Proteoglycans from Chick Limb Bud Chondrocyte Cultures

KERATAN SULFATE AND OLIGOSACCHARIDES WHICH CONTAIN MANNOSE AND SIALIC ACID*

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The precursors, [35S]sulfate and [2-3H]mannose, were used to study the biosynthesis of keratan sulfate and other oligosaccharides on proteoglycans isolated from Day 8 cultures of chick limb bud chondrocytes. After alkaline borohydride treatment, three fractions with sialic acid were separated by molecular sieve chromatography. The first contained keratan sulfate which was purified by digestion with chondroitinase to remove chondroitin sulfate, followed by molecular sieve and ion exchange chromatography. The purified keratan sulfate contained about 8% of the [35S] activity originally in monomer. The chains had an average length of about 40 monosaccharides and contained only trace amounts of mannose (less than 1 residue/three to four chains). The second fraction contained the majority of the [3H]mannose originally in monomer, but no [35S] activity. This fraction appears to contain oligosaccharide-peptides of the asparagine-N-glycosylamine type because there were no reduced sugars present and the alkaline borohydride treatment extensively degraded the core protein. The composition of the oligosaccharides, with high proportions of mannose, N-acetylgalactosamine, galactose, and sialic acid, was consistent with this suggestion. The third fraction consisted of a series of oligosaccharides with sizes between three to six saccharides. They contained N-acetylgalactosaminitol, indicating that they were attached to the core protein by O-glycoside bonds between N-acetylgalactosamine and hydroxyl groups on serine and threonine. Thus, proteoglycans contain two classes of oligosaccharides, a mannose-rich class characteristic of glycoproteins and an O-glycoside class characteristic of mucins, in addition to the chondroitin sulfate and keratan sulfate chains.

Previous work with chondrocyte cultures derived from stage 23 to 24 chick limb bud mesenchyme cells has indicated that the extracellular matrix synthesized by the cells contains a large proportion of proteoglycans which are characteristic of mature cartilage (1-4). The proteoglycans are large, contain large numbers of chondroitin sulfate and keratan sulfate chains, and are able to interact with hyaluronic acid and link proteins to form aggregates (1-4). The average size of the keratan sulfate chains in the molecules appears to vary, depending upon the stage of developmental maturation of the chondrocytes and their surrounding matrix (2).

The chemical properties of skeletal keratan sulfate have been partially described (5-12). Most, if not all, keratan sulfate chains in cartilage proteoglycans are linked to core protein by O-glycosidic bonds between N-acetylgalactosamine and hydroxyl groups on serine and threonine. Position 3 of most of the linkage N-acetylgalactosamine residues contains a disaccharide, N-acetylenuraminyl-(2 → 3)-galactose (11, 19). Position 6 of the linkage N-acetylgalactosamine residue appears to be the location where the keratan sulfate chains, consisting of the repeating disaccharide β-galactosyl-(1 → 4)-β-N-acetylglucosaminyl-(1 → 3), are attached (5, 6, 8, 13). Position 6 of the N-acetylgalactosamines and, variably, position 6 of the galactoses, are sulfated (14). The keratan sulfate chain lengths are highly variable and the nonreducing termini appear to be substituted with sialic acid residues (14). Evidence has also been reported suggesting that skeletal keratan sulfate contains mannose, although the location of mannose residues in the structure has not been defined (10, 14).

The present study was undertaken to investigate additional details of the synthesis of keratan sulfate in the chick limb bud chondrocyte cultures. During the course of the investigations, two additional classes of oligosaccharides present on the proteoglycan molecules were discovered, a class which contains mannose and a class which contains structures similar to those of the linkage region between keratan sulfate and the core protein. The chemical structures of the second class of oligosaccharides are described in more detail in an accompanying paper in which the oligosaccharides were purified from proteoglycans isolated from the Swarm rat chondrosarcoma (13).

EXPERIMENTAL PROCEDURES

Materials—Ultrapure guanidine HCl and cesium chloride were obtained from Bethesda Research Laboratories, Inc.; 6-aminohexanoic acid and benzamidine HCl were from Eastman Kodak Co.; papain (twice crystallized), diphenylcarbamyl chloride-treated trypsin, α-chymotrypsin (three times crystallized), and phenylmethylsulfonyl fluoride were from Sigma; chondroitinase ABC (Proteus vulgaris) was from Miles Laboratories; Bio-Gel P-10 and P-30 were from Bio-Rad; Sephadex and Sepharose were from Pharmacia Fine Chemicals; DEAE-cellulose (DE52) was from Whatman Ltd.; tissue culture supplies were from Grand Island Biological Co.; carrier-free [35S]sulfate, as the sodium salt (approximately 800
mCi/ml (sodium 14C), and Aqualon was from New England Nuclear. [2-14C]mannose (2 Ci/mmole) was from Amersham/Searle, and N-acetylamino acid was from Calbiochem.

**Cell Cultures**—The conditions for isolating and culturing stage 23 to 24 chick limb bud mesenchymal cells have been described elsewhere (1). Approximately 8 cultures, 54-60 mg of proteoglycan, sample (1), were used. For radioisotope labeling of the cultures, 10-15 ml Petri dishes initially seeded with 50 100 0.25 1 ml plus 12 0.5 ml and bathed in medium 37°C for 10 h. The leaves of the cells and matrix were washed twice with cold Tyrode's balanced salt solution and then collected and frozen as described previously (1, 2). Purified bovine nasal cartilage proteoglycans (A1-D1) were obtained as described previously (16).

**Enzymatic Treatments**—Chondroitinase ABC (0.05 unit/mg of sample) and trypsin and chymotrypsin (10 µg/mg of sample) digestions were done in 0.1 M sodium acetate, 0.1 M Tris, pH 7.3, at 37°C as described elsewhere (2). Papain digestion (30 µg/ml of sample) was done at 55°C for 4 h in 0.1 M sodium acetate, pH 7.0, containing 0.005 M sodium EDTA and 0.005 M cysteine hydrochloride. All digests were chromatographed immediately or after storage at -30°C.

**Analytical Procedures**—Hexosamine and hexosaminidase analyses were done with a Duromax analyzer (20). [35S]Sulfate (0.5 µCi/ml) was eluted with 0.1 M guanidine HCl extraction and purified by direct dissociative density gradient centrifugation as described previously (1, 2). Purified bovine nasal cartilage proteoglycans (A1-D1) were obtained as described previously (16).

**Column Chromatography**—Analytical Sepharose 2B, Sepharose CL-4B, Sepharose 6B, Sephadex G-75, and Sephadex G-200 gel filtration columns (10 x 0.6 cm) were prepared and eluted with 0.5 M sodium acetate, pH 7.0. Bio-Gel P-10 (200 to 400 mesh) and Bio-Gel P-30 (minus 400 mesh) analytical and preparative columns were eluted with 0.1 M pyridinium acetate, pH 6.5. DEAE-cellulose was washed with 0.5 M NaOH, 0.1 M HCl, H2O, 0.1 M NaOH and then extensively with H2O to neutrality. The columns (0.6 x 10 cm or 1.5 x 25 cm) were eluted with 0 to 1.0 M gradient of pyridine acetate, pH 6, in 0.1 M step increases followed by 3 column volumes of 0.5 M pyridinium acetate buffer.

**Alkaline Borohydride Treatment**—Treatment with alkaline borohydride was done according to Carlson (20) under conditions which prevent the degradation of sugar chains by the "peeling" reaction: 0.05 M NaOH, 1 M NaBH4, 45°C, 48 h. Excess borohydride was destroyed after the treatment by neutralization with glacial acetic acid. Samples treated with alkaline borohydride were chromatographed immediately or after storage at -30°C. For preparative purposes, 250 µg of unlabeled D1 proteoglycan isolated from Day 8 cultures was mixed with 5 µg of [35S]sulfate-labeled D1 (10 x 104 dpm) and 100 µg of [1-14C]mannose-labeled D1 (4 x 105 dpm). The mixture was treated with alkaline borohydride as described above (25 µg of untreated proteoglycan/ml of alkaline borohydride solvent). The digest was neutralized, clarified by centrifugation, and chromatographed on a preparative Bio-Gel P-10 column (2.5 x 155 cm) in two aliquots of approximately 125 mg each. Fractions of 4 ml were collected and monitored for radioactivity, sialic acid, and uronic acid contents. The three peak regions, I, II, and III, (see Fig. 4 below) were separately recovered by lyophilization and further purified.

**Isolation and Characterization of Keratan Sulfate**—The Peak I was dissolved in 10 ml of 0.1 M sodium acetate, 0.1 M Tris, pH 3.5, and dialyzed extensively against the same buffer. The sample then was digested with chondroitinase ABC (1 unit/ml, 37°C, 1.5 h) and the digest was chromatographed on Bio-Gel P-30 (0.9 x 145 cm) in four aliquots of approximately 2.5 ml each. The eluted fractions, 1 ml each, were analyzed for radioactivity. The keratan sulfate peak (Ks = 0.59) was recovered, lyophilized, and further purified by ion exchange chromatography on DEAE-cellulose. The keratan sulfate peak then was dialyzed against H2O and lyophilized. Hexosamines, neutral sugars, and sialic acid contents were determined. A portion of the sample was chromatographed on Sephadex G-200 in order to estimate the molecular size.

**Isolation and Characterization of Mannose-rich Oligosaccharides**—A small portion of the [3H]-labeled Peak II component (about 15,000 dpm) was hydrolyzed with 3 N HCl at 100°C for 3 h. The sample then was analyzed for its radioactive sugar content with a Hewlett-Packard model 5700A gas chromatograph equipped with a 101 stream splitter; eluent fractions were collected as described elsewhere (21). The sugars were detected as butane boronic acid derivatives of the hexitols, according to the procedure described by Eisenberg (22). Carrier hexoses as butane boronic acid derivatives were mixed with the radioactive sample just before analysis on the gas chromatograph.

The remainder of the Peak II component was dissolved in 1 ml of H2O and chromatographed on DEAE-cellulose with a step gradient of pyridinium acetate. A similar procedure has been used previously to separate sialic acid-containing oligosaccharides (23). Three [3H]-labeled peaks, eluting with H2O or 0.1 or 0.3 M pyridinium acetate, respectively, were separately lyophilized and analyzed for hexosamines, neutral sugars, and sialic acid contents.

**Characterization of the 0-Linked Oligosaccharides**—A portion of the Peak III oligosaccharide fraction was chromatographed on an analytical Bio-Gel P-10 column (0.5 x 110 cm). The fractions were analyzed for contents of sialic acid and radioactivity. The Bio-Gel P-10 column was calibrated with oligosaccharides of known size obtained by partial degradation of hyaluronic acid by testicular hyaluronidase (24). A separate portion of the Peak III sample was analyzed for neutral sugars, hexosamines, and sialic acid contents.

**RESULTS**

**Comparison of Keratan Sulfate in Proteoglycans from Bovine Nasal Cartilage and Chick Limb Bud Chondrocyte Cultures**—Proteoglycan monomer, D1, was isolated from Day 8 cultures labeled with [35S]sulfate. Carrier monomer, A1-D1, from bovine nasal cartilage was added, and an aliquot of the mixture was chromatographed on Sepharose 2B (Fig. 1a). The elution profiles for hexuronic acid (bovine cartilage monomer) and radioactivity (monomer from the cultures) were very similar, indicating that the size distribution of the intact molecules are nearly the same for both proteoglycans. The sizes of the keratan sulfate chains were studied by treating the proteoglycan mixture with chondroitinase ABC and then with alkaline borohydride to remove the individual keratan sulfate chains from the protein core. The digests were chromatographed on Bio-Gel P-30 (Fig. 1c). The borohydric reactive peak (shaded area) shows the elution position of keratan sulfate chains from the bovine proteoglycan (25) and corresponds to where an oligomer of hyaluronic acid with 13 residues would elute. Approximately 8% of the total 35S radioactivity eluted as an earlier peak in a position where hyaluronic acid oligomers with approximately 26 residues would elute. The remaining radioactivity eluted in the total column volume where the chondroitin sulfate disaccharide digestion products elute. In the solvent conditions used, the keratan sulfate chains interact weakly with the polyacrylamide support and, therefore, elute slightly later than their molecular size would dictate. When the labeled keratan sulfate sample was chromatographed on a calibrated Sephadex G-200, the elution peak (26) corresponded to the position where disaccharide products elute.

1 The abbreviations used are those introduced by Heinegibb (15) to denote proteoglycan fractions: D1 and A1-D1 are monomer preparations derived either directly from a dissociative gradient (D1) or from a dissociative gradient of an aggregate preparation (A1-D1).
$M_r = 9500$ chondroitin sulfate chains elute (26) (data not shown). If the keratan sulfate chains have similar hydrodynamic properties as chondroitin sulfate, this would correspond to average chain lengths of about 35 to 40 monosaccharides, a value consistent with the chemical analyses discussed below. The keratan sulfate chains on the chick proteoglycan, then, have an average size at least twice that of the keratan sulfate chains from the bovine cartilage proteoglycan.

Experiments by Heinégärd and Axelsson (25) have demonstrated that about 60% of the keratan sulfate chains on proteoglycans from bovine hyaline cartilages reside on a portion of the core protein, the keratan sulfate-rich region, where less than 10% of the total chondroitin sulfate chains are located. An experiment was done to determine if the proteoglycan from the cultures contained a similar region. The monomer mixture was digested sequentially with chondroitinase ABC, trypsin, and chymotrypsin, and the digest was chromatographed on Sepharose 6B (Fig. 1b). The first carbazole reactive peak (Fig. 1b, shaded area) shows the position of the keratan sulfate-rich peptides for the bovine proteoglycans (25). The second carbazole reactive peak contains some keratan sulfate chains on smaller peptides as well as the chondroitin sulfate attachment region oligosaccharide-peptides remaining after the chondroitinase digestion (25). The peak in the total volume contains the chondroitin sulfate disaccharide digestion products. Approximately 10% of the $^{35}$S radioactivity eluted as a broad peak skewed toward smaller molecular sizes.

The peak position (Fig. 1b, arrow) was more retarded than the first carbazole reactive peak. This indicates that a large proportion of the keratan sulfate on the proteoglycans from the cultures resides on peptides analogous to the keratan sulfate-rich peptides observed for the bovine proteoglycans. However, since the average size of these keratan sulfate peptides is smaller for the labeled proteoglycans whereas the average size of the keratan sulfate chains is larger, each peptide must contain far fewer chains.

**Analyses of Proteoglycans Labeled with [2-3H]Mannose—** Day 8 cultures were incubated with [2-3H]mannose and the monomer proteoglycans were isolated. When an aliquot was chromatographed on Sepharose 2B, an elution profile typical for monomer proteoglycans was observed (Fig. 2a, dashed line). For comparison, the solid line (Fig. 2a) shows the elution profile of a $^{35}$S-labeled monomer isolated from Day 8 cultures incubated with $[^3S]$sulfate and chromatographed on the same column. When the $^3$H-labeled monomer was digested with chondroitinase ABC and chromatographed on Sepharose CL-4B in the presence of 4 M guanidine HCl, the profile in Fig. 2b was obtained. About 20% of the $^3$H activity eluted in the total volume of the column; this $^3$H activity was present in disaccharide digestion products of chondroitin sulfate.

The fact that some of the $^3$H activity in the proteoglycan was present in chondroitin sulfate chains (and often in small amounts of contaminating glycan, see below) suggests that the radioactive precursor may have contained small amounts of $^3$H-labeled glucose or of $^3$H activity on positions other than position 2 of the mannose since the $^3$H would be lost if $[2$-[3H]$]$mannose were epimerized to glucose. The major $^3$H peak ($K_v = 0.47$) contained about 70% of the total activity and eluted in the same position as chondroitinase-digested core protein. This indicates that most of the $^3$H activity in the proteoglycans isolated from cultures labeled with [2-3H]mannose residues in structures which remain associated with the core protein after chondroitinase digestion. As discussed below, most of this $^3$H activity is still present in mannose residues.

Aliquots of monomer from cultures labeled with [3S]sulfate and from cultures labeled with [2-3H]mannose were mixed and portions were treated with (a) papain, (b) chondroitinase ABC followed by papain, (c) alkaline borohydride, and (d) chondroitinase ABC followed by alkaline borohydride. The treated samples were chromatographed on Sephadex G-75 (Fig. 3). In all cases, 57 to 65% of the total $^3$H label eluted as a peak in a region with no corresponding peak of $^{35}$S radioactivity (Fig. 3, shaded areas). As discussed below, more than 80% of the $^3$H label in this component was present in mannose residues. For the two samples treated with chondroitinase (Fig. 3, b and d), about 25% of the $^3$H label eluted in the total volume of the column as chondroitin sulfate digestion products. In both of these cases, a small amount of $^3$H label eluted in the excluded volume. This peak was shown to contain glycan and the $^3$H radioactivity resided primarily in glucose.

**Fig. 2.** Sepharose chromatography of labeled monomer proteoglycans. Panel a shows the elution profile on Sepharose 2B chromatography of [2-3H]mannose-labeled (---) and of $[^3S]$sulfate-labeled (-----) monomer proteoglycans. Panel b shows the elution pattern of [2-$^3$H]mannose-labeled monomer proteoglycans chromatographed on Sepharose CL-4B after digestion with chondroitinase ABC (---). The eluting solvent was 4 M guanidine HCl.
residues (data not shown). After chondroitinase ABC and papain (Fig. 3b), 11% of the $^{35}$S label was present as keratan sulfate chains. The elution position for the keratan sulfate peptides (from the papain digest, Fig. 3b) was earlier than for the free chains (Fig. 3d), suggesting that there is more than one keratan sulfate chain/peptide in the papain digest, consistent with previous observations (12, 28). Neither the keratan sulfate peptides (Fig. 3b) nor the keratan sulfate chains (Fig. 3d) elute with the major $^3$H-labeled peak, indicating that the [$^3$H]mannose residues are located in oligosaccharides which are distinct from the keratan sulfate. For the direct papain and alkaline borohydride treatments (Fig. 3, a and c), the majority of the $^{35}$S activity, located in chondroitin sulfate, was excluded from the column.

Isolation of Keratan Sulfate and Oligosaccharide Fractions—A large amount (250 mg) of unlabeled monomer was isolated from Day 8 cultures. This preparation was mixed with small amounts of $^{35}$S- and $^3$H-labeled monomer preparations. The sample was treated with alkaline borohydride and chromatographed on a preparative Bio-Gel P-10 column (Fig. 4). As is shown in the accompanying paper, this treatment extensively degrades the core protein (13). Eluent fractions were analyzed for $^3$H and $^{35}$S radioactivity and for sialic acid. The chondroitin sulfate and keratan sulfate chains eluted in and shortly after the excluded volume fraction, Peak I, where the large majority of the $^{35}$S radioactivity was recovered. The [$^3$H]mannose-labeled Peak II was also observed. The sialic acid profile yielded three peaks. The first, eluting in the Peak I region, contained about 14% of the total sialic acid and represents the sialic acid located in keratan sulfate chains (as is discussed below). The second, containing approximately 22% of the total sialic acid, eluted in the Peak II region with the majority of the included $^3$H activity. The third, Peak III, contained about 64% of the total sialic acid. No $^{35}$S radioactivity was observed in the Peak III region, but a shoulder of $^3$H radioactivity extending from the Peak II region was present.

Analyses of Keratan Sulfate—The Peak I fraction was recovered by lyophilization, dissolved in buffer, dialyzed, and digested with chondroitinase ABC. The digest was chromatographed on Bio-Gel P-30 (Fig. 5a). The keratan sulfate resolved in an included peak (Fig. 5a, shaded area) well separated from the chondroitin sulfate digestion products. Only small amounts of $^3$H radioactivity were found in the column, indicating that the dialysis step removed some of the residual contaminating Peak II fraction (the mannos oligosaccharides) from the keratan sulfate. The keratan sulfate peak was isolated from several identical column runs, pooled, and lyophilized (recovery = 9 mg). A portion of this keratan sulfate, 3 mg, was applied to DEAE-cellulose, followed by elution with a step gradient of pyridinium acetate, then 1 M KCl (Fig. 5b). The pyridinium acetate gradient removed a small amount of $^3$H-labeled material primarily in the 1 M pyridinium acetate fraction. The $^{35}$S-labeled keratan sulfate was quantitatively recovered in the 1 M KCl fraction (Fig. 5b,
neuraminic acid. Liquid chromatography as described in Ref. 13. GlcNAc, N-acetylgalactosamine; NeuNAc, N-acetylneuraminic acid.

Approximately 30% was recovered as galactosamine residues from the core protein is incomplete because the presence of either a free carboxyl or amino group on the serine or threonine to which the galactosamine is attached would prevent the β elimination step (29).

Table I shows the chemical composition of this purified keratan sulfate fraction. As expected, the ratio of N-acetylgalactosamine/galactose was 1. The ratio of N-acetylgalactosamine/N-acetylgalactosamine was 20, suggesting that the number average chain length for the keratan sulfate would be about 40 monosaccharides, assuming 1 N-acetylgalactosamine residue/chain. The ratio of sialic acid/N-acetylgalactosamine was about 2, suggesting the presence of about 2 sialic acid residues/chain. A trace amount of mannose, about 0.3 residue/N-acetylgalactosamine, was observed. No xylose was observed, indicating the absence of any chondroitin sulfate. In hexosaminol analyses, about 70% of the total N-acetylgalactosamine residues were recovered as galactosaminol as a result of β elimination of the chains from the core protein (see Footnote a in Table I).

Table II shows the chemical composition of the Peak II fraction recovered as indicated in Fig. 4 above was hydrolyzed for neutral sugar analysis. The boronic acid derivatives of the heptitols were separated by gas liquid chromatography and fractions were counted for radioactivity (21). Slightly more than 10% of the counts eluted ahead of the heptitols, some of which may have been in fucose. Of the remaining activity, 92% was present in mannose, 5% in glucose, and 3% in galactose. The remainder of the Peak II fraction was dissolved in water and applied to a DEAE-cellulose column (Fig. 5c). Approximately 55% of the H activity was not bound to the column (Peak IIa), whereas the remaining H activity was recovered primarily in two fractions eluting at 0.1 M pyridinium acetate (Peak IIb), and at 0.3 M pyridinium acetate (Peak IIc); with a trace of activity in the 1 M pyridinium acetate fraction. The proportions of H activity in Peaks IIb and IIc were 29 and 14%, respectively. The 35S activity in Peak II, representing residual chondroitin sulfate and keratan sulfate chains, remained bound to the column until 1 M KCl was used as the eluent.

The H-labeled peaks were recovered by lyophilization and their compositions were determined (Table II). The three major fractions from the DEAE-cellulose column presented a general similarity in their composition. Mannose, N-acetylgalactosamine, and galactose were the predominant sugars and fucose was a minor component observed in all three peaks. N-Acetylgalactosamine was either not detected or was present at less than 4% of the total sugars. The two fractions bound to the DEAE-cellulose (Peaks IIb and IIc) contained at least twice as much sialic acid as Fraction IIa, thus explaining, in part, the different behavior of these fractions on the ion exchange column. The alkaline borohydride treatment did not produce any sugar alcohol, thus excluding an O-glycoside linkage; the oligosaccharides, then, would still be bound to small peptides resulting from the degradation of the core protein by the alkaline borohydride treatment. The similarity in composition between these oligosaccharides and those typically linked to glycoproteins by N-asparagine glycosylaspartate bonds (30) suggest that these mannose-rich oligosaccharides are probably linked to the core protein by O-glycoside bonds between N-acetylgalactosamine and hydroxyl groups on serine or threonine residues. The major sugars in Peaks III are N-acetylgalactosaminotet, N-acetylgalactosamine, galactose, and sialic acid. A portion of the Peak III fraction was chromatographed on an analytical Bio-Gel P-10 column and the radioactivity and sialic acid contents of eluent fractions were determined (Fig. 6). The oligosaccharides partially resolved into a series of three to four oligosaccharide peaks eluting in the region where hyaluronic acid oligomers with between two and six monosaccharides elute. The predominant peak elutes near the position of a tetrasaccharide of hyaluronic acid and in the same region as Peaks IIIA and IIIB from the rat chondrosarcoma proteoglycan (13). The H activity did not elute with a similar profile as the sialic acid suggesting that some of the Peak II mannose-oligosaccharides with similar size may be contaminants in the Peak III fraction.

Table III shows the chemical composition of the Peak III sialate oligosaccharide fraction. The sugar composition was analyzed as described in Ref. 13. GlcNAc, N-acetylglucosamine; NeuNAc, N-acetylneuraminic acid; galactosamine. Analyses of Oligosaccharides in Peak III—The composition of the oligosaccharides recovered in Peak III (Fig. 4), is shown in Table III. All of the N-acetylgalactosamine was converted to N-acetylgalactosaminotet by the alkaline borohydride treatment, indicating that the predominant oligosaccharides in this fraction were linked to the core protein by O-glycoside bonds between N-acetylgalactosamine and hydroxyl groups on serine or threonine residues. The major sugars in Peaks III are N-acetylgalactosaminotet, N-acetylgalactosamine, galactose, and sialic acid. A portion of the Peak III fraction was chromatographed on an analytical Bio-Gel P-10 column and the radioactivity and sialic acid contents of eluent fractions were determined (Fig. 6). The oligosaccharides partially resolved into a series of three to four oligosaccharide peaks eluting in the region where hyaluronic acid oligomers with between two and six monosaccharides elute. The predominant peak elutes near the position of a tetrasaccharide of hyaluronic acid and in the same region as Peaks IIIA and IIIB from the rat chondrosarcoma proteoglycan (13). The H activity did not elute with a similar profile as the sialic acid suggesting that some of the Peak II mannose-oligosaccharides with similar size may be contaminants in the Peak III fraction.
This is consistent with the presence of a small amount of mannose in the Peak III fraction (Table III). Isolating sufficient amounts of proteoglycans from the chick limb bud cultures to purify the individual O-linked oligosaccharides in Peak III for further characterization is difficult because of the large number of cultures required. Because the proteoglycans from the Swarm rat chondrosarcoma contain a similar class of oligosaccharides, purification and structural analyses of these oligosaccharides were undertaken in that system as described in the accompanying report (13).

**DISCUSSION**

The keratan sulfate chains on the proteoglycans isolated from cultures of chick limb bud chondrocytes have an average estimated molecular weight of about 9,500, at least twice that of those present on proteoglycans isolated from bovine nasal cartilage. The chondroitin sulfate chains on the proteoglycans from the cultures used in these experiments had an average molecular weight of about 25,000$^6$ (28). Since 8% of the $^35$S radioactivity in the macromolecules resides in keratan sulfate, there would be about one keratan sulfate chain for every four chondroitin sulfate chains.

In addition to chondroitin sulfate and keratan sulfate, the proteoglycans from the chick limb bud chondrocyte cultures contain a class of oligosaccharides linked to the core protein through $\text{O}$-glycoside bonds between N-acetylgalactosamine and serine and threonine hydroxyl groups. There is about 4.5 times as much sialic acid in these oligosaccharides as there is in the keratan sulfate chains. Assuming that the average number of sialic acid residues/oligosaccharide is the same as for the keratan sulfate chains, this suggests that there would be slightly more than one oligosaccharide for every chondroitin sulfate chain. Thus, the total of keratan sulfate chains plus O-linked oligosaccharides would be almost 1.4/chondroitin sulfate chain, a value close to that estimated for the ratio of O-linked oligosaccharides/chondroitin sulfate chains in proteoglycans from the rat chondrosarcoma (see "Discussion" in accompanying paper (13)).

Approximately 20% of the sialic acid in the chick chondrocyte proteoglycan is located in the mannose oligosaccharides. These oligosaccharides were selectively labeled with [1H]mannose and contain the majority, if not all, of the mannose present in the macromolecules. They have a composition similar to that of a large number of glycoprotein oligosaccharides linked to protein by N-glycosylamine bonds to asparagine (30). For this reason, and because no reduced sugar moieties were detected after the alkaline borohydride treatment (31), it is likely that these oligosaccharides are linked to the core protein by N-glycosylamine bonds to asparagine. The average number of the mannose-rich oligosaccharides/core protein cannot be estimated accurately in the absence of additional information about their structure. However, if they contain the same average number of sialic acid residues as the keratan sulfate chains, then they would be at least as abundant, i.e. more than one/every four chondroitin sulfate chains.

In analogy with the results obtained for the rat chondrosarcoma proteoglycan (13), it is likely that most of these oligosaccharides are concentrated on the core protein near the hyaluronic acid-binding region.

At present, there is no evidence available on whether or not proteoglycans from the more thoroughly studied bovine hyaline cartilages contain the same or similar mannose-rich oligosaccharides. Such proteoglycans do contain mannose, and, further, keratan sulfate preparations isolated from them after proteolytic digestion procedures contain mannose (10, 12, 14). This has led to the suggestion that mannose is an integral part of the keratan sulfate structure (10, 14). However, the keratan sulfate isolated from the chick chondroitin proteoglycan was essentially devoid of mannose, less than 0.3 residues/chain. It is quite possible that the procedures used to isolate the keratan sulfate fractions from bovine cartilages in previous studies (5-12, 14) would not remove the mannose-rich oligosaccharides, and it is likely, then, that at least part of the mannose in the bovine proteoglycans resides in such oligosaccharides and not in keratan sulfate. Thus, the suggestion that mannose is an integral part of the keratan sulfate structure may need to be reconsidered when isolation techniques similar to those described in this report are applied to bovine cartilage proteoglycans.

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**REFERENCES**

Chick Chondrocyte Proteoglycans