Structural Heterogeneity among Unique Sulfated L-Galactans from Different Species of Ascidians (Tunicates)*

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The sulfated polysaccharides that occur in the tunic of ascidians differ markedly in molecular weight and chemical composition. A high molecular weight fraction (F-1), which has a high galactose content and a strong negative optical rotation, is present in all species. Several structural differences were observed among the F-1 fractions obtained from three species of ascidians that were studied in detail. Large numbers of α-L-galactopyranose residues sulfated at position 3 and linked glycosidically through position 1→4 are present in F-1 from all three ascidians. However, α-L-galactopyranosyl units, 1→3-linked and partially sulfated at position 4, comprise about half of the sugar units in the central core of F-1 from Ascidian nigra. In addition, L-galactopyranose nonreducing end units occur in F-1 from Styela plicata and A. nigra, but comprise only a minor fraction of F-1 from Clavelina sp. The combination of these various component units gives a complex structure for F-1 from S. plicata and A. nigra, whereas F-1 from Clavelina sp. possesses a simpler structure. The structures of these ascidian glycans are unique among all previously described sulfated polysaccharides, since they are highly branched (except that from Clavelina sp.), sulfated at position 3, and contain large amounts of L-galactose without its D-enantiomorph. These data show unusual examples of polyanionic glycans with structural function in animal tissues.

Sulfated polysaccharides are widespread in nature, occurring in a great variety of organisms. In marine algae, for example, there are the carrageenans and fucoidan, which are composed mainly of sulfated galactose and fucose, respectively. In the animal kingdom, sulfated glycosaminoglycans abound in vertebrate connective tissue (2) and, to a lesser extent, are also present in invertebrates (2, 3).

The glycosaminoglycans display many biological functions and in connective tissues they exert a crucial role in the maintenance of the structural integrity, mainly through interactions with other molecules of the extracellular matrix (4, 5).

In previous studies we have reported the isolation of novel sulfated polysaccharides from invertebrate tissues, namely the tunic of ascidians (6–8) and the body wall of sea cucumber (6, 9, 10). We speculated that the occurrence of high amounts of sulfated polysaccharides in these tissues indicated that they were playing important structural roles, perhaps like those of glycosaminoglycans in vertebrate connective tissue.

In this paper is described the purification and chemical analysis of the sulfated polysaccharides extracted from the tunics of various species of ascidians. The main polysaccharide found in all species was a high molecular weight sulfated L-galactan, which differs from all previously described galactose-rich glycans of marine algae and also from the animal glycosaminoglycans. Interestingly, marked structural variations were observed among the sulfated L-galactans from different species of ascidians. In this class of compounds there were differences in the proportion of nonreducing end units, in the content of 1→3- and 1→4-linked units and in the degree and position of sulfation.

EXPERIMENTAL PROCEDURES

RESULTS AND DISCUSSION

Sulfated Polysaccharides from Different Species of Ascidians

The sulfated polysaccharides extracted from the tunics of five species of ascidians were analyzed by agarose and polyacrylamide gel electrophoresis (Fig. 1).

The electrophoretic mobilities of the sulfated polysaccharides on agarose gel were characteristic for each species of ascidian (Fig. 1A). Two metachromatic bands with electrophoretic mobilities different from those of standard glycosaminoglycans are observed in the agarose gel electrophoretograms of the sulfated polysaccharides from Ascidian nigra, Botryllus sp., and Styela plicata. The glycans extracted from Clavelina sp. and Herdmania monus show a single widespread band.

Polyacrylamide gel electrophoresis of the ascidian polysaccharides (Fig. 1B) indicates that all the species have at least two fractions: one of high molecular weight that stays at the origin and another of low molecular weight that migrates into the gel. The glycans from A. nigra, Clavelina sp., and H. monus show a single low molecular weight fraction, while those from Botryllus sp. and S. plicata have two distinct bands of low molecular weight.

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Fractionation and Chemical Analysis of the Ascidian Polysaccharides

Gel Chromatography on Sepharose CL-4B and Sephadex G-200—The sulfated glycans from three species of ascidians were fractionated by gel filtration on Sepharose CL-4B and Sephadex G-200 (Fig. 2). A high molecular weight fraction, designated F-1, is observed in all species and accounts for at least 50% of the total polysaccharides. This fraction does not enter the polycrylamide gel due to its high molecular weight (Fig. 3B) and on agarose gels shows a single band (Fig. 3A). A low molecular weight fraction, designated F-2, is eluted near the total volume of the Sepharose CL-4B column (Fig. 2). In S. plicata, this fraction has two components, which were further purified by gel chromatography on Sephadex G-200 (fractions F-2-A and F-2-B in the insert to Fig. 2C). Each fraction F-2 (or F-2-A and F-2-B) migrates as a single band on the polycrylamide gel, but the average molecular weight differs for each species (Fig. 3B). Electrophoresis on an agarose gel confirms the homogeneity of the F-2 fractions (Fig. 3A).

Chemical Composition of the Fractions—Table II shows the chemical analysis and the specific rotation of the purified polysaccharides from the three species of ascidians. Fraction F-1 from all species is composed mainly of galactose and sulfate. Fraction F-2 shows a more heterogeneous chemical composition and a wide variation among the various species of ascidians. However, it always has a higher hexosamine content than F-1, and its predominant amino sugar is glucosamine, while galactosamine predominates in F-1. The sulfate content of both fractions increases from A. nigra to S. plicata and Clavelina sp. (Table II).

Sulfated polysaccharides from the other two species (Botryllus sp. and H. monous) were also purified by gel filtration and checked for purity by agarose and polycrylamide gel electrophoresis (results not shown). The high molecular weight fraction (F-1) of these two species is a sulfated galactan, with minor amounts of glucose and amino sugar, while the F-2 fractions have always a higher hexosamine content than F-1, as already reported for the other ascidians in Table II.

In the ascidian glycans, the presence of amino sugars, which are especially prominent in F-2 fractions, resembles the glycosaminoglycans from animal tissues. In fact, F-2 from A. nigra and Clavelina sp. possess equimolar proportions of galactose and glucosamine, as do keratan sulfate from mammalian cartilages and corneas (19). However, the specific optical rotation (Table II) and the resistance to the mucopolysaccharidases (6, 7) indicate that the ascidian glycans differ from all previously described glycosaminoglycans.

Fraction F-1 from the various species of ascidians forms broad peaks on Sepharose CL-4B columns (Fig. 2). In order to determine whether this fraction has a homogeneous chemical composition, F-1 from S. plicata was subdivided into F-1-A and F-1-B (Fig. 2C, see also Ref. 7). The chemical analysis of these two subfractions shows comparable chemical compositions with respect to hexoses, hexosamine, and sulfate, confirming previous data (7). This indicates that the high molecular weight fraction encompasses a broad range of molecular weights but is structurally homogeneous.

L-Galactose in the Ascidian Polysaccharides—The strongly negative specific rotations of the ascidian polysaccharides (Tables I and II), especially of the F-1 fractions (−100 to

In the present study we also methylated F-1-A and F-1-B and obtained similar proportions of methylated derivatives for both of them (data not shown).
Sulfated Polysaccharides from Ascidians

TABLE I

Chemical composition and specific optical rotation of the sulfated polysaccharides from the ascidians

| Species        | Gal | Man | Glc | Fuc | HexNH* | SO4/sugar | [α]D°/C |
|----------------|-----|-----|-----|-----|--------|-----------|---------|
| Clavelina      | 0.68| <0.01| 0.10| 0.09| 0.13   | 0.69      | -88°    |
| A. nigra       | 0.72| 0.04 | 0.04| <0.01| 0.01   | 0.20      | -84°    |
| S. plicata     | 0.49| 0.04 | 0.32| <0.01| 0.15   | 0.66      | -79°    |
| Botryllus      | 0.52| <0.01| 0.18| <0.01| 0.15   | 0.70      | -60°    |
| H. monus       | 0.80| <0.01| <0.01| <0.01| 0.20   | 0.94      | -30°    |

*HexNH, hexosamine.

**These analyses were carried out after purification of the polysaccharides on a DEAE-cellulose column.

Fig. 2. Fractionation of the sulfated polysaccharides extracted from the tunics of various species of ascidians on Sepharose CL-4B and Sephadex G-200 columns. About 40 mg of the sulfated polysaccharides from the tunic of A. nigra (A), Clavelina sp. (B), and S. plicata (C) were chromatographed on a Sepharose CL-4B column (115 × 1.5 cm), eluted with 0.5 M pyridine-acetate buffer (pH 6.0) at a flow rate of 6 ml/h. Fractions of 1.5 ml were collected and assayed by the DuBois reaction (O...O) and by the metachromatic property (●—●). The inset in C shows the Sephadex G-200 gel chromatography of fraction F-2 from S. plicata. About 15 mg of the fraction F-2 obtained from the Sepharose CL-4B column were applied to a Sephadex G-200 column (90 × 1.5 cm), eluted with 0.5 M pyridine-acetate buffer (pH 6.0) at a flow rate of 2 ml/h. Fractions of approximately 1 ml were collected and assayed by the metachromatic property (●—●). The columns were calibrated using blue dextran as a marker for $V_0$, and cresol red as a marker for $V_v$.

Fig. 3. Electrophoresis of the purified fractions of sulfated polysaccharides from the tunics of various species of ascidians. About 25 µg of fractions F-1 and F-2 (or F-2-A and F-2-B) from the columns shown in Fig. 2 were subjected to agarose (A) and polyacrylamide (B) gel electrophoresis, as described in the legend of Fig. 1. The molecular weight (M.W.) markers used were chondroitin 6-sulfate (C-6-S), average $M_v = 40,000$; dermatan sulfate (D-S), average $M_v = 19,000$; dextran sulfate (Dex-S), average $M_v = 8,000$.

—132°), is compatible with residues of $\alpha$-L-galactopyranose, since the specific rotation of methyl $\alpha$-L-galactopyranoside is $-179°$. Furthermore, the presence of $\alpha$-L-galactopyranosyl units in the ascidian polysaccharides was demonstrated unequivocally by the finding that galactose in these glycans occurs only in the L-enantiomeric form and that its D-enantiomorph is lacking (7, 20). This finding indicates that the ascidian polysaccharides are unique among known galactose-rich polysaccharides (1, 21-24), in that their major constituent is L-galactose, the D-enantiomorph being entirely absent.

**Main Structural Features of Fraction F-1**

In spite of their chemical similarities (Table II) the very different electrophoretic mobilities of the various F-1 fractions (Fig. 3A) suggest important structural differences. These were examined in detail for A. nigra, Clavelina sp., and S. plicata, using periodate oxidation, methylation, $^1H$ NMR, and $^{13}C$ NMR, as described below.

**Periodate Oxidation**—The products obtained by acid hydrolysis of the periodate-oxidized F-1 fractions after borohy-
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**TABLE II**

| Species | Fraction | Gal | Man | G1c | Fuc | HexNH | SO/sugar | [a]θ°C |
|---------|----------|-----|-----|-----|-----|-------|----------|--------|
| Clavelina | F-1 | 0.72 | <0.01 | 0.19 | 0.09 | <0.01 | 0.70 | -104° |
| F-2 | 0.52 | <0.01 | 0.05 | 0.03 | 0.40* | 0.68 | -32° |
| A. nigra | F-1 | 0.67 | 0.09 | 0.17 | <0.01 | 0.11* | 0.17 | -100° |
| F-2 | 0.48 | 0.12 | 0.02 | <0.01 | 0.38* | 0.57 | -66° |
| S. plicata | F-1 | 0.82 | <0.01 | 0.14 | <0.01 | 0.04* | 0.50 | -132° |
| F-2-A | 0.20 | <0.01 | 0.57 | <0.01 | 0.23* | 0.73 | -32° |
| F-2-B | 0.21 | 0.12 | 0.27 | <0.01 | 0.40* | 0.67 | -13° |

* Approximately 90% of the hexosamine (HexNH) is glucosamine.
* Approximately 80% of the hexosamine is galactosamine.

**TABLE III**

| Product | tR | Molar ratio |
|---------|-----|------------|
| Glycerol | 1.0 | Clavelina | A. nigra | S. plicata |
| Erythritol | 4.1 | <0.01 | 0.05 | 0.04 |
| Threitol | 4.4 | 0.02 | 0.04 | 0.03 |
| Fucose | 5.6 | 0.14 | <0.01 | <0.01 |
| Mannose | 11.9 | <0.01 | 0.02 | <0.01 |
| Galactose | 13.3 | 0.84 | 0.48 | 0.62 |
| Glucose | 14.3 | <0.01 | 0.08 | 0.02 |
| Hexosamine | 21.8 | <0.01 | 0.05 | <0.01 |

* Retention time (tR) relative to glycerol on a packed 2% NPGS column on Chromosorb W 80/100 mesh.

mers. Regarding the amino sugars, it is possible that they occur at nonreducing ends or are 1→6 glycosidically linked units, since most of them disappear from F-1 of S. plicata and A. nigra after periodate oxidation.

**Methylation Studies**—The methylation studies of fraction F-1 from Clavelina sp. indicate that this polysaccharide is constituted mainly of a core of galactose-linked glycosidically through position 1→4 and sulfated at position 3. That is, 2,3,4,6-tetra-O-methylgalactose as aldito acetate derivative is the main methyl ether obtained from desulfated F-1, whereas 2,6-di-O-methylgalactose is a good candidate. Thus, small amounts of 6-mono-O-methylgalactose were obtained from desulfated F-1 of S. plicata. The sum of these derivatives does not amount to the proportion of tetra-O-methyl derivatives. This underestimation could be due to a low recovery of branching residues under our hydrolysis conditions. However, even when the methylated F-1 was hydrolyzed under milder conditions (90% formic acid, 1 h, 100 °C, followed by 0.15 M H2SO4, 16 h, 100 °C), the same proportion of methylated sugars as that reported in Table IV was obtained. Furthermore, we methylated and hydrolyzed glycogen under the same experimental conditions used for ascidian polysaccharides. The formation of equivalent proportions of 2,3,4,6-tetra-O-methylglycogen indicates that the underestimation of the branching residues in the ascidian polysaccharides cannot be attributed to our experimental protocol.

The presence of 2,4,6-tri-O-methylglactose in the methylation of desulfated F-1 from S. plicata (Table IV) indicates the presence of small but reproducible amounts of galactose glycosidically linked through position 1→3, and sulfated at position 4, whereas this type of unit is absent in F-1 from Clavelina sp. Methylation of F-1 from A. nigra produces essentially the same products as reported for F-1 from S. plicata, but the relative proportions of the methylated derivatives differ considerably between these two species. The distribution among the various methylated derivatives, without the preponderance of any type of linkage, suggests that this fraction in A. nigra may have a more heterogeneous structure. However, some distinctive features are observed. More 2,4,6-tri-O-methylgalactose is obtained from intact F-1 of A. nigra, and the level increases 2-fold after desulfation. Therefore, F-1 from A. nigra includes large amounts of 1→3-linked galactose residues that are partially sulfated at position 4.

For A. nigra a better recovery of the branching galactose units may account for the higher yields of 6-mono-O-methylgalactose, which after desulfation yields 3,6-di-O-methyl-
protons of the sulfate-substituted carbons. Overall, the
in the vicinity of were recorded with 99.8% D₂O as the solvent, at 60
field by about 0.42-0.74 ppm (25, 26), these signals in the
or totally disappear (Fig. 4E) after chemical desulfation. Since
however, they strongly decrease in relative intensity (Fig. 4C)
a-@-galactopyranoside (4.3-4.6 ppm) (25, 27) are also found;
derivatives of galactose, glucose and/or mannose, which did
increase after desulfation, indicates that many nonsul-
fated end groups are present in F-1 from
CZuzeZina
intact
degradation
file at position 3 of the galactose residues and indicates that
the branching occurs only in the 14-linked units. As already
cross-hatched.
The formation of large amounts of 2,3,4,6-tetra-O-methyl
methylated sugars (as alditol acetates) of the ascidian polysaccharides
Table IV
Methylation analysis of the ascidian polysaccharides

| Methylated sugars | 6s | F-1 from Clavelina sp. Sulfated | Desulfated | F-1 from A. nigra Sulfated | Desulfated | F-1 from S. plicata Sulfated | Desulfated |
|-------------------|----|-------------------------------|------------|----------------------------|------------|----------------------------|------------|
| 2,3,4,6-Glc       | 1.00 | 0.03                          | 0.04       | 0.21                       | 0.19       | 0.05                       | 0.05       |
| 2,3-Fuc           | 1.08 | ND                            | 0.10       | ND                         | ND         | ND                         | ND         |
| 2,3,4,6-Gal       | 1.11 | 0.10                          | 0.08       | 0.20                       | 0.16       | 0.19                       | 0.35       |
| 2-Fuc             | 1.22 | 0.10                          | ND         | 0.20                       | 0.16       | ND                         | ND         |
| 2,4,6-Gal         | 1.47 | 0.09                          | 0.36       | 0.18                       | 0.07       | 0.09                       | 0.34       |
| 2,3,6-Gal         | 1.57 | 0.14                          | 0.58       | 0.18                       | 0.07       | 0.08                       | 0.34       |
| 2,3,6-Glc         | 1.63 | 0.06                          | 0.18       | 0.07                       | 0.10       | 0.13                       | 0.05       |
| 2,6-Gal           | 1.98 | 0.45                          | 0.02       | 0.05                       | 0.10       | 0.40                       | 0.03       |
| 3,6-Gal           | 2.32 | ND                            | ND         | ND                         | ND         | ND                         | ND         |
| 6-Gal             | 2.44 | 0.03                          | ND         | 0.11                       | 0.02       | 0.06                       | 0.02       |

*The identity of each peak was established by mass spectrometry. Major derivatives in each species are underlined.

Retention time (tR) on AN-600 capillary column relative to 2,3,4,6-tetra-O-methyl glucitol.

The molar ratios are based on the area of each peak compared with total area.

This peak may contain 2,3,4,6-tetra-O-methyl mannitol, which has the same retention time as 2,3,4,6-tetra-O-
methyl glucitol.

ND = not detected.

The 1H NMR spectra also show a signal at approximately δ = 2.0 ppm in the F-1 fraction from S. plicata (Fig. 4A)
and A. nigra (Fig. 4F), but it is absent in F-1 from Clavelina sp. (Fig. 4D). Since the chemical analyses show amino sugars
only in F-1 from S. plicata and A. nigra (Table II), and since these polymers resist deaminative cleavage by nitrous acid (6,
7), we attribute this signal at δ = 2.0 ppm to acetamido methyl. Another peak at approximately δ = 1.3 ppm, which is attributed
to deoxymethyl, occurs only in F-1 from Clavelina sp. This signal is rather large in the 1H spectrum, more consistent
with about 20% of fucose than the 9-10% that we found by
calorimetric analysis of this fraction (Tables II and IV). However, we have no explanation for this discrepancy.

Interestingly, F-1 from S. plicata after Smith degradation shows a 1H NMR spectrum with much sharper peaks (Fig.
4B), which strongly resembles the spectrum of F-1 from Clavelina sp. (Fig. 4D). In fact, the chemical shifts of the
protons in the anomeric region are very similar for both polysaccharides, except for the peak at δ = 4.78, which is absent in the
1H NMR spectrum of F-1 from Clavelina sp.

13C NMR—The 13C NMR spectra obtained from F-1 of the three species of ascidians (Figs. 5, A, C, and D) show a major signal
in the region of anomeric carbon, which resonates at δ = 101.3 ppm. This result shows that the major anomeric
Carbon of F-1 is more strongly shielded than is the correspond-
ing carbon of β-galactopyranoside, which resonates at δ = 103.4-105.2 ppm (28, 29). In addition, the anomeric carbon of
F-1 resonates in the range expected for an α-galactopyranoside (97.7-101.5 ppm) (28, 29). The 13C NMR spectra show
also an intense signal attributed to nonsubstituted carbon 6,
which resonates at δ = 62.4-60.7 ppm. This result agrees with the
methylation studies in showing the absence of substitution
at carbon 6.

Fraction F-1 from Clavelina sp. affords a well resolved 13C
spectrum (Fig. 5D). Six signals are clearly distinguished. From
the shapes of these signals and their relative intensities, it is possible to conclude that this is a polysaccharide containing
mainly six nonequivalent carbons. Because of their distinctive

FIG. 4. Proton magnetic resonance spectra at 400 MHz of
intact F-1 from S. plicata (A), F-1 from S. plicata after Smith
degradation (B) or chemical desulfation (C), intact F-1 from
Clavelina sp. (D), F-1 from Clavelina sp. after chemical de-
sulfation (E), and intact F-1 from A. nigra (F). The spectra
were recorded with 99.8% D₂O as the solvent, at 60 °C. D₂O peak is
cross-hatched. HOD, hydrogen oxygen deuterium.

Table IV
Methylation analysis of the ascidian polysaccharides

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Galactose. This result also confirms the presence of sulfate
ester at position 3 of the galactose residues and indicates that
the branching occurs only in the 1→4-linked units. As already
mentioned for F-1 from S. plicata, these branching derivatives are probably underestimated.

The formation of large amounts of 2,3,4,6-tetra-O-methyl
derivatives of galactose, glucose and/or mannose, which did
not increase after desulfation, indicates that many nonau-
fated end groups are present in F-1 from A. nigra.

1H NMR—The 1H NMR spectra of F-1 fractions from the	hree species of ascidians (Figs. 4, A, D, and F) show signals in the
vicinity of δ = 4.7 and 5.3 ppm, which are consistent with
the anomeric protons of α-galactopyranosides (25, 26).

Several other signals that resonate in the range expected for
a β-galactopyranoside (4.3-4.6 ppm) (25, 27) are also found;
however, they strongly decrease in relative intensity (Fig. 4C)
or totally disappear (Fig. 4E) after chemical desulfation. Since
protons from carbons bearing a sulfate ester are shifted down-
field by about 0.42-0.74 ppm (25, 26), these signals in the
region of the β-anomeric proton are probably attributable to
protons of the sulfate-substituted carbons. Overall, the 1H
NMR analysis confirms the preponderance of α-anomeric
protons in all F-1 fractions. These observations agree with
the proposal that the F-1 fractions are composed mainly of α-
galactopyranosyl units, as already suggested by their strong
negative optical rotations (Table II).
chemical shifts, signals at δ = 101.3 and 60.7 ppm are readily attributable to the anomeric carbon and nonsubstituted carbon 6, respectively. Based on the methylation studies (Table IV), it is possible to conclude that most of carbons 2 and 5 in F-1 from Clavelina sp. are neither sulfated nor substituted by glycosidic linkage. Therefore, signals at δ = 68.6 and 72.6 ppm, which resonate in the region of nonsubstituted secondary carbons, may be attributed tentatively to carbons 2 and 5, respectively (29). Two other signals ascribed to substituted secondary carbons resonate at δ = 77.7 and 77.1 ppm. From the methylation data (Table IV), it is possible to attribute these signals to carbons 3 and 4, which are sulfated and substituted by glycosidic linkage, respectively. However, the specific assignment of these two peaks is not possible because a similar downfield displacement with respect to the parent sugars is observed for the shifts of carbon atoms carrying a sulfate group (30–32), or substituted by a glycosidic linkage (29).

For fractions F-1 from S. plicata (Fig. 5A) and A. nigra (Fig. 5C), more complex 13C NMR spectra were obtained. The 13C nuclei of these polysaccharides resonate at δ = 72.4–68.2 ppm and a large variety of signals attributable to glycosidically linked or sulfated secondary carbons resonate at δ = 80.2–74.3 ppm. The complexity of the spectra in the region of substituted secondary carbons does not permit the identification of the main glycosidically or sulfate-substituted carbons. However, the spectra demonstrate that the 13C nuclei of both F-1 fractions are more strongly shielded than are the corresponding nuclei of α- or β-galactofuranosides, which resonate at 84.7–71.7 ppm (34). The 13C nuclei of both F-1 fractions resonate in the range expected for an α- or β-galactopyranoside (71.6–69.2 ppm) (35, 36).

The 13C spectrum of A. nigra (Fig. 5C) shows small signals of acetamido sugar, as suggested by the chemical analysis (Table II). However, the spectrum of S. plicata (Fig. 5A) has only a minor signal for acetamido methyl and the expected signal corresponding to carbon 2 of the 2-deoxy-2-acetamido hexose at about δ = 55 ppm appears to be lost in the noise.

The methylation studies (Table IV) suggest that the major heterogeneity of F-1 from S. plicata occurs in the sugar residues at the nonreducing end, while the carbohydrate core of this fraction is similar to that of F-1 from Clavelina sp. Therefore, it is expected that the Smith degradation, which oxidizes the periodate-sensitive sites and removes the polyalcohols formed by mild acid hydrolysis, would produce from F-1 of S. plicata a polysaccharide similar to F-1 of Clavelina sp. In fact, F-1 from S. plicata after Smith degradation exhibits a 13C NMR spectrum (Fig. 5B) that closely resembles the 13C spectrum of intact F-1 from Clavelina sp. (Fig. 5D).

Furthermore, F-1 from S. plicata after Smith degradation produces a simpler 1H NMR spectrum, with much sharper peaks and a lower acetamido methyl signal (Fig. 4B), which resembles the 1H NMR spectrum of intact F-1 from Clavelina sp. (Fig. 4D). In addition, the chemical analysis of F-1 from S. plicata after Smith degradation shows an increase in the relative proportions of L-galactose and sulfate, lower hexosamine and glucose contents, and a stronger negative optical rotation (Table V). These results indicate that the Smith degradation removes mainly the nonreducing end groups, which are mostly nonsulfated, and the intrachain glucose residues, which are linked through position 1→4, increasing the relative proportion of 3-sulfated α-L-galactopyranosyl units in the polymer.

### Table V

| F-1 Treatment | Molar ratios | [α]D (°) |
|---------------|-------------|---------|
| Intact        | Gal         | Glc     | HexNH⁺ | Sulfate/total sugar | [α]D (°) |
| Smith degradation | 0.84       | 0.10     | 0.06  | 0.50                           | -132°      |
|               | 0.97      | 0.03     | <0.01 | 0.80                           | -160°      |

*HexNH⁺, hexosamine.

### Conclusion

In the present work sulfated polysaccharides were extracted from the tunics of several species of ascidians and fractionated by gel filtration (Fig. 2). A high molecular weight fraction (F-1), which contains high amounts of L-galactose, is obtained from all species. The other fraction (F-2 or F-2-A and F-2-B) has a molecular weight and a chemical composition that vary greatly among the different species.

It is interesting that F-1 from Clavelina sp. differs from F-1 of the other two ascidians in the amounts of branching units. Clavelina sp. is considered to be a more primitive tunicate, as compared to S. plicata and A. nigra (36). Therefore, we can speculate that the addition of nonreducing end units, possibly by a different biosynthetic enzyme, occurred only in the later evolution of the tunicates.

This comparison is based on analysis of the main structural features of fraction F-1 from the three species of ascidians. Fig. 6 summarizes the main galactose units found in these polysaccharides. Large amounts of α-L-galactopyranose residues, sulfated at position 3 and linked glycosidically through positions 1 and 4 (Fig. 6), are present in all F-1 fractions. However, α-L-galactopyranose units, 1→3-linked and partially sulfated at position 4, are present in large amounts only in F-1 from A. nigra (3, Fig. 6C). They occur in small amounts in F-1 from S. plicata (Fig. 6A) and are absent in F-1 from Clavelina sp. (Fig. 6B). The methylation studies (Table IV) show high amounts of L-galactopyranose nonreducing end units in F-1 from A. nigra and S. plicata, indicating that both F-1 fractions are highly branched polymers. However, methylation (Table IV) and 13C NMR (Fig. 5D) show that F-1 from Clavelina sp. is distinguished by a paucity of nonreducing end units.

Fraction F-1 from S. plicata subjected to Smith degradation, which oxidizes the periodate-sensitive sites and removes the polyalcohols formed by mild acid hydrolysis, gives a 13C NMR spectrum (Fig. 5B) similar to that of intact F-1 from Clavelina sp. (Fig. 5D). Both 13C NMR spectra contain mainly six
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Fig. 6. Hypothetical structure for the main galactose units in F-1 fraction from *S. plicata* (A), *Clavelina sp.* (B) and *A. nigra* (C). Fractions F-1 from all species contain high amounts of α-D-galactopyranose residues, sulfated at position 3 and linked glycosidically through position 1→4 (1). Furthermore, α-L-galactopyranose units 1→3-linked and partially sulfated at position 4 (3) are present in high amounts in F-1 from *A. nigra* (C), but only in minor amounts in F-1 from *S. plicata* (A), and are absent in F-1 from *Clavelina sp.* (B). Non sulfated L-galactopyranose nonreducing end units occur in high concentration in F-1 from *A. nigra* (C) and *S. plicata* (A), but only in minor amounts in F-1 from *Clavelina sp.* (B). Our studies did not determine the branching point of these polymers, although the O-2 position is a good candidate. The methylation studies (Table IV) of F-1 from *A. nigra* show some evidence that the branching point is the O-2 position of the α-1-galactopyranose units, 1→4-linked (1). *Galp*, galactopyranose; *GlcP*, glucopyranose; *ManP*, manno.pyranose.

non-equivalent carbons. Two of them resonate in the region of the substituted second carbons, as expected for a linear polysaccharide whose units are sulfated. Such results confirm that the major heterogeneity of F-1 from *S. plicata* is the presence of nonreducing end units.

The F-1 fractions from the various ascidians are unique among previously described sulfated glycanas. The main galactose-rich sulfated polysaccharides described in living tissues are keratan sulfate and carrageenans. Keratan sulfate, which occurs mainly in mammalian cartilages and corneas, is composed of β-D-galactopyranose units 1→4-linked glycosidically to N-acetyl-D-glucosamine 6-sulfate (19). The algal carrageenans present a more heterogeneous structure (1). They are linear chains of β-D-galactopyranose residues linked glycosidically through position 1→3 to α-galactopyranose. The α-galactopyranose can occur in either D or L form or can be wholly or partly converted to 3,6-anhydro forms. The sulfate ester may occur at positions 2, 4, or 6 of the galactose residues in the carrageenans.

Fraction F-1 from the ascidians differs from these previously described sulfated polysaccharides not only in the types of linkages and position of sulfation, but also in the extensive branching (except F-1 from *Clavelina sp.*), while both keratan sulfate and carrageenans are linear polysaccharides. Furthermore, the ascidian glycans are the first group of polysaccharides that contain large amounts of α-L-galactose and not its D-enantiomorph.

The presence of extensive branching and the abundance of sulfate ester in these polysaccharides may increase their water binding capacity. As in the cartilaginous proteoglycans of vertebrates, this property would contribute to the resilience of the tissue.

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Extraction of the sulfated polysaccharides from the tissue of ascidians: The polysaccharides were extracted from the tissue by papain as previously described for S. plumosum (7).

Fractionation of the sulfated polysaccharides

1) DEAE-cellulose countercurrent. About 200 mg of the polysaccharides extracted from the tissue were applied to a DEAE-cellulose column (7 x 2 cm) equilibrated with 0.1 M sodium acetate buffer (pH 5.0) and washed with 100 ml of the same buffer. The column was developed by a linear gradient prepared by mixing 0 ml of 0.1 M sodium acetate buffer (pH 5.0) with 40 ml of 1.0 M NaCl and 50 ml of 2.0 M NaCl in the same buffer. The flow rate of the column was 12 ml/hr, and fractions of 30 ml were collected. They were basified with the Dubois et al. reaction (11) and for ultraviolet-visible and nuclear material. The fractions containing the sulfated polysaccharides were pooled, dialyzed against distilled water, and lyophilized.

2) Sephadex CL-4B. About 40 mg of the sulfated polysaccharides were applied to a Sephadex CL-4B column (1.5 x 14 cm), eluted with 0.5 M sodium acetate buffer (pH 6.0) at a flow rate of 2 ml/hr. Fractions of approximately 1.5 ml were collected and assayed by the Dubois et al. reaction (11) and by the anthrone method (17).

Sulfated polysaccharides were analyzed by electrophoresis. The sulfated polysaccharides were estimated by polyaniline gel electrophoresis (7,32). For evaluation of relative charge density, the average molecular weights of the sulfated polysaccharides were estimated by polyaniline gel electrophoresis (7,32).

Chemical analysis. Total hexose was measured by the phenol-sulfuric acid method of Dubois et al. (11). After acid hydrolysis (6.0 M trifluoroacetic acid, 100°C for 3 hr), the sulfated polysaccharides were treated with a modified Bunslow-Morgan reaction (14) and by the Vanillin-sulfuric acid method (13), respectively. Standard curves for hexuronic acid were constructed from glucuronic acid and by the hydrolysis of the corresponding Als suit (16) and by the Vanillin-sulfuric acid method. The proportions of the different hexosamine and hexuronic acids were determined by gas-liquid chromatography of the corresponding Alc acetates (18) and by paper chromatography in butanol-acetic acid-water (7:6:1, v/v/v) for 3 hr or in isopropyl alcohol-1.0 M NH₄OH (5:3, v/v). The sulfates were detected on the chromograms by silver nitrate staining.

Physical modification of the polysaccharides

1) Methylation. About 10 mg of the intact or of the desulfated F-1 fraction was methylated by the method of(17) with the modifications introduced by (18). The methylated polysaccharides were hydrolyzed with 0.5 M trifluoroacetic acid for 2 hr at 100°C and reduced with borohydride, and the alditols were acetylated (18). The alditol acetates from the methylated sugars were analyzed by a Jeol D-300 600-MHz spectrometer. Spectra were scanned in the region from 2.5 to 6.0 ppm.

2) Molecular rotation. About 5 mg of the intact or desulfated F-1 fraction was dissolved in a 0.05 M NaCl solution. The rotation was measured in the region from 2.5 to 6.0 ppm.

3) NMR spectra. 300 MHz spectra (600 MHz on a JEOL D-300 spectrometer) with respect to internal sodium 4,4-dimethyl-4-diphenylmethane-1-sulfonate as internal standard were measured in CDCl₃.

4) Other methods. Optical rotations were measured with a digital polarimeter (Perkin-Elmer, model 241-B). Infrared spectra were recorded with a Perkin-Elmer infrared spectrometer, model 248.