Occurrence of a Unique Fucose-branched Chondroitin Sulfate in the Body Wall of a Sea Cucumber*

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The sulfated polysaccharides in the body wall of the sea cucumber occur as three fractions that differ markedly in molecular mass and chemical composition. The fraction containing a high molecular mass component has a high proportion of fucose and small amounts of galactose and amino sugars, whereas another fraction contains primarily a sulfated fucan. The third fraction (F-2), which represents the major portion of the sea cucumber-sulfated polysaccharides, contains approximately equimolar quantities of glucuronic acid, N-acetyl galactosamine, and fucose, and has a sulfate content higher than that in the other two fractions.

The structure of fraction F-2 was examined in detail. This polysaccharide has an unusual structure composed of a chondroitin sulfate-like core, containing side chain disaccharide units of sulfated fucopyranosyl linked to approximately half of the glucuronic acid moieties through the O-3 position of the acid. These unusual fucose branches obstruct the access of chondroitinases to the chondroitin sulfate core of F-2. However, after partial acid hydrolysis, which removes the sulfated fucose residues from the polymer, fraction F-2 is degraded by chondroitinases into 6-sulfated and nonsulfated disaccharides.

During the last few years we have searched for sulfated polysaccharides in different invertebrate connective tissues (1–4). Structural studies of these polysaccharides have revealed interesting structural differences between them and the well-known glycosaminoglycans that occur in vertebrate connective tissues. Among the macromolecular components of vertebrate connective tissues, the glycosaminoglycans interact with proteins in the tissue matrix, this association being fundamental for the maintenance of most of the unique properties of these tissues (5, 6). In the invertebrates that have been studied, the structurally important glycans are sulfated like those of vertebrates but are much larger (2, 7); in ascidians the principal core component is polysaccharide rather than polypeptide, and the component glycans are highly branched (3).

Other structural differences have begun to appear. From the tunic of ascidians we extracted a novel sulfated polysaccharide composed of L-galactopyranose units (3), and in the body wall of sea cucumbers we found large amounts of sulfated polysaccharides rich in fucose (4).

In the present work we report a more detailed examination of the sulfated polysaccharides from the sea cucumber body wall. We find that the main fraction of these polysaccharides has a chondroitin sulfate-like structure, containing unexpectedly large numbers of sulfated fucose branches.

A chondroitin sulfate containing small amounts of glucose branches substituted at carbon 6 of the hexosamine moieties was previously found in squid cartilage (7). This glycosaminoglycan can be degraded by prolonged incubation with chondroitinase ABC. In contrast, the fucose branches found in the polysaccharide from sea cucumber obstruct the access of this enzyme to the chondroitin sulfate core.

**EXPERIMENTAL PROCEDURES**

**Materials**

The sea cucumber *Ludia ciliata* was collected in Guanabara Bay (Urca), Rio de Janeiro. Chondroitin 4-sulfate, chondroitin 6-sulfate, dextran sulfate (average Mr = 50,000), dextran sulfate (average Mr = 10,000), pork liver L-fucose dehydrogenase, chondroitin 4-sulfatase (chondroitin 4-sulfatase, chondroitin 6-sulfatase, 1-ethyl-3-(3-dimethylaminopropyl)carboimide, and Sepharose CL-4B were purchased from Sigma. Dermatan sulfate, chondroitinase AC, and chondroitinase ABC were from Miles Laboratories (Elkhart, IN), papain and N-cetyl-N,N,N-trimethylammonium bromide from E. Merck A.G. (Darmstadt, Federal Republic of Germany), toluidine blue from Fisher, agarose (standard low Mr) from Bio-Rad and DEAE-cellulose from Whatman Chemical Separation Ltd. (England).

**Isolation of Acidic Glycans from the Body Wall of Sea Cucumber**

The body wall of *L. grisea* was carefully separated from other tissues, immersed immediately in acetone, and kept for 24 h at 4 °C. The dried tissue (50 g) was cut in small pieces, suspended in 1000 ml of 0.1 M sodium acetate buffer (pH 6.0) containing 5 g of papain, 5 mM EDTA, and 5 mM cysteine, and incubated at 80 °C during 24 h. The incubation mixture was then centrifuged (2000 × g for 10 min at 10 °C), and the clear supernatant was precipitated with 2 volumes of 95% ethanol. After maintenance at −10 °C for 24 h, the precipitate formed was collected by centrifugation (2000 × g for 15 min at 10 °C), vacuum dried, dissolved in 50 ml of distilled water, exhaustively dialyzed against distilled water, and lyophilized. About 5 g (dry weight) of crude extract was obtained after these procedures.

**Fractionation of the Acidic Glycans**

**DEAE-Cellulose Chromatography**—About 400 mg of the crude extract from the body wall of *L. grisea* was applied to a DEAE-cellulose column (7 × 2 cm) equilibrated with 0.1 M sodium acetate buffer (pH 5.0) and washed with 100 ml of the same buffer. The column was developed by a linear gradient prepared by mixing 80 ml of 0.1 M sodium acetate buffer (pH 5.0) with 80 ml of 0.6 M NaCl and 80 ml of 1.2 M NaCl in the same buffer. The flow rate of the column was 12 ml/h, and fractions of 3.0 ml were collected. They were checked by the DuBois et al. (8) and carbazole (9) reactions, and conductivity was measured. Two main fractions of sulfated glycans (F-1 and F-2) were obtained, dialyzed against distilled water, and lyophilized. Sepharose CL-4B—About 40 mg of each fraction of sulfated glycans purified by DEAE-cellulose chromatography dissolved in 1.5 ml of

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†To whom correspondence should be addressed.
**Fucose-branched Chondroitin Sulfate**

0.3 M pyridine/acetic acid buffer (pH 5.0) was chromatographed on a Sepharose CL-4B column (115 × 1.5 cm). Columns were eluted with the same buffer at a flow rate of 6 ml/h and aliquots of approximately 1.5 ml were collected. The fractions were assayed by the DuBois et al. (8) and baricose (9) reactions and by the metachromatic property (2), as previously described. Columns were calibrated using blue dextran as a marker for V₀ and creosol red as a marker for Vₑ.

_Agarose and Polyacrylamide Gel Electrophoresis_

Sulfated polysaccharides were analyzed by agarose gel electrophoresis, as previously described (10). About 25 µg of sulfated glycans was applied to a 0.5% agarose gel in 0.05 M acetic acid. (8) and carbazole (9) reactions and by the metachromatic property were fixed with dextran as a marker for Sepharose CL-4B column (115 × 5.0 mm). The glycan molecular weights were determined by polyacrylamide gel electrophoresis (11). About 50 µg of the sulfated glycans was applied to a 6% polyacrylamide slab gel, and after electrophoresis the gel was stained with 0.1% toluidine blue in 1% acetic acid/ethanol/water (0.1:5:5, v/v). The glycan molecular weights were determined by polyacrylamide gel electrophoresis (11). About 50 µg of the sulfated glycans was applied to a 6% polyacrylamide slab gel, and after electrophoresis the gel was stained with 0.1% toluidine blue in 1% acetic acid. After staining, the gel was washed for about 12 h in 1% acetic acid.

_Chemical Modifications of the Sea Cucumber Polysaccharides_

**Mild Acid Hydrolysis**—About 100 mg of the sea cucumber polysaccharide was subjected to partial acid hydrolysis in 10 ml of 75 mm H₂SO₄ at 100 °C for 30 min. A saturated solution of Ba(OH)₂ was added to 5 ml of the hydrolysate, the mixture was neutralized with sodium metaperiodate and kept for 5 days at 4 °C.

**Desulfation**—Desulfation of the sulfated polysaccharides was performed as described by Nagasawa et al. (12). About 100 mg of the fraction F-2 in 10 ml of water was mixed with 1 g (dry weight) of Dowex 50-W (H⁺, 200–400 mesh). After neutralization with pyridine, the solution was lyophilized. The resulting pyridinium salt was dissolved in 10 ml of dimethyl sulfoxide/methanol (8:2, v/v). The mixture was heated at 80 °C for 4 h, and the desulfated products were dialyzed against 3 liters of distilled water. The extent of desulfation was estimated by the molar ratio of sulfate/total sugars and by the decrease in the carbazole reaction (9) and the formation of glucose. After 20 mg of carboxyl-reduced polysaccharide was obtained.

**Reduction of Carboxyl Groups**—Reduction of hexuronic acid carboxyl groups in the polysaccharides was performed as described by Taylor et al. (13). About 25 mg of the polysaccharide was dissolved in 4 ml of water, and the pH of the solution was adjusted to 4.75 with 0.1 M HCl. Solid 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (75 mg) was added over a period of 10 min, while the pH was maintained at 4.75 with 0.1 M HCl. About 300 mg of solid NaBH₄ was added slowly with stirring, and the solution was heated at 50 °C for 24 h. After addition of several drops of acetic acid to destroy the excess borohydride, the solution was dialyzed against distilled water and lyophilized. The extent of reduction of the carboxyl groups was estimated by the decrease in the carboxylate reaction (9) and the formation of glucose. About 20 mg of carboxyl-reduced polysaccharide was obtained.

**Periodate Oxidation**—About 5 mg of the polysaccharides was mixed with 2 ml of 0.1 M sodium metaperiodate and kept for 5 days at 4 °C in the dark. The excess metaperiodate was destroyed by the addition of several drops of ethylene glycol. The solution was dialyzed against distilled water and lyophilized. About 10 mg of sodium borohydride in 1 ml of NH₄OH was added to the lyophilized polysaccharide, and the reaction mixture was maintained overnight at room temperature. After addition of several drops of acetic acid to destroy the excess borohydride, the solution was dialyzed against water and lyophilized. The polysaccharides obtained after periodate oxidation and sodium borohydride reduction were hydrolyzed (4.0 M HCl, 100 °C for 6 h) and redosed with borohydride, and the alditols were acetylated with 1:1 acetic anhydride/pyridine (14). The products were analyzed by gas-liquid chromatography in a packed 2% NPGS column on Chromosorb W 80/100 mesh (200 × 0.4 cm). The column was programmed to run at 140 °C for 10 min, then raised to 230 °C at 2 °C/min and held for 5 min.

**Methylation**—About 10 mg of the desulfated and carboxyl-reduced fraction F-2 was methylated by the Hakomori method (15), with the modifications introduced by Conrad (16). The methylated polysaccharides (10 mg) were hydrolyzed with 4.0 M trifluoroacetic acid for 6 h at 100 °C, reduced with borohydride, and the alditol acetates were observed by gas chromatography/mass spectrometry unit, model 5972A. Injected was made in the split mode with SE-54 capillary column (25 m × 0.3 mm). The column was programmed to run at 120 °C for 2 min, then raised to 250 °C at 2 °C/min and held for 5 min.

**Enzymatic Degradation**

Incubation with Chondroitinas and Chondro-sulfatases—About 100 µg of the acidic oligosaccharide obtained by partial acid hydrolysis of fraction F-2 and 100 µg of standard chondroitin 4-sulfate were incubated with 0.01 units of chondroitinase AC or ABC (17) in 0.05 M ethylene-diamine-acetate buffer (pH 8.0) at 37 °C for 8 h. In some experiments, about 0.01 units of chondro-4-sulfatase or 0.01 units of chondro-6-sulfatase were added to the incubation mixtures. The reaction mixtures were spotted on Whatman No. 1 paper and chromatographed in isobutyrinic acid, 1 M NH₄OH (8:3, v/v) for 4 h. The unsaturated disaccharides formed were visualized by silver nitrate staining and quantitated by densitometry using a Quick Scan densitometer (Helena Laboratories, Beaumont, TX).

Incubation with 1-Fucose Dehydrogenase—Deriving amounts of fucose isolated from the sea cucumber polysaccharides, and authentic samples of D- or L-fucose, were incubated with 0.2 units of porcine liver 1-fucose dehydrogenase (18) and 2.5 µmol of NAD⁺ in 2 ml of 0.01 M glycine/NaOH buffer (pH 8.0). The reaction mixtures were incubated at 30 °C for different times and the formation of NADH was followed by measuring the absorbance at 340 nm.

**Chemical Analyses**

Total hexose was measured by the method of DuBois et al. (8), hexuronic acid by the carbazole reaction (9), total methylpentose by the method of Dische and Shettles (19), and reducing sugars by the method of Park and Johnson (20). After acid hydrolysis of the polysaccharides (6.0 M HCl at 100 °C for 6 h), total hexosamine was measured by a modified Elson-Morgan reaction (21), and sulfate by the BaCl₂/gelatin method (17). Standard curves for hexosamine were constructed with glucosamine subjected to exactly the same hydrolytic conditions as the biological samples. The percentages of hexoses, methylpentoses, and hexosamines in the acid hydrolyzates were estimated by gas-liquid chromatography of the corresponding alditol acetates (14) and by paper chromatography in ethyl acetate/pyridine/water (8:2:1, v/v) for 24 h (22). The periodate consumption of the sulfated fucose was determined spectrophotometrically (23, 24). The course of the oxidation was followed by measuring consumption of periodate with time at room temperature. The unreactive periodate which was not involved in the oxidation reaction was destroyed by arsenite and the amounts of formaldehyde (25), acetaldheyde (26), and formic acid (27) were measured. Acetol groups (as acetic acid) were measured after acid hydrolysis (2.5 M HCl, at 100 °C for 3 h) by gas-liquid chromatography. A coiled glass column (200 × 0.5 cm) packed with Porapak Q 100/200 mesh was used as support medium. Vaporization, column, and detector temperatures were 150, 156, and 200 °C, respectively.

**Other Methods**

Optical rotations were measured with a digital polarimeter (Perkin-Elmer model 245-B) and conductivity with a conductometer, model E-527 (Metrohm Herisau, Switzerland). Infrared spectra were recorded with a Perkin-Elmer infrared spectrometer, model 298.

**RESULTS AND DISCUSSION**

Fractionation and Chemical Analysis of the Sulfated Polysaccharides from Sea Cucumber—The sulfated polysaccharides from the body wall of _L. grisea_ were purified by DEAE-cellulose and Sepharose CL-4B columns (Fig. 1). The DEAE-cellulose chromatography (Fig. 1A) separated the sulfated polysaccharides into two DuBois reaction peaks, designated F-1 and F-2. Hexuronic acid was detected only in the fraction F-2. Two other peaks containing sugars, according to the DuBois reaction, were observed in the DEAE column. One was a major peak that was not retained in the DEAE column.
Fucose-branched Chondroitin Sulfate polyanhydride from the body wall of DEAE-cellulose column was obtained, for the procedures shown in Methods. The fractions were checked by the DuBois method. F-1 (40 mg) was chromatographed on a Sepharose CL-4B column in the figure, designated F-1 and F-2, and dialyzed against distilled water and lyophilized. About 40 mg of F-1 and 100 mg of F-2 were obtained, for the procedures shown in B and C. B, the DEAE-purified F-1 (40 mg) was chromatographed on a Sepharose CL-4B column (115 × 1.5 cm), eluted with 0.3 m pyridine/acetate buffer (pH 6.0), and the fractions were checked for methylpentose, hexuronic acid (C--O) and metachromatic property (Δ--Δ). About 9 mg of F-1-x and 20 mg of F-1-y were obtained. C, the DEAE-purified F-2 (40 mg) was chromatographed on a Sepharose CL-4B column, as described above. About 30 mg of Sepharose-purified F-2 were obtained.

and probably corresponds to nonacidic oligosaccharides. The other peak was eluted at the beginning of the salt gradient, and it was not included in this study because it corresponds to only a small portion of the total sugars obtained from sea cucumber.

When the DEAE-cellulose-purified F-1 was chromatographed on Sepharose CL-4B (Fig. 1B), a broad metachromatic peak designated as F-1-y and containing methylpentose was eluted with a K_m between 0.35 and 0.85. Another sharp peak designated as F-1-x was eluted near the void volume. Gel filtration chromatography on Sepharose CL-4B separated fraction F-2 from a minor contaminant containing fucose but lacking the metachromatic property (Fig. 1C), and shows the coincidence between the fucose and hexuronic acid peaks in this fraction.

Fig. 2 shows the electrophoretic mobilities of the purified polysaccharides on agarose and polyacrylamide gels. Fraction F-1-x did not migrate into the polyacrylamide gel due to its high molecular weight (Fig. 2B) while F-1-y showed a broad band, as expected from its gel filtration elution on Sepharose CL-4B. Fraction F-2 had a single band on the polyacrylamide gel with an average molecular mass of 35,000 (compared with glycosaminoglycan standards). Electrophoresis on agarose gels confirmed the homogeneity of the fractions (Fig. 2A).

The chemical analysis of the purified fractions of sulfated polysaccharides is shown in Table I. Fucose and sulfate were detected in all fractions; however, the proportions of these components varied from one fraction to another. F-1-y consisted of a single sulfated fucan, while small amounts of hexosamines and galactose were found in F-1-x. Fraction F-2 has approximately equimolar proportions of fucose, glucuronic acid, and galactosamine. Besides the sugars listed in Table I no other sugar was detected in these polysaccharides at a sensitivity of 0.02 mg/mg of polysaccharides.

The strongly negative specific rotation of F-1-x and F-1-y (−93° and −158°, respectively) is compatible with residues of α-L-fucopyranosyl. This structure type is supported by the finding that the fucose obtained by acid hydrolysis of F-1 (a mixture of F-1-x and F-1-y) was exclusively of the L-configuration. However, the finding that 10% of the fucose obtained by acid hydrolysis of F-2 was not oxidized by L-fucose dehydrogenase and that the specific rotation of this fucose solution is −50°, suggests the presence of small amounts of the D-enantiomer of this sugar in fraction F-2.

Partial Acid Hydrolysis of Fraction F-2—The time course of partial acid hydrolysis of fractions F-1-y and F-2 (Fig. 3) indicates that F-1-y was almost totally hydrolyzed in 60 min while in the same period only 37% of the sugars from F-2 were liberated.

Since fucose forms a glycosidic linkage that is more sensitive to acid than that formed by glucuronic acid or by hexosamine (28), it is possible to speculate from the experiment
of the sulfated fucose (-57°) is more negative and that of the sulfated fucose, when compared with that of -70°, suggests a preponderance of the L-isomer. Regarding the hexosamine, it might resist the partial acid hydrolysis of F-2. Attempts were made to isolate these compounds on a DEAE-cellulose column (Fig. 4A). Indeed, partial acid hydrolysis of F-2 produced a glucuronic acid-rich product which was eluted at the beginning of the salt gradient and a fucose-rich product which was not retained on the column.

Paper chromatography (Fig. 4B) of the fucose-rich product obtained by partial acid hydrolysis of F-2 shows a minor component with the same mobility as standard fucose and a major component which migrated close to the anode on paper electrophoresis (Fig. 4C). The chemical composition of this major product identifies it as a sulfated fucose (Table II). Upon periodation, it consumed 2.34 mol of periodate/mmol sugar, forming 1.18 mol of acetaldehyde/mol sugar and 1.30 mol of formaldehyde/mol sugar. These results suggest that the sulfate groups are attached at position 2 or 3 of the fucose. To distinguish between these two possibilities, the sulfated fucose was reduced with NaBH₄, and the sulfated fucitol obtained was oxidized with NaOH. The production of 1.18 mol of acetaldehyde/mol sugar and 1.30 mol of formaldehyde/mol sugar after these treatments demonstrated that the sulfate is linked to the 0-3 position of the fucose.

Polyacrylamide gel electrophoresis of the glucuronic acid-rich product formed by partial acid hydrolysis of F-2 showed only one band with an average molecular mass of 10,000 (compared with glycosaminoglycan standards). This compound, which was denominated acidic oligosaccharide, possesses approximately equimolar proportions of glucuronic acid and galactosamine (Table II).

Another observation worth noting is that the specific rotation of the sulfated fucose (-57°) is more negative and that of the acidic oligosaccharide (-8°) is less negative when compared with the specific rotation of F-2 (-30°). The rotation of the sulfated fucose, when compared with that of -70° recorded for a mutarotated solution of authentic L-fucose, suggests a preponderance of the L-isomer. Regarding the rotation of the acidic oligosaccharide, it is very close to that recorded for chondroitin 6-sulfate (-11°), which is composed of repeating disaccharide units of β-D-glucuronic acid 1→3 linked glycosidically to N-acetyl β-D-galactosamine (29).

**Degradation of the Acidic Oligosaccharide by Chondroitinase AC and ABC**—Structural similarity between chondroitin 4/6-sulfate and the acidic oligosaccharide obtained from F-2 is suggested by the chemical composition and specific rotation of the latter (Table II). This hypothesis is confirmed by the degradation of the acidic oligosaccharide by chondroitinase...
AC and ABC, while the intact F-2 is resistant to both chondroitinases (Fig. 5A). Based on its hexosamine content, about 82% of the acidic oligosaccharide was degraded by chondroitinase AC and ABC, and 60% of the products were an unsaturated 6-sulfated disaccharide (ΔGlcUA-GalNAc6S) while 40% were an unsaturated nonsulfated disaccharide (ΔGlcUA-GalNAc).

The chondroitinase experiment included a control (not shown in the figure), which demonstrates the formation of the same products by chondroitinase AC digestion of the acidic oligosaccharide in the presence and absence of intact F-2, to ensure that the latter contained no inhibitor of the enzyme. Consequently, it is possible to conclude that the presence of sulfated fucose branches in intact F-2 obstruct the access of the enzyme to the chondroitin sulfate-like core.

Sequential degradation of the acidic oligosaccharide by chondroitinase AC and chondro-4-sulfatase or chondro-6-sulfatase demonstrated that the disaccharide with chromatographic migration similar to ΔGlcUA-GalNAc6S (product 2, Fig. 5B) was digested only by chondro-6-sulfatase, forming nonsulfated disaccharide (product 3, Fig. 5B). A small amount of another product with a lower chromatographic migration than ΔGlcUA-GalNAc6S was also observed after chondroitinase AC digestion of the acidic oligosaccharide (product 1, Fig. 5B). This compound had a chemical composition similar to that of ΔGlcUA-GalNAc6S (0.68 mol of sulfate/mol glucuronic acid, 0.77 mol of acetyl groups/mol glucuronic acid, \( \varepsilon_{232}^{\text{nm}} = 7547 \)) and was degraded by chondro-6-sulfatase but

### Table II

<table>
<thead>
<tr>
<th>Chemical composition, average molecular weight, and specific optical rotation of the main products obtained by partial acid hydrolysis of fraction F-2 (Fig. 4A)</th>
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<tbody>
<tr>
<td><strong>Method of characterization</strong></td>
</tr>
<tr>
<td>-------------------------------</td>
</tr>
<tr>
<td><strong>Chemical composition</strong> (as molar ratios)</td>
</tr>
<tr>
<td>Fucose</td>
</tr>
<tr>
<td>Glucuronic acid</td>
</tr>
<tr>
<td>Galactosamine</td>
</tr>
<tr>
<td>Sulfate</td>
</tr>
<tr>
<td>( [\alpha]_{B}^{20}^\circ )</td>
</tr>
<tr>
<td><strong>Average molecular mass</strong></td>
</tr>
</tbody>
</table>

\(^{a}\) Average molecular mass compared with glycosaminoglycan standards.

\(^{b}\) N.D., not determined.

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**Fig. 5.** Paper chromatograms of the degradation products formed by the action of chondroitinase AC, chondroitinase ABC, chondro-4-sulfatase, and chondro-6-sulfatase on intact F-2 and on the acidic oligosaccharide of Fig. 4A. A, about 100 μg of intact F-2 or of the acidic oligosaccharide was incubated with 0.01 units of chondroitinase AC or ABC in 0.05 M ethylenediamine/acetate buffer (pH 8.0), in a final volume of 100 μl. After incubation for 8 h at 37 °C, the mixtures were spotted on Whatman No. 1 paper and subjected to chromatography in isobutyric acid, 1 M NH₄OH (5:3, v/v) for 24 h. The products were located on the chromatograms by silver nitrate staining. B, about 100 μg of the acidic oligosaccharide from Fig. 4A (left) or of standard chondroitin 4-sulfate (right) was incubated with: a, 0.01 units of chondroitinase AC, b, 0.01 units each of chondroitinase AC and chondro-4-sulfatase, c, 0.01 units each of chondroitinase AC and chondro-6-sulfatase, and d, 0.01 units each of chondroitinase AC, chondro-4-sulfatase, and chondro-6-sulfatase, in a final volume of 100 μl. After incubation for 8 h at 37 °C, the products were analyzed by paper chromatography as described above.
not by chondro-4-sulfatase (Fig. 5B). Probably, it is a minor degradation product of the chondroitin core under the conditions of partial acid hydrolysis. In fact, with chondroitinase AC digestion of chondroitin 6-sulfate subjected to the same hydrolytic procedure, a small amount of the same product was formed (data not shown).

$^{13}$C NMR of Fraction F-2—The comparison of the $^{13}$C NMR spectrum of fraction F-2 with that of standard chondroitin 4-sulfate shows the coincidence among various signals of both polysaccharides. Indeed, the $^{13}$C NMR spectrum of F-2 shows several signals in the vicinity of $\delta = 75-85$ ppm which have chemical shifts similar to the $^{13}$C nuclei of standard chondroitin 4-sulfate (Fig. 6, A and B). The spectra of both compounds show signals attributable to carbonyl (CO), methyl (COCH$_3$) and ring carbon-2 (A-2) of the N-acetyl galactosamine moiety, which resonate at approximately $\delta = 178, 54$, and 25 ppm, respectively.

However, the additional complexity of the $^{13}$C NMR spectrum of F-2, which may be due to the fucose residues linked to the chondroitin sulfate core, does not afford reliable information about the site of sulfation or substituted carbons of the fucose residues.

The detection of a signal at $\delta = 63$ ppm, which can be attributed to nonsubstituted carbon-6 (A-6, Fig. 6A), agrees with the chondroitinase experiments (Fig. 5) in showing the partial desulfation of the chondroitin sulfate core in F-2. Furthermore, the signal at approximately $\delta = 69$ ppm, which is obscured by the overall complexity of the spectrum in the region of fucose signals, may be attributed to sulfated carbon-6 of the N-acetylgalactosamine (30). In addition, the $^{13}$C NMR spectrum of the desulfated F-2 (Fig. 6C) shows an integral increase of the signal at $\delta = 63$ ppm with a concomitant integral decrease of the peaks in the region of $\delta = 69$ ppm.

The anomeric region of the $^{13}$C spectrum of F-2 shows peaks coincident with carbon-1 of both the $\beta$-D-glucuronic acid (U-1) and the N-acetyl-$\beta$-D-galactosamine (A-1) units, together with several other signals of $\alpha$- and $\beta$-glycosidically linked carbons.

Periodate Oxidation and Methylation Studies—From the $^{13}$C NMR and chondroitinases studies, it was not possible to deduce the position at which the fucose residues are attached to the chondroitin sulfate core of F-2. However, the periodate studies (Table III) indicate that the fucose residues are linked to position 2 or 3 of the glucuronic acid in F-2. That is, most of the glucose residues in the carboxyl-reduced F-2, either before or after desulfation, are resistant to periodate oxidation, while the glucose residues in the carboxyl-reduced chondroitin 6-sulfate are oxidized by periodate forming erythritol.

In order to distinguish whether the fucose residues are linked to position 2 or 3 of the glucuronic acid, the desulfated and carboxyl-reduced F-2 was methylated by the Hakomori procedure (15, 16). The formation of large amounts of 2,6-di-O-methyl glucitol (Table IV) indicates that the fucose is linked to position 3 of the glucuronic acid residues.

The observation that approximately equimolar proportions of 2,3-tri-O-methyl- and 2,6-di-O-methyl-glucitol were formed after methylation of desulfated and carboxyl-reduced F-2 suggests that only half of the glucuronic acid residues are fucosylated. This result, together with the presence of equimolar proportions of fucose and glucuronic acid in fraction F-2, suggests that only half of the glucuronic acid residues are fucosylated. This result, together with the presence of equimolar proportions of fucose and glucuronic acid in fraction F-2, suggests that only half of the glucuronic acid residues are fucosylated.

**Table III**

<table>
<thead>
<tr>
<th>Product</th>
<th>F-2</th>
<th>Desulfated F-2</th>
<th>Chondroitin 6-sulfate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Galactosamine</td>
<td>0.34 ($&lt;5$)</td>
<td>0.29 ($&lt;5$)</td>
<td>0.41 ($&lt;5$)</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.36 ($&lt;5$)</td>
<td>0.26 (12)</td>
<td>0.08 (86)</td>
</tr>
<tr>
<td>Fucose</td>
<td>0.30 ($&lt;5$)</td>
<td>0.08 (86)</td>
<td>0.01 ($&lt;5$)</td>
</tr>
<tr>
<td>Erythritol</td>
<td>&lt;0.01</td>
<td>0.09</td>
<td>0.51</td>
</tr>
<tr>
<td>Glycerol and/or</td>
<td>&lt;0.01</td>
<td>0.28</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>butanetriol$^b$</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ The values in parentheses are the percentages of sugar residues that disappeared after periodate oxidation.

$^b$ Glycerol and butanetriol have a very close retention time on gas-chromatography column and could not be distinguished under our conditions of analysis.

**Table IV**

<table>
<thead>
<tr>
<th>Methylated sugars$^a$ (as alditol acetates)</th>
<th>$t_R$</th>
<th>Molar ratios$^d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,3,4-Fuc</td>
<td>1.00</td>
<td>0.15$^d$</td>
</tr>
<tr>
<td>2,3-Fuc</td>
<td>1.28</td>
<td>0.09$^d$</td>
</tr>
<tr>
<td>3,4-Fuc</td>
<td>1.36</td>
<td>0.15$^d$</td>
</tr>
<tr>
<td>2,3,6-Glc</td>
<td>1.69</td>
<td>0.14</td>
</tr>
<tr>
<td>2,6-Glc</td>
<td>1.94</td>
<td>0.18</td>
</tr>
<tr>
<td>4,6-GalNH</td>
<td>2.55</td>
<td>0.21</td>
</tr>
<tr>
<td>6-GalNH</td>
<td>2.86</td>
<td>0.10</td>
</tr>
</tbody>
</table>

$^a$ The identity of each peak was established by mass spectrometry.

$^b$ Retention time ($t_R$) on SE-54 capillary column relative to 2,3,4-tri-O-methyl fucitol.

$^c$ The molar ratios were based on the area of each peak compared with total area.

$^d$ The relative proportions of the methylated derivatives from fucose were not modified after a second methylation.

FIG. 6. $^{13}$C magnetic resonance spectra at 300 MHz of intact fraction F-2 (A), standard chondroitin 4-sulfate (B), and desulfated F-2 (C). The spectra were recorded with deuterium oxide as the solvent, at 90°C. The chemical shifts ($\delta$) were measured with respect to the dioxane internal standard.
F-2 (Table I), indicates that the glucuronic acid must be substituted mainly by disaccharide composed of fucopyranosyl. In fact, the presence of fucopyranose units linked glycosidically though position 1 → 4 and/or 1 → 2 was confirmed by the formation of 2,3-di-O-methyl-fucitol and 3,4-di-O-methyl-fucitol after methylation of desulfated F-2 (Table IV). Further evidence for the presence of fucopyranose linked glycosidically though position 1 → 4 and/or 1 → 2 was obtained by the periodate studies. That is, glycerol and/or butanetriol were formed after periodate oxidation of the desulfated F-2 (Table III).

Finally, the formation of 2,3,4-tri-O-methyl-fucitol confirms the presence of fucopyranose end-groups in this polysaccharide. The amount of nonreducing terminal fucose is slightly below that expected for the number of branching points, as estimated by the proportions of 2,6-di-O-methyl-glucitol or dimethylated derivatives from fucose. However, the proportions of the various methylated derivatives from fucose remain constant after a second round of methylation (Table IV). Thus, one may exclude the possibility that the dimethylated fucitol is merely a partially methylated product, and that this derivative accounts for an underestimation of the nonreducing terminal fucose residues. It may be that, compared with other methylated sugars, a slightly higher proportion of 2,3,4-tri-O-methyl-fucitol is destroyed during the acid hydrolysis. This is especially noteworthy with our material, where hydrolysis conditions more drastic than is customary for hydrolyzing methylated polysaccharides must be used due to the presence of high amounts of hexosamine moieties in the polysaccharide (28).

Regarding the position of the sulfate ester in the fucose residues of F-2, the periodate oxidation data (Table III) agree with the partial acid hydrolysis studies in showing that the sulfate is located at position 3 of the fucose. Thus, the fucose residues were not oxidized by periodate in the carboxyl-reduced F-2, whereas after chemical desulfation, most of the fucose residues were attacked by periodate.

CONCLUSION

Fig. 7 shows the proposed structure for the major components of fraction F-2. This fraction is a branched polysaccharide containing a central core of repeating disaccharide units of β-D-glucuronic acid 1 → 3 linked glycosidically to N-acetyl-β-D-galactosamine, which are partially sulfated at carbon 6 of the hexosamine moiety. Approximately half of the glucuronic acid residues are not fucosylated (A, Fig. 7). Branches of disaccharides composed of fucopyranosyl units are linked to position 3 of the other glucuronic acid residues. The methylation (Table IV) and the periodate (Table III) studies indicate two possibilities for the branches that occur frequently, formed by 1 → 2 (B, Fig. 7) and 1 → 4 (C, Fig. 7) linked disaccharides of fucopyranose units.

The presence of fucose branches obstructs the access of the chondroitinases to the chondroitin sulfate core of fraction F-2 (Fig. 5). However, after partial acid hydrolysis, which removes the sulfate fucose residues from the polysaccharide, the acidic oligosaccharide obtained is degraded by chondroitinase AC or ABC (II), forming 6-sulfated and nonsulfated disaccharides (Fig. 5). Further evidence for the structure of the core is given in Fig. 6. The methylation studies (Table IV) indicate that approximately half of the glucuronic acid units are not fucosylated (A). The 0–3 positions of the other glucuronic acid residues are substituted by disaccharide units formed by fucopyranose linked glycosidically through position 1 → 2 (B) or 1 → 4 (C). Further evidence for this structure of the branches is given in Table III.

FIG. 7. Hypothetical structure for the major components of fraction F-2. Fraction F-2 is composed of a chondroitin sulfate-like core, containing branches of sulfated fucose. After partial acid hydrolysis (I) (Fig. 4), which removes the sulfated fucose residues from the polysaccharide, the acidic oligosaccharide obtained is degraded by chondroitinase AC or ABC (II), forming 6-sulfated and nonsulfated disaccharides (Fig. 5). Further evidence for the structure of the core in given in Fig. 6. The methylation studies (Table IV) indicate that approximately half of the glucuronic acid units are not fucosylated (A). The 0–3 positions of the other glucuronic acid residues are substituted by disaccharide units formed by fucopyranose linked glycosidically through position 1 → 2 (B) or 1 → 4 (C). Further evidence for this structure of the branches is given in Table III.

position of the β-D-glucuronic acid units from the chondroitin sulfate core.

A chondroitin sulfate containing small amounts of glucose branches linked to position 6 of the N-acetyl-β-D-galactosamine moieties was found in squid cartilage (7). Compared with this chondroitin sulfate, the polysaccharide from sea cucumber differs in the presence of fucose instead of glucose, in the presence of sulfate ester in the branches, in the higher proportion of branched disaccharide units and in the linkage of the fucose to position 0–3 of the β-D-glucuronic acid units.

The body wall of the sea cucumber is formed by a network of collagen fibers embedded in an amorphous substance. A large number of small, irregular microfibrils, which resemble the proteoglycans of vertebrate cartilages, form bridges among collagen fibers (31). Alcianophilic cells containing large secretory granules have also been observed in this tissue. Interestingly, several species of sea cucumbers have been reported to
undergo, when stimulated or disturbed, a process of quick degradation of the body wall called autotomy (32). This phenomenon is accompanied by loss of granules of the alcianophilic cells and by digestion of the microfibrils (31).

If, as we suspect, these microfibrils are the source of the polysaccharides characterized in this study, it will be interesting to identify the enzymes responsible for their degradation during autotomy.

The function of the sulfated fucose branches in this unique chondroitin sulfate is a matter of speculation. The striking decrease the degradation of the glycosaminoglycan chain. A similar function might be attributed to the sulfated fucose branches of the sea cucumber chondroitin sulfate.

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REFERENCES