The Occurrence of a Wide Variety of Dermatan Sulfate-Chondroitin Sulfate Copolymers in Fibrous Cartilage

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SUMMARY

A large fraction with the properties of a dermata n sulfate-chondroitin sulfate copolymer was obtained from the meniscus (semilunar cartilage) of the human knee joint. This fraction appeared to be homogeneous with respect to charge density, as judged by its behavior on DEAE-cellulose chromatography, but it was further resolved into a number of subfractions by ethanol fractionation, by electrophoresis on cellulose acetate, and by chromatography on Sephadex G-200. Analyses of these subfractions showed that the copolymer was composed of a series of variants whose properties could be accounted for by a gradual variation in the proportion of L-iduronosyl-N-acetylgalactosamine 4-sulfate units and D-glucuronosyl-N-acetylgalactosamine 6-sulfate units.

These results indicate that meniscus mucopolysaccharides represent a separate family of mucopolysaccharides that may be specifically adapted to the function of fibrous (collagen-rich) cartilages, and further suggest that any attempt to obtain a mucopolysaccharide sample for structural study must take into consideration this type of polymorphism as well as the scarcity of reliable methods for purification of such mixtures.

Among the sulfated mucopolysaccharides of connective tissue, a family of polysaccharides containing α-1,3-L-iduronic acid and β-1,4-N-acetyl-β-galactosamine 4-sulfate as the main repeating units is designated as chondroitin sulfate B (1) or dermata n sulfate (2). It has further been shown that multiple minor variations exist in the composition of dermata n sulfate chains of proteoglycans isolated from different tissue sources. Some examples of such variations are the presence of additional sulfate groups on uronic acid residues (3) or on hexosamine residues (4), and the presence of D-glucuronic acid besides L-iduronic acid (5-12). We do not know, however, how many of the variations are random, as, for example, caused by variable epimerization and sulfation and which, if any, are oriented in an orderly fashion. Fratussom et al. (12) have pointed out that some regularities and tissue specificities in the distribution of D-glucuronosyl-N-acetylgalactosamine 4-sulfate and D-glucuronosyl-N-acetylgalactosamine 6-sulfate units.

These results indicate that meniscus mucopolysaccharides represent a separate family of mucopolysaccharides that may be specifically adapted to the function of fibrous (collagen-rich) cartilages, and further suggest that any attempt to obtain a mucopolysaccharide sample for structural study must take into consideration this type of polymorphism as well as the scarcity of reliable methods for purification of such mixtures.

1 Synonym: semilunar cartilage. This tissue has been recognized by histologists as a special type of cartilage concerned chiefly with the facilitation of knee joint movements; the predominant collagen fibrils arranged in parallel bundles make this cartilage readily discernible from hyaline and elastic cartilages. Several workers (15, 16) have studied its mucopolysaccharide component and described that chondroitin 4- and 6-sulfate, but not dermatan sulfate, are present as the principal components.

2 The abbreviations used are: IdUA, L-iduronic acid; ΔGlcUA, Δ-glucuronic acid; GalNAc(4S) and GalNAc(6S), N-acetylgalactosamine 4-sulfate and 6-sulfate, respectively; ΔGlcUA-GalNAc(4S), 3-O-Δ-glucuronosyl-N-acetylgalactosamine (i.e. unsaturated disaccharide derived from hyaluronic acid by lyase reaction); ΔGlcUA-GalNAc(6S), 3-O-Δ-glucuronosyl-N-acetylgalactosamine (i.e. unsaturated disaccharide derived from chondroitin by lyase reaction); ΔGlcUA-GalNAc(4S) and ΔGlcUA-GalNAc(6S), derivatives of ΔGlcUA-GalNAc bearing a sulfate at position 4 and a sulfate at position 6, respectively, on the N-acetylgalactosamine moiety (for the structural formula, see Ref. 17).
permit a formulation of the unique property of dermatan sulfate in this tissue. Several lines of evidence have now been obtained to indicate that the meniscus contains a great variety of hybrid molecules with different proportion and distribution of \( \text{IdUA-GalNAc}(4S) \) and \( \text{GluUA-GalNAc}(6S) \) units. In this paper we wish to present the evidence that leads to this conclusion.

**EXPERIMENTAL PROCEDURE**

_Materials—_The following commercial materials were used: hyaluronic acid (human umbilical cord) from Sigma, St. Louis; human \( \gamma \)-globulin and bovine serum albumin from Mann Research Laboratories, Inc., New York; AG I-X8, 200 to 400 mesh, from Calbiochem, Los Angeles; Sephadex G-150, Sephadex G-200, and blue dextran (for void volume determination) from Pharmacia, Uppsala; DEAE-cellulose from Brown Co., Keene, N. H.; and Separax (cellulose acetate film) from Fuj Film Co., Tokyo.

The following materials were prepared by previously described methods: chondroitinase-ABC from _Proteus vulgaris_ NCTC 4636 (18); chondroitinase-AC and a glucuronidase (\( \Delta^4 \)-glucuronide hydrolase) from _Flavobacterium heparinum_ ATCC 13125 (16); \( \Delta \text{GluUA-GlcNAc}, \Delta \text{GlcUA-GalNAc}, \Delta \text{GlcUA-GalNAc}(4S), \) and \( \Delta \text{GlcUA-GalNAc}(6S) \) from hyaluronic acid, chondroitin, chondroitin 4-sulfate, and chondroitin 6-sulfate, respectively (18); and \( N \)-acetylgalactosamine 6-sulfate from \( \Delta \text{GluUA-GalNAc}(6S) \) (3).

Generous gifts of the following materials are acknowledged: dermatan sulfate (average molecular weight = 27,000) with an \( L \)-iduronic to \( D \)-glucuronic acid ratio of 19:1 (hog intestinal mucosa) (10) from Dr. M. B. Mathews and Dr. J. A. Cifonelli, University of Chicago; partially depolymerized dermatan sulfate (average molecular weight = 16,000) and chondroitin 6-sulfate (average molecular weight = 15,000) prepared from pig skin and shark cartilage, respectively, by digestion with testicular hyaluronidase followed by fractionation with Sephadex G-200 columns, chondroitin 4-sulfate with a 4-sulfate to 6-sulfate ratio of about 4:1 (whale cartilage) (20), chondroitin 6-sulfate (average molecular weight = 30,000) with a 6-sulfate to 4-sulfate ratio of about 9:1 (shark cartilage) (21), and heparin from SEikagaku Kogyo Co., Tokyo; a hyaluronate lyase from _Streptomyces hyalurolyticus_ (22) from Dr. T. Ohya, Amano Pharmaceutical Co., Nagoya; and Pronase-P (45,000 units per g) from Kaken Kagaku Co., Tokyo.

_Chemical Analysis—_Analyses for total hexuronic and total carboxyl groups were carried out by the carbazole (23) and orcinol (24) methods; the latter procedure. The final recovery of \( N \)-xylose (98%) then provided internal standards with which the sugars liberated from the polysaccharide sample were compared. The buffer used was pyridine-acetic acid-water (1:9:115, by volume), pH 3.5. The strips were stained, according to the method of Park and Johnson (29). In order to determine the losses of the neutral sugars that occurred during hydrolysis and chromatography, known amounts of \( N \)-xylose and \( N \)-galactose were added to one set of the mucopolysaccharide sample before hydrolysis. The final recovery of \( N \)-galactose (96%) and that of \( N \)-xylose (92%) then provided internal standards with which the sugars liberated from the polysaccharide sample were compared.

_Detectors—_Optical rotation was determined with a Jasco photoelectric spectropolarimeter, model ORD/UV-5.

_Electrophoresis and Chromatography—_Electrophoresis of mucopolysaccharide samples was carried out on 6-cm long strips of Separax (cellulose acetate film) in a glass apparatus (Jokosangyo Co., Tokyo) at a constant current of about 1 ma per cm for 30 min. The buffer used was pyridine-acetic acid-water (1:9:115, by volume), pH 3.5. The strips were stained, according to the method of Sano et al. (30), with 0.5% Alcian Blue in 3% acetic acid for about 20 min. After washing with water for 10 min, the strips were blotted dry, let stand at room temperature for several minutes, and then pressed between sheets of filter paper.

_Electrophoresis of unsaturated oligosaccharides was carried out on 60-cm long strips of Toyo No. 51A filter paper in the apparatus described by Markham and Smith (31) at a potential gradient of 30 volts per cm for 45 min. The buffer used was 0.05 M ammonium acetate-acetic acid, pH 5.0. The oligosaccharides containing \( \Delta^4 \)-glucuronic acid were detected by viewing under ultraviolet light or by staining with \( \text{AgNO}_3 \) (26).

Chromatography of uronic acid on AG 1-X8 (anion exchange resin) was carried out as described by Fransson et al. (32) after hydrolysis with 1 M \( \text{H}_2\text{SO}_4 \) for 2 hours at 100°.
The solution was treated with 90 mg each of Pronase as described above (three cycles) and dialyzed against running tap water for 2 days. Trichloroacetic acid was added at 4°C to a concentration of 5%. The precipitate was removed by centrifugation at 12,000 x g for 10 min. The supernatant solution was dialyzed against distilled water at 4°C. Potassium acetate was added to the dialyzed solution to a concentration of 1%. The mucopolysaccharide mixture was precipitated with 2 volumes of ethanol, centrifuged, washed with ethanol, ether, and dried over P₂O₅ in a vacuum. The yield was 1.35 g (1.4 nmols as carbazole glucuronic acid). This preparation will be referred to as “crude mixture.”

RESULTS

Survey of Mucopolysaccharides in Crude Mixture—It has been shown previously that a separation of small amounts of mucopolysaccharides is possible by electrophoresis on cellulose acetate strips with appropriate buffer systems. Indeed, by the use of pyridine-acetic acid buffer, pH 3.5, it was possible to separate hyaluronic acid (human umbilical cord), dermatan sulfate (hog intestinal mucosa), chondroitin 4-sulfate (whale cartilage), and heparin (d) in a vacuum. The yield was 1.35 g (1.4 nmols as carbazole glucuronic acid). This preparation will be referred to as “crude mixture.”

PREPARATION OF MUCOPOLYSACCHARIDE MIXTURE FROM HUMAN MENISCUS—Human menisci were collected from normal subjects aged 60 to 75 years in the autopsy room of the Medical School for their assistance. In each case, about 290 g of tissue was minced and homogenized using a metal mortar and pestle. The homogenate was then centrifuged, washed with ethanol, ether, and dried over P₂O₅ in a vacuum. The yield was 1.35 g (1.4 nmols as carbazole glucuronic acid). This preparation will be referred to as “crude mixture.”

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Preparation of Mucopolysaccharide Mixture from Human Meniscus—Human menisci were collected from normal subjects aged 60 to 75 years in the autopsy room of the Medical School for their assistance. In each case, about 290 g of tissue was minced and homogenized using a metal mortar and pestle. The homogenate was then centrifuged, washed with ethanol, ether, and dried over P₂O₅ in a vacuum. The yield was 1.35 g (1.4 nmols as carbazole glucuronic acid). This preparation will be referred to as “crude mixture.”

The solution was treated with 90 mg each of Pronase as described above (three cycles) and dialyzed against running tap water for 2 days. Trichloroacetic acid was added at 4°C to a concentration of 5%. The precipitate was removed by centrifugation at 12,000 x g for 10 min. The supernatant solution was dialyzed against distilled water at 4°C. Potassium acetate was added to the dialyzed solution to a concentration of 1%. The mucopolysaccharide mixture was precipitated with 2 volumes of ethanol, centrifuged, washed with ethanol, ether, and dried over P₂O₅ in a vacuum. The yield was 1.35 g (1.4 nmols as carbazole glucuronic acid). This preparation will be referred to as “crude mixture.”
This component disappeared after treatment of the crude mixture with Streptomyces hyaluronidase, a lyase which degrades hyaluronic acid, but not chondroitin, chondroitin 4- and 6-sulfate, and dermatan sulfate (22). There is little doubt, therefore, that the slow component represents hyaluronic acid.

The other component appeared on the electrophoretogram as a broad band extending from the dermatan sulfate zone to the chondroitin sulfate zone (Fig. 2). The profile was not changed by treating the sample with 1.0 N NaOH at 4°C for 15 hours. This component proved resistant to Streptomyces hyaluronidase, but susceptible to degradation with chondroitinase-ABC. Digestion with chondroitinase-AC, on the other hand, caused a partial reduction in the metachromatic activity of this component; those portions near the chondroitin sulfate area began to exhibit exceedingly feeble Alcian Blue reactions whereas the portions near the dermatan sulfate area were less affected by the enzyme digestion. It was assumed from these properties that the fast-moving component of the crude mixture must contain, in addition to typical dermatan sulfate and chondroitin 4- or 6-sulfate, dermatan sulfate-chondroitin sulfate copolymers with a wide range of chemical heterogeneity.

It has been established previously (18) that chondroitinase-ABC cleaves dermatan sulfate and chondroitin 4- and 6-sulfate at their β-hexosaminidic bonds to either L-iduronic or D-glucuronic acid residues to produce unsaturated disaccharides, while chondroitinase-AC cleaves only the bonds to D-glucuronic acid residues. Digestion of dermatan sulfate-chondroitin sulfate copolymer with chondroitinase-AC should yield oligosaccharides with one or more L-iduronic acid residues and one nonreducing terminal Δ1-glucuronic acid. Previous studies on the mucopolysaccharides from HeLa-S and L-929 cells (35) as well as those from horse aorta (11) have shown that this was indeed the case.

This method was applied to the analysis of the crude mucopolysaccharide sample from meniscus. Aliquots of the test sample were separately subjected to digestion with chondroitinase-ABC and chondroitinase-AC. The digests were chromatographed on paper in Solvent A (Fig. 3). The chromatogram of the chondroitinase-ABC system showed the formation of four main spots; comparison of their mobilities with standard disaccharides permitted the conclusion that they, in the order of their relative mobility (RF values). The chromatographic mobilities of Oligo-I and Oligo-II, Oligo-III, Oligo-IV, and Oligo-V in the order of their RF values, were 90 and 30% less, respectively, in the chondroitinase-AC system than in the chondroitinase-ABC system. The results suggest that the mixture contains a high proportion of N-acetylgalactosamine 6-sulfate residues linked to l-iduronic acid. Also notable in the chromatogram of the chondroitinase-AC system (Fig. 3) was the appearance of five slow spots (referred to as Oligo-I, Oligo-II, Oligo-III, Oligo-IV, and Oligo-V in the order of their RF values). The chromatographic mobilities of Oligo-I and Oligo-II were similar to those of the known disulfated disaccharides bearing an extra sulfate on position 6 of the hexosamine moiety (4) or on the uronic acid moiety (3), respectively, but chemical analyses (see below) indicated that none of these products from the meniscus contains such extra sulfates. Therefore, this pattern of chondroitinase products seems reasonable only if it is assumed that such slower moving spots as those produced with chondroitinase-AC, but not with chondroitinase-ABC, represent oligosaccharides which are derived from hybrids containing both D-glucuronic and L-iduronic acid residues in the same polysaccharide chain. This interpretation is in agreement with the prediction made from the electrophoretic analysis (see above) and further suggests that studies of these oligosaccharides will permit a formulation of the general hybrid properties of the parent polysaccharides.

Characterization of Oligosaccharides Obtained after Chondroitinase-AC Degradation. Each oligosaccharide was prepared on a large scale as outlined in Table I. The results of chemical analyses shown in Table I are in reasonable agreement with the assigned structures.

Each of the oligosaccharides had the characteristic ultraviolet absorption spectrum of an α,β-unsaturated acid (36). With the
The crude mucopolysaccharide preparation, 200 mg, was dissolved in 40 ml of 0.05 \( \text{M} \) Tris-acetate, \( \text{pH} \) 8.0, containing 4 mg of bovine serum albumin, and 100 units of chondroitinase-AC. The mixture was incubated at 37° for 6 hours, and then heated in a boiling water bath for 2 min. The precipitate was removed by centrifugation and discarded. The supernatant solution was concentrated to about 0.1 volume over \( \text{P}_2\text{O}_5 \) in a vacuum. The solution was chromatographed as zones (16 cm) on 20 sheets of Toyo No. 50 filter paper (20 × 60 cm) in Solvent A. The products were located by viewing under ultraviolet and by staining several guide strips with \( \text{AgNO}_3 \), after which the products were eluted from the chromatograms with water. Chromatography in Solvent A was repeated with each product to remove small amounts of contaminants. The samples were then desalted separately by paper chromatography for 2 days in Solvent B, in which all the samples had little mobility. After the chromatograms were dried, the ultraviolet-absorbing zones at the origin were cut out and eluted with water. The samples thus obtained were further purified by paper chromatography in Solvent B as described above. Afterwards, the samples were eluted from the papers with water and lyophilized.

Table I: Characterization of oligosaccharides

| Abbreviation | Structure (→ reducing end) | Yield* | Molar ratio to galactosamine | Degree of polymerization* | Unsaturated disaccharides produced by chondroitinase-ABC
|--------------|---------------------------|--------|-----------------------------|-------------------------|--------------------------------------------------------|
| Oligo-I      | \( \Delta \text{GlcUA-GalNAc-IdUA-GalNAc(4S)} \) | 0.47   | 0.51                         | 1.98                    | 0.94 0.93 0.00                                       |
| Oligo-II     | \( \Delta \text{GlcUA-GalNAc(6S)-IdUA-GalNAc(4S)} \) | 9.46   | 0.86 0.98                   | 2.03                    | 0.00 1.02 0.08                                       |
| Oligo-III    | \( \Delta \text{GlcUA-GalNAc(6S)-IdUA-GalNAc(4S)} \) | 5.13   | 1.12 1.04                   | 2.08                    | 0.00 2.14 1.02                                       |
| Oligo-IV     | \( \Delta \text{GlcUA-GalNAc(6S)-IdUA-GalNAc(4S)} \) | 3.90   | 1.02 1.06                   | 4.19                    | 0.00 3.01 1.03                                       |
| Oligo-V      | \( \Delta \text{GlcUA-GalNAc(6S)-IdUA-GalNAc(4S)} \) | 1.16   | 1.10 0.90                   | 6.07                    | 0.00 4.80 1.10                                       |

* Expressed as the amounts (micromoles) obtained from 200 mg of the crude mucopolysaccharide preparation.

** Estimated by the orcinol reaction (24).

* Absorbance at 292 nm after chondroitinase-ABC digestion to assumption that each has \( \Delta \)glucuronic acid residue, which can be quantitatively determined by ultraviolet absorption, the degree of polymerization of each compound was calculated as indicated in Table I. Since the separation of oligosaccharides higher than disaccharides has been difficult, the proposed structure of Oligo-V may represent only a statistical model of the higher oligosaccharides.

The presence of L-iduronic acid as well as the absence of D-glucuronic acid (saturated form) were confirmed with all the samples by mild acid hydrolysis followed by paper chromatography in Solvent E (34) and by chromatography on a column of AG 1-X8 (32).

The oligosaccharides were subjected to chondroitinase-ABC degradation and the resulting disaccharides were determined as described in the previous paper (25). As shown in Table I, each of the oligosaccharides gave \( \Delta \text{GlcUA-GalNAc(4S)} \) and either \( \Delta \text{GlcUA-GalNAc(6S)} \) or \( \Delta \text{GlcUA-GalNAc(6S)} \) in yields which were consistent with those calculated from the proposed structures.

In order to obtain information regarding the sequence of the disaccharide units, each oligosaccharide was subjected to digestion with Flavobacterium glucuronidase, an enzyme which catalyzes a hydrolytic release of the \( \Delta \)glucuronic acid adjacent to N-acetylgalactosamine or N-acetylgalactosamine 4-sulfate, but not the \( \Delta \)glucuronic acid adjacent to N-acetylgalactosamine 6-sulfate (3, 18). The resulting mixture was then cleaved with chondroitinase-ABC and chromatographed as described above. The chromatogram revealed two reducing sugars corresponding to \( \Delta \text{GlcUA-GalNAc(4S)} \) (from all the oligosaccharides) and either \( \text{N-acetylgalactosamine} \) (from Oligo-I) or \( \text{N-acetylgalactosamine} \) 6-sulfate (from Oligo-II, III, IV, and V) (for \( R_p \) values of these sugars, see Reference 3). It should be pointed out that no spots corresponding to either \( \Delta \text{GlcUA-GalNAc(4S)} \) or \( \Delta \text{GlcUA-GalNAc(6S)} \) could be observed. Since such disaccharides as those produced from the intact oligosaccharides, but not from the glucuronidase-treated oligosaccharides should originate from the nonreducing portion, it was deduced that the intact oligosaccharides contained disaccharide units in the sequences shown in Table 1.

Preparation of Hyaluronic Acid-free Mixture—As described above, electrophoresis of the crude mucopolysaccharide mixture showed two major components, with the slow component corresponding to standard hyaluronic acid. To purify the copolymer fraction for further characterization, the crude mixture was fractionated with DEAE-cellulose, and this enabled the mixture to be separated into two major fractions (Fig. 4).

The 0.4 M \( \text{NaCl} \) fraction was indistinguishable from standard hyaluronic acid in its chemical composition, electrophoretic mobility (Fig. 5), and behaviors toward chondroitinase-AC and Streptomyces hyaluronidase. Therefore, no further investigation of this fraction has been carried out.

The 0.7 M \( \text{NaCl} \) fraction, by electrophoresis, was shown to correspond to the fast component in the crude mixture (Fig. 5). Those compounds yielding oligosaccharides by chondroitinase-AC digestion were exclusively recovered in this fraction.

All the oligosaccharides had low molar extinction coefficients at 885 nm in the modified Morgan Elson reaction (26), indicating that position 4 of the reducing terminal N-acetylgalactosamine of each oligosaccharide is substituted. The substituting group appears to be removed by treating the oligosaccharides with excessive amounts of Proteus vulgaris chondro-4-sulfatase (18), because the resultant products all had much higher extinction coefficients (885 nm = 13,000 ~ 20,000) than those of the parent materials and, upon exhaustive digestion with chondroitinase-ABC, gave a new product corresponding to \( \Delta \text{GlcUA-GalNAc(4S)} \), with a compensatory decrease in the yield of \( \Delta \text{GlcUA-GalNAc(6S)} \). In contrast, all the sulfate residues in the oligosaccharides (including those located at position 6) were completely resistant to hydration by chondro-6-sulfatase.
The major portion of this peak (tubes 89 to 142) were pooled, washed, and dried as described above. The yield was 0.67 g (0.92 mmole as carboxyl-positive glucuronic acid). This preparation will be referred to as “hyaluronic acid-free mixture.”

The profile in Fig. 4 (see the inset) showed that this fraction is fairly homogeneous with respect to charge density. Nevertheless, the same fraction displayed a considerable heterogeneity in the electrophoretic profile (the profile was similar to that of Sample 2 in Fig. 5 and is not shown here), suggesting that heterogeneity of uronic acid composition in the copolymers may be reflected in their electrophoretic properties.

**Alcohol Fractionation of Copolymers—** It has been shown previously (37) that dermatan sulfate and chondroitin 6-sulfate (calcium salts) need different concentrations of ethanol to be precipitated. Both the sulfate location as well as the uronic acid composition might be expected to play a role in determining the solubility characteristics. Indeed, it was possible to separate standard dermatan sulfate (heteroglucom) from standard chondroitin 6-sulfate (shark cartilage) by the ethanol fractionation method; the former was quantitatively precipitated at ethanol concentrations as low as 20%, the bulk of the latter between 30 and 40%.

This approach was used to study the properties of copolymers in the hyaluronic acid-free mixture. The results of the ethanol fractionation are listed in Table II. It can be seen that the sample of human meniscus is not simply a mixture of dermatan sulfate and chondroitin 6-sulfate, i.e. the sample gave a large fraction between 20 and 30% ethanol concentration, where neither standard dermatan sulfate nor standard chondroitin 6-sulfate are precipitated.

The 20% ethanol precipitate which from now on will be called “Fraction 20” produced on electrophoresis a band with the same mobility as standard dermatan sulfate, and the 30 to 40% precipitate (“Fraction 40”) a somewhat extended band close to the chondroitin 6-sulfate zone (Fig. 6). However, the 20 to 30% precipitate (“Fraction 30”) differed markedly from these standard polysaccharides, i.e. it occupied an intermediary position in the electrophoretic profile.

Fractions 20, 30, and 40 had different rotations and carbazole to orcinol ratios (Table II). As far as these properties are concerned, Fractions 20 and 40 are similar to standard dermatan sulfate and chondroitin 6-sulfate, respectively, and Fraction 30 appears to be a copolymer with intermediate properties.

Taking advantage of the selective action of chondroitinase-ABC and chondroitinase-AC on galactosaminide linkages to L-iduronic and D-glucuronic acid residues, it is possible to determine the molar ratio of D-glucuronic acid to L-iduronic acid in a given mucopolysaccharide sample (25). The chondroitinase method was applied to the analysis of the three ethanol fractions. The results, shown in Table II, suggested that Fraction 30 contains 62% of total uronic as L-iduronic acid and 38% as D-glucuronic acid, whereas Fractions 20 and 40 are much richer in L-iduronic acid and in D-glucuronic acid, respectively.

The yields of unsaturated di- and tetrasaccharides in the three ethanol fractions were compared after chromatographic separation (Table II). Most notable in this comparison is the high yield of the tetrasaccharide from Fraction 30, i.e. about 18% of the total uronic acid residues (corresponding to about 24% of the total D-glucuronic acid residues) was recovered as AGeUa-GalNAc(6S)-IdUA-GalNAc(4S) after chondroitinase-AC treatment. This indicates that in Fraction 30 about one-half of the total D-glucuronic acid residues is located in a sequence of AGeUa-GalNAc(6S)-IdUA-GalNAc(4S)-GlCUA-.
TABLE II
Comparison of properties of three fractions obtained by ethanol fractionation

| Preparationa | Yieldb (mg) | [α]_20°Cc | Carbazole to orcinol ratio | Uronic acid compositionc | Products of chondroitinase-AC digestiond |
|--------------|-------------|-----------|---------------------------|-------------------------|-----------------------------------------|
|              |             |           |                           |                         |                                         |
| Fraction 20  | 77          | -60°      | 0.33                      | 88% Glucuronic          | 0.16 4 3 8                            |
| Fraction 30  | 81          | -51       | 0.56                      | 62% Glucuronic          | 1.16 6 14 18                           |
| Fraction 40  | 381         | -19       | 1.37                      | 7% Glucuronic           | 4.16 6 79 6                            |

a The fraction numbers indicate the final concentrations of ethanol (%) at which each fraction precipitated.
b Expressed as the amounts (milligrams) obtained from 72 g of dried meniscus.
c Values for D-glucuronic acid were calculated from the amounts to the A4-glucuronic acid residues formed by chondroitinase-AC digestion. Values for L-iduronic acid were calculated from the differences in the amounts of the A4-glucuronic acid residues formed by chondroitinase-ABC digestion and those formed by chondroitinase-AC digestion.
d Expressed as the amounts (moles of uronic acid) obtained from 100 moles (as uronic acid) of each polysaccharide fraction.

Fig. 7. Gel chromatography on Sephadex G-150 of Fraction 20 (a), Fraction 30 (b), and Fraction 40 (c) before (O—O) and after (●—●) treatment with chondroitinase-AC. Samples (1.0 µmole as uronic acid) were dissolved in 1 ml of 0.2 M NaCl and applied to a column (1 × 130 cm) of Sephadex G-150. Elution was carried out with 0.2 M NaCl at a rate of 5 ml per hour. One-milliliter fractions were collected and analyzed for uronic acid by the orcinol reaction. Solid bars above curve indicate fractions containing blue dextran (void volume marker) and unsaturated disaccharides.

Fig. 8. Gel chromatography on Sephadex G-200 of Fraction 20 (O—O), Fraction 30 (●—●), and Fraction 40 (X—X). Samples (10 µmole as uronic acid) were dissolved in 1 ml of 0.2 M NaCl and applied to a column (1 × 110 cm) of a Sephadex G-200. Elution was carried out with 0.2 M NaCl at a rate of 5 ml per hour. One-milliliter fractions were collected and analyzed for uronic acid by the orcinol reaction. The peak positions of internal markers (HGG, human γ-globulin; BSA, bovine serum albumin) are indicated. In order to compare the properties of different areas (see the text), the material in each peak was subdivided as indicated in the upper part.

Fig. 7 shows the effect of chondroitinase-AC upon the three ethanol fractions, followed by chromatography on Sephadex G-150. Under the conditions used, the intact samples were all eluted near the void volume (tubes 31 to 69), while unsaturated disaccharides emerged between tubes 90 and 105.

In the case of Fraction 40, a rather simple profile with a major peak in the region expected for disaccharides was obtained, although small amounts of higher oligosaccharide fragments also appeared.

In contrast, Fractions 20 and 30 gave dispersed profiles with major peaks in the region expected for higher oligosaccharides. Apparently, their elution profiles differ with respect to the molecular size distribution of the fragments. The difference may reflect the fact that the iduronic acid-containing sections of the Fraction 20 polysaccharides are larger in average size than those of the Fraction 30 polysaccharides.

Heterogeneity of Copolymers—The heterogeneity of meniscus copolymers was further illustrated by the behavior of the three ethanol fractions on fractionation with Sephadex G-200 (Fig. 8). For each experiment, human γ-globulin and bovine serum albumin were run as internal markers. The materials in each ethanol fraction displayed a considerable polydispersity. Furthermore, the elution profiles of the three fractions were significantly different; the peak of Fraction 30 was located at an earlier...
position than that of Fraction 20, while the peak of Fraction 40 was located at a later position. In every case, the behavior on gel chromatography was not affected by treatment of the material with 1 N NaOH at 4°C for 15 hours, indicating that neither mucopolysaccharides associated with large peptides nor multichain mucopolypeptides were present in the samples. The question was raised whether these profiles reflect a chain length polydispersity or a diversity of molecular hybrids. In order to evaluate the contribution of these factors, each peak was subdivided into four fractions as indicated in Fig. 8; these were dialyzed against distilled water to remove salt, and subjected to analyses for the determination of chain length and uronic acid composition.

Qualitative analysis of neutral sugars by paper chromatography in Solvent C showed the presence of galactose and xylose in all of the subfractions. The quantitative analysis of Subfractions 20-IV and 30-I (Table III) showed that the molar ratio of serine to xylose to galactose was approximately 1:1:2. Treatment of the preparations with 1 N NaOH at 4°C for 15 hours resulted in 45% destruction of the serine residues. No significant destruction of other amino acids was observed. The failure with these mucopolysaccharides to attain complete destruction of the serine residues suggests that approximately one-half of the serine residues occupied COOH- or NH₂-terminal positions in the peptides, since the work of Stern et al. (38) has indicated that cleavage of the xylosidic linkage does not occur if the amino or carboxyl groups of serine are free.

Estimates based on the molar ratios of total uronic acid to total xylose gave values close to 91 (the number of disaccharide units per molecule) for Subfraction 20-IV and 95 for Subfraction 30-I. Since the difference in degree of polymerization between the earliest component and the latest component is within the limits of precision of the methods employed, it seems fair to conclude that the extent of hybridization, and not chain length, is the principal parameter involved in these elution profiles.

The analytical data for D-glucuronic acid composition (Fig. 9) indicate that the elution profiles of Fractions 20 and 30 reflect the D-glucuronic to L-iduronic acid ratio; i.e. the higher the D-glucuronic acid content, the faster the elution rate. For Fraction 40, however, the reverse is true. It seems likely, therefore, that

### Table III

| Components     | 20-IV | 30-I |
|----------------|-------|------|
| Aspartic acid  | 3.83  | 5.10 |
| Threonine      | 2.81  | 2.40 |
| Serine         | 9.43  | 9.83 |
| Glutamic acid  | 5.40  | 4.89 |
| Proline        | Trace | 0.48 |
| Glycine        | 13.5  | 11.4 |
| Alanine        | 7.75  | 6.95 |
| Leucine        | 0.91  | Trace|
| Xylose         | 10.5  | 10.9 |
| Galactose      | 23.4  | 21.4 |

*a* Determined by the Park-Johnson reaction (29) after hydrolysis with 1 N HCl for 3 hours followed by chromatographic separation (see the text for details).

the elution rate in gel chromatography may mirror the degree of hybridization in the sense that the elution rate becomes faster as the D-glucuronic to L-iduronic acid ratio become closer to unity.

The arguments presented so far indicate that the material in Fractions 20, 30, and 40 was composed of hybrid molecules with various proportions of IduA-GalNAc(6S) units and GlcUA-GalNAc(6S) units. This inference was validated by the finding that the proportions of AGlcUA-GalNAc(4S) and AGlcUA-GalNAc(6S) produced by chondroitinase-ABC digestion also varied gradually from about 96% AGlcUA-GalNAc(4S) (Subfraction 20-IV) to about 86% AGlcUA-GalNAc(6S) (Subfraction 40-IV) (Fig. 9). In addition, all the subfractions on gel chromatography differed with respect to their contents of the Oligo-II after chondroitinase AC digestion; the values (per cent of total uronic acid) being increased progressively from about 3% for Subfractions 20-IV and 7% for 40-IV to about 40% for Subfraction 30-I.

The multiplicity of hybrid molecules was further illustrated by the electrophoretic profiles of these subfractions (Fig. 10). From 20-IV to 30-I, the subfractions could be arranged serially; the higher the D-glucuronic acid content, the greater the mobility. Subfractions 40-1 to 40-IV, on the other hand, did not show such a separation on the electrophoretogram. This is to be expected since their ratios of glucuronic to iduronic acid did not seem to differ to any large extent as compared to those of Subfractions 20-IV to 30-I.

**Mucopolysaccharide Patterns of Menisci from Subjects Aged 20 and 25 Years**—Changes in the proportion of 4- and 6-sulfate units in cartilage chondroitin sulfates have been noted during growth of higher vertebrates (39) as well as in many pathological conditions. The question is then raised as to whether the structures of human meniscus copolymers may change with age or with some other physiological factors. We, therefore, examined the materials obtained from two young patients (20-year-old female and 23-year-old female dying without any known skeletal dis-
acid compositions of dermatan sulfates from various sources
pressive microheterogeneity revealed by analysis of the uranic
mucopolysaccharide (40). The second hypothesis is that ini-
mucopolysaccharide chains, the occurrence of a series of dermatan
hybridization appears to be a direct consequence of how L-
dermatan sulfate sample for structural study must take into con-
reliable methods for purification of such mixtures.
sulfate-chondroitin sulfate copolymers with varying degrees of
ation of n-glucuronic acid- and L-iduronic acid-containing sections.
formation of L-iduronic acid is governed primarily by the availa-
bility of an enzyme that catalyzes the 5-epimerization of o-gluc-
rate, these findings leave little doubt that the meniscus contains
synthesized and that subsequently some of the n-glucuronic acid
is that UDP-n-glucuronic acid is converted to UDP-n-iduronic
acid which might serve as the source of the L-iduronic acid in
mucopolysaccharide chains. One hypothesis hypotheses have been advanced to explain the occurrence of
various hybrid molecules with different proportion and distribu-
tion of GlcUA-GalNAc (6X) and IdUA-GalNAc (4S)
units. Sephadex G-200 chromatography provided the resolution
required to separate copolymers differing in extent of hybridiza-
tion. There was a general trend toward a decrease in the extent
of hybridization as the elution volume increased. A simple
theory to account for this behavior is to assume that a copolymer
chain would tend to take up a more complex conformation as
compared with the same length of homopolymer chains. At any
rate, these findings leave little doubt that the meniscus contains
various hybrid molecules with different proportion and distribu-

d- glucuronic acid- and L-iduronic acid-containing sections.
It should be stressed, therefore, that any attempt to obtain a
dermatan sulfate sample for structural study must take into con-

deration this type of polymorphism as well as the scarcity of
reliable methods for purification of such mixtures.

When examined from the point of view how cells synthesize
mucopolysaccharide chains, the occurrence of a series of dermatan
sulfate-chondroitin sulfate copolymers with varying degrees of
hybridization appears to be a direct consequence of how L-
iduronic acid resides are introduced into the molecule. Two
hypotheses have been advanced to explain the occurrence of
L-iduronic acid in mucopolysaccharide chains. One hypothesis
is that UDP-d-glucuronic acid is converted to UDP-L-iduronic
acid which might serve as the source of the L-iduronic acid in
mucopolysaccharide (40). The second hypothesis is that ini-
ially a polymeric intermediate containing d-glucuronic acid is
synthesized and that subsequently some of the d-glucuronic acid
is converted enzymatically to L-iduronic acid (41). The im-
pressive microheterogeneity revealed by analysis of the uronic
acid compositions of dermatan sulfates from various sources
may argue in favor of the second hypothesis, that the
formation of L-iduronic acid is governed primarily by the availa-

DISCUSSION

It is clear from the results presented in this paper that the
human meniscus contains a great variety of dermatan sulfate-
chondroitin sulfate copolymers. Thus, electrophoresis in pyri-
dine-acetic acid as well as chromatography on Sephadex G-200
were shown to separate the copolymer into a series of variants
whose mobilities could be accounted for by a gradual variation
in the proportion of GlcUA-GalNAc (6S) and IdUA-GalNAc (4S)
units. Sephadex G-200 chromatography provided the resolution
required to separate copolymers differing in extent of hybridiza-
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The Occurrence of a Wide Variety of Dermatan Sulfate-Chondroitin Sulfate Copolymers in Fibrous Cartilage

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