Distribution of Keratan Sulfate in Cartilage Proteoglycans*

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After chondroitinase digestion of bovine nasal and tracheal cartilage proteoglycans, subsequent treatment with trypsin or trypsin followed by chymotrypsin yielded two major types of polypeptide-glycosaminoglycan fragments which could be separated by Sepharose 6B chromatography. One fragment, located close to the hyaluronic acid-binding region of the protein core, had a high relative keratan sulfate content. This fragment contained about 60% of the total keratan sulfate, but less than 10% of the total chondroitin sulfate present in the original proteoglycan preparation. The weight average molecular weight of the keratan sulfate-enriched fragment was 122,000, as determined by sedimentation equilibrium centrifugation. The chemical and physical data indicate that this fragment contains an average of 10 to 15 keratan sulfate chains, if the average molecular weight of individual chains is assumed to be about 8,000, and about 5 chondroitin sulfate chains attached to a peptide of about 20,000 daltons. The other population of fragments was derived from the other end of the proteoglycan molecule, the chondroitin sulfate-enriched region, and contained mainly chondroitin sulfate chains. About 90% of the total chondroitin sulfate, but only 20 to 30% of the total keratan sulfate was recovered in these fragments. On the average, approximately 5 chondroitin sulfate chains and 1 keratan sulfate chain could be linked to the same peptide. Another 10 to 20% of the total keratan sulfate, originally found in or near the hyaluronic acid-binding region, was not separated from the chondroitin sulfate-enriched fragments. Hydroxylamine could be used to liberate a large molecular size, chondroitin sulfate-enriched fragment ($K_v$ 0.54 on Sepharose 2B) from the proteoglycan aggregates. The remainder of the protein core, containing the keratan sulfate-enriched region, was bound to hyaluronic acid with the link proteins and recovered in the void volume on the Sepharose 2B column.

The current model for the structure of proteoglycan monomer molecules isolated from hyaline cartilages is that of a central protein core, with an average molecular weight of 200,000, to which approximately 100 chondroitin sulfate and 50 keratan sulfate side chains are covalently attached (1, 2). About one-third of the protein, located at one end of the core, contains few or no polysaccharide side chains (3). This portion of the protein (the hyaluronic acid-binding region) exhibits a specific, noncovalent interaction with hyaluronic acid (3-5). This interaction is essential for the formation of proteoglycan aggregate structures. Such aggregates appear to be the predominant way in which the proteoglycans are organized in cartilage extracellular matrices (6). Available data indicate that the chondroitin sulfate chains are attached to the protein core in clusters which contain from 1 to as many as 10 individual, closely spaced chains (7). The peptide sequences between the chains within the clusters are short, whereas the peptide sequences separating the clusters are considered to be longer. The peptide sequences within the clusters are not hydrolyzed by treatment with a combination of trypsin and chymotrypsin, while those between the clusters are.

Little is known about the distribution of the keratan sulfate side chains along the protein core, although some evidence has been presented which indicates that the region of the core protein isolated with the hyaluronic acid-binding region is enriched in keratan sulfate chains relative to chondroitin sulfate chains (3). However, many details about the keratan sulfate chains, their chemical structure, their mode of attachment to the protein core, and their average chain size remain to be determined. The available evidence suggests that most of the keratan sulfate chains are attached to the protein through glycosidic bonds between galactosamine and the hydroxyl groups of serine and threonine residues (8, 9), although another type of linkage to glutamic acid or glutamine has also been proposed (9, 10). The galactosamine moiety often appears to be substituted on position 3 with a neuraminylgalactosyl disaccharide and on position 6 with the characteristic keratan sulfate chain (9) which consists of about 10 to 15 repeat units (2, 11) of the disaccharide (β-1,3-galactose β-1,4-2-deoxy-2-acetamido-β-galactose 6-sulfate). The keratan sulfate-peptide fragments isolated from papain digests of proteoglycans are polydisperse, with molecular weights of 5 to $10 \times 10^7$ (2, 9), whereas keratan sulfate chains isolated from alkali-treated proteoglycans are considerably smaller (2). Previously it was shown that at least 30% of the keratan sulfate chains are present in the chondroitin sulfate peptide fractions isolated by cetylpyridinium chloride precipitation of trypsin/chymotrypsin-digested proteoglycans (7). These keratan sulfate frag-

* Unpublished observations.
ments could not be separated from the chondroitin sulfate fragments by gel chromatography. Because of the selectivity of the cetylpyridinium chloride precipitation step for chondroitin sulfate, it was proposed that these keratan sulfate chains are located on peptide fragments which contain at least one chondroitin sulfate chain (7).

The purpose of this paper is to describe data which indicate that a large proportion of the keratan sulfate chains in proteoglycan molecules are located in a region of the protein which is located near the hyaluronic acid-binding portion of the core and that this region contains only a small proportion of the chondroitin sulfate chains present in the macromolecules. In contrast, the chondroitin sulfate chains are located primarily on the portion of the protein core furthest away from the hyaluronic acid-binding region. A preliminary account of some of the data discussed here has been given elsewhere (12).

EXPERIMENTAL PROCEDURES

Chemicals—All reagents used were analytical grade. Chondroitinase ABC was purchased from Miles. Diphenylcarbamyl chloride-treated trypsin was obtained from Sigma and 8-chymotrypsin from Koch-Light. The hydrochloric acid used for acid hydrolysis was ARISTAR grade (British Drug Houses). Gels for gel chromatography were obtained from Pharmacia Fine Chemicals. Guanidinium chloride was obtained from British Drug Houses. Gels for gel chromatography were obtained from Pharmacia Fine Chemicals.

Column Chromatography—The preparation of buffers and columns and the elution conditions were essentially as described previously (6). Preparative columns of Sepharose 2B (176 × 2.2 cm) and Sepharose 6B (192 × 2.2 cm and 146 × 2.0 cm) were eluted at 18°C with 0.5 M sodium acetate, pH 7.0. 10-ml fractions were collected, and Sephadex G-200 column (142 × 1.3 cm) was eluted with 4 M guanidinium chloride, 0.05 M sodium acetate, pH 5.8, into 3.7-ml fractions. Analytical columns of Sepharose 6B (145 × 8 cm) were eluted with 0.5 M sodium acetate, pH 7.0. Fractions of about 1.3 ml were collected with a drop counter.

Analytical Procedures—Protein was determined according to Lowry et al. (13). The carbazole method of Bitter and Muir (14) was used for uronic acid determinations. Column effluents were monitored for content of protein (Bolin), uronic acid (carbazole), and hexose (anthrone) using automated procedures described elsewhere (15). Amino acids were determined using an automatic amino acid analyzer. Samples were hydrolyzed for 24 h at 110°C in 6 M HCl under nitrogen. Hexosamines were determined using the automatic amino acid analyzer. Samples were hydrolyzed for 3 h in 8 M HCl at 95°C under nitrogen. Neutral sugars were determined after hydrolysis of samples in 2 M trifluoroacetic acid for 2 h at 100°C or in 0.01 M HCl-Dowex 50-X2 (in hydrogen form) for 30 h at 100°C. Gas chromatography of alditol acetates, as described elsewhere (16), was used for quantitation. Sodium dodecyl sulfate-polyacrylamide electrophoresis was performed on 7.5% gels according to Weber and Osborn (17). The weight average molecular weight of the keratan sulfate-enriched peptide fraction (peak 1, Fig. 1) was determined by sedimentation equilibrium ultracentrifugation essentially as described previously (7). Four solute concentrations, 0.15, 0.21, 0.27, and 0.33 mg/ml, in 0.25 M NaCl were used separately at 14,000 rpm, 20°C, 24 h, in a model E ultracentrifuge. The conditions were sufficient to achieve equilibrium and to obtain meniscus deflections at the air/solution interfaces as defined by Yphantis (18). Values for the apparent Mr, at each solute concentration were determined from best least square lines of ln c versus 1/M graph as described elsewhere (7). The ideal weight average molecular weight, Mr, was determined by a best least square line of the reciprocal of the apparent Mr values plotted against solute concentration. The partial specific volume, υ, was estimated from the chemical composition to be 0.63 ml/g by assuming a value of 0.90 ml/g for keratan sulfate (2) and 0.78 ml/g for the peptide.

Preparation of Proteoglycans—Fresh bovine nasal septum cartilage was obtained at a local abattoir. The cartilage was immediately cleaned and pulverized in liquid nitrogen as described previously.

Analytical Ultracentrifugation—^7 The weight average molecular weight of the keratan sulfate-enriched peptide fraction (peak 1, Fig. 1) was determined by sedimentation equilibrium ultracentrifugation essentially as described previously (7). Four solute concentrations, 0.15, 0.21, 0.27, and 0.33 mg/ml, in 0.25 M NaCl were used separately at 14,000 rpm, 20°C, 24 h, in a model E ultracentrifuge. The conditions were sufficient to achieve equilibrium and to obtain meniscus deflections at the air/solution interfaces as defined by Yphantis (18). Values for the apparent Mr, at each solute concentration were determined from best least square lines of ln c versus 1/M graph as described elsewhere (7). The ideal weight average molecular weight, Mr, was determined by a best least square line of the reciprocal of the apparent Mr values plotted against solute concentration. The partial specific volume, υ, was estimated from the chemical composition to be 0.63 ml/g by assuming a value of 0.90 ml/g for keratan sulfate (2) and 0.78 ml/g for the peptide.

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^2 The abbreviations used for proteoglycans and fragments in this report are essentially those described elsewhere (6, 19).
with chondroitinase ABC followed by trypsin. The digest was then chromatographed on Sepharose 6B.

The bottom 4 ml from the associative CsCl density gradient centrifugation contained more than 50% of the uronic acid in the gradient. The material was dialyzed against water at 4°C and freeze-dried. Sodium dodecyl sulfate-polyacrylamide electrophoresis of a portion of this material failed to detect any proteins entering the gel. Another portion of this sample (200 mg) was digested with chondroitinase, and the digest was chromatographed on Sepharose 6B.

Treatment of Proteoglycans with Hydroxylamine (Fig. 3) — Samples of the A1 fraction (10 mg/ml) were dissolved in 1 M hydroxylamine, 0.05 M 2-(N-morpholino)ethanesulfonic acid, pH 6.5, and incubated for 120 h at 25°C. Aliquots of the incubation mixture with about 50 mg of sample were subsequently fractionated on the preparative Sepharose 2B column. It was shown in preliminary experiments that prolonged treatment with hydroxylamine did not significantly change the chromatographic patterns. Another aliquot of the incubation mixture was dialyzed against 0.05 M sodium acetate, pH 5.8, and 2-ml fractions were collected. The fractions were analyzed for content of uronic acid (carbazole) and protein (absorbance at 280 nm); density was determined by pycnometry using a 200-μl constricted pipette.

RESULTS AND DISCUSSION

Identification of Keratan Sulfate-enriched Peptide Fraction — A sample of nasal cartilage proteoglycan monomers (A1-D1) was digested first with chondroitinase and then with the trypsin/chymotrypsin combination. The digest was chromatographed on a preparative Sepharose G-200 column. All of the keratan sulfate present in the original proteoglycan preparation was present in the effluent from the column. The elution profile, Fig. 1, shows three peaks: (a) a large molecular weight component (peak 1); (b) a smaller molecular weight component (peak 2); and (c) a peak of chondroitin sulfate oligosaccharides eluting near the total volume column. The peak 1 and 2 components were pooled as indicated in Fig. 1 and recovered. Analytical data for neutral sugars, hexosamines, and amino acids are given in Tables I and II. The chemical composition of the first component (peak 1) indicates that it contains primarily keratan sulfate and about 17% protein. The ratios of glucosamine to galactosamine and glucosamine to galactose are typical for keratan sulfate isolated from similar proteoglycan preparations. Further, the amino acid composition, with high contents of glutamic acid, proline, and phenylalanine, is very similar to that of keratan sulfate isolated from cartilage by papain digestion (2, 10). Xylose was present in amounts which indicated that the original peptides contained an average of about one chondroitin sulfate chain for every two keratan

* It was assumed that the xylose recovered in material from peaks 1 and 2 originated from the linkage region of chondroitin sulfate chains of the same average size (20).

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** Table I

| Glucosamine/protein (w/w) | 0.90 | 0.24 |
|---------------------------|------|------|
| Galactosamine/protein (w/w) | 0.23 | 5.90 |

* Indicates percentage of total xylose recovered.

** Indicates percentage of total glucosamine recovered.

N.D., not determined.
sulfate chains, assuming a molecular weight of about 8000 for the keratan sulfate chains (2, 9). It has been observed, however, that extensive treatment of keratan sulfate with alkali will yield molecules of considerably lower molecular weight (2, 10). Therefore, the ratio of the number of keratan sulfate chains to chondroitin sulfate chains in the original proteoglycans was calculated from the chemical data given in this paper. It has been shown that chondroitinase ABC digestion of cartilage proteoglycans will leave a minimum of 1 disaccharide unit from the chondroitin sulfate still attached to the protein. Consequently, in the peak 1 component the galactosamine derived from chondroitin sulfate would equal the xylose contents. The ratio of the remaining galactosamine to glucosamine would be 0.207. Assuming 1 galactosamine/keratan sulfate chain (9), the $M_r$ for the keratan sulfate chain would be about 2500. This would give a ratio of the number of keratan sulfate chains to the number of chondroitin sulfate chains in the original proteoglycans of about 4 to 5. The chemical composition of the peak 2 component indicates that most of the chondroitin sulfate chains in the undigested sample were originally present in these peptides. Further, the high contents of glycine, serine, and glutamic acid very much resemble that of chondroitin sulfate isolated after trypsin, chymotrypsin, or papain digestion of cartilage or of proteoglycans (7, 21). The ratio of glucosamine to xylose of about 1.65:1 indicates that these peptides contain few keratan sulfate chains, approximately 1 for every 5 to 10 chondroitin sulfate chains (assuming a $M_r$ of about 8000 for keratan sulfate), that is, an average of no more than one keratan sulfate chain for every cluster of chondroitin sulfate chains (7, 22). Alternatively, the peptides may contain one keratan sulfate chain with a $M_r$ of about 2500 for every two to three chondroitin sulfate chains.

The apparent molecular weight of the peak 1 fraction was determined at several solute concentrations by sedimentation equilibrium centrifugation as described under "Experimental Procedures." The graphs of $\ln c$ against $r^2/2$ yielded straight lines for each solute concentration (Fig. 4). However, this apparent ideal behavior appears to be the result of a balance between the nonideality from charge effects of the polyanion solute molecules and the inherent polydispersity of molecular weights present in the sample. This is indicated by the fact that the apparent $M_\text{r}$, determined from the slope of the graph, was different for each solute concentration. The ideal weight average $M_\text{r}$ was estimated to be about 122,000 from the plot of the reciprocal of the apparent $M_\text{r}$ values against solute concentration (inset in Fig. 4). If the apparent $M_\text{r}$ of a keratan sulfate chain is either 8,000 (2, 9) or 2,500 as calculated above, the chemical composition and the $M_\text{r}$ of the peak 1 fraction indicate that each peptide would have an average $M_\text{r}$ of about 20,000, and be substituted with about 12 or 30 to 40 keratan sulfate chains, respectively. Since the material in the fractions was recovered quantitatively, the data could be used for determining the stoichiometry of the fractions. It can be calculated from the figures given in Tables I and II that 50 to 60% of the keratan sulfate in the proteoglycan A1-D1 occurred in the larger size peak 1 fragment, while the remainder was found in the smaller peak 2 peptides. The chondroitin sulfate, as estimated from the xylose values, was originally primarily linked to the peptides recovered in peak 2, which contained about 90% of the total xylose.

Another sample of nasal cartilage proteoglycans by action of chondroitinase, trypsin, and chymotrypsin (A1-D1) was digested with chondroitinase and then only with diphenylcarbamyl chloride-treated trypsin. The digest was chromatographed on a preparative Sepharose 6B column. Two major polysaccharide-peptide peaks were obtained, I ($K_\text{av} = 0.32$) and II ($K_\text{av} = 0.69$) (Fig. 5). The elution positions of the corresponding peaks observed when both trypsin and chymotrypsin were used are indicated by arrows 1 ($K_\text{av} = 0.35$) and 2 ($K_\text{av} = 0.51$) in Fig. 5. In addition to the two major peaks, a minor component chromatographed between peaks I and II. The compositions (Table III) indicate that peak I contains the keratan sulfate-enriched fragment, since the contents of glucosamine, glutamic acid, and proline are high. The elution position indicates that the keratan sulfate-enriched fragments liberated by trypsin alone are somewhat larger than those liberated by the combined action of trypsin and chymotrypsin. Both the glucosamine and the galactosamine to protein ratios were lower in keratan sulfate-enriched fragments liberated by trypsin, compared with those isolated after trypsin/chymotrypsin digestion. It is possible, then, that the chymotrypsin treatment primarily liberates peptide-rich fragments from the keratan sulfate-enriched trypsinized peptides.

![Fig. 4. Analytical sedimentation equilibrium centrifugation of the keratan sulfate-enriched peptide at various concentrations. $C_1 = 0.33, C_2 = 0.27, C_3 = 0.21$, and $C_4 = 0.12$ mg/ml.](image_url)
The composition of the peak II material is very similar to that of the chondroitin sulfate-enriched peptides (Tables II and III). The fragments obtained after digestion with trypsin containing no chymotryptic activity were considerably larger than the corresponding peptides isolated from the trypsin/chymotrypsin digests. Also the glucosamine to protein ratio was considerably lower in the trypsinized peptides (II), suggesting that subsequent chymotrypsin digestion not only splits the peptide to yield smaller oligosaccharide-peptide fragments, but also liberates some unsubstituted peptides.

The minor peak between peaks I and II, Fig. 5, has a composition intermediate to that of the peak I and II materials (data not shown).

Treatment of A1 Proteoglycan Fraction with Hydroxylamine—The position of the keratan sulfate-enriched fragment along the protein core was established by characterizing the products produced when proteoglycan aggregates, A1 fraction from nasal cartilage, were treated with hydroxylamine as described under "Experimental Procedures." When an aliquot of hydroxylamine-treated A1 was chromatographed on Sepharose 2B, the elution pattern shown in Fig. 6 was obtained. Fractions 1 and 2 indicated on the figure were isolated. The void volume peak (Fraction 1) contained the link proteins as shown by electrophoresis on sodium dodecyl sulfate polyacrylamide gels (data not shown). Proteoglycan fragments and hyaluronic acid were also present in this fraction. Therefore, this sample was chromatographed on Sephadex G-200 in 4 M guanidinium chloride and the elution profile is shown in Fig. 7. The link proteins were recovered in the included peak and all the proteoglycan fragments and hyaluronic acid were recovered in the void volume peak. The excluded material was analyzed for chemical composition and the data are shown in Table IV. Its keratan sulfate and protein contents were high, with keratan sulfate representing about 66% of the total present in the original A1 preparation. The high relative proportion of glutamic acid and proline is also indicative of the presence of keratan sulfate, since these amino acids are predominant in keratan sulfate peptide fragments. The contents of serine and glycine, which are usually associated with chondroitin sulfate, on the other hand were low. The material

**Table III**

| Residue/1000 residues | A1-D1-CB-T-6B1 | A1-D1-CB-T-6BII |
|-----------------------|----------------|-----------------|
| Aspartic acid         | 27             | 70              |
| Threonine             | 58             | 59              |
| Serine                | 132            | 139             |
| Glutamic acid         | 198            | 148             |
| Proline               | 209            | 85              |
| Glycine               | 96             | 136             |
| Alanine               | 52             | 67              |
| Cysteine              | 43             | 78              |
| Valine                | 3              | 3               |
| Methionine            | 25             | 40              |
| Isoleucine            | 44             | 86              |
| Leucine               | 7              | 12              |
| Tyrosine              | 65             | 24              |
| Phenylalanine         | 31             | 11              |
| Lysine                | 3              | 11              |
| Histidine             | 7              | 28              |
| Glucosamine/protein   | 0.80           | 0.22            |
| (w/w)                 |                |                 |
| Galactosamine/protein | 0.19           | 0.21            |
| (w/w)                 |                |                 |

**Fig. 5.** Preparative Sepharose 6B chromatogram of chondroitinase ABC- and trypsin-digested proteoglycan monomers (A1-D1).

**Fig. 6.** Sepharose 2B (preparative) gel chromatogram of hydroxylamine-treated nasal septum cartilage A1. The horizontal bars indicate the fractions pooled.

**Fig. 7.** Sephadex G-200 (eluted with 4 M guanidinium chloride, pH 5.8) chromatogram of material recovered in the Sepharose 2B void volume (Fraction 1 in Fig. 6) when hydroxylamine-treated nasal cartilage A1 was chromatographed.
Table IV

| Amino acid composition of two fragments obtained from proteoglycan aggregates by treatment with hydroxylamine and then separated by Sepharose 2B chromatography |
|---------------------------------|
| Excluded fragment | Included fragment |
| residues/1000 residues |
| Aspartic acid | 66 | 68 |
| Threonine | 61 | 50 |
| Serine | 77 | 116 |
| Glutamic acid | 172 | 174 |
| Proline | 127 | 107 |
| Glycine | 84 | 121 |
| Alanine | 75 | 61 |
| Cysteine | 6 |  |
| Valine | 59 | 61 |
| Methionine | 4 | 2 |
| Isoleucine | 35 | 36 |
| Leucine | 63 | 75 |
| Tyrosine | 26 | 13 |
| Phenylalanine | 44 | 32 |
| Lysine | 27 | 21 |
| Histidine | 16 | 24 |
| Arginine | 49 | 27 |

Glucosamine (% of hexosamines) | 26.4 (63%*) |
Galactosamine (% of hexosamines) | 73.6 (22%*) |
Protein (% of dry weight) | 17.9 |

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FIG. 8. Distribution of uronic acid and protein (absorbance at 280 nm) on associative CsCl density gradient centrifugation (starting density, 1.73 g/ml) of hydroxylamine-treated nasal septum cartilage aggregates (A1).

The different fractions obtained by gel chromatography and by CsCl density gradient centrifugation of the hydroxylamine-treated proteoglycan aggregates (A1) were digested with chondroitinase followed by trypsin. The digests were then chromatographed on Sepharose 6B, yielding the chromatograms shown in Figs. 10 and 11. The general pattern described above for the chondroitinase-trypsin digest of proteoglycan monomers can be identified. The gel chromatograms show that the fragments capable of interacting with hyaluronic acid (recovered from Sepharose 2B, Fraction 1, or from the top of the density gradient) contained a large proportion of the large size keratan sulfate-enriched peptide. The smaller sized fragments (recovered from Sepharose 2B, Fraction 2, or the bottom of the density gradient), on the other hand, only contained small amounts of this keratan sulfate-enriched peptide. These latter fragments, then, which contained most of the chondroitin sulfate chains, would be located on portions of the protein core of proteoglycan mono-
Aspartic acid
Threonine
Serine
Glutamic acid
Proline
Glycine
Alanine
Valine
Galactosamine (% of hexosamines) 28 (61%) 2.6 (20%)
Galactosamine (% of hexosamines) 72 (18%) 97.4 (64%)

a Percentage of total glucosamine in the CsCl gradient.
b Percentage of total galactosamine in the CsCl gradient.

The presence of the keratan sulfate-enriched region in the bottom fraction of the CsCl gradient of the trypsin digest (A1-T1A1) was established. This fraction, which does not contain the hyaluronic acid-binding region, as discussed above, has a glucosamine to galactosamine ratio of 0.061. A sample was digested with chondroitinase ABC and chromatographed on Sepharose 6B (Fig. 13). The first peak represents the keratan sulfate-enriched region, as could be established by amino acid analysis and determination of the hexosamine ratio. The material had a high content of glutamic acid and proline (18.9% and 20.0% of the total amino acids, respectively) and the glucosamine to protein ratio was 0.75, while the galactosamine to protein ratio was 0.25. The major protein peak (peak 3) eluting at about 600 ml had the same $K_v$ as the chondroitin sulfate-enriched peptides isolated from trypsin digests of proteoglycan monomers. The material in this peak had high contents of serine, glutamic acid, and glycine (16.7%, 14.9%, and 16.7% of the total amino acids, respectively). The galactosamine to protein ratio was 0.32, while the glucosamine to protein ratio was 0.11. This latter value is about half that of the value from the corresponding material isolated from trypsin digests of A1-D1 (6B2, Table III). The difference is most likely due to the absence of the keratan sulfate from the hyaluronic acid-binding region in the A1-T-A1 preparation. Therefore, all of the keratan sulfate recovered with the chondroitin sulfate-enriched peptides is probably derived from the chondroitin sulfate-enriched region of the proteoglycan.
values in Tables II and IV indicate that the keratan sulfate content of the chondroitin sulfate-enriched region of the proteoglycan probably is about 20% of the total keratan sulfate content.

Keiser and DeVito (23) fractionated trypsin/chymotrypsin digests of proteoglycans (A1-D1) on DEAE-cellulose columns to yield two major fractions: the first, which eluted at low ionic strength, contained primarily keratan sulfate, while the second eluted at high ionic strength and contained primarily chondroitin sulfate. When sodium acetate is used to elute chondroitinase ABC, trypsin, and chymotrypsin digests of proteoglycans, both the peak 1 (keratan sulfate-enriched) and peak 2 (chondroitin sulfate-enriched) materials are eluted between 0.7 and 0.9 M sodium acetate while the keratan sulfate from the hyaluronic acid-binding region appears to be eluted with lower strength salt solutions. Therefore the keratan sulfate-peptide fragment recovered in the low salt fraction by Keiser and DeVito (23) would not contain the keratan sulfate-enriched peptide, but may be derived from the keratan sulfate located in the hyaluronic acid-binding region fragment, as these authors tentatively suggested. The keratan sulfate-peptide antigen, then, discussed by Keiser in a subsequent paper (24) are not derived from the keratan sulfate-enriched region.

GENERAL DISCUSSION

Although it has been well documented that keratan sulfate and chondroitin sulfate side chains are attached to the same protein core (2, 10, 25), little has been published about the distribution of keratan sulfate in cartilage proteoglycans. In a previous report (10) a peptide fragment of relatively large molecular size was isolated from tracheal cartilage proteoglycan monomers by using hyaluronidase and trypsin digestion. This fragment was very much enriched in keratan sulfate compared with chondroitin sulfate, and the amino acid composition was similar to that of the keratan sulfate-enriched peptide. In addition smaller peptide fragments containing a large proportion of chondroitin sulfate-oligosaccharides, and a
small amount of keratan sulfate were isolated (10), in accordance with the data presented in this report.

It has been claimed that small molecular weight proteoglycans with very low keratan sulfate and protein contents can be isolated from cartilage by extraction with 0.15 M potassium acetate or potassium chloride (5, 26). The very low keratan sulfate contents of 2 to 4% of the glycosaminoglycans in these proteoglycans strongly indicate that they do not contain the keratan sulfate-enriched peptide region discussed above. The low protein content, the chemical composition, and the Sepharose 2B $K_v$ values of the low salt-extracted proteoglycans, $K_v = 0.54$ (51, are very similar to that of the low molecular weight fragment liberated from proteoglycan aggregates by the action of hydroxylamine as is discussed above. Therefore it is possible that the low salt-extracted proteoglycan is a degradation product of the normal proteoglycan monomer. In support of such a hypothesis, as is discussed in a subsequent report (27), the low salt-extracted proteoglycans cannot be identified in nasal cartilage guanidinium chloride extracts when procedures which minimize proteolysis are used.

The data discussed are consistent with the model shown in Fig. 14. In this model the keratan sulfate-enriched region occupies a part of the protein core very near the portion of the proteoglycan interacting with the hyaluronic acid. This keratan sulfate-enriched region contains about 60% of the keratan sulfate in the proteoglycan monomer, but only 10% of the chondroitin sulfate. The chondroitin sulfate-enriched region is located at the other end of the monomer, away from the hyaluronic acid-binding region. The chondroitin sulfate-enriched region contains 90% of the chondroitin sulfate chains, but only about 20% of the total number of keratan sulfate chains in the proteoglycan. The remainder of the keratan sulfate chains are attached to the protein recovered with the hyaluronic acid-binding region.

In separate experiments it has been shown that the proteoglycan monomers from bovine tracheal cartilage contain a keratan sulfate-enriched region with the same size and composition as observed in bovine nasal cartilage.

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