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# A sulfated α-L-fucan from sea cucumber

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## ABSTRACT

A purified sulfated α-L-fucan from the sea cucumber body wall was studied, before and after almost complete desulfation, using methylation analysis and NMR spectroscopy. NMR analysis indicates that 2,4-di-*O*-sulfo-L-fucopyranose and unsubstituted fucopyranose are present in equal proportions, and that 2-*O*-sulfo-L-fucopyranose is present in twice that proportion. There is some NMR evidence that a regular repeating sequence of four residues comprises most or all of the polysaccharide chain.

## INTRODUCTION

Sulfated polysaccharides are abundant[1–3] in the body wall of the sea cucumber (Echinodermata, Holothuroidea). Three fractions were isolated that differ markedly in molecular mass and chemical composition. The main fraction has a chondroitin sulfate-like structure (**1**), containing unexpectedly large numbers of α-L-fucopyranosyl branches linked to 0–3 of the β-D-glucuronic acid[2]. Besides the fucose branches, the sea cucumber chondroitin sulfate contains sulfate esters at 0–3 of the uronic acid units[3]. Our results suggest high heterogeneity of the glycosidic linkage and the sites of sulfation in the fucosyl branches. We suggested a preponderance of disaccharide units formed by 3,4-di-*O*-sulfo-α-L-fucopyranosyl units glycosidically linked through position 1 → 2 to 4-*O*-sulfo-α-L-fucopyranose. Another fraction, which represents a small portion of the sulfated polysaccharides, contains a heterogeneous polymer of high molecular mass, composed of fucose, galactose, and amino sugars. Finally, the third fraction is primarily a sulfated fucan[2,3].

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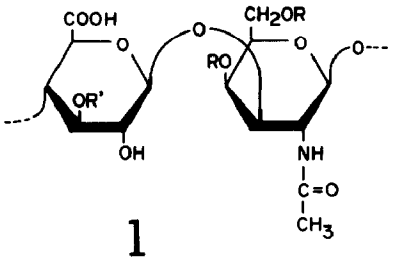


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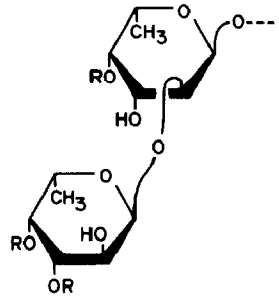
R = H or $SO_3^-$

R' = H or $SO_3^-$ or




Sulfated fucans have already been found in other organisms, namely the cell walls of marine brown algae[4–6] and the jelly coat from sea urchin eggs[7–9]. The structure of the polysaccharide from sea urchin has not been investigated in detail, whereas the sulfated fucan from brown seaweed was reported to be composed mainly of 4-sulfated and 2-linked α-L-fucopyranosyl units[4,5]. Studies on the sulfated fucan from other species of brown algae are consistent with a polysaccharide composed mainly of 3-linked and 4-sulfated fucose units[6].

Biological activities of the sulfated fucans from brown algae and sea urchin have been reported. For example, the polysaccharide from seaweed exhibits blood anticoagulant properties[10–13]. However, a possible relationship between physical properties and chemical structure of these sulfated fucans with their anticoagulant activity remains to be established[10–13]. The sulfated fucan from sea urchins induces the acrosome reaction upon contact of spermatozoa with the jelly layer of the egg[9]. This reaction involves exocytosis from an apical vesicle, called the acrosome granule, and protrusion of the vesicular membrane at the end of a rapidly polymerized actin[9]. Species-specific induction of the acrosome reaction resides solely in the sulfated fucan, suggesting that there must be structural differences in this polysaccharide in the various species of sea urchins[7].

Structurally similar compounds, the sulfated fucoidans, have been shown to inhibit the binding of human sperm to human zona pellucida[14]. Fucoidans also have other interesting properties, for example as anti-angiogenic agents[15] and as inhibitors of retroviral infection of T-cells[16].

The biological activities of the sulfated fucan from sea cucumber have not yet been examined. Its extracellular location and its high tissue concentration, which

resembles the amounts of glycosaminoglycans found in vertebrate connective tissues[17], suggest that this sulfated fucan is necessary for maintaining the structural integrity of the sea cucumber body wall.

In the present work we report a detailed examination of the sulfated fucan from the sea cucumber body wall. We found a unique structure, which differs substantially from that proposed for other fucose-rich polymers found in brown seaweed[4–6].

## EXPERIMENTAL

*Extraction of the sulfated polysaccharides from the body wall of the sea cucumber.*—The body wall of the sea cucumber *Ludwigothurea grisea* was carefully separated from other tissues, immersed immediately in acetone, and kept for 24 h at 4°C. The sulfated polysaccharides were extracted from the dried tissue (25 g) by papain digestion, and partially purified by cetylpyridinium chloride and ethanol precipitation, as previously described[3]. About 2.5 g (dry weight) of crude extract was obtained after these procedures.

*Purification of the sulfated fucan.*—(*a*) *DEAE-cellulose column.* The crude polysaccharides (200 mg) were applied to a DEAE-cellulose column (9 × 2 cm) equilibrated with 50 mM sodium acetate buffer (pH 5.0) and washed with 100 mL of the same buffer containing 0.8 M NaCl and 10 mM EDTA. Thereafter, the column was eluted by a linear gradient prepared by mixing 120 mL of 50 mM sodium acetate buffer (pH 5.0) containing 0.8 M NaCl and 10 mM EDTA with 120 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 phenol–$H_2SO_4$ (ref 18) and carbazole[19] reactions, and by metachromatic assay using 1,9-dimethyl Methylene Blue[20]. The NaCl concentration was estimated by conductivity. The fractions containing the sulfated polysaccharides, as indicated by the hexose and the metachromatic-positive tests, were pooled, dialyzed against distilled water, and lyophilized.

(*b*) *Sephacryl S-400 chromatography.* The DEAE-cellulose-purified polysaccharides (40 mg) were applied to a Sephacryl S–400 column (90 × 1.5 cm) and eluted with 50 mM sodium acetate buffer (pH 5.0), containing 8 M urea, 0.5 M NaCl, and 10 mM EDTA at a flow rate of 8 mL/h. Fractions of 1.5 mL were collected and assayed by the reaction of Dubois et al.[18] and by their metachromasia[20]. The column was calibrated using blue dextran and Cresol Red as markers for $V_0$ and $V_t$, respectively. In addition, chondroitin 6-sulfate from shark cartilage (60 kDa), chondroitin 4-sulfate from whale cartilage (40 kDa), and dextran sulfate (500 kDa) were used as molecular weight markers.

*Agarose gel electrophoresis.*—Sulfated polysaccharides were analysed by agarose gel electrophoresis, as previously described[2,21]. Sulfated glycans (~ 15 μg) were applied to a 0.5% agarose gel in 0.05 M 1,3-diaminopropane–acetate (pH 9.0). After electrophoresis, the glycans were fixed with aqueous 0.1% cetyltrimethylam-

monium bromide and stained with 0.1% Toluidine Blue in 0.1:5:5 acetic acid–EtOH–water.

*Chemical analyses.*—Total hexose was measured by the method of Dubois et al.[18], hexuronic acid by the carbazole reaction[19], and total "methylpentose" (6-deoxyhexose) by the method of Dische and Shettles[22]. After acid hydrolysis of the polysaccharides (4.0 M HCl at 100°C for 6 h), total hexosamine was measured by a modified Elson–Morgan reaction[23], and sulfate by the $BaCl_2$–gelatin method[24]. The presence of contaminant free sulfate in the polysaccharide solution was excluded by the negative $BaCl_2$–gelatin reaction previous to the acid hydrolysis of the polysaccharide. A recently prepared $BaCl_2$–gelatin solution was always used to assure a precise sulfate estimation. The percentages of hexoses, 6-deoxyhexoses, and hexosamines in the acid hydrolysates were estimated by paper chromatography in 3:2:1 *n*-butanol–pyridine–water for 48 h and by gas–liquid chromatography of derived alditol acetates[25]. Optical rotations were measured with a digital polarimeter (Perkin–Elmer model 243-B) and infrared spectra recorded with a Perkin–Elmer infrared spectrometer model 298.

*Oxidation with* L-*fucose dehydrogenase.*—Fucose obtained by acid hydrolysis of the sea cucumber polysaccharides, and authentic samples of D- or L-fucose, were incubated with 0.2 units of porcine liver L-fucose dehydrogenase[26] and 2.5 μmol of $NAD^+$ in 2 mL of 0.01 M glycine–NaOH buffer (pH 8.0). The mixtures were incubated at 30°C for different times and the formation of NADH was followed by measuring the absorbance at 340 nm.

*Desulfation.*—Desulfation of the sulfated fucan was performed by solvolysis in $Me_2SO$ (ref 27), as described previously for desulfation of other polysaccharides from sea cucumber[2,3]. Different times of desulfation were tested in order to minimise molecular mass reduction or loss of material. The best results were obtained using 6 h at 80°C. Loss of sulfate was monitored by disappearance of the S=O band at 1240 $cm^{-1}$ in the infrared spectra. In addition, desulfation was estimated from the molar ratio of sulfate:fucose.

*Methylation analysis.*—The native and desulfated fucans were methylated as follows[28,29]. To a solution of the dry polysaccharides (4 mg) in $Me_2SO$ (1.28 mL) were added powdered NaOH (102 mg) and methyl iodide (90 μL). The mixture was stirred for 60 min at room temperature, cooled, mixed with 1 M acetic acid (3.3 mL) and distilled water (1.28 mL), dialysed against distilled water, and lyophilized. This procedure was repeated three times. Sulfate groups are not removed during the methylation reaction. This is supported by the fact that comparison of the infrared spectra of the original and methylated polysaccharide showed no apparent loss in the band corresponding to sulfate at 1240 $cm^{-1}$.

Each methylated polysaccharide was hydrolysed with 4 M $CF_3CO_2H$ for 5 h at 100°C, and the products reduced with $NaBH_4$ and acetylated. The resulting alditol acetates were analysed by GLC as described above and by GLC–MS on an SE-54 capillary column (30 m × 0.3 mm i.d.). The column was programmed to run at 110°C for 5 min, then raised to 200° at 2°C/min and held for 5 min.

*NMR spectroscopy.*—$^1$H NMR spectra were recorded at 500 MHz and $^{13}$C NMR spectra at 125 MHz using a Varian Unity 500 spectrometer in the FT mode. The sulfated polysaccharide (~ 15 mg) was lyophilized three times from 99.8% $D_2O$ (Goss Scientific, Ingatestone, UK) and dissolved in 0.6 mL 100% $D_2O$ (Aldrich Chemical Co., Poole, UK) for NMR spectroscopy in a 5-mm i.d. NMR tube. The whole of the same sample, after solvolytic desulfation as described above, was exchanged and prepared for NMR spectroscopy in the same way. $^1$H NMR spectra of both polysaccharides were recorded at both 60 and 40°C using a spectral width of ~ 10 ppm; 16K data points were collected giving an acquisition time of 2.0 s, with a relaxation delay of 1.5 s. No solvent suppresion techniques were used. $^{13}$C NMR spectra were recorded at 60°C, with a spectral width of 220 ppm and 16K data points giving an aquisition time of 0.3 s. A short relaxation delay of 0.1 s was used to optimise the length of time needed to acquire the spectra of the polysaccharides. Signals due to low molecular weight impurities with long relaxation times were reduced in intensity by this rapid repetition rate. Two-dimensional spectra, double-quantum-filtered COSY[30] and NOESY[31] spectra were recorded in the phase-sensitive mode using the pulse programs provided by the spectrometer manufacturer, with a spectral width of ~ 5 ppm for the double-quantum-filtered COSY spectrum at 60°C, 256 × 32 transient spectra of 2K data points were accumulated; for the NOESY, at 40°C with a mixing time of 100 ms, 512 × 16 transient spectra of 2K data points were accumulated. The spectra were transformed after zero-filling to 1024 points in the sulfated fucan, with shifted sine-bell weighting applied in both dimensions. All the spectra were referenced to external 3-(trimethylsilyl)propionic acid 2,2,3,3-$d_4$ sodium salt.

## RESULTS AND DISCUSSION

*Fractionation of the sulfated fucan.*—Anion exchange chromatography on a DEAE-cellulose column separated the sulfated polysaccharides from sea cucumber body wall into two peaks (Fig 1A). The major one was eluted at the beginning of the salt gradient and contained fucose and hexuronic acid. Its electrophoretic mobility on agarose gel (Fig 1B) and chemical composition (Table I) indicate that it corresponds to fucose-branched chondroitin sulfate which was characterised in our previous studies[2,3]. The other peak, containing fucose but lacking hexuronic acid, was eluted at high NaCl concentration. It corresponds to a mixture of the sulfated fucan and high-molecular-mass sulfated polysaccharide. The latter can be identified as a left shoulder not totally separated from the sulfated fucan (Fig. 1B). Total separation of these two polysaccharides was achieved by gel filtration chromatography on Sephacryl S-400 (Fig. 2A). The sulfated heterogeneous polysaccharide was eluted near the void volume, whereas the sulfated fucan was eluted with a $K_{av}$ of ~ 0.5. These two polysaccharides can be easily distinguished by their elec-

trophoretic mobilities on agarose gel (Fig. 2B) and their chemical composition (Table I) *.

The strongly negative specific rotation of the sulfated fucan (Table I) is compatible with residues of α-L-fucopyranose. This structure is supported by the finding that the fucose obtained by acid hydrolysis of the sulfated fucan was exclusively of the L-configuration, as shown by oxidation with L-fucose dehydrogenase.

*Structural analysis.*—(*a*) *Methylation studies.* Methylation of sulfated polysaccharides does not always yield reliable proportions of methylated alditols[32–34], but it may offer valuable information concerning the position of the glycosidic linkage and the site of sulfation. Methylation of the sulfated fucan from sea cucumber (Table II) can be rationalized as follows. The native polymer yielded mainly unmethylated fucitol. After partial desulfation of the fucan, the proportion of unmethylated fucitol decreased sharply, whereas 2,4-di-*O*-methyl and 2-*O*-methyl fucitol became the predominant *O*-methyl fragments (Table II). Thus, some fucosyl residues are unequivocally 2,4-disulfated and 3-linked, structures which yield unmethylated and 2,4-di-*O*-methyl derivatives from the native and desulfated polysaccharides, respectively. The formation of 2-*O*-methyl fucitol after methylation of the partially desulfated fucan may be ascribed to incomplete removal of sulfate esters substituted at O-4. In the same way, the unmethylated fucitol can be ascribed to incomplete removal of sulfate esters at both O-2 and O-4 positions. The formation of 2-*O*-methyl fucitol after methylation of the native polymer may be ascribed either to incomplete reaction or to a small desulfation of the polysaccharide during the methylation. Infrared spectroscopy, which was used to follow the polysaccharide during the methylation reaction, does not exclude removal of small amounts of sulfate esters from the polymer. The relative proportions of the methylated derivatives from fucose were not modified after an additional methylation. Methylation of the sulfated and partially desulfated fucan was repeated with two additional samples after that of Table II and the proportions of methylated alditols were similar. Thus, the possibility that the unmethylated fucitol is merely a product of incomplete reaction is unlikely. Nevertheless, it is possible that the sulfated fucan cannot be methylated fully due to steric hindrance and the degree of sulfation determined by methylation analysis may be overestimated.

(*b*) *$^1$H NMR spectra.* The $^1$H NMR spectra at 40°C of the polysaccharides are shown in Fig. 3. At 60°C some of the resonances were obscured by that of HOD. The spectra were assigned with the help of 2D spectroscopy. Starting from four H-1 doublets at 5.0–5.4 ppm, the DQCOSY spectrum (Fig. 4) shows H-1–H-2

* Since the heterogeneous sulfated polysaccharide and the sulfated fucan are not well separated on DEAE-cellulose column (Fig. 1A) the proportions of these two polysaccharides that contaminate each other on the fractions from DEAE-column vary according to the experiment. The preparation of partially purified sulfated fucan in Fig. 2B (lane 1) contains higher proportion of contaminant heterogeneous sulfated polysaccharide than the preparation shown in lane 4, Fig. 1B.

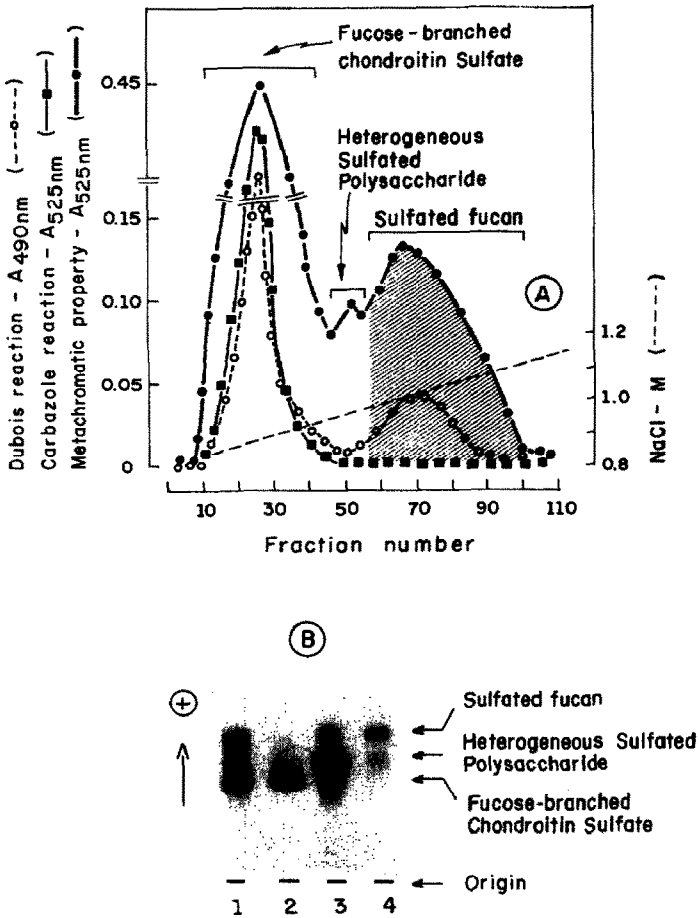



Fig. 1. Fractionation of the sulfated polysaccharides from sea cucumber body wall on DEAE-cellulose. (A) The crude polysaccharides were purified on a DEAE-cellulose column as described in the Experimental section. The fractions were checked by the phenol–$H_2SO_4$ (Dubois) (– – o – –) and carbazole (—■—) reactions, for metachromasia (—●—) and for NaCl concentration (– – –). The fractions indicated by horizontal bars were pooled, dialysed against distilled water, and lyophilized. The fractions corresponding to the sulfated fucan are cross-hatched. (B) Crude sulfated polysaccharides from sea cucumber body wall (lane 1), and sulfated polysaccharides purified on the DEAE-cellulose column, namely the fucose-branched chondroitin sulfate (lane 2), the partially purified heterogeneous sulfated polysaccharide (lane 3), and the partially purified sulfated fucan (lane 4) (25 $\mu$g of each) were analysed by agarose gel electrophoresis as described in the Experimental section. The electrophoretic mobilities of the various sulfated polysaccharides are indicated by arrows at the right side of B. See also ref. 2.

cross-peaks of four fucose spin-systems A, B, C and D, with H-3–H-4 cross-peaks for systems B and C. Starting from four methyl H-6 doublets at 1.21–1.25 ppm, four clear H-6–H-5 cross-peaks are seen in the same DQCOSY spectrum. No H-4–H-5 cross-peaks can be seen, as expected since $^3J_{H4,H5}$ is typically very small ($\sim$ 1 Hz). The assignment was completed using a NOESY spectrum (Fig. 5), in which cross-peaks connect protons nearby in space; in this case cross-peaks are

TABLE I

Chemical composition, specific optical rotation and average molecular mass of the sulfated polysaccharides from sea cucumber body wall

| Polysaccharide [a] | Composition (molar ratios) | | | | | | $[\alpha]_D^{20}$ (°) | Average molecular mass [b] (kDa) |
|---|---|---|---|---|---|---|---|---|
| | Fuc | GlcUA | GalNH | GlcNH | Gal | sulfate/total sugar | | |
| Fucose-branched chondroitin sulfate [c] | 0.32 [d] | 0.30 | 0.33 | n.d. [e] | n.d. | 0.88 | −30 | 40 |
| High-molecular-mass sulfated polysaccharide | 0.68 | n.d. | 0.04 | 0.11 | 0.17 | 0.55 | n.d. | >100 |
| Sulfated fucan | 1.00 [d] | n.d. | n.d. | n.d. | n.d. | 1.10 | −76 | 30 |

[a] See Figs. 1A and 2A for the purification of these polysaccharides. [b] Average molecular mass compared with glycosaminoglycans standards. [c] See also refs 2 and 3. [d] Fucose occurs entirely in the L-enantiomeric form since this sugar is totally oxidized by L-fucose dehydrogenase. [e] n.d., Not detected. The detection threshold of the methods used is ~0.01 mg per mg of polysaccharide.

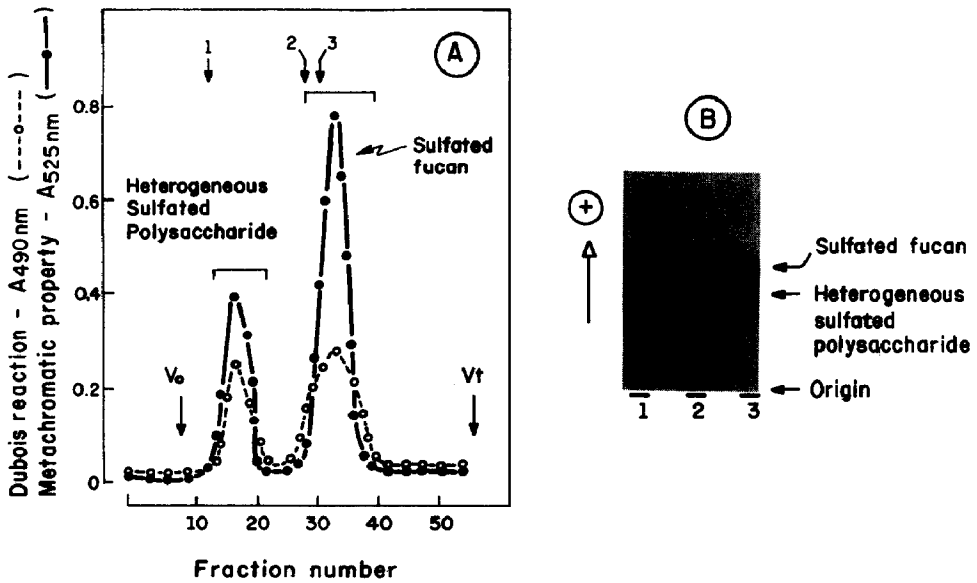



Fig. 2. Purification of the sulfated fucan on Sephacryl S–400. (A) The sulfated fucan, partially purified on a DEAE-cellulose column (see Fig. 1), was chromatographed on a Sephacryl S–400 column. Fractions of 1.5 mL were collected and assayed by the phenol–$H_2SO_4$ method (Dubois) (– – o – –) and for metachromasia (—●—). The fractions indicated by horizontal bars were pooled, dialysed against distilled water and lyophilized. The arrows indicate the elution of standard dextran sulfate (lane 1, 500 kDa), chondroitin 6-sulfate from shark cartilage (lane 2, 60 kDa), and chondroitin 4-sulfate from whale cartilage (lane 3, 40 kDa). (B) About 25 μg of the sulfated fucan partially purified on DEAE-cellulose (lane 1), the heterogeneous sulfated polysaccharides (lane 2) and the purified sulfated fucan (lane 3) obtained on the Sephacryl S–400 column, were subjected to agarose gel electrophoresis, as described in the legend of Fig. 1.

TABLE II

Methylated sugars obtained from native and partially desulfated fucan

| Alditol [a] | $t_R^b$ | % of total peak area | |
|---|---|---|---|
| | | Native [c] | Partially desulfated [c] |
| 2,4-$Me_2$-Fuc | 1.00 | 6 | 31 |
| 2-Me-Fuc | 2.44 | 6 | 21 |
| 4-Me-Fuc | 3.78 | 12 | 18 |
| Fuc | 5.75 | 73 | 28 |

[a] The identity of each peak was established by mass spectrometry. [b] Retention time on an SE-54 capillary column relative to that of 2,4-di-*O*-methylfucitol. [c] The sulfate:fucose molar ratios of the native and desulfated fucans were 1.10 and 0.30, respectively.

expected for the equatorial–axial proton pairs H-1–H-2, H-3–H-4 and H-4–H-5, and for H-3–H-5, but H-2–H-3 cross-peaks are not expected. NOESY cross-peaks are present linking H-4 of spin systems B and C with two of the H-5 resonances identified from the DQCOSY spectrum; the expected H-3C–H-5C cross-peak is also present, but as H-3B and H-5B are nearly coincident a separated H-3B–H-5B cross-peak is not seen. A similar pattern of H-4–H-5 and H-3–H-5 cross-peaks can be seen linking H-3D with another of the established H-5 resonances, establishing the chemical shift of H-4 (almost coincident with H-2D), and completing the assignment of system D. The remaining H-5 resonance is connected to H-4 at 4.91 ppm; by elimination this is assigned to spin system A. As H-5A and H-3A are almost coincident, the H-3A–H-4A cross-peak overlaps the H-4A–H-5A cross-peak. The chemical shifts and approximate coupling constants for the four types of fucose residue giving rise to spin systems A, B, C, and D are listed in Table III.

Other cross-peaks and features in the spectra are assigned to minor components and impurities in the sample.

Integration of regions of the $^1$H NMR spectrum indicated that the four types of residues are present in equal proportions. The values of the measured integrals are shown in Fig. 3 and show similar values for the four H-1 signals and for the H-4A signal. One H-2 signal is obscured by the HOD peak, and the integral over the region 4.15–3.9 ppm is about 15% larger than the calculated value, but, allowing for the broad lines in the spectrum, and the presence of minor components, measured and calculated integrals are in agreement.

The NOESY spectrum of the native fucan displays some interresidue NOE cross-peaks. H-1 of residue A shows a cross-peak to H-3 of residue D and to H-2/H-4D; H-1D shows cross-peaks to H-3C and H-4C; H-1C shows a cross-peak to H-3B, and H-1B to H-3A. This is evidence to support the presence of (1 → 3)-linkages thoughout the polysaccharide, as suggested by the methylation studies (Table II). NOEs from H-1 to H-3 and H-4 of the glycosylated residues are characteristic of (1 → 3)-linkages for galactose residues[35]; (1 → 4)-linkages would be expected to give H-1–H-6 NOEs; neither (1 → 4)- nor (1 → 2)-linkages would be expected to give rise to strong H-1–H-3 NOEs.

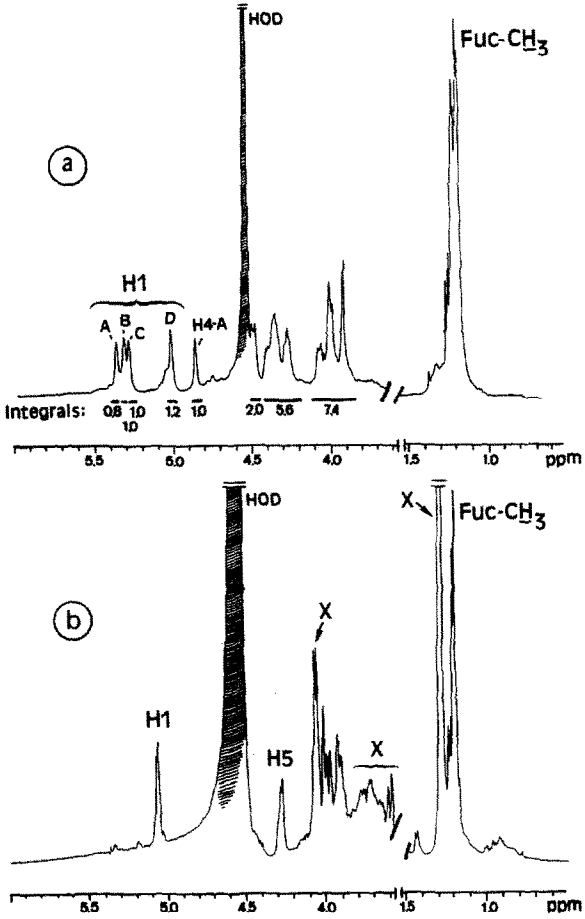



Fig. 3. $^1$H NMR spectra (500 MHz) of the sulfated fucan from sea cucumber before (a) and after (b) desulfation. The spectra were recorded for solutions in $D_2O$ at 40°C. The integrals listed under the anomeric and ring proton regions of the spectrum are normalized to a total of 20 protons. The HOD peak is cross-hatched. Peaks marked X are due to contaminants.

The $^1$H NMR spectrum of a desulfated sample of the sea cucumber fucan is shown in Fig. 3b. The sample was partially degraded during the desulfation procedure, and signals from contaminants are prominent in the spectrum (particularly at 1.25 ppm and ~4.05 ppm, consistent with the presence of isopropyl alcohol or possibly lactate). However, some features of the spectrum of the fucan itself are clear. Only one H-1 signal remains at 5.08 ppm. A well-resolved multiplet at 4.27 ppm, attributable to H-5 by analogy with the native fucan, is also seen, as is one major H-6 doublet ($J$ 6.6 Hz) at 1.21 ppm, and a minor H-6 doublet (~25% of the major doublet in intensity) at 1.23 ppm.

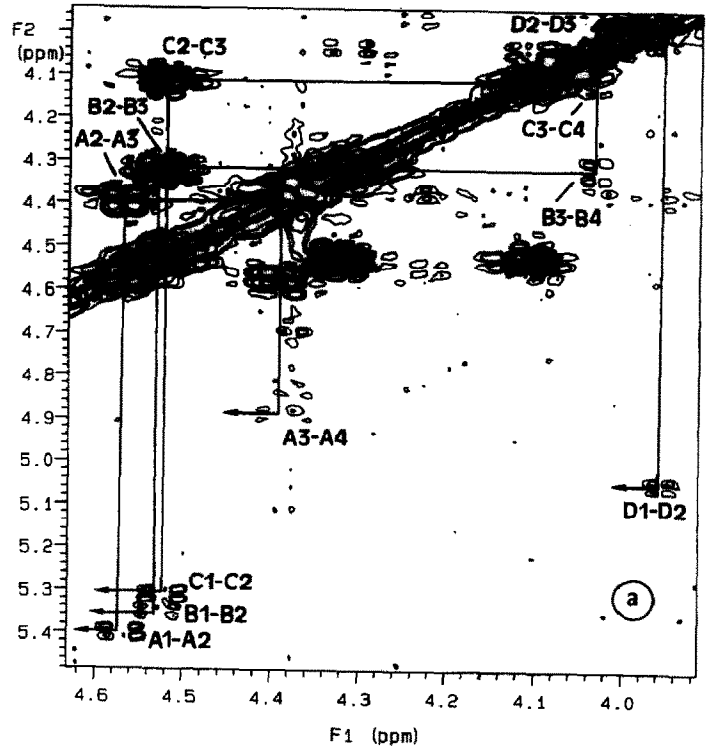
Measured shifts and coupling constants of multiplets at 3.92 and 4.00 ppm are consistent with assignment to H-2 and H-3 (taking the chemical shifts of residue D in the spectrum of the native fucan as a rough guide). These assignments are summarised in Table IIIb. Approximate coupling constants given in Tables IIIa and b are estimated from multiplet splittings in the 1D spectrum, and are accurate to $\sim \pm 0.5$ Hz.

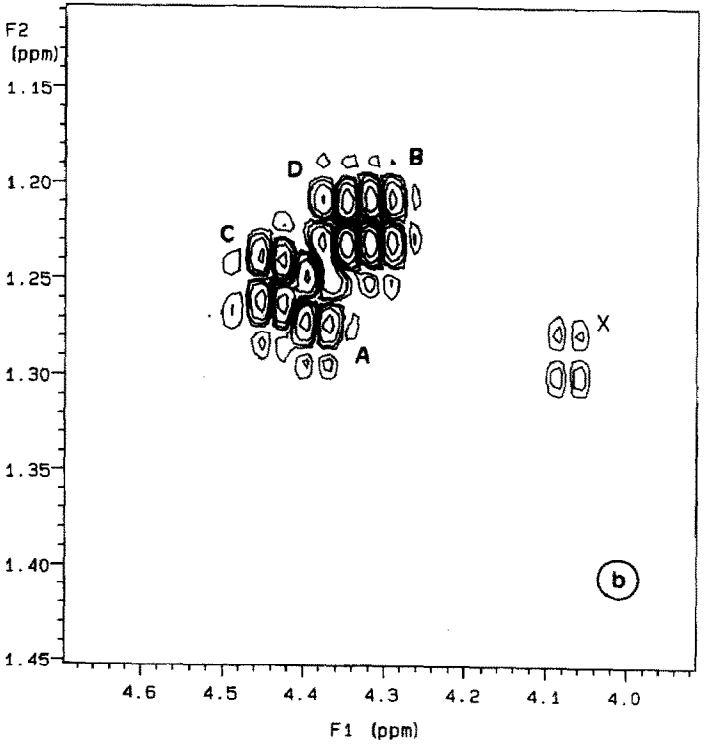
*(c) $^{13}C$ NMR spectra.*—The $^{13}C$ NMR spectrum of the native material (Fig. 6a) contains three signals in the anomeric region with shifts consistent with the $\alpha$ configuration of the fucose residues (100.7, 98.44, and 96.3 ppm); one of which (at 96.3 ppm) is about twice as intense as the other two and may result from the coincidence of two resonances. Ring carbon resonances fall into three groups: an isolated peak at 83.1 ppm, a group between 75 and 78 ppm, and a third group at 68–72 ppm. This third group can be assigned to unsulfated and unglycosylated carbons in the fucose ring, while the first and second groups arise from glycosylated and sulfated positions. Carbon chemical shifts for sulfated and unsulfated fucose residues have been assigned by Jain and Matta[36], who report downfield shifts of 6–9 ppm for 0-sulfated carbons. This interpretation is supported by the integrals over these areas of the spectrum shown in Fig. 6a.

Methyl C-6 resonances are present in the spectrum at 17.9–18.3 ppm, at reduced intensity due to the rapid repetition rate used in accumulation of the spectrum.

The $^{13}C$ NMR spectrum of the desulfated fucan shown in Fig. 6b has poor signal-to-noise due to the small amount of sample remaining after the desulfation procedure. However, signals from C-1 (98.16 ppm), C-5 (68.98 ppm), and C-3 (77.56 ppm) can be assigned by analogy with literature values[36], assuming a glycosylation shift of 5–6 ppm for the C-3 signal. The relatively intense peak at 71.06 ppm may arise from C-2 and C-4. The C-1 signal at 98.1 ppm is consistent with the $\alpha$ configuration. The C-6 signal at 17.87 ppm is reduced in intensity by the rapid repetition rate necessary for this scarce sample, as is the singlet at 22.67 ppm presumably arising from the contaminant giving an intense signal at 1.25 ppm in the $^1H$ NMR spectrum. Contributions to the spectrum from minor components are lost in the noise.

*(d) Interpretation of NMR spectra.*—The contributions to the $^1H$ NMR spectrum of the residues A, B, C, and D vary most significantly in their H-2 and H-4 shifts. Assuming a (1 → 3)-linkage throughout, these differences may be attributed to sulfation shifts. The value of $\delta_{H\text{-}4}$ for A, at 4.91 ppm, is shifted $\sim 0.9$ ppm downfield of $\delta_{H\text{-}4}$ for B, C and D, indicating that residue A is 4-*O*-sulfated. The value of $\delta_{H\text{-}2}$ of residue D is $\sim 0.6$ ppm upfield of $\delta_{H\text{-}2}$ for A, B, and C, indicating that only residue D is not 2-*O*-sulfated. So residue A bears two sulfate groups, at positions 4 and 2; residue D is unsulfated, and B and C are both 2-*O*-sulfated residues in different environments. This interpretation explains the downfield shifts of H-1 and H-3A, H-1 and H-3B, and H-1 and H-3C with respect to their shifts in residue D as relating to sulfation at position 2 of those residues. The $^1H$


F2 (ppm)
C2-C3
D2-D3
B2-B3
A2-A3
C3-C4
B3-B4
A3-A4
D1-D2
C1-C2
B1-B2
A1-A2
a
F1 (ppm)


F2 (ppm)
D
B
C
A
X
b
F1 (ppm)

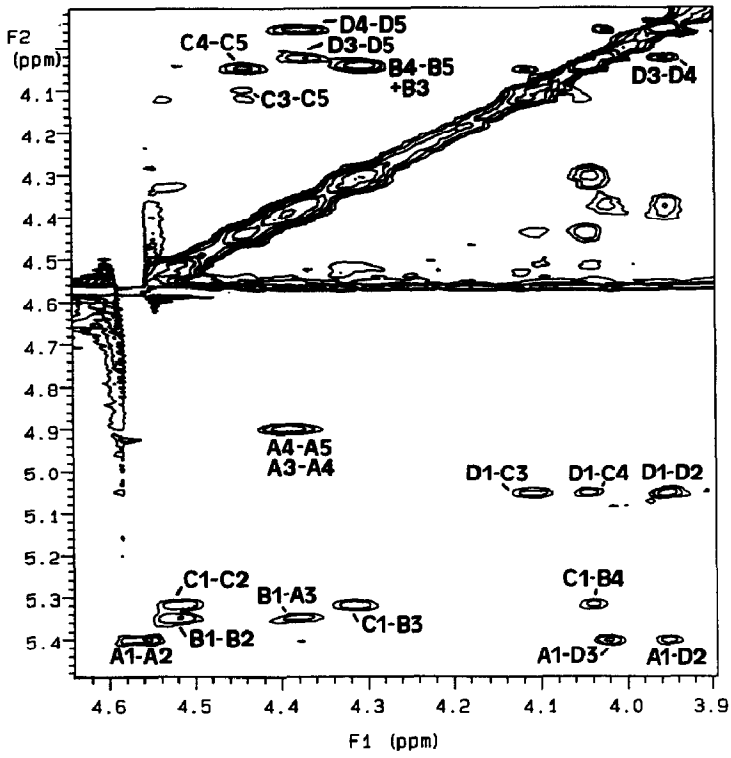
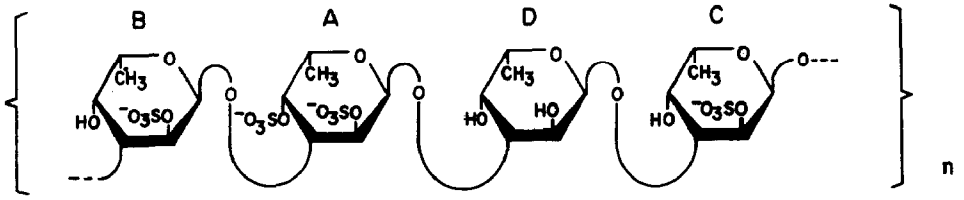



Fig. 5. Expansion of the phase-sensitive NOESY spectrum of the native fucan from sea cucumber, recorded at 500 MHz and 40°C as described in the Experimental section. Only positive contours are shown. Both intra- and inter-residue NOE cross-peaks are labelled.




2

Fig. 4. Double-quantum filtered phase-sensitive COSY spectrum of the native fucan from sea cucumber recorded at 500 MHz and 60°C as described in the Experimental section. Both positive and negative contours are shown. (A) Expansion of the spectrum over the anomeric and ring proton region of the spectrum showing cross-peaks used to assign the four spin-systems corresponding to four types of fucose residue. (B) Expansion showing H-5–H-6 peaks for the four spin systems as labelled. The cross-peak marked X arises from a contaminant.

TABLE III

$^1$H NMR chemical shifts and approximate $^3J_{H,H}$ values for the native fucan from sea cucumber and its desulfated derivative

| (a) | Native fucan | | | | | |
|---|---|---|---|---|---|---|
| | Chemical shifts (ppm) [a] | | | | | $^3J_{H,H}$ (Hz) [b] |
| | A [c] | B | C | D | | |
| H-1 | 5.40 | 5.35 | 5.32 | 5.03 | H-1,H-2 | 4.0 |
| H-2 | 4.58 | 4.53 | 4.53 | 3.96 | H-2,H-3 | 10.0 |
| H-3 | 4.39 | 4.31 | 4.11 | 4.01 | H-3,H-4 | n.d. [d] |
| H-4 | 4.91 | 4.03 | 4.05 | 3.96 | H-4,H-5 | n.d. |
| H-5 | 4.37 | 4.30 | 4.46 | 4.35 | H-5,H-6 | 7.0 |
| H-6 | 1.25 | 1.21 | 1.24 | 1.21 | | |

| (b) | Desulfated fucan | | |
|---|---|---|---|
| | Chemical shifts (ppm) [a] | | $^3J_{H,H}$ (Hz) [b] |
| H-1 | 5.08 | H-1,H-2 | 4.0 |
| H-2 | 3.92 | H-2,H-3 | 10.0 |
| H-3 | 4.00 | H-3,H-4 | n.d. |
| H-4 | n.d. | H-4,H-5 | n.d. |
| H-5 | 4.27 | H-5,H-6 | 6.3 |
| H-6 | 1.21 | | |

[a] Relative to 3-(trimethylsilyl)propionic acid 2,2,3,3-$d_4$ sodium salt ($\delta = 0$), at 40 or 60°C; no temperature induced shifts were observed. [b] Measured from multiplet splitting in the resolution-enhanced 1D spectrum; ±0.5 Hz. [c] A–D: spin systems; see structure **2**, labels in Fig. 3 and explanation in text. [d] n.d., Not determined.

NMR spectrum of residue D is similar to that of fucose in other biological oligosaccharides (see for example Rao et al.[37]).

The fact that the anomeric protons of each residue show interresidue NOE connections to one and only one of the other residues, in a neatly circular fashion, may well indicate that the polysaccharide has a linear repeating unit of four fucose residues, all (1 → 3)-linked, but systematically differing in their pattern of sulfation at the 2- and 4-positions. The ratios of the integrals of the four anomeric signals in the $^1$H NMR spectrum is near 1 : 1 : 1 : 1 consistent with this interpretation.

$^{13}$C NMR spectra of both native and the desulfated polysaccharides are consistent with the above interpretation.

## CONCLUSIONS

The present study concerns the structure of another sulfated polysaccharide from the connective tissue of the sea cucumber. It is essentially a linear polysaccharide, composed of 3-linked α-L-fucopyranosyl units, but with a highly heterogeneous sulfation pattern at the O-2 and O-2,4 positions (**2**). NMR analysis indicates that 2,4-di-*O*-sulfo-L-fucopyranose and unsubstituted fucopyranose are present in equal proportions, and that 2-*O*-sulfo-L-fucopyranose is present in twice that proportion. There is some NMR evidence that a regular repeating sequence of four residues comprises most or all of the polysaccharide chain.

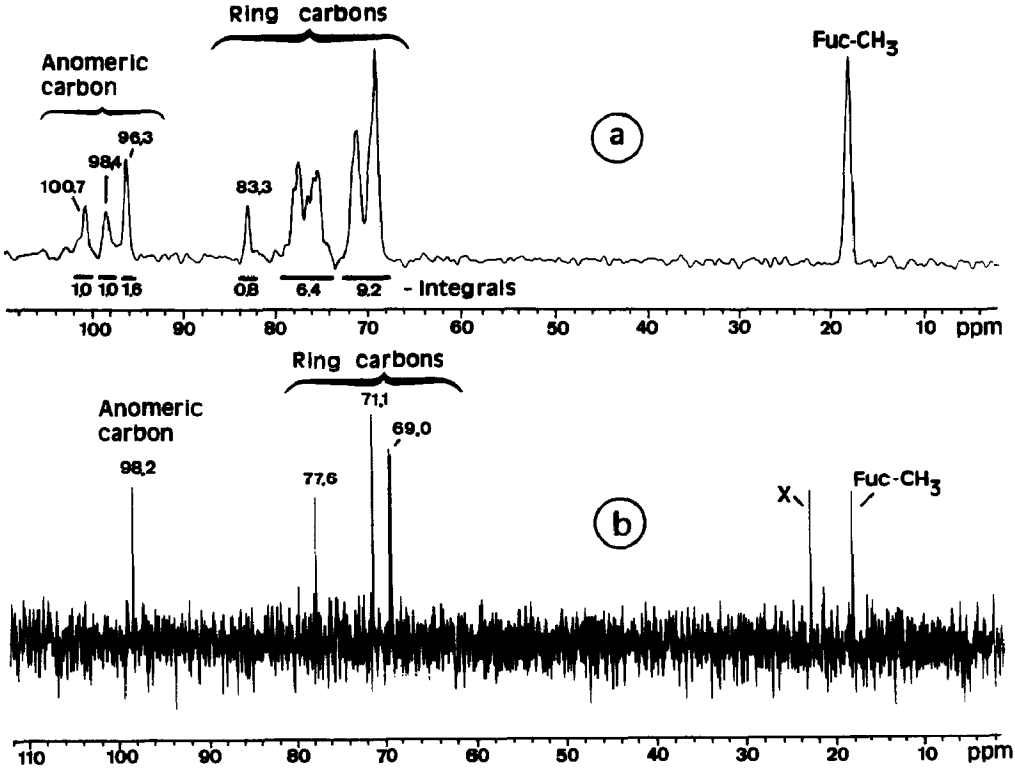



Fig. 6. $^{13}C$ NMR spectra (125 MHz) of the sulfated fucan from sea cucumber before (a) and after (b) desulfation. The spectra were recorded for solutions in $D_2O$ at 60°. The integrals listed under the anomeric and ring carbon regions of the spectrum are normalized to a total of 20 carbon atoms. The peak marked X is due to a contaminant.

The fucosyl branches in the fucosylated chondroitin sulfate (**1**) and the fucose residues in the sulfated fucan (**2**) show considerable differences in respect to glycosidic linkage and site of sulfation. These two structures, although found in the same tissue and composed of α-fucopyranosyl residues, must be synthesized by different pathways.

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