The structure of a unique fucose-branched chondroitin sulfate isolated from the body wall of a sea cucumber was examined in detail. This glycosaminoglycan contains side chain disaccharide units of sulfated fucopyranosyl units linked to approximately one-half of the glucuronic acid moieties through the O-3 position of the acid. The intact polysaccharide is totally resistant to chondroitinase degradation, whereas, after desulfation, it is partially degraded by the enzyme. However, only after an additional step of desulfation, the chondroitin from sea cucumber is almost totally degraded by chondroitinase AC or ABC. This result, together with the methylation and NMR studies of the native and chemically modified polysaccharide, suggest that besides the fucose branches, the sea cucumber chondroitin sulfate contains sulfate esters at position O-3 of the β-D-glucuronic acid units. Furthermore, the proteoglycan from the sea cucumber chondroitin sulfate is recognized by anti-Leu-7 monoclonal antibody, which specifically recognizes 3-sulfoglucuronic acid residues. In analogy with the fucose branched units, the 3-O-sulfo-β-D-glucuronosyl residues are resistant to chondroitinase degradation. Regarding the position of the glycosidic linkage and site of sulfation in the fucose branches, our results suggest high heterogeneity. Tentatively, it is possible to suggest the preponderance of disaccharide units formed by 3,4-di-O-sulfal-α-L-fucopyranosyl units glycosidically linked through position 1 → 2 to 4-O-sulfo-α-L-fucopyranosyl. Finally, the presence of unusual 4/6-disulfated disaccharide units, together with the common 6-sulfated and non-sulfated units, was detected in the chondroitin sulfate core of this polysaccharide.

During the last few years we have searched for sulfated polysaccharides in different invertebrate connective tissues. The main purpose of such studies is to compare these polysaccharides with the well known glycosaminoglycans that occur in vertebrate tissues and to relate their structure with physicochemical and biological properties. In previous studies, we have isolated novel sulfated polysaccharides from these invertebrate tissues, namely the tunic of ascidians (1–5) and the body wall of the sea cucumber (1, 6, 7).

In the body wall of a sea cucumber, we found that the main fraction of the polysaccharide has a chondroitin sulfate-like structure, containing unexpectedly large numbers of α-L-fucopyranose branches linked to position 3 of the β-D-glucuronic acid residues (7). Some other chondroitin sulfates containing branches have been described. Thus, a chondroitin sulfate containing small amounts of glucose branches substituted at carbon 6 of the hexosamine moieties was previously found in squid cartilage (8). In addition, a polysaccharide consisting of a desulfated chondroitin backbone, to which β-fructofuranosyl is linked at carbon 3 of the D-glucuronic acid, was isolated from the capsular polysaccharide of a uropathogenic Escherichia coli (9). The squid glycosaminoglycan can be degraded by prolonged incubation with chondroitinase ABC (8). In contrast, the α-L-fucopyranose and β-fructofuranose branches obstruct the access of the chondroitinases to the chondroitin core. However, after partial acid hydrolysis, which removes these branches, the polymers obtained are degraded by chondroitinases (7, 9).

In the present work, we report new data on the structure of the fucose-branched chondroitin sulfate from the sea cucumber body wall. Methylation and chondroitinase degradation of the native and chemically modified polysaccharide, together with immunoreaction with a monoclonal antibody, suggest that besides the fucose branches, this chondroitin contains sulfate esters at position 3 of the β-D-glucuronic acid. In analogy with the fucose branched units, the 3-O-sulfo-β-D-glucuronosyl residues are resistant to chondroitinase degradations.

**EXPERIMENTAL PROCEDURES AND RESULTS AND DISCUSSION**

**CONCLUSION**

Fig. 7 shows a hypothetical structure for the major components of the fucose-branched chondroitin sulfate from sea cucumber body wall. Portions of this paper (including "Experimental Procedures," "Results and Discussion," Figs. 1–6, and Tables I–V) are presented in miniprint at the end of this paper. The abbreviations used are: ΔGlcUA-GalNAc, 2-acetamido-2-deoxy-3-O-(β-D-glucopyranosyluronic acid)-D-galactose; ΔGlcUA-GalNAc4S, ΔGlcUA-GalNAc6S, and ΔGlcUA-GalNAc4/6diS, derivatives of ΔGlcUA-GalNAc bearing a sulfate ester at position 4, at position 6, and at both positions, respectively, of the hexosamine moiety, GlcUA-GalNAc, 2-acetamido-2-deoxy-3-O-(β-D-glucopyranosyluronic acid)-D-galactose, GlcUA-GalNAc4S and GlcUA-GalNAc6S, derivatives of GlcUA-
The presence of sulfation, the methylation (Table VI) and Fig. 6) studies suggest high heterogeneity. Tentatively, it is proposed that the presence of non-sulfated glucuronic acid units (left) further evidence for the presence of 3-O-sulfate units in this chondroitin sulfate is given by the immunoreaction of the native, desulfated, and defucosylated carboxyl-reduced chondroitin after carboxyl reduction (upper portion of the figure) suggests that sulfate esters are linked to the O-3 position of non-fucosylated glucuronic acid units (left). Further evidence for the presence of 3-O-sulfate units in this chondroitin sulfate is given by the immunoreaction of the native, desulfated, and defucosylated carboxyl-reduced chondroitin after carboxyl reduction (upper portion of the figure) suggests that sulfate esters are linked to the O-3 position of non-fucosylated glucuronic acid units (left).

The biological relevance of this unusual polysaccharide is still unclear. Possibly, the presence of fucose branches and sulfate esters linked to the O-3 position of the β-D-glucuronic acid, which make this glycosaminoglycan resistant to degradation by hyaluronidase and chondroitinase, serves to prevent digestion of the sea cucumber body wall by microorganisms present in the marine environment.

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Fucos-Branch Cordrin Sulfate

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Supplement Material to

SYNTHESIS OF A FUCOS-BRANCHED CHONDROITIN SULFATE FROM SEA CUCUMBER EVIDENCE FOR THE PRESENCE OF 3-0-SULFO-2-O-GLUCURONIDIC RESIDUES

Ricardo P. Vieira, Barbara Malloy and Paulo A. Moura

EXPERIMENTAL PROCEDURES

Materials: The sea cucumber Lucernariopsis baltica (Schlumberger Holothuridae) was collected in Guanabara Bay (Uras, Rio de Janeiro, Brazil). Chondroitin 4-sulfate, chondroitin 6-sulfate, chondroitin 4,6-sulfate (disaccharide-4-sulfate, disaccharide-6-sulfate, disaccharide-4,6-sulfate) were purchased from Sigma Chemical Company (St. Louis, MO, USA). Desulfated Chondroitin sulfate from sea cucumber, after carboxyl reduction, was methylated by the method described (7). The resulting partially methylated alditol acetates were dissolved in 2 ml of ice-cold 300 m M NaCl:ethanol (100:1 v/v) solution and precipitated with 3 ml of CHCl₃:ethanol (5:1 v/v). The precipitate was washed three times with cold acetone and lyophilized to give about 25 mg of the desulfated polymer. The extent of desulfation was estimated by the molar ratio of sulfate:total sugar and by the infrared spectrum.

Leaves and stems of the experimentally grown seaweed were collected in Guanabara Bay (Uras, Rio de Janeiro, Brazil). The seaweed was cut into small pieces, suspended in 5 ml of 0.1 M sodium acetate buffer (pH 5.5) containing 100 mg/ml papain, 4 µ M EDTA and 5 µ M cysteine, and incubated at 60°C for 24 h. The incubation mixture was centrifuged at 10,000 x g for 10 min, and the supernatant was precipitated with 1.6 ml of 95% ethanol. The resulting precipitate was dissolved in 10 ml of 150 m M sodium acetate buffer (pH 5.5) containing 0.1 M NaCl:ethanol (100:1 v/v) solution and precipitated with 3 ml of 95% ethanol. After standing at 4°C for 24 h, the mixture was centrifuged (1000 x g for 15 min), washed three times with 95% ethanol, and once with 5% ethanol. The final precipitate was dried at 60°C for 2 h, dissolved in 3 ml of distilled water and lyophilized.

Leaves of the experimentally grown seaweed were collected in Guanabara Bay (Uras, Rio de Janeiro, Brazil). The seaweed was cut into small pieces, suspended in 5 ml of 0.1 M sodium acetate buffer (pH 5.5) containing 100 mg/ml papain, 4 µ M EDTA and 5 µ M cysteine, and incubated at 60°C for 24 h. The incubation mixture was centrifuged (2000 x g for 15 min at 10°C), and the sulfated polysaccharides in the clear supernatant were precipitated with 1.6 ml of 10% tetraethylammonium chloride solution. After standing at room temperature for 24 h, the mixture was centrifuged (2000 x g for 15 min). The sulfated polysaccharides were dissolved with 15 ml of 1 N NaOH (100:13:v/v) solution and precipitated with 30 ml of 95% ethanol. After 24 h at 4°C, the precipitate formed was collected by centrifugation (1000 x g for 15 min), washed twice with 95% ethanol, and once with 5% ethanol. The final precipitate was dried at 60°C for 2 h, dissolved in 3 ml of distilled water and lyophilized.

Purification of the Fucos-Branch Cordrin Sulfate: The fucos-branch cordrin sulfate was purified using a DEAE-cellulose column, as previously described (7). The resulting partially methylated alditol acetates were dissolved in 2 ml of 0.1 M sodium acetate buffer (pH 5.5) containing 0.1 M NaCl. The aqueous phase of the column was 12 ml/h, and fractions of 3.0 ml were collected. They were assayed by the Dubois et al. reaction (11), by the carboxyl reaction (12) and by

Nehemchroomic acid using 1,1-dimethylmethylenediamine blue (14). The HL concentration in the fractions was estimated by conductivity. The fractions containing the fucos-branch cordrin sulfate, as indicated by a positive carboxyl test, were pooled, dialyzed against distilled water and lyophilized. About 100 mg of this sample was applied to a new DEAE-cellulose column and repurified as described above. The purity of this polysaccharide was checked by agarose gel electrophoresis, as previously described (7).

Chemical modifications of the polysaccharide

a) Mild hydrolysis with acid: This experiment was performed in order to remove the fucose branches from the sea cucumber polysaccharide. The fucos-branch cordrin sulfate (100 mg) in 10 ml of 0.001 N HCl was heated to 100°C for 30 min. After addition of 10 ml of ice-cold 300 m M NaCl to give a pH of 4.5, the solution was dialyzed against distilled water and lyophilized. About 65 mg of deacetylated cordrin sulfate was obtained.

b) Desulfation: Desulfation of the polysaccharide was performed as described previously (7). The fucos-branch cordrin sulfate (100 mg) in 10 ml of 0.001 N HCl was heated to 100°C for 30 min. After addition of 10 ml of ice-cold 300 m M NaCl to give a pH of 4.5, the solution was dialyzed against distilled water and lyophilized. About 30 mg of deacetylated cordrin sulfate was obtained.

c) Reductive deamination of the carbohydrate groups: Reduction of hexosamine acid carboxyl groups in the polysaccharide was performed as described by Taylor et al. (15). 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 HC1. Solid 1-ethyl-3-[3-dimethylaminopropyl]carbodiimid (75 mg) was added slowly with stirring, and the solution was maintained at 90°C for 2 h. After addition of several drops of acetic acid to destroy the excess borohydride, the solution was dialyzed against distilled water and lyophilized to give 20 mg of the carboxyl-reduced polymer. The extent of reduction of the carbohydrate groups was estimated by the decrease in the carbohydrate reaction (13) and the formation of glucose.

d) Methylation: The native, desulfated and deacetylated chordrin sulfate from sea cucumber, after reductive deamination, was methylated with the Nakamoto method (14), with the modifications introduced by Conrad (15). The molar ratio of sulfate:total sugar and by the infrared spectrum was estimated in the fractions estimated by the decrease in the carbohydrate reaction (13) and the formation of glucose.

Flux}\models
sea cucumber (20 µg as hexuronic acid) were incubated with 5 µM of chondroitin AC in 5 mM Tris-HCl buffer (pH 8.0) at 31°C for different times. Since the reaction proceeds as a B-elimination from the glucuronic acid residue, the extent of the reaction was monitored by the increase in absorbance at 232 nm. Assay 3: Standard chondroitin 4-sulfate, chemically demodified chondroitin, fucoidan from sea cucumber or chondroitin 4-sulfate, and native and chemically modified chondroitin sulfates from sea cucumber (100 µg of each) were incubated separately with 0.05 M ethylenediaminetetraacetic buffer (pH 8.0) at 31°C for 8 h. The reaction mixtures were spotted on Whatman No. 1 paper and chromatographed in isobutyric acid:0.1 M MgCl₂ (11:3, v/v) for 48 h. The products formed were visualized by silver nitrate staining and quantitated by densitometry using a Quick Scan densitometer (Helena Laboratories, Beaumont, TX, USA). The unsaturated disaccharides were also visualized by a short wave ultraviolet lamp.

**RESULTS AND DISCUSSION**

**Chemical modifications of the fucose-branched chondroitin sulfate from sea cucumber: Table 1 shows the chemical analyses of the fucose-branched chondroitin sulfates before and after the various chemical modifications. The fucose branches were removed from the polymer by mild hydrolysis with acid. Since fucose forms a glycosidic linkage that is more sensitive to acid than that of the glucuronic acid residue, hexosamine was analyzed by a modified Elson-Morgan reaction (22), and sulfate by the BaCl₂/gelatin method (19). Standard curves for hexosamine were constructed with galactosamine subjected to exactly the same hydrolytic conditions as the biological samples. The percentages of hexose, methylpentoses and galactosamine were constructed with gas-liquid chromatography of the corresponding alditol acetates (18) and by paper chromatography in butanol-pyridine-water (3:2:1, v/v) for 48 h.**

**Table 1:** Chemical composition, specific optical rotation, and average molecular mass of native and chemically modified chondroitin sulfate from sea cucumber.

| Polysaccharide | Method employed | Molar ratio | mean [a] (°) | Average molecular mass (kDa) |
|----------------|----------------|-------------|--------------|----------------------------|
| Native         |                  |             |              |                            |
| Deacetylated   | Microbial deácetation | 0.95          | -7.0±5.0     | 4.08                        |
| Deacetylated   | Microbial deácetation | 0.50          | 4.0±5.0      | 3.90                        |
| Deacetylated   | Microbial deácetation | 0.25          | 3.9±5.0      | 3.12                        |

**Analysis of the products formed by chondroitinase AC degradation of the native, deacetylated and deacetylated fucose-branched chondroitin sulfate from sea cucumber.** The analysis of the degradation products was performed by paper chromatography after digestion of the deacetylated chondroitin sulfate with AC. Six distinct products can be visualized on paper chromatograms after digestion of the deacetylated chondroitin sulfate with AC.**

**Figure 2** shows the time course of chondroitinase AC action on native and chemically modified samples of chondroitin sulfate from sea cucumber. The native polymer is totally resistant to chondroitinase AC whereas the deacetylated and deacetylated chondroitins are both partially degraded by the enzyme. Combined deacetylation and deacetylation produces a polymer that is almost totally degraded by chondroitinase AC. Similar results were obtained when the experiment of Fig. 2 was done with chondroitinase ABC (not shown).
Chondroitin AC action on the chemically modified chondroitin from sea cucumber. Equal amounts (35 μg) of disaccharides were compared with standard disaccharides of chondroitin sulfate (Fig. 3 and Table I). Since they are ascribed to non-reducing end units of chondroitin sulfate, the high yield of saturated disaccharides may reflect the low molecular weight of the chondroitin sulfate core in the sea cucumber polysaccharide (see also Table I). In addition, it is possible that the internal units are more resistant to chondroitin AC than the non-reducing ends, due to the presence of residual fucose branches or sulfate esters that survive the denaturation and disulfonation procedures.

In an earlier study, we did not detect disulfated disaccharide in the defucosylated chondroitin sulfate from sea cucumber. In that study, the isolation of defucosylated polysaccharide obtained by partial acid hydrolysis was neutralized with saturated aqueous barium bicarbonate. However, the barium salts of acidic poly saccharides may be insoluble and this characteristic could account for the low recovery of the defucosylated polysaccharide. In the present experiments, the barium neutralization procedure was omitted and a high yield of defucosylated chondroitin sulfate was obtained. With this new procedure for isolating defucosylated chondroitin sulfate, more disulfated disaccharides were also formed.

Table I: Distribution of products formed by the action of chondroitin AC on native and chemically modified chondroitin sulfate from sea cucumber.

| Product Formed | Native | Defucosylated | Desulfated | Defucosylated and Desulfated |
|----------------|--------|---------------|------------|-----------------------------|
| GlcUA-GalNAc   | 0.01   | 0.01          | 0.01       | 0.01                        |
| GlcUA-GalNAc-2S| 0.01   | 0.01          | 0.01       | 0.01                        |
| GlcUA-GalNAc-4S| 0.01   | 0.01          | 0.01       | 0.01                        |
| GlcUA-GalNAc-6S| 0.01   | 0.01          | 0.01       | 0.01                        |
| GlcUA-GalNAc-2S| 0.01   | 0.01          | 0.01       | 0.01                        |

The products formed by chondroitin AC action on disaccharide chondroitin sulfate were separated by paper chromatography and characterized by densitometry. The products of the polysaccharide reaction in the presence of barium salts were characterized by paper chromatography after neutralization by paper chromatography and comparing the barium salts equivalents at the origin with the total number of monosaccharides.

Table II: Gas chromatography/mass spectrometry analysis of methyl derivatives of native, defucosylated and desulfated chondroitin sulfate from sea cucumber after carboxyl reduction.

| Methylated sugars | tf | Molar ratio |
|-------------------|----|-------------|
| GlcUA-GalNAc-2S   | 1.00 | 0.00       |
| GlcUA-GalNAc-4S   | 1.00 | 0.00       |
| GlcUA-GalNAc-6S   | 1.00 | 0.00       |
| GlcUA-GalNAc-2S   | 1.00 | 0.00       |
| GlcUA-GalNAc-4S   | 1.00 | 0.00       |
| GlcUA-GalNAc-6S   | 1.00 | 0.00       |
| GlcUA-GalNAc-2S   | 1.00 | 0.00       |
| GlcUA-GalNAc-4S   | 1.00 | 0.00       |
| GlcUA-GalNAc-6S   | 1.00 | 0.00       |

1 The molar ratios are based on the area of each peak compared with total area of the fucose and glucose derivatives shown. Methyl ethers of the aldonic sugars are not included.

2 Retention time (tf) on SE-54 capillary column relative to 2,3,4,6-tetra-O-methyl fucose.

3 See also reference 2.

In order to investigate this possibility, the native, defucosylated and desulfated chondroitin sulfate from sea cucumber, after carboxyl reduction, were methylated by the Hakomori procedure (10,17). The formation of 2,3,4,6-tetra-O-methyl fucose from the native polysaccharide (Table II) indicates that...
the O-3 position of the glucuronic acid is substituted by fucose branches or sulfate esters. However, after either defucosylation or desulfation approximately equimolar proportions of 2,3,4-tri-Q-methyl and 2,4-di-Q-methyl-glucitol are formed, which suggests that approximately half of the O-3 position of the glucuronic acid is fucosylated while the other half is sulfated.

Concerning the fucose branches, the presence of equimolar proportions of fucose and glucuronic acid in the sea cucumber chondroitin sulfate (Table I), together with the formation of approximately equimolar proportions of 2,3,6-tri-Q-methyl and 2,6-di-Q-sulfated 0-3 position of the glucuronic acid is fucosylated while the other half is sulfated.

It is difficult to obtain conclusive evidence regarding the position of the glycosidic linkages and the site of sulfation in the fucose branches from the methylation experiment, especially since the formylated sulfate esters appear to be more resistant than other sulfated p-glucosides to hydrolysis in dimethyl sulfoxide. Evidence for the resistance of the fucose-linked sulfate ester to sulfonation in dimethyl sulfoxide was obtained during desulfation of fucan. Thus, total desulfation of chondroitin sulfate was obtained after heating its pyridinium salt at 80°C for 90 min (10,11), whereas fucan required more than 3 h. Nevertheless, the formation of 2- and 3-Q-methylated after methylation of the desulfated and carboxyl-reduced polysaccharide, indicates that disaccharide units composed of fucose must be linked to half of the glucuronic acid residues.

Concerning the fucose branches, the presence of equimolar proportions of 2.3.6-tri-Q-methyl and 2.6-di-Q-sulfated of the glucuronic acid residues. The proportions of 2.3.6-tri-Q-methyl and 2.6-di-Q-sulfated of the glucuronic acid residues after methylation of the desulfated and carboxyl-reduced polysaccharide, indicate that disaccharide units composed of fucose must be linked to half of the glucuronic acid residues.

Signals from fucose are almost completely absent in the 1H NMR spectrum of the defucosylated chondroitin sulfate from sea cucumber (Fig. 4C) and the spectrum resembles that of standard chondroitin 6-sulfate (Fig. 4D). These major signals were assigned by analogy with that of chondroitin 6-sulfate, and using a 2-D COSY spectrum (data not shown). The chemical shifts of the major 1H resonances of the defucosylated polysaccharide are shown, together with those of standard chondroitin 6-sulfate in Table IV. An extra, minor set of resonances for N-acetyl-galactosamine residue can also be seen. Several additional signals in this spectrum, which compared with that of standard chondroitin 6-sulfate reflect the heterogeneity of the chondroitin sulfate core, as already indicated by the chondritic acid experiments (Table II).

The two signals at δ 4.13 ppm, attributable to H-5 and H-4, respectively, of non-substituted glucuronic acid residues (Fig. 4D), are almost absent in the 1H NMR spectrum of native chondroitin from sea cucumber (Fig. 4A), but increases in intensity after defucosylation (Fig. 4C) or desulfation (Fig. 4B). This result agrees with the methylation experiment (Table III), and reinforces the suggestion that fucose branches and sulfate esters are substituted in the glucuronic acid units of the chondroitin from sea cucumber. Finally, the intense signal at δ 4.21 ppm in the 1H NMR spectrum of defucosylated chondroitin (Fig. 4C) and its absence in the spectrum of desulfated polysaccharide (Fig. 4A) confirms that most of the O-6 position of the chondroitin core is sulfated.

Concerning the fucose residues in the sea cucumber polysaccharide, the presence of signals in the vicinity of δ 5.00 - 5.70 ppm in the 1H NMR spectra of native (Fig. 4A) and desulfated (Fig. 4B) polymers are consistent with the anionic protons of -Fucopyranosyl residues (12-14). As already suggested by comparison between the optical rotation of the native and defucosylated polysaccharides (Table I), three sets of signals attributable to systems traced on the COSY spectrum from 5-5 of -Fucopyranosyl (Table V). The chemical shifts of these signals differ somewhat from those of fucose branches of blood group oligosaccharides (Table V). Figure 4 shows a defucosylated shift for H-2 of about 0.4 ppm, so it is possible to assign this signal tentatively to 2-glycosidically linked fucose residues. The fucose chemical shifts could be rationalized by hypothesizing that some of the fucose residues are single fucose residues. In this case there would be three types of fucose signals: i.e., 1-fucosylated (a and b) and 2-fucosylated (b and c). Since desulfation of the sea cucumber polysaccharide reduces the fucose content slightly (Table I), it is possible that the amount of single disaccharide side chains (c) in Fig. 4B and Table V is underestimated by the analysis of the desulfated polymer. The three fucose H-5/H-6 systems show δ 6.0 of 4.13 ppm (a) and about 4.1 ppm (b,c). The use of 1H chemical shifts of structural

![Figure 4: 1H NMR spectra (500 MHz) of the sea cucumber polysaccharide before (A) and after desulfation (B) or defucosylation (C) and standard (D) and desulfated (E) chondroitin 6-sulfate. The spectra were recorded in solutions of 09.5% D2O at 30°C. Signals designated by X refer to those produced by over-condensed glucomannan residue, whereas those of uronyl residues are labelled Y.](image)

![Table IV: 1H chemical shifts (δ ppm) for defucosylated chondroitin sulfate from sea cucumber and for standard chondroitin 6-sulfate](table)

| Proton | Defucosylated chondroitin sulfate from sea cucumber | Standard chondroitin 6-sulfate |
|--------|-----------------------------------------------------|-----------------------------|
| H-5    | 4.13                                                | 4.12                        |
| H-4    | 4.21                                                | 4.20                        |
| H-3    | 3.69                                                | 3.70                        |
| H-2    | 3.40                                                | 3.41                        |
| H-6    | 3.96                                                | 3.95                        |
| H-6    | 4.21                                                | 4.20                        |

1 Minor peaks are less than 10% of the major ones.
2 Data from Matsuura et al., 1979 (31).
3 Data from Kajitani and Tagami, 1980 (32).
4 n.d., not detected.

The fucose branches show considerable heterogeneity in the native polysaccharides (Fig. 5A). In different fucose branches, noticeably slightly different H-5/H-6 pairs are distinguishable in the COSY spectrum (data not shown). This heterogeneity may be due to variations in sulfation, and perhaps to the presence of some monoaccharide fucose sidechains in the native polymer.

![Figure 5: 1H NMR spectra of native, deacetylated, and defucosylated chondroitin sulfate (Fig. 5B) and standard (Fig. 5A) polysaccharides.](image)

![Table V: 1H chemical shifts (δ ppm) for defucosylated chondroitin sulfate from sea cucumber and for standard chondroitin 6-sulfate](table)
In addition, the $^{13}$C NMR spectrum of chondroitin sulfate from sea cucumber shows several signals in the vicinity of $\delta = 75 - 80$ ppm, which have chemical shifts similar to the $^{13}$C nuclei of standard chondroitin sulfate (Fig. 5b). Finally, the spectra of both compounds also show signals attributable to carbonyl ($\delta$ 178.25 ppm), acetamidomethyl ($\delta$ 25.55 ppm), and ring carbon-2 ($\delta$ 63.5 ppm) of the N-acetyl-D-glactosamine moiety, which resonate at approximately $\delta = 178.25$ and 55 ppm, respectively. However, the $^{13}$C spectrum of the chondroitin sulfate from sea cucumber (Fig. 5a) shows additional complexity attributable to anomeric carbons of 4-linked fucose, ring carbons of fucose and deoxy Gal, which resonate in the vicinity of $\delta = 102, 99, 71.7, 49.2$ and 38.8 ppm, respectively. The detection of a signal at $\delta = 0$ ppm in the $^{13}$C spectrum of the native polysaccharide (Fig. 5a), which is attributed to non-substituted carbon 4 (see spectra of Figs. 5a and 5b), agrees with the chondroitinase experiments (Table II) in showing the partial desulfation of the chondroitin sulfate core in the sea cucumber polysaccharide. In fact, this signal increase sharply after desulfation of the polysaccharide (Fig. 5b). The presence of several signals in the anomeric region of 4-linked fucose in the $^{13}$C spectrum of this desulfated polysaccharide (as well as in the spectrum of the native polysaccharide) confirms the heterogeneity of the fucose residues, as already expected from the $^1$H NMR studies (Fig. 4). 

Finally, the small decrease in the deoxy Gal $\delta$ 55 signal observed after desulfation agrees with the chemical analysis (Table I) showing a small decrease in the fucose residues after desulfation of the polysaccharide. Deacetylated chondroitin sulfate from sea cucumber shows a $^{13}$C NMR spectrum (Fig. 5c) which closely resembles that of standard chondroitin sulfate (Fig. 5b), and the signals attributable to fucose residues almost disappear from the spectrum. However, several other signals are observed (some of them are indicated by the arrows in Fig. 5c), whose chemical shifts do not coincide completely with the chemical shifts of the standard (Fig. 5b) or deacetylated (Fig. 5c) chondroitin sulfate. This observation suggests additional complexity in the structure of the chondroitin sulfate core from the sea cucumber polysaccharide, as already indicated by the chondroitinase (Fig. 2 and Table I) and methylation experiments (Table III).

Immunoreaction of the fucose-branched chondroitin sulfate with monoclonal antibody anti-Le$^a$-7 (MEM-1). The occurrence of 3-0-sulfate R-$\beta$-glucuronosyl residues in the chondroitin sulfate from sea cucumber was supported by the chondroitinas (Figs. 2 and Table II) and methylation (Table III) experiments. However, it could not be confirmed by the $^1$H and $^{13}$C NMR studies, possibly due to the high heterogeneity of this polysaccharide. In order to obtain further evidence for this type of unit, we studied the immunoreaction of the sea cucumber proteoglycans with monoclonal antibody anti-Le$^a$-7, which specifically recognizes 3-sulfocateuronic acid residues (38-41). Fig. 6 shows the correspondence between the antibody-stained band in immunoblot and the fraction of fucose-branched chondroitin sulfate, stained by toluidine blue. This result confirms the presence of 3-sulfocateuronic acid epitope in the sea cucumber polysaccharide.

In addition, the $^{13}$C NMR spectrum of polysaccharide from sea cucumber (Fig. 5a) shows several signals in the vicinity of $\delta = 75 - 80$ ppm, which have chemical shifts similar to the $^{13}$C nuclei of standard chondroitin sulfate (Fig. 5b). Finally, the spectra of both compounds also show signals attributable to carbonyl ($\delta$ 178.25 ppm), acetamidomethyl ($\delta$ 25.55 ppm), and ring carbon-2 ($\delta$ 63.5 ppm) of the N-acetyl-D-glactosamine moiety, which resonate at approximately $\delta = 178.25$ and 55 ppm, respectively. However, the $^{13}$C spectrum of the chondroitin sulfate from sea cucumber (Fig. 5a) shows additional complexity attributable to anomeric carbons of 4-linked fucose, ring carbons of fucose and deoxy Gal, which resonate in the vicinity of $\delta = 102, 99, 71.7, 49.2$ and 38.8 ppm, respectively. The detection of a signal at $\delta = 0$ ppm in the $^{13}$C spectrum of the native polysaccharide (Fig. 5a), which is attributed to non-substituted carbon 4 (see spectra of Figs. 5a and 5b), agrees with the chondroitinase experiments (Table II) in showing the partial desulfation of the chondroitin sulfate core in the sea cucumber polysaccharide. In fact, this signal increase sharply after desulfation of the polysaccharide (Fig. 5b). The presence of several signals in the anomeric region of 4-linked fucose in the $^{13}$C spectrum of this desulfated polysaccharide (as well as in the spectrum of the native polysaccharide) confirms the heterogeneity of the fucose residues, as already expected from the $^1$H NMR studies (Fig. 4). Finally, the small decrease in the deoxy Gal $\delta$ 55 signal observed after desulfation agrees with the chemical analysis (Table I) showing a small decrease in the fucose residues after desulfation of the polysaccharide. Deacetylated chondroitin sulfate from sea cucumber shows a $^{13}$C NMR spectrum (Fig. 5c) which closely resembles that of standard chondroitin sulfate (Fig. 5b), and the signals attributable to fucose residues almost disappear from the spectrum. However, several other signals are observed (some of them are indicated by the arrows in Fig. 5c), whose chemical shifts do not coincide completely with the chemical shifts of the standard (Fig. 5b) or deacetylated (Fig. 5c) chondroitin sulfate. This observation suggests additional complexity in the structure of the chondroitin sulfate core from the sea cucumber polysaccharide, as already indicated by the chondroitinase (Fig. 2 and Table II) and methylation experiments (Table III).