The Molecular Weight Distribution of the Chondroitin Sulfates of Bovine and Whale Nasal Septum

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Received for publication, September 12, 1979

Chondroitin sulfates were quantitatively extracted from bovine and whale nasal septum without indiscriminate depolymerization by treatment with 0.5 M sodium hydroxide containing 0.26 M sodium borohydride at 3-5° for 10 days. The reducing-end xylitol of the glycosaminoglycans was accurately determined by a microchemical method which had been developed for the separation and determination of the reducing-end alditols of reduced polysaccharides with high molecular weight (Yamaguchi, H., Inamura, S., & Makino, K. (1976) J. Biochem. 79, 299-303; Yamaguchi, H. & Makino, K. (1977) J. Biochem. 81, 563-569). The xylitol proportion of each glycosaminoglycan was in fair agreement with the xylose proportion of the corresponding chondroitin sulfate prepared by proteolytic digestion, suggesting that the xylose residues of the chondroitin sulfate chains are all situated in the reducing-end position and are quantitatively converted to xylitol by reductive β-elimination during the extraction with alkaline sodium borohydride. Accordingly, the molecular weight of the glycosaminoglycans was readily derived from the ratio of xylitol content to polysaccharide weight. The combined use of this method for molecular weight estimation and gel chromatography made it feasible to analyze the molecular weight range of chondroitin sulfates on relatively small amounts of materials. The chondroitin sulfates prepared by alkali extraction were fractionated on Sephadex G-200 into 12 fractions. The molecular weight determination of the glycosaminoglycan in each fraction revealed that the molecular weight ranges of the chondroitin sulfates are appreciably wider than those previously reported. Linear relationships were found for every chondroitin sulfate preparation when the partition coefficient $K_{av}$ and the elution volume of each fraction were plotted against the log of the corresponding molecular weight determined by the present method.

Abbreviations: A-chondroitin sulfate, chondroitin sulfate prepared by alkali extraction; P-chondroitin sulfate, chondroitin sulfate prepared by Pronase digestion; Mn, number-average molecular weight.
glycosaminoglycan chains, therefore, offers important insight into the fine structure of the proteoglycan.

The determination of the molecular weight of glycosaminoglycans has generally been accomplished by physical methods such as ultracentrifugal analysis (3, 5, 6), light scattering (7), and osmetry (5), or by measuring the reducing power of the terminal residue (8–10). These physical methods, however, are laborious and require relatively large amounts of material, especially for the analysis of molecular weight distribution, in which glycosaminoglycans are usually fractionated by gel chromatography. To get over this difficulty, Robinson and Hopwood have recently used NaB₃H₄ for labeling of the reducing-end of glycosaminoglycan chains in their sensitive method for molecular weight determination (11). On the other hand, the method based on estimation of reducing activity as an end group analysis is simple to use, but might be erroneous in the event of contamination with keratan sulfate, which is a common contaminant in chondroitin sulfate preparations.

To examine the molecular weight and molecular weight range of glycosaminoglycans, it is first necessary to isolate them from the proteoglycan complexes. These glycosaminoglycans, however, may be partially degraded during the isolation, and the results from the methods currently used for molecular weight determination might be seriously affected by even minor depolymerization.

One way out of these difficulties seems to be to determine the original reducing-end group of the glycosaminoglycans, prepared without degradation of their linkage region, by a rapid and sensitive method. Therefore, estimation of the xylose content may provide a basis for calculating the average chain length of chondroitin sulfates (12), if it is assumed that no xylose residues are situated in positions other than the reducing-end of the glycosaminoglycans. In the previous papers (13, 14), the author has demonstrated that the reducing-end sugar residues of some glucans with high molecular weight can be quantitatively separated as alditols by treatment with a strongly basic resin after reduction and acid hydrolysis of the polysaccharides. It is well known that chondroitin sulfate chains are attached to the core protein by the glycosidic linkage between xylose and serine and that the reducing-end xylose may be converted to xylitol by reductive β-elimination cleavage. The reducing-end estimation of chondroitin sulfates, therefore, might be achieved by the application of the above technique to the determination of the reducing-end xylitol.

The present investigation describes a convenient method for accurate determination of the molecular weight of chondroitin sulfates. Application of the present method to chondroitin sulfate fractions obtained by gel filtration on Sephadex G-200 revealed wide distributions of molecular size of the glycosaminoglycans.

**EXPERIMENTAL PROCEDURE**

*Materials*—Sulfuric acid was a super special grade reagent. All other reagents used were of analytical grade. A strongly acidic resin (Dowex 50, X8, 100–200 mesh) was retreated with 2 M hydrochloric acid immediately before use. A strongly basic resin (Dowex 1, X8, 200–400 mesh) and a weakly basic resin (Amberlite IR-4B) which had been stored in the chloride form were converted to the basic form by washing with 1 M sodium hydroxide and 2 M ammonium hydroxide, respectively, just before use.

*Analytical Methods*—Hexuronic acid was determined as described by Bitter and Muir (15), and hexosamine was assayed by means of the modified Elson-Morgan reaction (16) or with an amino acid analyzer. Sulfate was determined by the method of Terho and Hartiala (17). Amino acid analysis was performed with a Hitachi KLA-5 amino acid analyzer. Neutral sugars were analyzed by GLC as their alditol acetates. Peracetylation of alditols was achieved by heating them together with 0.1 ml each of anhydrous pyridine and acetic anhydride at 100°C for 2 h (18). The acetylation reagent was removed by repeating the addition of 0.5 ml of water and evaporation of the solution under reduced pressure. The acetylated products thus obtained were dissolved in 30 μl of chloroform and chromatographed. GLC was carried out on a Shimadzu 4APF gas chromatograph, equipped with a hydrogen-flame ionization detector, using a glass column (200 × 0.3 cm i.d.) of 3% OV-225 on Gas Chrom Q (100–120 mesh) at 200°C. The analytical values were corrected for destruction due to acid hydrolysis.

*J. Biochem.*
Preparation of Chondroitin Sulfates—Bovine nasal septum cartilage was obtained from a single animal (about 2 years old) at a slaughterhouse and immediately frozen. Nasal septa of adult whales (Balaenoptera acutorostrata and Physeter catodon) were supplied in a frozen state by Seikagaku Kogyo Co., Tokyo, Japan. The cartilage was freed of adhering noncartilaginous tissue, cut into small cubes, and crushed with solid CO2 in a large porcelain mortar. The roughly broken cartilage was ground to a fine powder in acetone by means of a Biotron homogenizer (Biotrona Co., Küsnacht, Switzerland). The milled cartilage was defatted by repeated extraction with acetone, dried in vacuo, and then stored at -20°C.

(a) Preparation of chondroitin sulfates by extraction with alkali: The acetone-dried powder of cartilage (1 g) was stirred with 20 ml of 0.5 M sodium hydroxide containing 0.26 M sodium borohydride at 3-5°C for 10 days and then the excess sodium borohydride was decomposed by adding acetic acid. To the acidified reaction mixture, trichloroacetic acid was added to a final concentration of 5% with external cooling in an ice-water bath, and the turbid solution was kept in the cold for 2 h. The precipitate was removed by centrifugation and the supernatant was dialyzed against distilled water. The glycosaminoglycan mixture thus obtained was subjected to ethanol fractionation on a cellulose column, essentially according to the method of Gardell (19). After washing the cellulose column with 40% ethanol containing 0.3% barium acetate, the chondroitin sulfate fraction was eluted with water. Further purification of the chondroitin sulfate preparation was achieved by anion-exchange chromatography according to Schiller et al. (20). The Dowex 1 (×2, 200-400 mesh, Cl- form) column was first washed with 0.5 M NaCl, and then chondroitin sulfate was eluted with 1.5 M NaCl, dialyzed thoroughly, and lyophilized. The yields of chondroitin sulfate from bovine, B. acutorostrata, and P. catodon were 0.27 g, 0.34 g, and 0.25 g, respectively.

(b) Preparation of chondroitin sulfates by proteolytic digestion: The acetone-dried powder of cartilage (1 g) was suspended in 20 ml of water, and Pronase (40 mg) was added. The suspension was covered with toluene and allowed to stand with shaking at 40°C. The pH was maintained at 7.8-8.0 by occasional addition of 1 M sodium hydroxide. After 1 day, Pronase (8 mg) was added to the reaction mixture and the proteolytic digestion was continued overnight. The turbid solution was subjected to centrifugation to remove insoluble material. The supernatant was treated with trichloroacetic acid and subjected to the procedures for isolation and purification of chondroitin sulfates exactly as described above. The yields of chondroitin sulfates from the three kinds of cartilage were very close to those from the alkali extraction.

Isolation and Determination of Xylitol of Chondroitin Sulfates—The isolation of the xylitol from the chondroitin sulfates was performed essentially according to the previously proposed method (13, 14), but with some improvements. A chondroitin sulfate (0.2-0.8 mg) prepared by the extraction with alkaline sodium borohydride (A-chondroitin sulfate) was heated with 0.3 ml of 0.5 M sulfuric acid in vacuo at 100°C for 6 h. To the hydrolysate was added 0.1 ml of an internal standard solution containing 5 μg of 2-deoxy-D-glucitol. The solution was made weakly acidic by adding 1 M barium hydroxide, then neutralized with barium carbonate. After centrifuging, the supernatant was evaporated to a small volume and passed through a small column of Dowex 50 (×8, H+ form, 0.2 ml) to remove barium ions. The resin was eluted with 0.8 ml of water and the effluent was evaporated to dryness. The residue was dissolved in 0.2 ml of methanol and allowed to stand at 50°C for 15 min. The alcoholic solution was evaporated to dryness under reduced pressure, and the residue was subjected to two further evaporations with methanol to ensure the removal of trace amounts of borate. The residue was dissolved in 0.2 ml of water and immediately passed through a small column of Dowex 1 (×8, OH- form, 0.2 ml) to isolate alditols. The resin was eluted with 1.2 ml of water. The effluent containing alditols was evaporated to a small volume and treated again with Dowex 50 (×8, H+ form, 0.2 ml) as just described. The effluent was evaporated to dryness in a glass tube (5×1.4 cm) with a Teflon-lined screw cap and the residue was subjected to repeated additions and evaporations of methanol. The alditols were acetylated and analyzed by GLC as already described.

Isolation and Determination of Neutral Sugars of Chondroitin Sulfates—A chondroitin sulfate
(0.2–0.8 mg) was hydrolyzed and freed of sulfuric acid after addition of the internal standard, as described above. The hydrolysis time was 6 h for xylose analysis and 9 h for galactose analysis. The effluent from the acidic resin was evaporated to a small volume, and quickly passed through a small column of Amberlite IR-4B (OH⁻ form, 0.2 ml) at 3–5°C. The resin was immediately eluted with 1.2 ml of water and the effluent containing neutral sugars was evaporated to dryness. The residue was dissolved in 0.1 ml of water and treated with 60 µl of 0.05 M sodium hydroxide containing 0.84 mg of sodium borohydride at 40°C for 2 h. The excess borohydride was decomposed by adding 0.1 ml of 0.4 M acetic acid and the resulting acidic solution was passed through Dowex 50 (×8, H⁺ form, 0.2 ml). The resin was eluted with 0.8 ml of water and the effluent was evaporated to dryness in a glass tube (5 × 1.4 cm) with a Teflon-lined screw cap. The residual boric acid was removed by repeated additions and evaporations of methanol. The products were worked up exactly as described above for GLC analysis. By this procedure, xylitol is quantitatively separated with other neutral sugars from the acid hydrolysate.

Gel Chromatography of Chondroitin Sulfates—Fractionation of chondroitin sulfates was carried out on a column (110 × 2.0 cm) of Sephadex G-200 (superfine grade) at room temperature. A chondroitin sulfate (20 mg) was dissolved in 1 ml of 0.2 M NaCl and applied to the column. The column was pre-equilibrated and eluted with 0.2 M NaCl at a flow rate of 5 ml/h. Five ml portions of the effluent were collected and assayed for uronic acid content. The elution peak obtained was divided into 12 fractions. Each fraction was passed through Dowex 50 (×8, H⁺ form, 2–7 ml) and the resin was eluted with water (10–35 ml). The acidic effluent was evaporated to dryness and subjected to compositional studies.

RESULTS AND DISCUSSION

Recovery and Composition of Chondroitin Sulfates—Extraction of chondroitin sulfates with alkali from tissue was carried out under conditions similar to those used by Hopwood and Robinson (21). However, borohydride was used of a higher concentration in order to secure a rapid progress of the reduction reaction throughout the prolonged extraction, since sodium borohydride is not very stable in dilute alkali. From a consideration of the hexuronic acid content of the residues obtained from cartilage after extraction of glycosaminoglycans, it was concluded that chondroitin sulfates were exhaustively released from the tissue by alkali extraction or by Pronase digestion. A hexose-positive material, keratan sulfate, was nearly quantitatively removed from the glycosaminoglycan mixtures by the subsequent ethanol fractionation, and all of the hexuronate-containing materials retained on the cellulose were recovered. Every chondroitin sulfate obtained after chromatography on Dowex 1 (×2, Cl⁻) contained equimolar amounts of hexuronic acid, 2-amino-2-deoxy-D-galactose, and sulfate, together with small amounts of neutral sugars. The chondroitin sulfates prepared by Pronase digestion (P-chondroitin sulfates) contained a small proportion of amino acids, whereas A-chondroitin sulfates were virtually free from them.

Determination of Xylitol and Xylose of Chondroitin Sulfates—are all of the xylose residues of chondroitin sulfates situated in the reducing-end position? The xylitol and xylose of A- and P-chondroitin sulfates were carefully determined in order to settle this point. An accurate determination of trace sugar components of a polysaccharide with high molecular weight may be achieved by GLC only if they can be quantitatively separated from the poly saccharide. A-Chondroitin sulfates, therefore, were subjected to the microchemical method previously developed for the isolation and determination of the reducing-end alditol of reduced polysaccharides (13, 14). This method, in which alditols can be quantitatively separated from mixtures with large amounts of reducing monosaccharides in a high state of purity by treatment with a strongly basic resin, makes it feasible to determine the reducing ends of polysaccharides of high molecular weight. In the present investigation, model studies gave excellent recoveries of xylitol from known mixtures with large amounts of hexosamine and hexuronate upon such treatment with a basic resin.

A typical gas chromatogram obtained from the A-chondroitin sulfate of bovine nasal cartilage is shown in Fig. 1A. The A-chondroitin sulfates of whale nasal cartilage also afforded only xylitol on similar analysis. The failure to detect galactitol...
suggests that A-chondroitin sulfates may have been prepared without scission of the glycosidic bonds within the linkage region. In contrast, no alditols were found from any of P-chondroitin sulfates.

With the aim of assessing the xylitol contents estimated above, the xylose of both A- and P-chondroitin sulfates was determined by GLC after separation from the polysaccharides. Since xylose was analyzed as xylitol acetate, the amount of xylose of A-chondroitin sulfates was derived by subtracting the xylitol content from the analytical value obtained. No significant difference, however, was detected between the values obtained by the two analytical methods from each A-chondroitin sulfate, indicating that A-chondroitin sulfates are virtually free from xylose (Table I). This result was supported by the observations that no appreciable amounts of xylose acetate were detected when A-chondroitin sulfates were analyzed for xylose without carrying out the reduction reaction. On the other hand, the analysis of P-chondroitin sulfates afforded peaks derived from xylose and galactose on gas chromatograms (Fig. 1B). It should be noted that the xylitol proportion of an A-chondroitin sulfate is in fair agreement with the xylose proportion of the corresponding P-chondroitin sulfate (Table I).

In earlier investigations dealing with the mode of the glycosaminoglycan-protein linkage, alkaline cleavage of chondroitin sulfate-peptide resulted in the detection of xylitol in the presence of sodium borohydride, suggesting the presence of xylose residues situated in the reducing-end position of the polysaccharide (22, 23). However, quantitative β-elimination of glycosaminoglycan-peptide linkage could not be effected. In the course of studies of the protein moieties of chondroitin sulfate-protein complexes, Baxter and Muir (24) observed that the molar ratio of xylose to serine residues destroyed on alkaline β-elimination is nearly unity. Another observation suggesting that all of the xylose residues of a chondroitin sulfate are situated in the reducing-end position has recently been reported by Seno and Sekizuka (25). They found that β-elimination-reduction of a chondroitin sulfate-peptide substituted at both the terminal amino and carboxyl groups caused conversion of almost all of the xylose into xylitol and concomitant loss of an equivalent amount of serine.

In the present study, quantitative β-elimination reduction combined with the sensitive estimation

<table>
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<tr>
<th></th>
<th>Xylitol (mol/100 mol of glucuronic acid)</th>
<th>Xylose</th>
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<tbody>
<tr>
<td>Bovine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A-Chondroitin sulfate</td>
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<td>+ a</td>
</tr>
<tr>
<td>P-Chondroitin sulfate</td>
<td>0</td>
<td>2.81</td>
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<tr>
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<td></td>
<td></td>
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<tr>
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<td>0</td>
</tr>
<tr>
<td>P-Chondroitin sulfate</td>
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</tr>
<tr>
<td>Whale, <em>P. catodon</em></td>
<td></td>
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</tr>
<tr>
<td>A-Chondroitin sulfate</td>
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</tr>
<tr>
<td>P-Chondroitin sulfate</td>
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<td>2.57</td>
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a Trace.
of xylitol and xylose gave results in support of the above suggestions. It is clear from Table I that all of the xylose of the chondroitin sulfate chains was situated in the reducing-end position and was quantitatively converted to xylitol by reductive \( \beta \)-elimination during the extraction with alkaline sodium borohydride. Consequently, the number-average molecular weight (Mn) of the chondroitin sulfates was calculated from the ratio of polysaccharide weight to xylitol content, assuming that one xylose residue occupies the reducing-end position of each glycosaminoglycan chain of chondroitin sulfate-protein complexes. The weight of polysaccharide was derived from the molar proportion of hexuronate assayed. The molecular weight (Mn=18,600, sodium salt) thus obtained from the A-chondroitin sulfate of bovine nasal cartilage is in reasonable agreement with the values previously reported by osmometry (Mn=20,800) (4) and by the estimation of the end-group labeled with NaB\( \text{H}_4 \) (Mn=21,000) (21). It is noteworthy that the molecular weight obtained by the present method is not higher than those previously obtained by other methods. This finding may permit the following deductions. (a) A selective degradation of the reducing-end xylose can be ruled out, at least under the present conditions of preparing the chondroitin sulfates. (b) No other type of chondroitin sulfate-protein linkage involving xylose is present. These suggestions are in harmony with those previously presented by Robinson and Hopwood (21, 11). On the other hand, the molecular weights found for the A-chondroitin sulfates of whale nasal cartilage (B. acutorostrata, Mn=22,400; P. catodon, Mn=20,600) were higher than that of the bovine A-chondroitin sulfate.

Though the amounts of xylose of P-chondroitin sulfates gave molecular weights very close to those derived from the xylitol estimation, the xylose determination is relatively laborious and is inferior to the xylitol analysis method in reproducibility.

**Fractionation of Chondroitin Sulfates by Gel Chromatography and Chemical Properties of the Fractions**—Gel chromatography of A-chondroitin sulfates produced broad and symmetrical peaks as shown in Fig. 2. The elution characteristic of each A-chondroitin sulfate was very similar to that of the corresponding P-chondroitin sulfate, suggesting that random degradation of the chondroitin sulfate chains did not occur to any significant extent during their extraction with alkali. The effluent was divided into 12 fractions as indicated by Roman numerals I–XII. In the compositional studies after desalting, these fractions were all found to contain equimolar amounts of hexuronate acid and 2-amino-2-deoxy-D-galactose as major components. Further characterization of these fractions was performed not only by determination of xylitol but also by analysis of other neutral sugars (Table II). The combined totals for xylitol and xylose are given together with the xylitol and galactose contents in Table II, but it is clear that every fraction is virtually free from xylose, in agreement with the analytical values for the unFractionation of A-chondroitin sulfates of bovine (A) and whale (B. acutorostrata) (B) nasal septum by gel chromatography on Sephadex G-200 (superfine grade). The eluates were divided into 12 fractions as indicated.
molecules have the sequence Gal-Gal-xylitol in the reducing-end region, regardless of their molecular size. The relatively high proportion of galactose observed for the fractions at the tail end of the elution peaks may be attributed to contamination with a small amount of keratan sulfate. Similar contamination with keratan sulfate has previously been observed for chondroitin sulfate subfractions of lower molecular weight (21). The data presented in Table II, therefore, support the above deductions that all of the xylose residues were situated in the reducing-end position of the chondroitin sulfate chains and that the linkage region of the glycosaminoglycans was quantitatively converted to Gal-Gal-xylitol by reductive β-elimination during the treatment with alkaline sodium borohydride.

The macromolecular properties of A-chondroitin sulfates are shown by the elution profiles from Sephadex G-200 (Fig. 3). The xylitol content of each fraction was plotted against the elution volume, defined as the position dividing the elution

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Bovine</th>
<th></th>
<th>Whale (B. acutorostrata)</th>
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<tbody>
<tr>
<td></td>
<td>Xylitol</td>
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<tr>
<td>I</td>
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<tr>
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</table>

Fig. 3. Elution profiles from Sephadex G-200 of bovine (A) and whale (B. acutorostrata) (B) A-chondroitin sulfate. ○, hexuronic acid; ●, xylitol; Φ, calculated molecular weight.
The molecular weight of the polysaccharide in each fraction was calculated from the molar proportion of xylitol as described above. Figure 3 clearly shows that the progress of elution of the polysaccharides from the gel column was accompanied by an increase of the xylitol proportion and, consequently, by a decrease of the molecular weight. The 12 fractions of the A-chondroitin sulfate of bovine nasal septum showed molecular weights ranging from 8,200 to 43,900. This range of molecular weight, which was derived from the chondroitin sulfate of a single animal, is appreciably wider than those previously reported for chondroitin sulfates prepared from pooled nasal septa (4, 5, 21). This difference may be largely ascribed to the fact that the chondroitin sulfates eluted from gel columns have generally been fractionated into comparatively large fractions since the physical methods for molecular weight estimation, including osmometry, require relatively large amounts of material (4, 5). In addition, the end-group estimation combined with NaB3H4 reduction of the reducing ends was not successful for the chondroitin sulfate fractions of lower molecular weight, owing to contamination with keratan sulfate (21). Judging from the present result, the polysaccharides with molecular weight lower than 10,000 may comprise a significant part of the bovine chondroitin sulfate preparation. The chondroitin sulfate of whale nasal cartilage (B. acutorostrata) was also observed to have a high degree of polydispersity (10,100–61,300). Similarly, a wide range of molecular weight was observed for the chondroitin sulfate of another whale, P. catodon.

In order to correlate elution behavior in gel chromatography and molecular weight, the partition coefficient $K_{av}$ (26) and the elution volume of each fraction were plotted against the log of the corresponding molecular weight. The void volume and the total volume of the Sephadex G-200 column were determined by chromatography of a high molecular weight dextran and glucose, respectively. As shown in Fig. 4, linear relationships were observed for the chondroitin sulfates of both bovine and whale. These results appear to support the above observation that indiscriminate degradation of the chondroitin sulfate chains did not occur to any significant extent during their isolation. This interpretation is compatible with the suggestion by Robinson and Hopwood (11). Additionally, these relationships of the fractions are again suggestive of the absence of any type of chondroitin sulfate-protein linkage not involving xylose. It may be concluded, therefore, that Fig. 3 faithfully reflects the molecular weight distribution of the chondroitin sulfate chains in vivo.

Compared with the physicochemical methods, the present procedure has the advantage of permitting ready determination of the molecular weights of chondroitin sulfates with relatively small amounts of them. Further, this method is not affected by contamination with keratan sulfate, since neither xylose nor hexuronic acid is present in keratan sulfates.

Every fraction obtained by gel chromatography of the chondroitin sulfates is in a narrow
molecular weight range and gives a sharp elution peak from a Sephadex G-200 column. These fractions could therefore be used as reference compounds for the calibration of a gel column to be used for estimating the molecular weights of glycosaminoglycans.

The author is very grateful to Mr. M. Sato, Okura Kasei, Co., for very kindly arranging to supply bovine nasal cartilage. The whale nasal septa were generously supplied by Seikagaku Kogyo Co. The technical assistance of Mr. K. Makino is also gratefully acknowledged.

REFERENCES