Sulfated Fucans from Echinoderms Have a Regular Tetrasaccharide Repeating Unit Defined by Specific Patterns of Sulfation at the 0-2 and 0-4 Positions*

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Sulfated fucans from echinoderms (sea cucumber and sea urchin) have a linear backbone of 1→3-linked α-L-fucopyranose with some sulfite substitution at the 2- and 4-positions. NMR spectroscopy indicates that both polysaccharides have a tetrasaccharide repeat unit in which the separate residues differ only in the extent and position of their sulfite substitution. The sea urchin fucan has the structure,

\[
[3\alpha-L-Fucp-2(\text{OSO}_3)-1\rightarrow3\alpha-L-Fucp-4(\text{OSO}_3)-1\rightarrow]_n
\]

and the sea cucumber has the structure,

\[
3\alpha-L-Fucp-2,4(\text{OSO}_3)-1\rightarrow3\alpha-L-Fucp-2(\text{OSO}_3)-1\rightarrow]_n
\]  

This type of regular structure has not previously been described, and is in contrast with the random arrangement of substituents on the similar 1→3-linked α-L-fucopyranose backbone of the fucoidans from brown algae.

Sulfated fucans are among the most widely studied of all the sulfated polysaccharides of non-mammalian origin that exhibit biological activities in mammalian systems. Such compounds have been isolated from the cell walls of marine brown algae (1–3), the jelly coat from sea urchin eggs (4–6), and the sea cucumber body wall (7, 8).

Most investigations of biological activity have involved the fucoidans from brown algae; a preparation of the fucan from Fucus vesiculosus is available commercially. These polysaccharides have antiangiogenic activity as measured in several different assays (9–13) and are potent activators of both antithrombin III and heparin cofactor II (13). The F. vesiculosus fucoidan demonstrates differential binding to interleukins 1α and 2, and 6 (14). Algal fucoidans are also inhibitors of retroviral infection (15), blocking the infection of human cell lines with, for example, human immunodeficiency virus, herpes, and cytomegalovirus (16). They are also inhibitors of natural (17) and recombinant (18) human immunodeficiency virus reverse transcriptase activity in vitro. Fucoidans can act as anti-angiogenic agents (19) and can block selectin-mediated cell-cell binding (20).

The sulfated fucan from sea urchin plays an important role in fertilization events in that species, inducing the acrosome reaction on contact of the spermatozoa with the jelly layer of the egg (6). Species-specific induction of the acrosome reaction is said to reside solely in the sulfated fucan, suggesting that there may be differences in the structures of fucans from different species of sea urchin (4). It is interesting to note that algal fucoidans can block sperm-egg binding in diverse animal (including mammalian) species (21–25), also affecting recognition events necessary for penetration of the zona pellucida (26, 27).

In spite of the level of interest shown in functional aspects of sulfated fucans, their structural properties have been relatively little studied.

The structure originally proposed for the sulfated fucan from F. vesiculosus was composed mainly of 4-sulfated and 2-linked α-L-fucopyranosyl units (1–2) and has only recently been superseded by a revised structure in which the α-L-fucopyranosyl units are 1→3-linked (3, 28). This structure resembles that determined for a fucan from Ecklonia kurome, another brown seaweed (29). A preliminary study on the structure of the sulfated fucan from sea cucumber, also containing 1→3-linked units, was reported recently (30). The basic structure of the backbone units of this family of sulfated fucans from marine organisms is shown in Fig. 1. The structure of the sea urchin fucan has not yet been described in detail. Relationships between chemical structure of these sulfated fucans and their biological activities therefore remain to be established.

In this study we have compared the sulfated fucans from echinoderms with those from two species of brown algae. Surprisingly, we find that the sulfated fucans from echinoderms have a previously unrecognized type of structure, composed of tetrasaccharide repeating units in which the 4 residues are 1→3-linked α-L-fucopyranosyl units differing only by specific patterns of sulfation at the O-2 and O-4 positions. The sulfated fucans from brown algae have more randomly heterogeneous structures. There is a preponderance of 3-linked units and sulfation at O-4 position, but we have found no evidence for a regular repeating structure, as observed for the fucans from echinoderms. Comparative studies of the biological activities of sulfated fucans from echinoderms and brown algae will allow a clearer link to be established between the chemical structures and biological action of these groups of sulfated polysaccharides.

EXPERIMENTAL PROCEDURES

Sulfated Fucan from Sea Cucumber

The sulfated fucan was extracted from the body wall of the sea cucumber Luidwigothurea grisea and purified by a combination of anion exchange chromatography on DEAE-cellulose and gel filtration on
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**FIG. 1.** The basic backbone structure of 1-3-linked fucose residues in many sulfated fucans from marine organisms. R may be hydrogen, sulfite, or a galactose or fucose side chain.

Sephacryl S-400 column, as described (30).

**Sulfated Fucan from Sea Urchin**

**Extraction—**Twelve mature specimens of the sea urchin *Lytechinus variegatus* were collected in Guanabara Bay (Rio de Janeiro, Brazil) and gametes were isolated by intracelomic injection of 0.5 M KCl (5 ml per specimen). Eggs were collected in 50 mM sodium acetate buffer (pH 8.0) containing 0.5 M NaCl and 10 mM EDTA, and stored in an ice bath during transportation to the laboratory. The egg jelly was separated by pH shock, as described previously (4). About 1.8 g (dry weight) of crude jelly coat was obtained after these procedures.

The acidic polysaccharides were extracted from the jelly coat by papain digestion, and partially purified by cetylpyridinium chloride and ethanol precipitation, as described (31). About 240 mg (dry weight) of crude extract was obtained after these procedures.

**Purification—**The crude polysaccharides (250 mg) were applied to a DEAE-cellulose column (9 x 2 cm) equilibrated with 50 mM sodium acetate buffer (pH 5.0) containing 10 mM EDTA and washed with 200 ml of the same buffer. The column was eluted in three different steps. Initially the column was eluted by a linear gradient prepared by mixing 50 ml of 50 mM sodium acetate buffer (pH 5.0) containing 10 mM EDTA with 50 ml of 1.0 M NaCl in the same buffer. The flow rate of the column was 5 ml/h and fractions of 5 ml were collected. Then the column was washed with 100 ml of the sodium acetate buffer containing 1.0 M NaCl, at a flow rate of 20 ml/h, and fractions of 5 ml were collected. Finally, the column was eluted by a linear gradient prepared by mixing 100 ml of 1.0 M NaCl with 100 ml of 5.0 M NaCl, both in the same sodium acetate buffer. The flow rate was 12 ml/h and fractions of 3 ml were collected. Fractions from the different elution steps were checked for methylpentose and sialic acid by the Diache and Shettles reaction (32) and by the thiobarbituric assay (33), respectively, and by their metachromasia (34). The NaCl concentration was estimated by conductivity. Fractions containing the sulfated fucan and the sialic acid glycoprotein, as indicated by fucose- and sialic acid-positive tests, respectively, were pooled, dialyzed against distilled water, and lyophilized.

**Extraction—**A commercial crude fucoidan, extracted from *F. vesiculosus*, was obtained from Sigma. The brown alga *Laminaria brasiliensis* was collected at Macaé (Rio de Janeiro, Brazil), immersed immediately in acetone, and kept for 24 h at 4°C. The sulfated polysaccharides were extracted from the dried tissue (2 g) by papain digestion, and partially purified by cetylpyridinium chloride and ethanol precipitation, as described (31). About 120 mg (dry weight) of crude extract was obtained after these procedures.

**Purification—**The crude polysaccharides (150 mg) were applied to a DEAE-cellulose column (9 x 2 cm) equilibrated with 50 mM sodium acetate buffer (pH 5.0) and washed with 200 ml of the same buffer. The column was eluted by a linear gradient prepared by mixing 150 ml of 50 mM sodium acetate buffer (pH 5.0) containing 0.2 M NaCl and 10 mM EDTA with 150 ml of 1.2 M NaCl in the same buffer. The flow rate of the column was 12 ml/h. Fractions of 3 ml were collected and checked by the phenol-H$_2$SO$_4$, Dubois (C) and carbazole (D) reactions, for metachromasia (O) and NaCl concentration (—). The fractions indicated by horizontal bars were pooled, dialyzed against distilled water, and lyophilized. Those corresponding to the sulfated fucan are shaded. The arrows in B indicate the elution of standard dextran sulfates (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).

1.2 mM NaCl, both solutions were prepared in the acetate buffer containing 10 mM EDTA. The flow rate of the column was 12 ml/h and fractions were collected and checked as described above for the first DEAE-cellulose column.

The DEAE-cellulose purified fucan (40 mg) was applied to a Sephacryl S-400 column and chromatographed as described for the sulfated fucan from sea cucumber (30).

**Agarose Gel Electrophoresis**

Sulfated fucans were analyzed by agarose gel electrophoresis, as described (8, 31).

**Chemical Analyses**

Total hexose was measured by the method of Dubois et al. (35), hexuronic acid by the carbazole reaction (36), and total methylpentose (6-deoxyhexose) by the method of Diache and Shettles (32). 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 (37), and sulfate by the BaCl$_2$/gelatin method (38). The percentages of hexoses and 6-deoxyhexoses in the acid hydrolysates were estimated by...
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Each sulfated polysaccharide was lyophilized from the crude polysaccharides from the sea urchin jelly coat were purified on DEAE-cellulose and Sephacryl S-400 columns as described under "Experimental Procedures." Fractions were checked by the methylpentose (Dische and Shettles) and sialic acid (thiobarbituric) assays, for metachromasia and NaCl concentration. The fractions indicated by horizontal bars were pooled, dialyzed against distilled water, and lyophilized. Those corresponding to the sulfated fucan are shaded. The arrows in B indicate the elution of molecular weight standards, as described in Fig. 2B.

**Fig. 3. Purification of the sulfated fucan from the sea urchin L. variegatus on DEAE-cellulose (A) and Sephacryl S-400 (B).** The crude polysaccharides from the sea urchin jelly coat were purified on DEAE-cellulose and Sephacyr S-400 columns as described under "Experimental Procedures." Fractions were checked by the methylpentose (Dische and Shettles) and sialic acid (thiobarbituric) assays, for metachromasia and NaCl concentration. The fractions indicated by horizontal bars were pooled, dialyzed against distilled water, and lyophilized. Those corresponding to the sulfated fucan are shaded. The arrows in B indicate the elution of molecular weight standards, as described in Fig. 2B.

**Fig. 4. Purification of the sulfated fucan from the brown alga L. brasiliensis on DEAE-cellulose (A and B) and Sephacryl S-400 (C).** Fractions were checked by the phenol-H$_2$SO$_4$ (Dubois) and carbazole (Volhardt and Debus) reactions, for metachromasia and NaCl concentration. The fractions corresponding to the sulfated fucan are shaded. Further details are described under "Experimental Procedures" and in the legend of Fig. 2.

- **Oxidation with L-Fucose Dehydrogenase**
  - Fucose obtained by acid hydrolysis of the sulfated fucans, and authentic samples of D- or L-fucose, were incubated with 0.2 units of porcine liver L-fucose dehydrogenase as described.

- **Desulfation and Methylation**
  - Desulfation of the sulfated fucans was performed by solvolysis in dimethyl sulfoxide as described previously for desulfation of other types of polysaccharides. Methylation was performed as described.

**NMR Spectroscopy**

- $^1$H spectra were recorded at 500 MHz and $^{13}$C spectra at 125 MHz using a Varian Unity 500 spectrometer in the FT mode. 10–20 mg of each sulfated polysaccharide was lyophilized from 99.8% D$_2$O (Goss Scientific, Ingatestone, United Kingdom) and dissolved in 0.7 ml of 100% D$_2$O (Aldrich Chemical Co., Poole, United Kingdom) for NMR spectroscopy. $^1$H spectra of the polysaccharides were recorded at 60 °C and sometimes 40 °C (where resonances were obscured by the HOD signal at 60 °C). $^{13}$C spectra were recorded at 60 °C. Signals due to low molecular weight impurities with long relaxation times were reduced in intensity by using a rapid repetition rate. Two-dimensional spectra, double-quantum-filtered COSY and NOESY spectra were recorded in the phase-sensitive mode using the pulse programs provided by the spectrometer manufacturer. For the NOESY spectra a mixing time of 100 ms was used. All the spectra were referenced to internal or external trimethylsilylpropionic acid.

**RESULTS AND DISCUSSION**

**Fractionation of the Sulfated Fucans**

The sulfated fucans from echinoderms and brown algae were fractionated by a combination of ion exchange and gel filtration chromatographies. Anion exchange chromatography on a DEAE-cellulose column (Fig. 2A), and gel filtration chromatography on Sephacryl S-400 (Fig. 2B) were used to separate the sulfated polysaccharides from sea cucumber body wall as has been previously described (30); both techniques were necessary in order to achieve complete separation of the sulfated fucan from the fucose-branched chondroitin sulfate which was characterized in our previous studies (8, 31, 46), and from a further high-molecular mass sulfated polysaccharide (30).
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**Fig. 5.** Purification of the sulfated fucan from the brown alga *F. vesiculosus* on DEAE-cellulose (A) and Sephacryl S-400 (B). Fractions were checked by the phenol-H$_2$SO$_4$ (Dubois) (C) reaction, for metachromasia (○) and NaCl concentration (---). Further details are described under "Experimental Procedures" and in the legend of Fig. 2.

**Fig. 6.** Agarose gel electrophoresis of the sulfated fucans from sea cucumber, sea urchin, and brown algae. Purified sulfated fucans (15 μg) were applied to a 0.5% agarose gel and run for 1 h at 110 V in 0.05 M 1,3-diaminopropane/acetate buffer (pH 9.0). The sulfated fucans in the gel were fixed with 0.1% N-cetyl-N,N,N',N'-trimethylammonium bromide solution. After 12 h, the gel was dried and stained with 0.1% toluidine blue in acetic acid-ethanol-water (6:1:5:5, v/v).

### Chemical composition, specific optical rotation, and average mass of the sulfated fucans from echinoderms and brown algae

<table>
<thead>
<tr>
<th>Sulfated fucan</th>
<th>Composition (molar ratios)</th>
<th>[α]$^2$</th>
<th>Average molecular mass $^a$</th>
<th>kDa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sea cucumber</td>
<td>1.00</td>
<td>&lt;0.01</td>
<td>1.10</td>
<td>-76</td>
</tr>
<tr>
<td><em>L. grisea</em></td>
<td>1.00</td>
<td>&lt;0.01</td>
<td>1.46</td>
<td>-58</td>
</tr>
<tr>
<td><em>L. variegatus</em></td>
<td>0.90</td>
<td>0.10</td>
<td>0.90</td>
<td>-134</td>
</tr>
<tr>
<td>Brown alga</td>
<td>0.75</td>
<td>0.25</td>
<td>0.40</td>
<td>-128</td>
</tr>
<tr>
<td><em>F. vesiculosus</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Fucose occurs entirely in the L-enantiomeric form since this sugar is totally oxidized by L-fucose dehydrogenase.

### Structural Analysis

**Methylation Studies**—Methylation of sulfated polysaccharides does not always yield reliable proportions of methylated alditols (28, 48–50). This may be a consequence of steric hindrance due to the sulfate esters, which do not allow complete methylation of these polymers. The more drastic conditions necessary to remove sulfate esters may also destroy some of the

1 The proportions of galactose are the same in the sulfated fucans from various purification steps (Figs. 4, A–C, and 5, A and B). It is therefore unlikely that this sugar arises from a different polysaccharide which contaminates the sulfated fucans.
methylated fucose derivatives (28). Alternative methods involving methylation of the desulfated polysaccharide are not always possible since it is difficult to obtain totally desulfated fucans. The fucose-linked sulfate esters appear to be more resistant than other sulfated polysaccharides to solvolysis in dimethyl sulfoxide (31). Nevertheless methylation analysis may offer valuable information concerning the position of the glycosidic linkage and the site of sulfation (31, 50–53).

Methylation of the sulfated fucans from echinoderms and brown algae yielded mainly unmethylated fucitol. After partial desulfation of the fucans, the proportions of unmethylated fucitol decreased sharply, whereas 2,4-di-O-methyl and 2-O-methyl fucitol became the predominant O-methyl fragment (Table II). These results indicate the presence of 2- and/or 4-sulfated fucosyl units. The absence or very low proportion of methyl ether substituted in carbon 3 in the native and partially desulfated fucans from echinoderms suggests the presence of 3-linked structures. The presence of 2,3,4-tri-O-methyl fucitol in the methylation of the partially desulfated fucan from *L. brasiliensis* indicates the presence of a branched polymer with fucosyl unit at nonreducing ends. This methylated derivative is not detected in the methylation analysis of the fucans from echinoderms.

**TABLE II**

<table>
<thead>
<tr>
<th>Alditol*</th>
<th>Sea cucumber</th>
<th>Sea urchin</th>
<th>Brown alga</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>L. grisea</em></td>
<td><em>L. variegatus</em></td>
<td><em>L. brasiliensis</em></td>
</tr>
<tr>
<td>Native</td>
<td>Partially desulfated</td>
<td>Native</td>
<td>Partially desulfated</td>
</tr>
<tr>
<td>% of total peak area</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2,3,4-Me₃-Fuc</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>2,3-Me₂-Fuc</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>2,4-Me₂-Fuc</td>
<td>6</td>
<td>31</td>
<td>3</td>
</tr>
<tr>
<td>2-Me-Fuc</td>
<td>6</td>
<td>23</td>
<td>31</td>
</tr>
<tr>
<td>4-Me-Fuc</td>
<td>12</td>
<td>15</td>
<td>26</td>
</tr>
<tr>
<td>Fuc</td>
<td>73</td>
<td>28</td>
<td>40</td>
</tr>
</tbody>
</table>

* The identity of each peak was established by mass spectrometry.
* The sulfate:fucose molar ratios of the partially desulfated fucans from sea cucumber, sea urchin, and brown alga were 0.30, 0.38, and 0.42 respectively.

**NMR Spectroscopy**—The *¹H* spectra at 500 MHz of the fucans from *F. vesiculosus* (both crude, as supplied, and purified), *L. brasiliensis*, sea cucumber, and sea urchin are shown in Fig. 7, a–e. The algal sulfated fucans give complex spectra with broad signals hampering resolution. The spectrum of the crude *F. vesiculosus* fucan yields relatively little information, as has been noted (28). The chemical shift of the envelope of anemic signals at 5.0–5.5 ppm (Fig. 7a) is consistent with the presence of *α*-l-fucopyranosyl units (as is an envelope of methyl signals at 1.2–1.8 ppm in the spectra of all the algal fucans, not shown in Fig. 7). Purification of the fucan leads to an altered spectrum in which several more distinct anemic signals can be seen.

**FIG. 7.** Expansions of the 6.0–3.5 ppm regions of the *¹H* spectra at 500 MHz of a crude commercial sample of sulfated fucan from *F. vesiculosus* (a); purified sulfated fucan from *F. vesiculosus* (b); purified sulfated fucan from *L. brasiliensis* (c); and expansions of the 6.0–3.5 ppm and 2.0–0.0 ppm regions of the spectra of purified sulfated fucan from sea cucumber (d); purified sulfated fucan from sea urchin (e), both with integrals marked for the low field resonances. Resonances marked A–D are anemic (*H1*) signals. All the spectra were recorded at 60 °C for samples in D₂O solution. Chemical shifts are relative to internal or external trimethylsilylpropionic acid at 0 ppm. The HOD signal has been suppressed by pre-irradiation in *a*, *b*, and *c*, and is marked with an asterisk in *d* and *e*. Signals marked *x* arise from contaminants.
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FIG. 8. Expansions of the 120-0 ppm regions of $^{13}$C NMR spectra at 125 MHz of a crude commercial sample of sulfated fucan from *F. vesiculosus* (a); purified sulfated fucan from *F. vesiculosus* (b); purified sulfated fucan from *L. brasiliensis* (c); and expansions with integrals of anomeric carbon signals of the spectra of purified sulfated fucan from sea cucumber (d); purified sulfated fucan from sea urchin (e), all at 60 °C in D$_2$O solution. Chemical shifts are relative to internal or external trimethylsilylpropionic acid at 0 ppm. The weighting functions for processing of all five spectra were similar. Signals marked x arise from contaminants.

There is a loss of intensity at 3.8 ppm on purification, ascribable to removal of other polysaccharides from the fucan. The high proportion of galactose remaining in the purified fucan (25%) must be responsible for some of the signals in the spectrum, but it is not possible to assign any particular signals to galactose. The increased relative intensity at about 4.6 ppm may be due to H4 and/or H2 of sulfated fucose residues (30), but may also arise from galactose residues (51). The *L. brasiliensis* fucan gives a spectrum (Fig. 7c) which is quite different from that of the *F. vesiculosus* fucan. In this case galactose is present at about 10% only, so it is safe to assign major features in the spectrum to fucose residues. Anomeric signals in the spectrum of the *L. brasiliensis* fucan are spread from 5.7 to 5.2 ppm, including signals at 5.6 and 5.7 ppm having no counterpart in any of the other fucans in this study. A feature at 4.9 ppm can tentatively be assigned to H4 of 4-sulfated fucose (30). An envelope at around 4.6 ppm may contain other signals from sulfated fucose residues.

It can safely be said that the $^1$H spectra of the algal fucans are complex, overlapping, and inconclusive for structural information. Attempts to record two-dimensional NMR spectra for these compounds gave no useful result.

The $^1$H spectra of the echinoderm fucans are in clear contrast to those of the algal fucans. Both the sea cucumber (Fig. 7d) and sea urchin fucans (Fig. 7e) give spectra which are simple, and although line widths are several Hz, as expected for polysaccharides of high molecular mass, several individual signals can easily be resolved. In particular both spectra contain four anomeric doublets with $^3$J$_{HH}$ less than 4 Hz between 5.0 and 5.5 ppm. These are labeled in Fig. 7, d and e, with the
FI. 9. Expansion of the double quantum filtered COSY spectrum of the sulfated fucan from sea urchin, at 500 MHz, 60 °C, in D2O. Cross-peaks between vicinal protons are labeled to demonstrate how the spectra of 4 distinct fucose residues can be traced from the four anomeric (H1) signals A, B, C, and D. The H5-H6 cross-peaks are not shown.

letters A–D, arbitrarily starting at the low-field (left-hand) end. Integration showed that these were present in the approximate ratio 1:1:1:1 for both polysaccharides.

In addition, sharp multiplets at 4.91 in the sea cucumber fucan spectrum and at about 4.78 ppm in the sea urchin fucan spectrum are consistent in their very small 3JH,H values with H4 of fucose, in the sea cucumber case with integrated intensity equivalent to one anomeric proton, and in the sea urchin case with intensity twice as great.

The 13C spectra of all five fucans are shown in Fig. 8, a–e. Comparison of the spectra of the crude (Fig. 8a) and purified (Fig. 8b) forms of the F. vesiculosus fucan shows that some signals of unknown origin are reduced in intensity in the purified material; these are at 111.2, 89.6, 87.1, 63.8, and 20.9 ppm. The signal at about 25 ppm in the spectrum of the purified material is probably due to contaminating acetate, introduced during the purification; this contaminant is present in all the purified fucans. The envelopes of signals from both anomeric carbons (106–96 ppm) and ring carbons (85–60 ppm) seem paradoxically to be simpler in the spectrum of the crude material, but this simplicity is merely the result of many signals overlapping to form broad envelopes. With the removal of impurities the underlying resonances from the fucan itself can be seen more clearly. In the 13C spectrum of the polysaccharide from L. brasiliensis (Fig. 8c) there are at least eight separate anomeric carbon signals. The analysis of monosaccharide components by chemical methods indicated only the presence of fucose and galactose (Table I), so the degree of heterogeneity indicated is high. The C6 signal of, presumably, galactose, is clearly visible at 64.2 ppm. Some signals, for example those at 59.4 and 54.3 ppm, do not originate from either galactose or fucose and are due to an unidentified impurity.

As in the case of the 1H spectra, the 13C spectra of the echinoderm fucans (Fig. 8, d and e) are well resolved compared with those of the plant fucans. The sea urchin fucan gives a spectrum in which four anomeric resonances with integral ratios of about 1:1:1:1 can be seen; in the case of the sea cucumber fucan two of these resonances overlap. Ring carbon signals can be seen for both unsubstituted positions (73–68 ppm) and carbons substituted either by glycosylation or sulfation (82–74 ppm) (30). C6 resonances at 17–19 ppm can be seen in the spectra of all the fucans (Fig. 8, a–e) as an envelope of overlapping signals for the algal fucans and as partly resolved groups for the echinoderm fucans.

Assignment of the 1H Spectra—The 1H spectrum of the sea urchin fucan was achieved by analysis of a double quantum filtered COSY spectrum, and expansions of the spectrum are shown in Fig. 9. It was possible in the case of this fucan to trace the entire coupling path round each one of four fucose spin systems, starting with the four H1 signals labeled A–D and ending with one of the H6 methyl signals, as shown in Fig. 9 (see Fig. 1 for the numbering conventions round the fucopyranose ring).

Each of these spin systems must originate from a fucose residue in a unique environment. Chemical shifts for these four types of fucose residue in the sea urchin fucan are listed in Table III, with some approximate 3JH,H values. Assignment of the 1H spectrum of the fucan derived from sea cucumber has
Previously been described (30). Chemical shifts for the four types of fucose residue in the sea cucumber fucan (from Ref. 30) are also shown in Table III for comparison.

The positions of sulfate substituents on these fucose residues can be deduced from sulfation shifts. The chemical shifts of protons at positions of sulfation are typically 0.6–0.7 ppm to low field of the equivalent proton at an unsulfated position. So, for residues A, B, and C, with H2 at 4.50–4.55 ppm, compared with residue D with H2 at 3.87 ppm, it can be concluded that there is sulfate substitution at position 2. Residues B and D have H4 at 4.77 and 4.78 ppm, respectively; residues A and C have H4 at 4.05 and 4.06 ppm, respectively. Residues B and D can therefore be deduced to be 4-sulfated. The same reasoning for the sea urchin fucan (30) showed that residue A was 4-sulfated, residues B, A, and C 2-sulfated, and residue D unsulfated.

Sequence of the Residues—NOESY spectra of both echinoderm fucans confirmed the assignments suggested by the DQFCOSY spectra, by means of the expected intra-residue NOEs, and provided evidence for a defined sequence for the 4-residue types by means of inter-residue NOEs. Expansions of the NOESY spectra of the sea cucumber and sea urchin fucans showing the inter-residue NOEs as well as some of the intra-residue interactions are shown in Fig. 10, a and b. Inter-residue interactions are seen between each anomeric signal A–D and an H3 signal from one and only one of the other residues. For the sea urchin fucan, H1 of residue A shows an NOE cross-peak to H3 of residue D; H1 of residue D to H3 of residue B; H1 of B to H3 of C; and to complete the cycle, H1 of residue C to H3 of residue A. Weaker peaks can be seen from H1 of A, B, and C to H4 of D, C, and A, respectively. This is strong evidence that the 4 residues are joined in a linear fashion by 1→3-linkages (as suggested by the methylation analysis), and that they are arranged in a regular tetrasaccharide repeating unit, defined by its pattern of substitution with sulfate esters. The same general interpretation has already been published for the sea cucumber fucan (30), and here the pattern of sulfation over the tetrasaccharide repeat unit is different; the sea urchin and sea cucumber fucan structures are shown in Fig. 11, a and b, respectively.

### Table III

<table>
<thead>
<tr>
<th></th>
<th>Sea urchin*</th>
<th>Sea cucumber*</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1</td>
<td>5.40</td>
<td>5.38</td>
</tr>
<tr>
<td>H2</td>
<td>4.51</td>
<td>4.54</td>
</tr>
<tr>
<td>H3</td>
<td>4.25</td>
<td>4.22</td>
</tr>
<tr>
<td>H4</td>
<td>4.05</td>
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<tr>
<td>H5</td>
<td>4.31</td>
<td>4.51</td>
</tr>
<tr>
<td>H6</td>
<td>1.23</td>
<td>1.25</td>
</tr>
</tbody>
</table>

Optimum values (Hz) for the 500 MHz, 60 °C in D_2O. Chemical shifts are referenced to internal TSP at 0 ppm.

*Measured at 500 MHz, 60 °C in D_2O. Chemical shifts are referenced to internal TSP at 0 ppm. *Taken from Ref. 30.

### Conclusions and General Comments

The present study concerns the structures of sulfated fucans from echinoderms and brown algae. Despite the extensive range of published studies on biological activities of these polysaccharides (4–6, 9–27), their chemical structures have rarely been investigated in detail. The work of Patankar et al. (28) on the fucan from F. vesiculosus has shown that the main α-L-fucose backbone is 1→3-linked, with an average structure containing some fucose branches and some sulfation at the 4-position. NMR analysis reveals that the sulfated fucans from echinoderms are essentially linear polymers, composed of a regular repeating sequence of 4 residues (Fig. 11, a and b). These 4-residue types may be distinguished by two-dimensional 1H-1H correlated spectroscopy (COSY), each residue giving rise to an individual spin system. Positions of sulfate substitution are revealed by characteristic sulfation shifts of signals from protons at sulfated positions in the one-dimensional proton spectrum. The sequence of residues is deduced from inter-residue NOEs evident from cross-peaks in the NOESY spectrum.

To the best of our knowledge, this is a type of polysaccharide which has not previously been described, in which the monosaccharide type and the glycosidic linkage remain constant and the repeating unit is defined only by the pattern of O-sulfation. It would be exceptionally difficult to determine these structures by degradative procedures without the aid of the NMR spectra of the whole polysaccharide.

The sulfated fucan from sea cucumber consisted of 2 residues sulfated at the O-2 position, one sulfated at both the O-2 and -4...
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FIG. 11. Proposed structures for the sulfated fucans from sea urchin (a), sea cucumber (b), and brown algae (c) and for the fucosylated chondroitin sulfate from sea cucumber (d). Sulfated fucans from echinoderms are linear polymers, composed of tetrasaccharides repeating units in which the 4 residues are 1→3-linked α-L-fucopyranosyl units differing only by specific patterns of sulfation at the O-2 and O-4 position (a and b). The sulfated fucans from brown algae have more heterogeneous structures. There is a preponderance of 3-linked units and sulfation at the O-4 position, but no evidence for a regular repeating structure. Some fucosyl residues are at nonreducing ends (c). Another fucose-rich polysaccharide found in echinoderms is a fucosylated chondroitin sulfate from sea cucumber (d). This polysaccharide has a chondroitin sulfate-like core structure, containing unexpectedly large numbers of sulfate α-L-fucopyranose branches linked to position 3 of the β-D-glucuronic acid residues.
positions, and one unsulfated residue (Fig. 11b). The fucan from sea urchin consisted of 2 residues sulfated at the O-2 position, one sulfated at both O-2 and -4 positions, and 1 residue sulfated at the O-4 position (Fig. 11a). The sulfated fucans from brown algae have a more heterogeneous structure. We found no evidence for a regular repeating structure. There is evidence for the presence of 3-linked units and sulfation at O-2 and/or -4 positions (Fig. 11c); in addition, some fucosyl residues are at nonreducing ends, as branching units. Both purified algal fucans in this study contain significant amounts of galactose: 10% in the case of the L. brasilensis fucan, and 25% in the fucan from F. vesiculosus.

Several of the studies of biological activity of algal fucoids cited in the Introduction to this article were carried out using the commercially available F. vesiculosus fucoidan without further purification. This preparation is by no means pure fucoidan; the present study corroborates the work of Nishino et al. (54), who find a wide range of other polysaccharides present. These substances may have their own biological activities, and at the very least will dilute the fucoidan itself. The purified algal fucoidan fractions described in this work, and in particular the clearly defined, simple structures of the echinoderm fucans may allow more definitive conclusions to be drawn about the biological activities of fucans in systems with possible therapeutic potential.

In addition to the sulfated fucan, the sea cucumber body wall contains high amounts of a fucosylated chondroitin sulfate (Refs. 8, 31, and 46, and Fig. 24). This polysaccharide has a chondroitin sulfate-like structure, containing unexpectedly a preponderance of disaccharide units formed by 3,4-di-O-sulfated fucosyl linkage and site of sulfation. These two structures, in addition to the sulfated fucan from sea cucumber has not been analyzed and NMR spectroscopy revealed that the position of the glycosidic linkage and the site of sulfation in the fucose branches are extremely heterogeneous. We proposed a preponderance of disaccharide units formed by 3,4-di-O-sulfated fucosylpyranosyl units glycosidically linked through position 1-2 to 4-O-sulfo-a-L-fucopyranose (Fig. 11d). These fucosyl branches in the fucosylated chondroitin sulfate and the fucose residues in the sulfated fucan from sea cucumber and sea urchin (Fig. 11, a and b) show considerable differences in respect to glycosidic linkage and site of sulfation. These two structures, although found in the same tissue and composed of a- fucopyranosyl residues, must be synthesized by different pathways.

Up to now it has not been possible to trace a relationship between the structures of the various fucans and their biological functions. The sulfated fucans from brown algae are found in cell walls, possibly as a space-filling structure (1). The function of the sulfated fucan from sea cucumber has not been defined. It occurs in the body wall of the animal, which is a connective-tissue-like tissue, possibly as an extracellular macromolecule (55). The sulfated fucan from sea urchin has a specific physiological role in the induction of the acrosome reaction (4-6). It may be that the occurrence of a sulfated fucan composed of a regular tetrasaccharide units is a unique feature shared by the echinoderms.

The species-specific induction of the acrosome reaction in sea urchin has been said to reside solely in the sulfated fucan, suggesting that there may be structural differences in this polymer in the various different sea urchin species (4). It is therefore possible that the specific patterns of sulfation at the O-2 and O-4 positions of the repeating tetrasaccharide units may signalize this species-specific reaction.

Finally, the biosynthesis of the sulfated fucans from echinoderms is possibly regulated in different ways from those of vertebrate glycosaminoglycans and other sulfated glycans from algae. In these cases the specificity of the substrate for the sulfotransferase is defined by the saccharide structure. However, the biosynthesis of the regular sulfated fucans from echinoderms involves incorporation of sulfate esters into defined positions of a saccharide chain containing similar monosaccharide and linkage types, which would require a different type of metabolic control from those reported for glycosaminoglycans.

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