymer [2-5].

Chondroitin sulfate occurs in nature as a proteoglycan. A small percentage of the sulfate present in chondroitin sulfate containing proteoglycans may be attributed to another sulfated glycosaminoglycan known as keratan sulfate. Multiple chains of chondroitin sulfate and keratan sulfate are attached to a single core protein to form a proteoglycan monomer which ranges in size from one to four million daltons in molecular weight [6]. Core protein, as found in hyaline
cartilage proteoglycans, is approximately 200,000 daltons in molecular weight and is rich in serine, glycine, and glutamine [7]. Chondroitin sulfate is covalently linked to serine residues of the core protein by β-glycosidic bonds. The attachments are concentrated to the amino terminus of this protein [7] and each occurs through a tetrasaccharide sequence referred to as the linkage region. The structure of the chondroitin sulfate linkage region [8,9] is shown in Figure 1 and is identical to the linkage regions of heparin [10-12] and dermatan sulfate [13].

Proteochondroitin sulfate (PCS) is a major component of cartilaginous tissues. It is produced and secreted in cartilage by chondrocytes. PCS is also found in non-cartilaginous tissues but differs from the form secreted by chondrocytes by several physical and chemical parameters [14]. One such difference is the tendency for the cartilaginous proteoglycans to form aggregates with hyaluronic acid [15], a glycosaminoglycan composed of alternating 4-linked β-D-glucuronic acid and 3-linked β,D-N-acetyl glucosamine. The aggregates, which exhibit a molecular weight of approximately 20 million daltons, are schematically drawn in Figure 2. Link proteins, also associated with the aggregate structure, are believed to contribute to the stability of the complexes [16].
**Figure 1: Structure of Chondroitin Sulfate.**

The three types of disaccharides found in the repeating portion of the chondroitin sulfate polymer and the linkage region sugars are shown here. Approximately 20 disaccharides including all three types may be found in a single polymer. The linkage region shown here is characteristic of serine linked glycosaminoglycans.
C₆S  C₄S  C₀S

LINKAGE REGION
Proteoglycan monomers are associated with hyaluronic acid chains through the carboxy terminal side of the core protein. Link proteins are believed to help stabilize the non-covalent interactions. The carbohydrate portions of the monomers are localized to the N-terminal portion of the core protein. Both keratan sulfate and chondroitin sulfate are linked to the same portein.
CORE PROTEIN

CHONDROITIN SULFATE

KERATAN SULFATE

LINK PROTEIN

HYALURONIC ACID
1.2 Possible significance of 6- or 4-sulfation.

The ability to secrete PCS, which is characteristic of cartilage tissues, has been used as a criterion for chondrogenic determination in developing cells. The development of chick limb buds and the changes in proteoglycan composition associated with endochondral bone formation have been intensely studied. The appearance of a form of PCS which sediments rapidly in sucrose density gradients (PCS-H) accompanied by an increase in the ratio of 6- to 4-sulfated GalNAc residues in chondroitin sulfate (hereafter referred to as 6:4 ratio) is indicative of chondrocyte differentiation [14,17]. In developing chondrocytes, the increase in 6:4 ratio occurs both in PCS-H and PCS-L, a "ubiquitous" form found in both chondrogenic and non-chondrogenic cells [18]. However, the 6:4 ratio is always higher in PCS-H than in PCS-L [19].

Although the exact role for the changes in 6:4 ratio which accompanies chick limb development is unknown, the suggestion that chondroitin sulfate serves as a calcium carrier for subsequent bone formation is often seen [20]. The possibility exists that the release of Ca++ in the region of bone formation is perhaps related to chondroitin sulfate catabolism. Evidence that the high 6:4 ratio of PCS-H may influence chondroitin sulfate catabolism has come from observations that the β1-4 linkages of C6S rich polymers are enzymatically hydrolyzed by testicular hyaluronidase, an endo-N-acetyl hexosaminidase, at a greater rate than are the same linkages of C4S rich chondroitin sulfate polymers [22,23].
1.3 Preparation of chondroitin sulfates for structural study.

Isolation of material for use in structural work on chondroitin sulfate has focused mainly on the preparation of proteoglycan monomers from cartilage tissue. Common sources of cartilage include bovine nasal septum, whale cartilage, shark cartilage, and chicken embryo limbs. Procedures for isolation of monomers have been published [24,25] and make use of dissociative reagents, such as guanidine hydrochloride, to disperse proteoglycan aggregates into hyaluronic acid, link proteins, and proteoglycan monomers. PCS monomers may then be removed from contaminating proteins by cetyl pyridinium chloride precipitation.

Dissociation of the hydroxyl-linked glycosaminoglycan portions of the monomers from the protein core may be accomplished by base catalyzed β-elimination (Figure 3). To prevent the alkaline degradation of the reducing terminal sugar which is exposed by this procedure, a reducing agent such as NaBH₄ is often introduced into the elimination mixture to convert the reducing terminal sugars into the corresponding sugar alcohol. The elimination also creates a highly reactive unsaturated amino acid from the former serine or threonine residues to which the sugars were linked. Quantitative conversion of this product to a more stable saturated amino acid is usually accomplished by addition of PdCl₂ to the reaction mixture which then becomes reduced to Pd metal by NaBH₄ [26]. The palladium metal serves as a heterogeneous catalyst for reduction of the double bonds in the unsaturated amino acid residues.
Figure 3: Mechanism of Base Catalyzed β-Elimination.

β-Elimination of chondroitin sulfate is initiated by extraction of a proton from the α-carbon of the serine residue. The reaction results in the production of a free reducing terminal sugar and an olefinic amino acid. Neither of these products are stable in base. The reducing sugar may undergo a second β-elimination reaction (peeling reaction), and the olefinic amino acid may also be destroyed in a multi-step process. The peeling reaction is prevented by reduction of the reducing sugar with NaBH₄. The unsaturated amino acid may be stabilized by reduction involving heterogeneous catalysis with in situ generated H₂ from NaBH₄ and Pd metal from reduction of PdCl₂ by NaBH₄.
with $H_2$ gas generated in situ from NaBH$_4$. Without PdCl$_2$, further
degradation of the olefinic amino acid may ensue (see Fig. 3).

1.4 Previous structural work on chondroitin sulfate.

Analysis of the distribution and quantity of 6- and 4-sulfated residues in chondroitin sulfate has relied heavily on enzymatic methods of depolymerization because of the loss of sulfate incurred upon depolymerization by acid hydrolysis; however, other chemical means by which oligosaccharides may be generated are currently under investigation.

Seno et al. [2,3] have used testicular hyaluronidase to generate sulfated tetrasaccharides from chondroitin sulfate obtained from various sources. To characterize the products, use was made of bacterial chondroitinase ABC [27] which is an eliminase that attacks at C5 of the uronic acid residues in chondroitin sulfate causing cleavage of the 1-4 GalNAc-GlA linkage and leaving an unsaturated 1-4,5 uronic acid residue. The tetrasaccharides were degraded by the chondroitinase treatment and a certain population of tetrasaccharides was found to give a 4-sulfated unsaturated disaccharide and a saturated 6-sulfated disaccharide. The presence tetrasaccharides with both 4- and 6-sulfated residues was also indicated by the behavior of the tetrasaccharide after extensive treatment with the chondro-4-sulfatase from Proteus vulgaris.
Delaney et al. [4] have also observed heterogeneity in oligosaccharides obtained from hyaluronidase digestion of chondroitin sulfate. Analysis of disaccharides obtained from chondroitinase ABC digestion of oligosaccharides which had been purified by HPLC showed that hybrid 6S,4S oligomers were present.

Hyaluronidase also catalyzes a transglycosylation reaction which has the potential for generating hybrid sequences from pure C6S and C4S polymers. Faltynek and Silbert [5] avoided this reaction by utilization of limited chondroitinase digestion for generation of oligosaccharides. These products also showed evidence of hybrid structures. Another method for avoiding transglycosylation is to couple β-glucuronidase digestion with the hyaluronidase reaction [22]. This exo-enzyme catalyzes the hydrolysis of the β1-3 GlcA-GalNAc linkage and will remove all nonreducing terminal glucuronic acid residues from a population of oligosaccharides, rendering them unsuitable as substrates for hyaluronidase-catalyzed transglycosylation.

1.5 Determination of regions of 4- and 6-sulfation in the chondroitin sulfate polymer.

There presently exist no good methods by which one could determine how the 4- and 6-sulfated residues are distributed in the polymer. In order to obtain such information, it is necessary to establish a reference point from which to base the locations of a 6- or 4-sulfated
GalNAc. Use of $\text{NaB}_3\text{H}_4$ in conjunction with base catalyzed $\beta$-elimination of chondroitin sulfate from its core protein can selectively convert the xylose residue of the linkage region to tritiated xylitol. Such treatment of proteoglycan monomers results in a population of end-labeled polymers. The work presented here describes the conditions necessary for quantitative elimination and conversion of xylose to $^3\text{H}$-xylitol and the characterization of linkage region fragments isolated following the procedure. The prospect of coupling these methods with hylauronidase/$\beta$-glucuronidase digestion to provide a method by which areas of 4- and 6-sulfation may be determined is also discussed.
2 Experimental Procedures

2.1 Materials

D-[\textsuperscript{14}C]-Glucose, 267 mCi/m mole, was obtained from New England Nuclear. Sodium borotritide (NaB\textsubscript{3}H\textsubscript{4}), 469 mCi/m mole, was from Amersham Radiochemicals.

Many materials were obtained from Sigma Chemical Company. These include hyaluronidase (Type V, 1100 turbidity reducing units/mg), bovine liver \( \beta \)-glucuronidase (Type B-10, 9000 Fishman units/mg), protease (thermolysin, Type X), chondroitinase ABC lyase, chondroitinase ACI lyase, and alkaline phosphatase (Type I) from calf intestinal mucosa. Chondroitin sulfate, Type A, was also obtained from Sigma.

N-acetylgalactosamine-6-sulfate sulfatase was a gift from Janet Glaser of our laboratory. Bacterial \( \Lambda4,5-\beta \)-glucuronidase was a gift from Abagail Salyers of the Microbiology Department at the University of Illinois at Urbana-Champaign. Chondroitin sulfate proteoglycan from bovine nasal septum was generously donated by Alex Kurizta of the Microbiology Department at the University of Illinois at Urbana-Champaign.
2.2 Methods

2.2.1 Preparation of chondroitin sulfate proteoglycan.

Proteochondroitin sulfate, obtained under dissociative conditions [24,25] from bovine nasal septa, was prepared by Alex Kurizta who followed a published procedure [28] described briefly here.

Cartilage tissue (150 g) from bovine nasal septa was ground in a blender and suspended with stirring in one liter of 4 M guanidine hydrochloride, 0.05 M Tris-HCl, pH 7.5, for 36 hrs. The suspension was filtered through cheese cloth and concentrated on an Amicon PM-30 filter. The retained material was dialyzed exhaustively against 12 liters of 0.4 M guanidine-HCl, 0.05 M Tris, pH 7.5. The chondroitin sulfate was then precipitated with 10% cetyl pyridinium phosphate in 1 M NaCl. The precipitate was dissolved in 100 ml 2M NaCl, 15% EtOH. The proteoglycan was then reprecipitated with 95% EtOH, and washed and resuspended in distilled water.

2.2.2 Enzymatic degradation with chondroitinases.

Two types of digestions were performed. One involves use of a mixture of ABC and ACII chondroitinases. The other was done in the presence of only one of the enzymes.
Samples to be digested (containing up to 1 mg of chondroitin sulfate polymer) were dissolved in 80 μl enriched Tris buffer, pH 7.3 [29]. To this was added 20 μl of enzyme solution—either a mixture of ABC and ACII, each at 5 units/ml, or either enzyme alone at 5 units/ml. The sample was incubated for two hrs at 37°C. A 20 μl booster of enzyme solution was added at the end of this period, and a second two hr incubation at 37°C followed.

2.2.3 Microdialysis

A 5 x 5 cm piece of twice boiled dialysis tubing was secured by a rubber band over a 6 x 50 mm borosilicate glass culture tube containing up to 200 μl of liquid. The tube was inverted and partially immersed in a beaker of distilled water while being held in place with a piece of metal wire hooked onto the edge of the beaker. Samples were dialyzed for 48 hrs at 4°C.

2.2.4 Reduction with NaB₃H₄.

50°C Reduction. Samples containing up to 200 nmoles of reducing termini were adjusted to 20 μl total volume, pH 9.0, with 1.0 M Na₂CO₃ in a fume hood. To this was added 10 μl of 0.25 M NaB₃H₄ in 0.1 N NaOH. Samples were heated to 50°C for 45 min. Excess NaB₃H₄ was then destroyed by acidification (to pH 0-1) with 6N H₂SO₄.
O C Reduction. Reduction of reducing termini containing alkaline labile α1-3 linkages requires milder reducing conditions [30]. Oligosaccharides, mixed with $^{14}$C-glucose as an internal standard, were dried under a gentle stream of air and resuspended in 10 μl of 1.0 M Na$_2$CO$_3$, pH 9.0. The sample was chilled on ice and 20 μl of pre-chilled 0.5 M NaB$_3$H$_4$ in 0.2 M Na$_2$CO$_3$, pH 10.2, was added. The sample was allowed to react for 90 min at 0°C. Excess NaB$_3$H$_4$ was then destroyed by adjustment of the pH to 0-1. Complete reduction was assayed by analysis of an aliquot of the reduction mixture in paper chromatography system III (described below).

2.2.5 β-elimination

This was a modified procedure of Seno and Sekizuka [31]. Two mg of chondroitin sulfate proteoglycan were dissolved in 100 μl of 0.3 M NaB$_3$H$_4$ in 0.4 M NaOH. The mixture was allowed to react for 24 hrs at room temperature. Excess NaB$_3$H$_4$ was destroyed by lowering the pH to 0 for at least one hr. The solution was neutralized with 2 M NaOH and dried under a gentle stream of air in a fume hood. Samples were then microdialyzed, using 2000 d MW cutoff dialysis tubing, for 72 hrs.

2.2.6 Paper chromatography and paper electrophoresis.

Three different solvent systems were used for descending paper
chromatography. In system I, radiolabeled samples were spotted 2.5 inches from one end of 1 x 22.5 inch strips of Whatman No. 3 paper and developed for 24 hrs in ethyl acetate: glacial acetic acid: formic acid: water (18:3:1:4, v:v:v:v). In system II, samples were spotted on 1 x 22.5 inch strips of cellulose phosphate paper (Reeve Angel) and were developed in ethyl acetate: pyridine: 5 mM boric acid (3:2:1, v:v:v) for 16 to 17 hrs. In system III, samples were spotted on Whatman 3 paper strips as in system I, and were developed in 1-butanol: glacial acetic acid: 1 M ammonium hydroxide (2:3:1, v:v:v) for 20 hrs. Systems I and II were used to resolve monosaccharides; solvent system III was used to resolve oligosaccharides.

Paper electrophoresis samples were spotted 4.0 inches from the ends of 1 x 22.5 inch strips of Whatman No. 3 paper. The paper strips were wetted with either pyridine: glacial acetic acid: water (1:10:400), pH 3.8, or formic acid: glacial acetic acid: water (2:7:70), pH 1.7. Electrophoresis took place at 1400 volts in a water-cooled apparatus for 2.5 hrs in the pH 3.8 system or for 3.0 hrs in the pH 1.7 system.

Following chromatography or electrophoresis, the paper strips were dried and cut into 1/2 inch segments which were counted in scintillation fluid containing 4 g diphenyloxazole per liter of toluene.
2.2.7 Assay for xylose content.

**Total xylose content.** A sample containing up to 4 mg of chondroitin sulfate proteoglycan was subjected to chondrointinase ABC/ACII digestion followed by microdialysis. Forty μl of 14C-glucose, 50 x 10 cpm/ml, was added to the dialyzed material to serve as an internal standard. The total volume was adjusted to 100 μl. A 20 μl aliquot was placed in a 0.4 x 40 mm tube and acidified to pH 0.0. The sample was covered with a layer of mineral oil and heated to 100 °C for 6 hrs. The hydrolyzed material was transferred to a 0.5 ml Eppendorf tube and reduced at 50 °C with NaB^3H_4. The reduction products were analyzed by paper chromatography.

\(^3H\)-Xylitol formed from β-elimination. Aliquots of microdialyzed, β-eliminated material were spiked with 14C-glucose and hydrolyzed as above. The hydrolysates were analyzed by paper chromatography.

2.2.8 Gel filtration chromatography.

**Preparative.** Samples were passed through a 1.9 (id) x 170 cm column packed with BioGel P-10 or BioGel P-2, 100-200 mesh. The column was eluted with 0.5 M NH_4HCO_3 and 3 ml fractions were collected. Radiolabeled material was detected in each fraction by counting a 0.1 ml aliquot to which was added 0.4 ml H_2O and 5 ml of a scintillation fluid.
containing 3 g diphenyloxazole per 1 of a mixture containing 250 ml Triton X-114 and 750 ml xylenes [32].

Analytical. Samples were passed through a 1.1 (id) x 117 cm column packed with BioGel P-2, 200-400 mesh. Samples were eluted in 0.5 M NaCl and collected in 1 ml fractions which were counted following the addition of 3 drops of concentrated HCl and 5 ml of scintillation fluid (described above).

2.2.9 Hyaluronidase/β-glucuronidase digestion.

This procedure has been described previously [22]. Substrates (1.0 mg) were incubated at 37 C with a mixture of 110 turbidity reducing units of ovine testicular hyaluronidase and 9000 Fishman units of β-glucuronidase in 1.0 ml of pH 5 buffer containing 0.05 M NaCl, 0.05 M Na₂SO₄, and 0.1 M NaOAc. Alternatively, samples were incubated at 45 C with 55 units of testicular hyaluronidase and 9000 units of β-glucuronidase in 1 ml of pH 6 buffer containing 0.06 M NaOAc. Under pH 6 conditions, C4S rich polymers are degraded at a 10X greater rate than are C6S rich polymers [22,23].

2.2.10 Analytical polyacrylamide gel electrophoresis.

This procedure has been described in detail by Knudson et al. [23]
who adapted it for use with oligosaccharides from the procedure of Maxam and Gilbert [33]. Tritium reduced oligosaccharides (1.2 x 10^6 dpm/sample), which had been desalted by paper chromatography system III or by gel filtration in NH_4HCO_3 followed by lyophilization, were dissolved in 10 µl of 5 mM Tris-borate, pH 8.3, 5mM EDTA, 30% glycerol, and 0.1 mg each of phenol red and bromophenol blue. Samples were layered at the bottom of a sample well of a 0.75 x 140 x 28 mm slab gel containing 25% acrylamide, 0.83% bisacrylamide, and 1.63 mM ammonium persulfate. Twenty microliters of N,N,N',N'-tetramethylenediamine were used to catalyze gel polymerization. Gels were electrophoresed at a constant voltage of 800 V (9.5 mA) in 50 mM Tris-borate, 1 mM EDTA, pH 8.3 in a water cooled electrophoresis apparatus.

Radioactivity was detected by fluorography [34,35] using as a flour sodium salicylate [36] which was introduced by shaking the gel in 1 M sodium salicylate, 65% EtOH for 30 min. The gel was dried under vacuum at 65°C between two pieces of slab gel backing (BioRad). Bands were visualized using pre-flashed [35] Kodak X-Omat AR5 film which was exposed to the dried gel at -70°C for 3 days.

2.2.11 Miscellaneous procedures.

1. Uronic acid content of proteoglycan.

The carbazole assay, as modified by Bitter and Muir [37], was used to determine the uronic acid content of chondroitin sulfate proteoglycan
from bovine nasal septum.

2. 6:4 ratios.

Proteoglycan was subjected to β-elimination followed by chondroitinase ABC digestion. Unsaturated disaccharides were analyzed by high performance liquid chromatography (HPLC) on a Parstil-10 SAX strong anion exchange column (Whatman) using a Perkin-Elmer Series 3 liquid chromatograph equipped with a Rheodyne 7105 injector. Samples were resolved using 40 mM KH₂PO₄/15% MeOH buffer at a flow rate of 1 ml/min [4]. Disaccharides were detected by absorbance at 232 nm.

3. Alkaline phosphate digestion.

This was performed as described by Kim and Conrad [38]. Samples were dissolved in 100 μl of 50 mM carbonate buffer, pH 10.5, containing 0.84 unit of calf intestine alkaline phosphatase. Following incubation at 37°C for 1 hr, the reaction was stopped by heating at 100°C for 10 min. The supernatant was analyzed by paper electrophoresis at pH 4.

4. Sulfatase treatment

Up to 43 nmole of substrate were dissolved in 85 μl of 0.05 M acetate buffer, pH 4.0, containing 10 mM dithiothreitol and 0.5 mM galacturonic acid lactone. Following addition of 15 μl of an enzyme mixture containing N-acetylgalactosamine-6-sulfate sulfatase activity, the samples were incubated for 5 hrs at 37°C.

5. Bacterial β-glucuronidase digestion.
Conditions used were described by Ototani and Yosizawa [39]. Samples were dissolved in 50 µl of 0.4 M NaOAc, pH 7.0. Fifty µl of a crude bacterial extract was added and the solution was incubated for one hr at 37 C. Reaction was terminated by heating to 100 C for 10 min. The precipitate was removed following centrifugation in a clinical centrifuge. Supernatants were analyzed by paper chromatography in system II and paper electrophoresis at pH 3.8.

6. Thermolysin digestion

Following β-elimination core protein was removed by thermolysin digestion. Substrate was dissolved in 90 µl of enriched Tris buffer [29]. Thermolysin (3 mg/ml) in enriched Tris was added (10 µl) and the mixture was incubated for three hrs at 37 C. The reaction was terminated by addition of 10 µl of 0.1 M Na₂EDTA, pH 7.
3 Results

3.1 Initial characterization of proteoglycan.

The proteoglycan solution used in these experiments was assayed for chondroitin sulfate by a modified carbazole assay [37] using commercial chondroitin sulfate as a standard. The solution was found to contain 8.88 mg/ml chondroitin sulfate. All hexuronic acid measurable by this assay was retainable on an Amicon PM-30 filter and was completely precipitable by 15% cetyl pyridinium chloride in 1 M NaCl. Furthermore, all uronic acid containing material eluted in the void volume when passed through a 1.0 x 115 cm column packed with BioGel A-5M.

The 6:4 ratio of chondroitin sulfate chains in the proteoglycan preparation was 13.7:86.3 as determined by HPLC analysis of disaccharides formed after chondroitinase ABC digestion of chondroitin sulfate released from the protein by β-elimination. This is in agreement with the 6:4 ratio found by other workers [3] for chondroitin sulfate from bovine nasal septum.

3.2 β-Elimination assay.
3.2.1 Xylose released by total hydrolysis.

To determine the total amount of xylose present per unit starting material, polysaccharides were converted to monosaccharides by acid hydrolysis. The monosaccharides were then analyzed by paper chromatography.

Because xylose represents only 1/50 of all monosaccharides from an intact chondroitin sulfate chain, a mixture of chondroitinases ABC and ACII was used to remove a majority of the polymer prior to hydrolysis. This enzymatic treatment has been reported to remove all repeating disaccharides from a protein linked chain, leaving only the four linkage region sugars attached to the core protein [41]. The released disaccharides were removed by microdialysis.

The core protein-linkage region complex was hydrolyzed, NaB₃H₄ reduced, and analyzed in chromatography system I. In this system, xylitol (Rg = 1.6) migrates as a single peak and is resolved from galactitol as is shown in Figure 4. The advantage of this system over system II is that it is relatively unsensitive to high salt concentrations in the sample. However, system II gives the best resolution of xylitol (Rg = 1.2), galactitol (Rg = 0.95), and uronic acids (Rg = 0.6). Uronic acids cannot be cleanly resolved in system I because of lactone formation in the acidic solvent. The control, which consisted of reduced but unhydrolyzed proteoglycan, served as a check on
**Figure 4:** Total Acid Hydrolysis of Chondroitin Sulfate Proteoglycan.

Chondroitin sulfate proteoglycan was digested with a mixture of chondroitinase ABC and chondroitinase ACII to remove the polymer. The remaining linkage region-protein complex was reduced and analyzed by chromatography system I as is shown in panel b. Another aliquot was hydrolyzed and then reduced and chromatographed as shown in panel c. A xylitol standard is shown in panel a. The amount of xylitol present in the original starting material was calculated from the amount found in c. (o----o) represents the $^{14}$C-glucitol internal standard.
"tritium blanks" and also to account for losses of material incurred during the hydrolysis step (the hydrolysis is the only step in these procedures requiring transfer of material from one tube to another). Partial loss of the sample was corrected for by comparison of the $^{14}$C-glucose present in hydrolyzed and unhydrolyzed samples.

The results reported in Table I represent the average of five trials. Based on the assumption that the chondroitin sulfate polymer is 20,000 daltons in molecular weight, the chondroitin sulfate concentration of the starting solution can be estimated as 6.35 mg/ml. This is close to the concentration calculated from the uronic acid content of 8.88 mg/ml.

3.2.2 Xylitol formed from $\beta$-elimination.

To quantitate the amount of $^3$H-xylitol formed during $\beta$-elimination, aliquots of the proteoglycan solution were treated as described in 2.2.5 and then subjected to total hydrolysis followed by analysis by paper chromatography. The radioactivity profiles in systems I and II are shown in Figure 5. Unhydrolyzed samples served as controls. The chromatograms indicate the release of xylitol following hydrolysis. However, other radioactivity remained at the origin. The amount of xylitol recovered by this procedure, shown in Table I, represents the average of five trials. The $\beta$-elimination was calculated assuming the amount of xylitol released by acid hydrolysis (3.2.1) was 100%.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Xylitol Recovery (µmoles/ml)</th>
<th>% Elimination</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Acid Hydrolysis</td>
<td>0.318</td>
<td>100</td>
</tr>
<tr>
<td>β-elimination</td>
<td>0.289</td>
<td>90.8</td>
</tr>
</tbody>
</table>
Chondroitin sulfate proteoglycan was subjected to base-catalyzed elimination in the presence of NaBH₄. Aliquots were analyzed directly by chromatography in system I, shown in panel b, and in system II, shown in panel e. Other samples were analyzed following hydrolysis in system I (panel c) and in system II (panel f). Panels a and d represent xylitol standards in system I and II, respectively. The amount of xylitol recovered in c and e was compared to the amount which would have been recovered by acid hydrolysis and reduction. (o--o) represents the ¹⁴C-glucitol internal standard.
In two of the trials used in the calculation for xylitol recovery following $\beta$-elimination, 12-14,000 d MW cutoff dialysis tubing was used in the dialysis step following elimination. The purpose of dialysis was to remove "blanks" present in the NaB$^3$H$_4$. For the other three trials, 2000 d MW cutoff tubing was used. The average $\beta$-elimination for the trials using the 12,000 d cutoff tubing was 77% while the average of the three trials using the lower MW cutoff tubing was 100.0%.

3.2.3 Time course.

The $\beta$-elimination reaction was terminated after various periods of time. When aliquots from 0, 2, 10, and 24 hr $\beta$-elimination were analyzed as described in 3.2.2, a progressive increase in the amount of $^3$H-xylitol recovered was observed. The results are shown in Table II and are displayed graphically in Figure 6.

3.3 Generation of linkage region fragments.

3.3.1 Purification

$\beta$-Elimination products, which had been exhaustively dialyzed using the 2000 d MW cutoff tubing, were divided into two fractions. One fraction was treated with chondroitinase ABC; the other was digested
<table>
<thead>
<tr>
<th>Time</th>
<th>Xylitol Recovery (μmolcs/ml)</th>
<th>% Elimination</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 hr</td>
<td>0.0917</td>
<td>29</td>
</tr>
<tr>
<td>10 hr</td>
<td>0.2420</td>
<td>76</td>
</tr>
<tr>
<td>24 hr</td>
<td>0.3034</td>
<td>95</td>
</tr>
</tbody>
</table>
Figure 6: Percentage of Elimination Incurred Over Time.

Chondroitin sulfate proteoglycan was subjected to base-catalyzed elimination in the presence of NaB\(_3\)H\(_4\) for various periods of time. The percent elimination (calculated as 100 x amount xylose recovered/amount xylose recovered by total hydrolysis) was plotted against reaction time.
with chondroitinase ACII. Each digestion mixture was passed through a 1.9 (id) x 175 cm column packed with BioGel P-2. The radioactivity elution profiles are shown in Figure 1. The peak radioactivity containing fractions were collected and pooled. The pools taken from the ABC digested material consisted of void volume eluting products and another major peak eluting in the included volume (Kav 0.17) which was called compound "I". The major tritium labeled product from the ACII digest which eluted in the included volume of the P-2 column was called compound "II" (Kav 0.41).

3.3.2 Size classing by gel filtration.

A 50:50 mixture of I and II was prepared and passed through a 1.1 x 117 cm column packed with BioGel P-2. Two peaks were observed in the elution profile as is shown in Figure 8a; the peaks exhibit retention values relative to glucitol of 0.58 and 0.71. The fraction I material was subjected to chondroitinase ACII digestion, mixed with and equal amount of II, and passed again through the column. One major peak, accounting for 94% of recovered radioactivity, was observed (Figure 8b). The result suggests that II can be derived from I by chondroitinase ACII digestion.

The approximate sizes of I and II can be estimated by comparison with standards resolved on the same column (data not shown). Fraction I, eluting with a retention value of 0.58, behaves like a
Figure 7: Preparative Gel Filtraion of Chondroitinase ABC and ACII Digests.

β-Eliminated chondroitin sulfate proteoglycan was digested by chondroitinase ABC (panel a) or chondroitinase ACII (panel b) and passed through a 1.9 (id) x 175 cm column packed with BioGel P-2. Vo represents the void volume of the column. I and II elute in the included volume with Kav values of 0.17 and 0.41, respectively. IIB in panel b represents a disulfated disaccharide used here as a standard. (o---o) represents the 14C-glucitol internal standard which is used here as a marker for the totally included volume of the column.
(a) A 50:50 mixture of I and II was passed through a 1.1 (id) x 117 cm column packed with Biogel P-2. I eluted with a retention value relative to glucitol (Rg) of 0.58 while the Rg for II was 0.71.

(b) I was treated with chondroitinase ACII and mixed with an equal amount of II. The mixture was passed through the same column used in (a) with 94% eluting as a single peak with an Rg of 0.71. (o-o-o) represents the $^{14}$C-glucitol internal standard which is used here as a marker for the totally included volume of the column.

Figure 8: Analytical Gel Filtration of I and II
hexasaccharide. Since II can apparently be derived from I following treatment with ACII, it is likely that II is a tetrasaccharide. However, the elution value of 0.71 is more characteristic of a trisaccharide. It should be pointed out that the standard oligosaccharides used were derived from the repeating portion of the polymer rather than the linkage region; therefore this size class assignment may not be valid.

3.4 Analysis of linkage region fragments.

3.4.1 Electrophoresis.

I and II were each subjected to paper electrophoresis at both pH 1.7 and 3.8. The radioactivity profiles of the electrophoreto grams are shown in Figure 9.

In the higher pH system, II separated into two peaks. The larger represented 81% of the radioactivity present in both peaks. Both peaks moved as if charged, although the larger only moved to segment 10. When II was electrophoresed at pH 1.7, two peaks were again observed in approximately the same proportions. In this system, the larger peak moved to segment 6 which is in the area where a non-charged molecule would diffuse under these conditions. The smaller peak moved to segment 16 which is the position of migration for a mono-sulfated pentasaccharide derived from chondroitin sulfate.
Figure 9: Radioelectrophoresis of I and II.

I and II were analyzed by paper electrophoresis at pH 3.8 (panels c and b respectively) and at pH 1.7 (panels f and e respectively). Two peaks were seen in both systems for II with the slower peak representing 80% of the material in both cases. Multiple peaks were seen for I at pH 3.8; the two major peaks were called Ic and Is as indicated. Three peaks were seen at pH 1.7. At both pH 1.7 and 3.8, the fastest moving peak represented 20% of the material. Panel a represents a fully sulfated pentasaccharide electrophoresed at pH 3.8; panel d represent a mixture of pentasaccharides with 0, 1, 2, and 3 sulfates electrophoresed at pH 1.7.
I was separated into multiple peaks by pH 3.8 electrophoresis. The fastest of these peaks accounted for 18% of the radioactivity, while the two other major peaks accounted for 31.2% and 38.3%. All the peaks moved as if they were charged in this system. Three peaks were seen when I was analysed in the lower pH system. Forty-one percent of the material moved to segment 8 as would be expected for uncharged material. Another 42% moved like a mono-sulfated pentasaccharide while the remaining 17% moved like a di-sulfated pentasaccharide.

The results indicate that although these oligosaccharide fragments appear homogenous by gel filtration, heterogeneity is seen on electrophoreograms and part of the heterogeneity is due to functional groups which remain charged at pH 1.7.

3.4.2 Chromatography in solvent system III.

Both I and II were chromatographed in chromatography system III. The resulting radioactivity profiles are shown in Figure 10. Both I and II chromatographed as single peaks in this system. No evidence of monosulfated disaccharides was seen.

3.4.3 Monosaccharide content of II.

II was subjected to hydrolysis and aliquots of the hydrolysate were
Figure 10: Chromatography of I and II in System III.

I and II were chromatographed in system III (panels a and b respectively). Panel a represents a reduced disulfated Δ-disaccharide. (o—-o) represents the 14C-glucitol internal standard.
analysed by chromatography systems I and II. An aliquot of the hydrolysate was re-reduced with NaB₃H₄ at 0°C and chromatographed in system II. The resulting radio-profiles are shown in Figure II.

The primary peak seen following hydrolysis is xylitol ([Rg = 1.26 in system II, R-glc = 1.6 in system I]). However, 25-30% of the counts remain at the origin in both systems. Upon re-reduction and chromatography in system 2, galactitol ([Rg = .97, 39,016 cpm] was seen in twice the proportion as xylitol on the same strip ([Rg = 1.3, 19,090 cpm]) suggesting that the molar ratio of galactose:xylose was 2:1. Blank counts present past segment 27 are also seen on this chromatogram; counts were also present at the origin.

The unsaturated A4,5-gluconic acid residue produced by the chondroitinase enzyme would be destroyed under the hydrolysis conditions. Consequently no evidence of a non-reducing terminal gluconic acid was seen.

3.4.4 Other enzyme treatments.

In order to determine the nature of the functional group in II which remains charged at pH 1.7, a variety of enzymatic treatments were used to attempt to alter the electrophoretic mobility at pH 1.7.

Alkaline phosphatase produced no changes in the electrophoretic
Figure 11: Monosaccharide content of II.

II was hydrolyzed and chromatographed in system I (panel a) and system II (panel b). Seventy-five percent of the radioactivity was represented by the xylitol peak. An aliquot was re-reduced and chromatographed in system II (panel c). The Gal:Xyl ratio was found to be 2:1 following re-reduction. (o---o) represents the $^{14}$C-glucitol internal standard.
migrations of II when compared before and after treatment. The same concentration of enzyme did alter glucose-6-phosphate migration, presumably by removal of the phosphate. Similarly N-acetylgalactosamine 6-sulfate sulfatase had no effect.

However, when II was treated with a bacterial A4,5-gluconidase and analysed by the pH 3.8 system, only one peak was observed which moved to segment 6. This is shown in Figure 12.

The failure of the phosphatase or the sulfatase to alter the electrophoretic mobility of II does not necessarily exclude the possibility of sulfate or phosphate existing on this compound since it may be that the enzymes merely do not recognize II as a substrate.

3.4.5 Polycrylamide gel electrophoresis (PAGE).

I was electrophoresed on polycrylamide slab gels. The gels were analyzed by fluorography. Comparison with standard markers present on the gel (Figure 13) shows that I runs much more slowly than do the oligosaccharides of corresponding size from the repeating portions of the polymer. Two major bands and one very faint band can be seen, consistent with the paper electrophoretograms. Fragment II migrates on PAGE like the slower moving major band (Iₙ) of I as is shown in Figure 14. A summary of the characteristics of I and II is presented in Table III.

Figure 12: Effects of Bacterial Δ4,5-β-Glucuronidase on II.

II was electrophoresed at pH 3.8 before (panel a) and after (panel b) treatment with a bacterial enzyme mixture. The mixture was able to cleave Δ4,5-glucuronic acid residue from a -disaccharide (not shown). The treatment eliminated migration of both the slow and fast moving peaks of II on pH 3.8 electrophoresis.
Figure 13: Polyacrylamide Gel Electrophoresis of I

I was analyzed by polyacrylamide gel electrophoresis (PAGE) as shown in lanes 2 and 3. Two major bands (I_f and I_r) were seen as well as one minor band which moved faster than either I_f or I_r. Lane 1 is a heptasaccharide standard. Lane 4 is a 15 min., pH 6 hyaluronidase/β-glucuronidase digest of commercial CNS.
Figure 14: PAGE of Larger Oligosaccharides Containing Linkage Region Sugars.

(a) Compounds I, I', II, and A through E were analyzed by PAGE methodology. The identity of the compound in each lane is indicated.

(b) P-10 gel filtration chromatography was used to prepare compounds A - E from a 1 hr, pH 5 hyaluronidase/glucuronidase digestion of -eliminated chondroitin sulfate polymers. D and E are actually pools of compounds representing at least three different size classes each. (o-o-o) represents the $^{14}$C-glucitol internal standard which is used here as a marker for the totally included volume of the column.
### TABLE III: Summary of Characteristics of I and II

<table>
<thead>
<tr>
<th>Treatment</th>
<th>I</th>
<th>II</th>
</tr>
</thead>
<tbody>
<tr>
<td>gel filtration (Kav)</td>
<td>0.17</td>
<td>0.41</td>
</tr>
<tr>
<td>size</td>
<td>hexasaccharide</td>
<td>tetrascarharide</td>
</tr>
<tr>
<td>ratio of products from total hydrolysis and re-reduction</td>
<td>galactitol: xylitol 2:1</td>
<td></td>
</tr>
</tbody>
</table>

**Electrophoresis**

**pH 3.8**

<table>
<thead>
<tr>
<th>R-penta</th>
<th>I</th>
<th>R-penta</th>
<th>I</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.79</td>
<td>18</td>
<td>0.63</td>
<td>19</td>
</tr>
<tr>
<td>0.67 (\text{I})</td>
<td>38.3</td>
<td>0.37</td>
<td>81</td>
</tr>
<tr>
<td>0.52 (\text{I})</td>
<td>31.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.37</td>
<td>12.5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**pH 1.7**

<table>
<thead>
<tr>
<th>R-penta</th>
<th>I</th>
<th>R-penta</th>
<th>I</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.79</td>
<td>17.1</td>
<td>0.61</td>
<td>20.4</td>
</tr>
<tr>
<td>0.56</td>
<td>42.0</td>
<td>0.23</td>
<td>79.6</td>
</tr>
<tr>
<td>0.31</td>
<td>40.9</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3.5 PAGE of larger fragments.

A solution containing β-eliminated polymer was treated with thermolysin followed by dialysis to remove core protein. The intact chondroitin sulfate chains were then digested with hyaluronidase/β-glucuronidase at pH 5 and the products were passed through a 1.9 (id) x 170 cm column packed with BioGel P-10. The radioactivity profile is shown in Figure 1b. The peak radioactivity containing fractions were pooled and lyophilized. The pools were designated "A" through "E" as is shown in Figure 1b. A, eluting closest to the fully included volume marker, had a retention value characteristic of a tetrasaccharide; each earlier eluting compound had retention values characteristic of the next larger "even-mere" (i.e. hexasaccharide, octasaccharide, and so forth). However as is the case for earlier gel filtration experiments, these may not be the correct size class designations because of the presence of neutral sugars in these compounds.

Compounds A through E were analyzed by PAGE methodology as is shown in Figure 1a. In each case, a multiple number of bands was seen. It is noted that the fastest moving band from C moves slower than the fastest moving band from E. Both D and E show even less mobility than does C. The major bands in I were separated by paper electrophoresis and run on a gel in parallel lanes as shown in Figure 1a. The faster compound was called If while the slower material was called Is. II, from
chondroitinase ACII digestion, was also run in a parallel lane. I, II, and bands from B and C all show equal mobilities.

3.6 Analysis of extra bands.

Hyaluronidase/β-glucuronidase digests of chondroitin sulfate have resulted in bands which do not correspond to purified odd numbered oligosaccharide standards when resolved by PAGE [23]. They are not even numbered oligosaccharides as can be seen by comparison of lanes 1 and 2 of the gel shown in Figure 15. In an attempt to determine the nature of these "extra bands", a NaB₃H₄ reduced, 24 hr, pH 6 hyaluronidase/β-glucuronidase digest of commercial CAS (the same material that appeared in lane 1 of Figure 15) was passed over a 1.9 (id) x 175 cm column packed with BioGel P-2. Fractions containing peak amounts of radioactivity were pooled as is indicated in Figure 16. The characteristics of the pooled fractions are listed in Table IV.

Electrophoretic behavior on polyacrylamide gels and on paper at pH 1.7 of the various pooled fractions is shown in Figure 17 and Figure 18. Three compounds were resolved on the gel and on paper for pool 2. Two of the paper peaks migrate slower than the fully sulfated pentasaccharide standard. The results indicate that undersulfation may account for the extra bands seen in the gels.

Commercial CAS was digested with hyaluronidase/β-glucuronidase at pH
Figure 15: PAGE of C4S Digested at pH 6 with Hyaluronidase and β-Glucuronidase.

Lane 1 represents a 24 hr, pH 6 hyaluronidase/β-glucuronidase digest of commercial C4S. Lane 2 contains 2 hour, pH 5 hyaluronidase digest of commercial C4S. Notice the spacing between the bands in lane 2 and the spacing of bands in lane 1. The bands in both lanes should represent oligosaccharides which differ in size by a disaccharide, although lane 1 should contain "odd-mers" while lane 2 should contain "even-mers". It is apparent that lane 1 contains "extra bands" which are not even numbered oligosaccharides.
Figure 16: Gel Filtration Profile of pH 6
Hyaluronidase/β-Glucuronidase Digest of Commercial C4S.

Digested C4S was passed through a 1.9 (id) x 175 cm column packed with BioGel P-2 resulting in the shown profile. Fractions were pooled (1-4) as indicated. (o--o) represents the 14C-glucitol internal standard which is used here as a marker for the totally included volume of the column.
Figure 17: PAGE of Size Classes.

Pools 2-4 (see Figure 16) were run in parallel lanes on a polyacrylamide gel. Lanes 1, 4, and 5 represent pools 2, 3, and 4 respectively. Lane 2 represents the unfractionated digest; lane 3 represents a fully sulfated pentasaccharide standard.
<table>
<thead>
<tr>
<th>Pool</th>
<th>Kav</th>
<th>% of Total</th>
<th>Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.342</td>
<td>12.8</td>
<td>trisaccharide</td>
</tr>
<tr>
<td>2</td>
<td>0.158</td>
<td>35.4</td>
<td>pentasaccharide</td>
</tr>
<tr>
<td>3</td>
<td>0.110</td>
<td>28.3</td>
<td>mixture of penta and hepta</td>
</tr>
<tr>
<td>4</td>
<td>0.070</td>
<td>23.5</td>
<td>hepta and larger</td>
</tr>
</tbody>
</table>
Figure 18: Paper Electrophoresis of Size Classes.

Pools 1-4 (see Figure 16) were analyzed by paper electrophoresis at pH 1.7. Panels b-e represent pools 1-4 respectively. In c, d, and e, multiple peaks are seen which run as slow or slower than the fully sulfated pentasaccharide standard (panel a). In panel c, three peaks are seen which most likely represent the tri-, di-, and monosulfated pentasaccharide.
6 for various periods of time. Fifteen min, 30 min, 1 hr, 4 hr, 12 hr, and 24 hr digestions were analyzed by PAGE as is shown in Figure 12. In all cases, the intermediate bands remained constant in intensity as compared to the major bands when visualized by fluorography.
Figure 19: PAGE of Hyaluronidase/β-Glucuronidase Digestions of C4S Performed for Various Amounts of Time.

Lanes 1, 2, 3, 5, and 6 represent commercial C4S samples digested at pH 6 for 1 hr, 30 min, 15 min, 4 hrs, and 24 hrs respectively. Lane 4 is a heptasaccharide standard.
9 References


There also exist problems associated with resolution of oligosaccharides with respect to size by polyacrylamide gels that must be overcome before the technique can be coupled with the above outlined above. Oligosaccharides will separate according to their relative sizes in the SDS system only if their charge densities are the same. Not presented here has indicated that this is not the case for smaller oligosaccharides with multiple linkage regions, but may be true for some oligosaccharides larger than monosaccharides. Another problem, namely undersulfation, may be encountered for the larger oligosaccharides needing separation if a single one place into multiple bands. Further studies with separate, monosaccharide sulfates have indicated that some of sulfates is not needed by apolipoprotein oligosaccharides digestion conditions and undersulfation may be an intrinsic feature of the molecule.

Some sources of electrophoretic sulfate may exist and cause charge heterogeneity that obscure. Recent report of the electrophoretic sulfate obvious from bovine adrenal cortex appear to consist negative charges associated with the linkage region moieties which are unexplained by the currently accepted models for linkage region structure. The charges are non-suppressable by leaving the gel in 1N HCl for hours, a sulfated or phosphatase functional group. Furthermore, a high degree of undersulfation is found on the electrophoretically isolated in the linkage region as evidenced by multiple banding patterns seen in polyacrylamide gel and paper electrophoresis of linkage region oligosaccharides resolved by gel chromatography.
The possibility remains that the bands from the p-30 region are contaminated with some amount of the neighboring peaks. This could partially explain the presence of multiple bands in each compound passed from what was supposedly a single peak on gel filtration. Another possibility is that variation in the degree of sulfation is responsible for the multiple bands.

The only work which was directed at determining the nature of "extra bands" appearing in the polyanion-gel electrophoretic patterns digested with a mixture of proteases and trypsin. These work electrophoretic separations solely on the basis of charge to mass ratio, the appearance of multiple bands from a single class of proteins (by gel filtration) was indicative of charge heterogeneity. If the electrophoretic is done at pH 4.7, only the sulfate groups retain charge and heterogeneity could indicate variations in sulfation. The sulfonated derivative shows in Figure 4 the same 3 major peaks, one of which run closer than a fully sulfated standard. These may be three possible states of sulfation in a polyanionic sulfate polyanion.
by Fiske methods (see Figure 1). Further, it also shows that \( B \) extracts fewer than \( A \) even though gel partition experiments have shown that \( A \) is smaller than \( B \). Apparently, \( B \) has the greater affinity to the Fiske system because of its higher charge density. It appears due to a sulfite and \( \beta \)-carotene residue. \( A \), although smaller, possesses only one charged functional group. \( C \), which presumably carries no sulfite group, has one more charged group than \( B \) and is also larger suggesting it is to be secreted by the gel to a greater degree. The combination of factors cause \( B \) and \( C \) to have equal solubility in the gel.

The oligosaccharides present from Fiske gel chromatography following gel chromatography purification, oligosaccharides and oligosaccharides' oligosaccharide oligosaccharide oligosaccharide oligosaccharide oligosaccharide oligosaccharide oligosaccharide oligosaccharide oligosaccharide oligosaccharide oligosaccharide oligosaccharide are even more difficult to interpret. Their exact scope are unknown at this point. \( A \) is known approximately to be a heterosaccharide, \( B \) is a homosaccharide, and \( C \) is a homosaccharide while \( D \) and \( E \) are mixtures of longer oligosaccharides. Again, as in previous gel partition experiments, the eluates due to more than one assignment are not clear, since they do not contain neutral sugars. These oligosaccharides are used in the preparation of \( A \) and \( C \), they should all be not rather than even numbered oligosaccharides. Further experimentation must be done to test these predictions.

The elution of these compounds by Fiske shows that the partition points vary from \( A \) through \( E \) according to their relative size as judged by their elution from the Fiske column. This is not accompanied
Attempts to characterize the nature of the extra charge in II have been unsuccessful. Addition of pyrophosphate and bis-pyrophosphate, as well as further studies with pyrophosphate buffer, have failed to affect the electrophoretic mobility of the compound. Unfortunately, we can only conclude from this that II is not a good substrate for either of the two enzymes, rather than that phosphate and pyrophosphate are not present in II. Phosphate has been reported to be associated with the linkage region area in approximately a 10:1 molar ratio of pyrophosphate (21). Material 10.4 g.s.-Glycine-OH abolished all mobility of II in an 0.01 electrophoretic buffer. This may be because the positive charge is located on the groups added at the end of the linkage region, and not on the phosphate or sulfate molecules themselves. However, a crude enzyme extraction was used and it should be possible that the 10.4 g.s.-Glycine-OH abstracts not only the phosphate, but also the sulfate phosphate activity and the only active of these preparations.

To even more heterogeneity than II is encountered in the extraelectrophoretic bands. This may be explained in part if the biopolymer contains the non-reducing terminal ester in eosin sulfated and desulfated forms. The presence of pyrophosphate, as well as its ability to affect the activity of the enzyme, suggests a possible modification, which is probably sulfated, could be expected to occur, thus an exchanged molecule. The other form could arise from a non-reduced monophosphate. For all 1.9 electrophoretic bands indicate two behave (Figures 11, 12). II and II are characterized by lower electrophoretic mobility at all pH and can be explained in terms of polycationic site I, abnormally similar to II. Figure 13. The bands corresponds to II, the bands emit also differences between I and II.
in possible that some chloride anion oxides are to be reduced to BrO$_3^-$.
The chlorophyll (67) has reported that quantitative conversion of the 
chlorine anions to the oxides may occur even in the absence of MoV.

For the internal standard ratio was not equal when 
generated before and after total separation. Since all chromatograms were developed till past the time since the actual point was made 
of the CDDP, it is possible that some oxidized components released 
during the separation were run off the paper strip.

Complete recovery of all that following initiation can only 
observed when from a 0.5 m chlorite was used in the digestion step. 
Some of the chloroalcohols, the anion crystals were able to pass through 
the 70,000-50,000 g cutoff setting. The anions more than half (for 
application) were recovered with the longer process, it is possible that 
the salt are exhibit a range of errors.

The chlorinated reagents were subjected to digestion for 
chromatography [7] or [8] to facilitate the sodium region. One of the 
produced a larger area of (67) [9] gave the use of MoV (67) 
graphite [7] [10]. Previous were by other investigators [9] have 
suggested that [1] to 6.2:1 (1,4) C$\text{H}_6$:Cl$\text{O}_5$ (1.6) Cl$\text{O}_3$ (1.4) Cl$\text{O}_4$ (1.4) Cl$\text{O}_5$ (1.4) Cl$\text{O}_6$ (1.4) Cl$\text{O}_7$ (1.4) Cl$\text{O}_8$ (1.4) Cl$\text{O}_9$. The data presented in this paper are consistent with 
these structures. The Phosphorus experiments were shown that [1] to 
larger than [1] by approximately one million. The data can be reported to
A Discussion

The results presented here have shown that high levels of protein can be recovered on HPLC in highly pure water and isotopes of stable, reduced disulfide and free cystine separated from the protein by high-performance liquid chromatography. The procedures described in this work did not allow the production of DTT, which has been used by other investigators [11]. Because the production of reduced protein in reduced to interfere with further analysis and because it was experimentally unnecessary for our purposes, the procedure of DTT is not necessary for the qualitative comparison of elution profiles with those produced during dialysis in more stable neutral forms (see Figure 6). However, the procedures of DTT were used to afford the substitution.

Although optimal for a major British containing protein released upon hydrolysis of the elution mixture, these radiolabeled materials were present in super disulfide groups, identified solely to the origin area. A possible source of these “substitutes” is protein surface. Surface protein changes are linked to the core protein through ionic and hydrogen bonds between similarly modified and the parts of the proteins. The results of 3H-3H exchange and the production of radioactive residues of the protein, upon hydrolysis, 3H-3H exchange indicates that a 3H group is from 3H-labeled amino acids remaining at the origin in chromatography system 2 and could serve to be - 2.7 in chromatography system 5 (data not shown). This is consistent with the position in Figure 3. Another possible source of origin could be in the protein if


