Oligosaccharides on Proteoglycans from the Swarm Rat Chondrosarcoma*

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Two classes of oligosaccharides were identified in alkaline borohydride digests of monomer proteoglycans from the Swarm rat chondrosarcoma. The first class consists of three similar oligomers, each of which is linked to the proteoglycan core protein by an O-glycosidic bond between N-acetylgalactosamine and hydroxyl groups on serine and threonine.

The structures of the two smallest oligosaccharides are:

(a) N-acetylneuraminyl-(2→3)-galactopyranosyl-(1→3)-N-acetylgalactosaminitol
(b) N-acetylneuraminyl (2→6)

The most probable structure of the largest is:

(c) N-acetylneuraminyl-(2→3)-galactopyranosyl-(1→4)-N-acetylgalactosaminitol

Proteoglycan monomers from the chondrosarcoma contain about 1.3 of these oligosaccharides for every chondroitin sulfate chain. Their structures and distribution on the core protein suggest that they are related to the linkage region between keratan sulfate chains and the core protein of cartilage proteoglycans. The second class of oligomers consists of oligosaccharide-peptides that contain mannoside and are probably linked to the core protein by N-glycosylamine bonds to asparagine. There appear to be about 15 of these oligosaccharides/proteoglycan molecule, and they are primarily located on the core protein nearer to the hyaluronic acid-binding site than both the oligosaccharides linked by O-glycosidic bonds and the chondroitin sulfate chains. Chondrocytes isolated from the chondrosarcoma and grown in culture synthesize both classes of oligosaccharides.

In the preceding paper, we have shown that the proteoglycans synthesized by chondrocytes in culture derived from chick limb bud mesenchyme cells contain two distinct classes of oligosaccharides in addition to chondroitin sulfate and keratan sulfate (1). Monomer proteoglycans from such cultures were treated with alkaline borohydride (2), and the digests were analyzed by molecular sieve chromatography. When column eluents were monitored for sialic acid, three components were identified. The largest in size contained about 15% of the total sialic acid and was identified as keratan sulfate. The second, intermediate component contained about 20% of the total sialic acid and was selectively labeled when [2-3H]mannose was used as a precursor. The smallest size component contained about 65% of the total sialic acid and consisted of oligosaccharides linked to the core protein by O-glycosidic bonds to N-acetylgalactosamine.

Because of the difficulty in obtaining large amounts of proteoglycans from the chick chondrocyte cultures, we have studied these classes of oligosaccharides in greater detail in proteoglycans purified from the Swarm rat chondrosarcoma. This system provides several advantages. The proteoglycans can be purified in large quantities (3-6) and the molecules do not contain keratan sulfate (3, 7) which, in the case of the chick chondrocyte proteoglycans, introduced difficulties in purifying the mannoside-rich oligosaccharide fraction. Finally, chondrocytes from the Swarm rat chondrosarcoma can be maintained in culture under conditions where they synthesize proteoglycans characteristic of those synthesized by the cells in the tumor in vivo (8), and these cultures can be used to study the biosynthesis of the oligosaccharides. This report describes experiments in which the structure, synthesis, and location of the oligosaccharides in the proteoglycan molecules were studied further.

EXPERIMENTAL PROCEDURES

Materials—D-[6-3H]Glucosamine HCl (20 to 38 Ci/mmol) was purchased from Amersham/Searle, neuramine lactose was from Calbiochem-Benning, and clostripain (from Clostridium histolyticum) was from Boehringer Mannheim. Other enzymes and reagents are described in the accompanying paper (1).

Analytical Procedures—Hexosamines and hexosaminitols were analyzed on a Durrum D-500 amino acid analyzer with buffers as
described elsewhere (1). Hexuronic acid, hexose, and protein contents in column effluents were determined with automated procedures (9).

Sialic acid was determined with an automated procedure adapted from the asparagine method (10). The flow diagram is shown in Fig. 1. The following reagents were utilized: periodic acid reagent, 0.4 M periodic acid diluted 1:20 with 0.02% sodium dodecyl sulfate before use, resorcinol reagent, 2% resorcinol (w/v) freshly dissolved in a solvent containing 575 ml of concentrated HCl, 45 ml of distilled water, and 62.4 mg of CuSO4·5H2O. At 60 samples/h and a 2:1 sample/wash ratio, a 10 Ag/ml standard of sialic acid gives an absorbance of about 0.20. The periodic acid oxidation conditions used gave nearly the same color yield for free sialic acid as for bound sialic acid (10). The sialic acid assays for the purified oligosaccharide fractions (Table 1 below) were done by the manual resorcinol method at 0°C using neuraminidase lactose as the standard.

Sugar Analysis—Oligosaccharides were hydrolyzed with 90% aqueous formic acid at 100°C for 5 h followed by hydrolysis with 0.25 M aqueous sulfuric acid at 100°C for 18 h and reduction with NaBD4. They were then analyzed as alditol acetates by gas-liquid chromatography (11) and mass spectrometry (12). Methylation analyses were done as previously described (13). A Hewlett-Packard 5890A gas chromatograph equipped with a flame ionization detector was used for gas-liquid chromatography. Separations were done on either: (a) a glass column (1 m x 2 mm) packed with 37% SE-30 on Supelcoport (100/120) on alditol acetates; or (b) SE-30 W/COT glass capillary column (0.30 mm x 24 mm) at 200°C for (partially methylated alditol acetates) and at 200-330°C for (permethylated oligosaccharide alditols). Gas-liquid chromatography/mass spectrometry was carried out on a Hewlett-Packard 5992A instrument fitted with a 0.25 mm x 2 mm column with 37% OV-1 on Chromosorb Q 100/120 at 180-240°C. The spectra were recorded at 70 eV and an ion source temperature at 130°C. Scintillation Counting—Radioactivities in samples were determined in a Beckman LS-5000 scintillation counter. Aliquots of 0.1 to 0.2 ml were diluted with 1 ml of water and 13 ml of Aquasol. For samples in 4 ml guanidine HCl, 1 ml of 70% ethanol was used instead of water.

Preparation of Proteoglycan Fractions—The aggregate (aAl) and monomer (aAl-D1) fractions were isolated from the rat chondrosarcoma as described previously (5). The M, 77,000 polypeptide, which contains the hyaluronic acid-binding site, and the chondroitin sulfate-peptide fragments were purified from clorstripain digests of proteoglycan aggregates by procedures similar to those described elsewhere (14) for trypsin digests. Samples of aAl (6 mg/ml) in 0.1 M sodium acetate, 0.1 M Tris, pH 7.3, were digested with 5 units/ml of clorstripain in the presence of 0.50 mm CaCl2, 0.12 M EDTA, and 0.1% Triton X-100 at 37°C. The reaction was stopped by the addition of excess iodoacetamide. The digests were centrifuged in an associative CsCl density gradient (initial density, 1.50 g/ml) for 68 h at 10°C and 37,000 rpm in a Beckman 50.2 Ti rotor. Peptides containing clusters of chondroitin sulfate were recovered at the bottom of the gradient.

The complex of hyaluronic acid-binding region, link protein, and chondroitin sulfate was digested with clostripain, with a single band of 67,000 polypeptide, which was purified on an Amicon PM-10 filter. Aliquots with about 40 mg of protein in 0.2 ml were diluted with unlabeled monomer proteoglycan peak were pooled, dialyzed against 0.5 M sodium acetate, pH 7, and then against water, and lyophilized. The labeled proteoglycans from the cell culture isolated by this technique were either used directly or diluted with unlabeled monomer proteoglycan from the chondrosarcoma before further treatment. A separate culture was incubated with [2-'H]mannose (100 Ci/ml) under the same conditions, and monomer proteoglycans were isolated by the same procedure described above.

Alkaline Borohydride Treatment and Oligosaccharide Preparation—A sample of aAl-D1 proteoglycan (1 g) containing purified [3H]labeled proteoglycan monomer was incubated with 1 M sodium hydroxide, 1 M sodium borohydride, 45°C, 48 h. After neutralization with concentrated acetic acid, samples were frozen until subsequent chromatography. Peroxidase-cleavable carbohydrate was recovered by procedures similar to those described elsewhere (12). A small aliquot (0.1 ml) was eluted on an analytical Bio-Gel P-10 column (0.6 x 300 cm) eluted with 0.5 M sodium acetate, pH 7, at a flow rate of 2 ml/h. Portions of each 0.5 ml fraction were analyzed for radioactivity, sialic acid content, and neutral sugar content. The remaining sample was chromatographed on a preparative Bio-Gel P-10 column (1.3 x 170 cm) eluted with 1 M pyridinium acetate, pH 6.5. For each column run, 5 ml of sample was used. Fractions of 4 ml were collected at a flow rate of 10 ml/h. Aliquots were withdrawn from each of the fractions, lyophilized, and reconstituted with water. The contents of peptide, hexose, and sialic acid were determined. Fractions corresponding to sialic acid positive peaks (II and III of Fig. 3) were either used directly or diluted with unlabeled monomer proteoglycan from the chondrosarcoma before further treatment. The separated fractions of glycopeptides were obtained in a yield ranging from 0.5 to 1.0 g of material, which contained a small amount of chondroitin sulfate, was digested with chondroitinase ABC as described below, and the digest was applied to a Bio-Gel P-10 column (0.6 x 300 cm) eluted with 1 M pyridinium acetate, pH 6.5. Fractions were collected at a flow rate of 2 ml/h. Crude Fraction II was applied to the same column without prior chondroitinase digestion. Fractions corresponding to peaks which contained sialic acid were pooled, lyophilized, redissolved in water, and lyophilized again to remove the last traces of pyridine, and dissolved in a small volume of 0.1 M Tris HCl, 0.1 M sodium acetate, pH 7.3. Fraction II, which contained a small amount of monosaccharide, was digested with chondroitinase ABC as described below, and the digest was applied to a Bio-Gel P-10 column (0.6 x 300 cm) eluted with 0.5 M sodium acetate, pH 7.3, to check the homogeneity of the final fractions. The composition of each purified fraction was determined.

Enzyme Treatment—Samples were dissolved in 0.1 M Tris HCl, 0.1 M sodium acetate, pH 7.3, and treated with one or more of the

![FIG. 1. Schematic diagram of automated procedure for sialic acid determination. SDS, sodium dodecyl sulfate.](image-url)
following enzymes: chondroitinase ABC (0.05 units/mg of sample), chondroitinase ABC (0.05 units/mg of sample), chondroitinase ABC (0.05 units/mg of sample), chondroitinase ABC (0.05 units/mg of sample), and proteinase K (0.8 unit/mg of sample), papain (25 μg/mg of sample in the presence of 5 mM disodium EDTA and 5 mM cysteine HCl).

Digestions were done at 37°C for 4 h, after which the digests were frozen or directly applied to columns as described under "Results."

RESULTS

Identification of Oligosaccharides—Monomer proteoglycan from the chondrosarcoma was mixed with monomer isolated from a culture in which [3H]glucosamine was used as a precursor. The mixture was treated with alkaline borohydride and then an aliquot was eluted on an analytical Bio-Gel P-10 column. The column was calibrated with defined oligomers of hyaluronic acid (8) which eluted at the positions indicated by the arrows (Fig. 2). Eluent fractions were monitored for contents of sialic acid, hexose, and radioactivity. The large peak of radioactivity and anthrone reactivity in the excluded volume indicates the elution position of the chondroitin sulfate chains which account for about 92% of the total radioactivity in the sample. No sialic acid was detected in this peak. A sialic acid peak, indicated as Peak II, eluted as a broad peak in the region where hyaluronic acid oligomers of about 10 to 14 monosaccharides elute. A shoulder containing about 3% of the total radioactivity and a small anthrone reactive peak were associated with this component. It contained about 15% of the total sialic acid and corresponds to the class of mannose-rich oligosaccharides identified previously in proteoglycans from the chick limb bud chondrocyte cultures (1). The remaining sialic acid in the sample eluted in three distinct peaks indicated as IIIA, IIIB, and IIIC, in the region where hyaluronic acid oligomers with between two and six monosaccharides elute. Hexose reactivity was observed in each of these three peaks with the ratio of sialic acid/hexose being higher in Peaks IIIA and IIIB than in Peak IIIC. Sialic acid was observed in each of the three peaks, accounting for 3:1, 1:1, and 0.5% of the total radioactivity, respectively. These oligosaccharides correspond to the class of O-linked oligosaccharides observed in the proteoglycans from the chick limb bud chondrocyte cultures (1). A late eluting peak of radioactivity in the region where monosaccharides elute was also observed. Since no hexose or sialic acid reactivity eluted in this peak, it could contain unincorporated precursor or, possibly, it could be derived from the presence of unsubstituted N-acetylgalactosamine residues covalently bound to the core protein in the labeled proteoglycans. Insufficient amounts of this fraction were recovered for further study.

A large amount of the alkaline borohydride digest, which contained about 1 g of a1-D1, was fractionated on a preparative Bio-Gel P-10 column eluted with pyridinium acetate, Fig. 3. The eluted fractions were analyzed for hexose, sialic acid, and protein. The two classes of sialic acid oligosaccharides are apparent, although the separate oligomers in the Peak III region were not resolved under these preparative conditions. The protein elution profile indicates that the alkaline borohydride procedure extensively degraded the core protein and that some peptide fragments co-eluted with the smaller oligosaccharides. Peaks II and III were recovered and lyophilized.

The Peak II fraction was digested with chondroitinase ABC and rechromatographed on Bio-Gel P-10 as described under "Experimental Procedures" to remove the contaminating chondroitin sulfate (data not shown). The mannose-rich oligosaccharide was recovered and a small portion of this purified sample was chromatographed on a small analytical Bio-Gel P-10 column, yielding the sialic acid elution profile in Fig. 4a. The different oligomers in the Peak II fraction were resolved by rechromatography on Bio-Gel P-10 as described under "Experimental Procedures" (data not shown). Small portions of the purified oligomers were chromatographed on an analytical Bio-Gel P-10 column and the sialic acid elution profiles are shown in Fig. 4, b, c, and d, for IIIA, IIIB, and IIIC, respectively.

The results of analyses for neutral sugars, hexosamines, and sialic acid for the purified subfractions are given in Table I. Component II containing primarily mannose, galactose, N-acetylgalactosamine, and sialic acid. No reduced sugar moieties were detected, suggesting that the oligosaccharides were bound to small peptides released from the protein by the alkaline borohydride treatment. Although they have somewhat higher sialic acid content, the properties and composition of this oligosaccharide fraction are similar to those of the mannose-rich oligosaccharides present in proteoglycans from chick limb bud chondrocytes as described elsewhere (1). All three of the smaller oligomers, IIIA, IIIB, and IIIC, contained N-acetylgalactosaminol after the alkaline borohydride treatment, indicating that they were linked to the core protein by O-glycoside bonds between N-acetylgalactosamine and hydroxyl groups of serine and threonine. The smallest, IIIC, contains 1 residue of galactose and 1 of sialic acid for each N-
Proteoglycan Oligosaccharides

**Purified Subfractions—P10**

**FIG. 4.** Purification of the mannose-oligosaccharides and of the O-linked oligosaccharides. Elution profiles on an analytical Bio-Gel P10 column are shown for: a, purified mannose-oligosaccharides (Peak I); b, c, and d, purified O-linked oligosaccharide subfractions IIIA, IIIB, and IIIC, respectively. The content of sialic acid (—NeuNAc) was monitored in every fraction.

<table>
<thead>
<tr>
<th>Table I</th>
<th>Sugar analysis of oligosaccharides isolated from rat chondrosarcoma tumor</th>
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<tbody>
<tr>
<td>Sugar</td>
<td>Residues/Man</td>
</tr>
<tr>
<td>---------</td>
<td>--------------</td>
</tr>
<tr>
<td>NeuNAc</td>
<td>1.9</td>
</tr>
<tr>
<td>Gal</td>
<td>1.6</td>
</tr>
<tr>
<td>Man</td>
<td>1.0</td>
</tr>
<tr>
<td>GlcNAc</td>
<td>1.9</td>
</tr>
<tr>
<td>GalNAc</td>
<td>0.3</td>
</tr>
<tr>
<td>Fuc</td>
<td>0.25</td>
</tr>
<tr>
<td>Xyl</td>
<td>0.06</td>
</tr>
</tbody>
</table>

1 Hexosamine analysis on the amino acid analyzer showed only galactosamine with no galactosaminitol present.
2 Hexosamine analysis on the amino acid analyzer showed only galactosaminitol with no galactosamine present.
3 ND, not detected.

The mass fragmentation pattern of the methylated disaccharide alditol obtained from mild acid hydrolysis of IIIC is shown in Fig. 5. The observed fragments are consistent with the proposed structure. The A series of fragments m/e 219, m/e 187, and m/e 155 shows the presence of a nonreducing hexose. The fragments m/e 130 and m/e 276 indicate the presence of an N-acetylgalactosaminitol. The 1 → 3 linkage is supported by the presence of fragments m/e 378 and m/e 133.

The next larger oligosaccharide alditol, IIIB, contained about twice as much sialic acid as IIIC but otherwise had a very similar composition (Table I). The small amounts of mannose in IIIB and also in IIIA probably indicate that small amounts of the mannose-rich oligosaccharides, Component II, which were not totally resolved in the purification procedure, were present. The methylation analyses (Table II) show the same changes in the methylation pattern for the galactose moiety in IIIB as for IIIC. However, the N-acetylgalactosaminitol residue was only methylated on positions 1, 4, and 5 prior to mild acid hydrolysis, after which position 6 was also methylated. This indicates that the additional sialic acid residue was linked to position 6 of the N-acetylgalactosaminitol, giving the structure:

\[
\text{NeuNAc-(2}\rightarrow\text{3)}\cdot\text{d-Galp-(1}\rightarrow\text{3)}\cdot\text{d-GalNAc-ol} \quad (1)
\]

The methylated disaccharide obtained from IIIB after mild acid hydrolysis gave the same mass fragmentation pattern as that obtained for desialylated, methylated IIIC (Fig. 5).

The largest O-linked oligosaccharide, IIIC, had the same

**Table II**

<p>| Methyl ethers obtained in methylation analysis of oligosaccharides in IIIA, IIIB and IIIC |
|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|</p>
<table>
<thead>
<tr>
<th>Sugar</th>
<th>T-value</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>NeuNAc (2 → 3) · d-Galp-(1 → 3) · d-GalNAc-ol</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NeuNAc (2 → 3) · d-Galp-(1 → 3) · d-GalNAc-ol</td>
<td></td>
<td></td>
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</table>

1 Retention times of the corresponding alditol saccharides relative to 1,4-di-O-acetyl-2,3,4,6-tetra-O-methyl-glucitol.
2 Desialylated by mild acid hydrolysis.
3 Quantitative determinations were not made since the response factors for the 2-(N-methylacetamido-2-deoxy-D-galactitol and 2-(N-methylacetamido-2-deoxy-D-glucitol derivatives are not known.

When intact IIIC was methylated, about 3% of the galactose residues were 2,3,4,6-tetramethylated, indicating the possible presence of a small amount of the disaccharide alditol, galactose-1-N-acetylgalactosaminitol, in IIIC.

![Fig. 5. Mass fragmentation pattern of the methylated disaccharide alditol obtained from mild acid hydrolysis of Subfraction IIIC.](image)
composition as IIIB, but with one more galactose and, in addition, 1 N-acetylgalactosamine residue. In contrast to IIIB, the methylation studies indicate that position 6 of the N-acetylgalactosaminol in IIIA is not methylated even after mild acid hydrolysis. Both galactose residues were methylated on positions 2, 4, and 6 before, and on positions 2, 3, 4, and 6 after, mild acid hydrolysis, indicating that both contained a sialic acid residue on position 3. The N-acetylgalactosamine was 3,6-dimethylated both before and after the mild acid treatment. This indicates that its position 4 was substituted. If position 3 of the N-acetylgalactosaminol contains the disaccharide, N-acetylneuraminylgalactose, in analogy with structures IIIB and IIIC, these results indicate that position 6 of the N-acetylgalactosaminol would contain a trisaccharide, N-acetylneuraminylgalactosyl-N-acetylgalactosamine. The structure of IIIA, then, would be:

\[
\text{NeuNAc-(2 → 3)-α-D-Galp-(1 → 4)-α-D-GlcNAc} \\
\text{(1 → 6)}
\]

The oligosaccharide remaining after mild acid hydrolysis of IIIA did not yield a derivative which could be analyzed directly with the mass fragment ion procedure. 

**Localization of the Oligosaccharides in the Proteoglycan**—Preparations of chondroitin sulfate-peptide clusters and of the hyaluronic acid-binding region were isolated from clastropain digests of aggregates. Samples of these preparations and of intact monomer were treated with alkaline borohydride, and the digests were chromatographed on a small analytical Bio-Gel P-10 column. For monomer, 15% of the total sialic acid eluted in the mannose-rich oligosaccharide region (Component II) and the remainder eluted in the three 0-linked oligosaccharide peaks (Fig. 6a). For the chondroitin sulfate-peptide clusters, only 3% of the sialic acid eluted in the mannose-rich oligosaccharide region; the remainder was recovered in the 0-linked oligosaccharide peaks (Fig. 6b). In this case, an equivalent amount of material, in terms of chondroitin sulfate, was used for the intact monomer. On the other hand, about 65% of the sialic acid in the preparation which contained the hyaluronic acid-binding site was present in the mannose-rich oligosaccharide fraction (Fig. 6c). In this case, about 10 times as much hyaluronic acid-binding region sample was used as would be present in the intact monomer sample. The recoveries indicate that at least 80% of the mannose-rich oligosaccharides, but less than 8% of the 0-linked oligosaccharides, that are present in intact monomer are located on the core protein fragment of M = 67,000 which contains the hyaluronic acid-binding site. Therefore, most, if not all of the mannose-oligosaccharides are located on the core protein closer to the active binding site for hyaluronic acid than are the 0-linked oligosaccharides and the chondroitin sulfate chains.

Monomer proteoglycans were purified from cultures labeled with [2-1H]mannose. The labeled monomer was mixed with unlabeled aA1-D1, and the mixture was treated with the alkaline borohydride procedure. The digest was chromatographed on Bio-Gel P-10 (Fig. 7a). The sialic acid elution profile shows the positions of the oligosaccharides. About 8% of the total radioactivity eluted in the region of the small O-linked oligosaccharides, and about 36% eluted in the region of the mannose-rich oligosaccharides and the chondroitin sulfate chains.

For oligosaccharide IIIA, we have recently analyzed the desialylated, permethylated derivative by direct probe mass-spectrometry. The pattern provides direct proof for the location of a Galp on position 3 of the GalNAc-ol and of Galp-(1 → 4)-GlcNAc on position 6 of the GalNAc-ol. Thus, the proposed structure for IIIA with the disaccharide NeuNAc-(2 → 3)-Galp on position 3 and the trisaccharide NeuNAc-(2 → 3)-Galp-(1 → 4)-GlcNAc on position 6 is correct.
directly with clostripain and chromatographed on Sepharose 4B (Fig. 8a). About 40% of the \(^3\)H radioactivity eluted with the chondroitin sulfate-peptide clusters which are indicated by the hexuronic acid peak (Fig. 8a, dotted line). A large proportion of the radioactivity in this peak is present in chondroitin sulfate chains as described above. The radioactivity that eluted later, 35% of the total, represents mannose-rich oligosaccharides and would account for 75 to 80% of the total originally present in the labeled monomer preparation. About 75% of the sialic acid eluted with the chondroitin sulfate-peptides (Fig. 8a, dashed lines), indicating that more than 80% of the total O-linked oligosaccharides are located in this fraction, consistent with the results discussed above. After digestion of a portion of the sample with trypsin and chymotrypsin, approximately 50% of the sialic acid eluted with the chondroitin sulfate-peptide fraction along with 25% of the \(^3\)H activity (Fig. 8b). Essentially all of the \(^3\)H-labeled mannose-rich oligomers eluted in the column total volume where 90% of the radioactivity and about 30% of the sialic acid eluted. After papain digestion and Sepharose 6B chromatography, the proportion of sialic acid eluting with the chondroitin sulfate-peptides decreased to 15% of the total, with the remainder distributing in two later eluting peaks (Fig. 8c). This suggests that about 15% of the O-linked oligosaccharides are located on peptides in close enough proximity to the chondroitin sulfate chains so that papain digestion does not remove them. The proportion of the \(^3\)H radioactivity associated with the chondroitin sulfate peak was the same as that observed for the trypsin, chymotrypsin digest. For all three enzyme digests, about 20% of the radioactivity eluted in the excluded column volume, probably representing labeled glycogen (1).

**Number of O-linked Oligosaccharides—**Amino acid, hexosamine, and hexosaminol analyses were done on monomer proteoglycan samples before and after alkaline borohydride treatment. The alkaline borohydride treatment destroyed 53% of the serine (73 residues serine destroyed/1000 residues of amino acids) and 41% of the threonine (36 residues threonine destroyed/1000 residues of amino acids) present in the proteoglycan, and the ratio of galactosamine/galactosaminitol in the treated sample was 37.8. The chondroitin sulfate chains in the proteoglycan have an average \(M_1 = 25,000\) (3, 8) or about 50 residues of N-acetylgalactosamine/chain. Therefore, the ratio of galactosaminol/chondroitin sulfate chain in the alkaline borohydride-treated sample was about 1.3, indicating that there would be about 1.3 O-linked oligosaccharides/chondroitin sulfate chain in the proteoglycan. The loss of serine and threonine after the alkaline borohydride treatment was consistent with this result. About 1.5 serine residues and 0.7 threonine residues/chondroitin sulfate chain were destroyed, a total excess of 1.2 alkali-labile and, therefore, substituted, residues/chain.

**Discussion**

The proteoglycan monomer from the Swarm rat chondrosarcoma contains a group of three sialic acid containing oligosaccharides which are bound to the core protein by O-glycosidic bonds between hydroxyl groups of serine and threonine residues and N-acetylgalactosamine. The structure of the smallest oligosaccharide, IIIC, consists of the trisaccharide shown in Formula 1, above. The next larger, IIIB, has the same trisaccharide with a sialic acid on position 6 of the N-acetylgalactosaminol (Formula 2, above). The largest, IIIA, appears to have the same trisaccharide structure, but with a trisaccharide substituent on position 6 of the N-acetylgalactosaminol (Formula 3, above). These structures appear to be closely related to the proposed linkage region for attaching keratan sulfate chains to the core protein. Bray et al. (16) observed a loss of N-acetylgalactosamine during alkaline treatment of human rib cartilage keratan sulfate which coincided with the appearance of a Kuhn chromogen precursor, indicating that the N-acetylgalactosaminol residues were substituted on position 3. It was concluded that a large proportion of the keratan sulfate chains were O-glycosidically linked to protein via a 3-substituted N-acetylgalactosamine. Since most of the generated chromogen remained attached to the keratan sulfate chains (see also Refs. 17 and 18), it was further suggested that the repeating disaccharide structure of the keratan sulfate was linked at a different position, most likely position 6. Subsequent work by Hopwood and Robinson (18, 19) provided evidence that a disaccharide, N-acetylneuraminylgalactose, was released from position 3 of N-acetylgalactosamine residues during alkali treatment of a keratan sulfate fraction isolated from bovine intervertebral disc. Further, Kiersa (20) isolated glycopeptides from mild acid hydrolysis of bovine nasal cartilage keratan sulfate and then oligosaccharides from the glycopeptides by alkaline borohydride treatments. A disaccharide alditol, galactosyl-(1 → 3)-N-acetylgalactosaminol was identified in the oligosaccharide fraction, indicating that this disaccharide was involved in the linkage region to the peptide. While interpretation of these studies (18–20) is somewhat complicated because of the likelihood that the keratan sulfate preparations used contained some O-glycosidically linked oligosaccharides of the type present on the rat chondrosarcoma, as well as some of the mannose-containing oligosaccharides (see Ref. 1 for discussion), the results, in sum (18–20), suggest that a significant proportion of the keratan sulfate chains in cartilage proteoglycans are linked to N-acetylgalactosamine residues which contain the disaccharide N-acetylneuraminylgalactose on position 3. The anomeric linkages between the galactose and the N-acetylgalactosamine and between the N-acetylgalactosamine and the serine and threonine residues are

![Fig. 8. The mixture of \(^3\)H-mannose-labeled monomer and unlabeled monomer was digested with different enzymes. a, clostripain followed by chromatography on an analytical Sepharose 4B column; b, trypsin plus chymotrypsin followed by chromatography on an analytical Sepharose 6B column; and c, papain followed by chromatography on an analytical Sepharose 6B column. The eluted fractions were monitored for \(^3\)H radioactivity (--.--), hexuronic acid (-----), and sialic acid (--.--).](image)
threonine hydroxyl groups remain to be determined. Since the structure of the O-linked oligosaccharides in the chondrosarcoma proteoglycan appear to be analogous to the keratan sulfate linkage region, the disaccharide linked to position 3 of the N-acetylgalactosamine postulated by Hopwood and Robinson (18) would be an N-acetylneuraminyl-(2→6)-galactose with the linkage being to position 3 of the galactose rather than the suggested positions 4 or 6. Further, the galactosyl-N-acetylgalcosamine on position 6 of the N-acetylgalactosaminyl in oligosaccharide IIIA would likely be the first disaccharide unit of the repeating backbone structure of keratan sulfate chains. The anomeric linkage between the N-acetylgalcosamine and the N-acetylglactosamine remains to be determined.

The data presented in the recent report by Thonar and Sweet (21) indicate that sialic acid-containing oligosaccharides, which are distinct from keratan sulfate, are present in proteoglycans from bovine articular cartilage. The oligosaccharides identified in their study have compositions and sizes consistent with the structure of the O-linked oligosaccharides described in this report.

The distribution of the O-linked oligosaccharides on the chondrosarcoma proteoglycan appears similar to that identified in the distribution of keratan sulfate chains on proteoglycans from bovine cartilages. The chondroitin sulfate-peptides generated from the chondrosarcoma proteoglycan by digestion with trypsin plus chymotrypsin contained about 50% of the keratan sulfate chains (22), suggesting that a proportion of the chondroitin sulfate-peptide clusters generated by this procedure contain some keratan sulfate chains. Further, following papain digestion, the chondroitin sulfate-peptides from the chondrosarcoma proteoglycan still contain 15% of the O-linked oligosaccharides. This is analogous to the “doublets” identified by Meyer and co-workers (23) and by Hopwood and Robinson (18) in which a proportion of chondroitin sulfate-peptides generated by papain digestion were found to contain a keratan sulfate chain as well. In previous studies (24) the molecular weight of keratan sulfate-peptides isolated by papain digestion from bovine nasal proteoglycans was shown to be 8 to 11 x 10^4. On the assumption that these represented single keratan sulfate chains, it was suggested that there were about 0.6 keratan sulfate chains/chondroitin sulfate chain in the proteoglycan. After alkaline borohydride treatment, the free keratan sulfate chains were found to have much smaller molecular weights, around 4000 (1). Thus, the number of keratan sulfate chains plus O-linked oligosaccharides in bovine cartilage proteoglycan molecules is probably nearly the same as that of the O-linked oligosaccharides in the chondrosarcoma proteoglycan molecules.

The reason why keratan sulfate is absent in the chondrosarcoma proteoglycan is not known. Two possibilities are that either (a) an enzyme, such as an N-acetylgalcosaminyl transferase, that is required for chain elongation is inactive or absent or (b) chain elongation on the oligosaccharide is blocked by the nonreducing terminal sialic acid residues on substituent 6 of the predominant IIIA and IIB oligosaccharides. It is of interest that there is a high proportion of the O-linked oligosaccharides on the proteoglycan from the chick limb bud chondrocyte cultures compared to keratan sulfate chains even though the keratan sulfate chains that are present are much longer than those observed in bovine nasal cartilage proteoglycans (1). This suggests that the elongation steps in keratan sulfate synthesis are not the limiting factors in determining the number of keratan sulfate chains for this case.

The linkage region of keratan sulfate in corneal proteoglycans involves an oligosaccharide which is linked to asparagine by an N-glycosylamine bond (25) and which appears to be synthesized using dolichol diphospho-N-acetylgalcosamine as a cofactor (26). Thus, oligosaccharides synthesized by quite different metabolic pathways appear to be used as acceptors for keratan sulfate synthesis in skeletal and corneal tissues. It is possible, however, that the structures within these distinctly different classes of oligosaccharides which serve as initiation sites for keratan sulfate synthesis are similar.

The trisaccharide IIC and the tetrasaccharide IIIb are probably identical to those originally described by Spiro and Bhoyroo (27) and more recently by Nilson et al. (28) as present on fetuin. The anomeric linkage of the galactosyl bond was shown to be β in these studies but remains to be determined for IIIb and IIIC. The fetuin trisaccharide has also been recently identified in the epiglycanin isolated from TA3-HA mouse ascites cells (29), and both the trisaccharide and tetrasaccharide appear to be present in a mouse melanoma glycoprotein (30) and in rat brain glycoproteins (31), while the tetrasaccharide appears to be present in a human erythrocyte glycoprotein (32). Thus, these structures appear to be widespread.

The rat chondrosarcoma proteoglycan also contains the class of mannosyl-oligosaccharides described for proteoglycans from chick limb bud chondrocytes in the accompanying paper (1). These oligosaccharides are probably in the N-glycosylamine family since the alkaline borohydride treatment did not generate any reduced sugars. The extensive degradation of the core protein by the alkaline borohydride procedure would release these as oligosaccharide-peptides. In the experiments described in this report, it was shown that most of these oligosaccharides are located on the M, = 67,000 polypeptide isolated from the hyaluronic acid-binding region after digestion of aggregates with clostripain, whereas the majority of the O-linked oligosaccharides were present in the chondroitin sulfate-peptide fragments. If there are about 2 sialic acid residues/mannose-oligosaccharide, the amount of sialic acid in these oligosaccharides, about 15% of the total, suggests that there would be about 0.2 of these oligosaccharides/chondroitin sulfate chain. Their function and details of their structure remain to be determined.

The results presented in this paper and the previous one (1) suggest that hyaline cartilage proteoglycans contain, in addition to the glycosaminoglycan chains, O-linked oligosaccharides common to the “mucin” class of macromolecules and mannos-rich oligosaccharides of the type found in most glycoproteins. A hypothetical average proteoglycan molecule of 2.5 x 10^5 molecular weight, then, would contain about 85 chondroitin sulfate chains of M, = 25 x 10^3, about 110 total keratan sulfate chains plus O-linked oligosaccharides, and about 15 mannos-rich oligosaccharides. It would be expected that the proportion of O-linked oligosaccharides/keratan sulfate would be quite high in proteoglycans, such as those from fetal cartilage, which have low keratan sulfate content and, conversely, would be low in proteoglycans such as those from older cartilages, which contain high proportions of keratan sulfate. Studies using procedures described in these reports should help clarify the variations observed for keratan sulfate structure and contents in different cartilaginous tissues.

The recent report by Sweet et al. (33) which appeared after this paper was submitted demonstrates that this is indeed true. Proteoglycans from immature bovine articular cartilage contained much higher proportions of the O-linked oligosaccharides than did proteoglycans from adult bovine articular cartilage.