Distribution of Sulfated Mucopolysaccharides in Invertebrates*

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The sulfated mucopolysaccharide composition of 22 species of invertebrates belonging to the phyla Arthropoda, Mollusca, Annelida, Tunicata, Echinodermata, Coelenterata, and Porifera was analyzed. It is shown that all the species contain variable amounts of one or more types of sulfated mucopolysaccharides, most of which similar to the ones found in vertebrates. It is shown also that each species has a characteristic composition, differing from each other regarding the relative amount and type of chondroitin sulfates A, B, and C, heparitin sulfate, and heparin. The possible biological role of the sulfated mucopolysaccharides in cell recognition or aggregation or both is discussed in view of the present findings.

It is now evident that all mammalian tissues as well as stabilized mammalian cell lines in culture contain a variety of sulfated mucopolysaccharides (1-3). It has also been recently observed that each mammalian tissue has a characteristic composition, differing from each other regarding the relative amount, type, and molecular size of chondroitin sulfates A and C, chondroitin sulfate B, and heparitin sulfate (4). Among the sulfated mucopolysaccharides, heparitin sulfate was present in all the tissues examined varying from one tissue to another in its relative concentration and molecular weight. The ubiquitous presence of these sulfated mucopolysaccharides in all the tissues, together with other data, led to the suggestion that heparitin sulfate, and to some extent other sulfated mucopolysaccharides, might be involved in the process of cell differentiation or maturation (or both) being responsible for cell adhesiveness and recognition, contact inhibition, etc. (4). If this were true, heparitin sulfate and other sulfated mucopolysaccharides should be present in all organisms that exhibit some tissue organization.

The presence of sulfated mucopolysaccharides in Chordata, besides mammals, is already well documented (1, 5). Conversely, data on the distribution of sulfated mucopolysaccharides in invertebrates are scarce and only some evidences for the presence of these compounds in a few organisms have been shown (1, 5-12). Thus, it seemed important to verify to what extent these compounds are distributed in such organisms.

This paper reports the main types of sulfated mucopolysaccharides found in some species of Insecta, Crustacea, Arachnida, Mollusca, Annelida, Tunicata, Echinodermata, Coelenterata, and Porifera. Preliminary communications of parts of this work have appeared (13, 14).

MATERIALS AND METHODS

Invertebrates—The following invertebrates were used in the present study: Insecta: Cornitermis cumulans (termite), Componotus rufipes (ant), and Periplaneta americana (cockroach); Crustacea: Penaeus brasiliensis (shrimp), Scyllarides brasiliensis (lobster), and Callinectes sapidus (crab); Arachnida: Nephila clavipes (spider); Mollusca: order Filobranchia, Aulocombia ater and Perun perna, order Eulamellibranchia, Meaddea lamarchii and Anomalocardia brasilienensis, order Dibranchia, Loligo brasilienensis and Octopus sp.; Annelida: Phertima hauoyana; Tunicata: Ascidia nigra and Styella pilosa; Echinodermata: Holothuria grisea and Tarethina variegata; Coelenterata: two species from the order Actiniaria, sub-order Actiniaria (not further classified); Porifera: two species from the order Spongiaria.

The exoskeleton of the species belonging to the class Crustacea and the shells of the organisms belonging to the orders Filobranchia and Eulamellibranchia were removed prior to the extraction of the sulfated mucopolysaccharides.

Extraction of Sulfated Mucopolysaccharides—The organisms were grinded with 10 volumes of acetone and kept overnight. The extracts were centrifuged and the precipitate washed once with acetone and dried under vacuum. The dried material (1 g) was incubated with 10 mg of trypsin in 0.05 M Tris/HCl buffer, pH 8.0, in a final volume of 20 ml, under a layer of toluene. After 24 h incubation, the pH of the suspension was brought to 11.0 and maintained at 25° for 8 h. The suspension was then brought to pH 6.9 and centrifuged. After the addition of 2 volumes of alcohol to the supernatant, the mixture was kept at 5° overnight. The precipitate formed was collected by centrifugation and resuspended in 5 ml of 90% of ammonium sulfate solution. The precipitate was removed by centrifugation and the supernatant dialyzed against distilled water and dried and further purified by preparative agarose gel electrophoresis as previously described (15). The fractions containing sulfated mucopolysaccharides were eluted from the gel by the freeze-thawing procedure (16), pooled, concentrated, and the sulfated mucopolysaccharides were precipitated with 2 volumes of alcohol. The precipitate formed was dried and resuspended in 100 µl of water. In some preparations DNA and RNA still contaminated the purified sulfated mucopolysaccharides, and were removed by treatment with DNase and RNase as previously described (4).

Identification and Quantitation of Sulfated Mucopolysacchari-
ridae. The sulfated mucopolysaccharides were identified and quantified by a combination of agarose gel electrophoresis and enzymatic degradation with specific mucopolysaccharidases as previously described (4, 17-20). Briefly, the method consisted in subjecting the sulfated mucopolysaccharides mixture to electrophoresis in agarose gel using barbital or 1,3-diaminopropane-acetate buffer (or both) before and after degradation with chondroitinase AC, chondroitinase ABC (Miles Laboratories), heparinas, and heparitinases. The disappearance of any sulfated mucopolysaccharides from the stained agarose gel is a first indication of the type of compound present. This is exemplified in Fig. 1 in which a mixture of heparitin sulfate, heparitin sul fate B, and chondroitin sulfate A were treated with the different enzymes. All four sulfated mucopolysaccharides are degraded by the crude extracts prepared from induced Flavobacterium heparinum cells that contain all the mucopolysaccharides. Heparitinase I plus chondroitinase ABC degrades all the sulfated mucopolysaccharides but heparin. Chondroitinase AC degrades only chondroitin sulfates A and C, and, finally, chondroitinase ABC degrades chondroitin sulfates A, B, and C, but not heparin and heparitin sulfate (Fig. 1). Also the sulfated mucopolysaccharides were further characterized by the type of degradation products formed by the action of the mucopolysaccharidases. The products were subjected to chromatography in isobutyric acid:1 m NH₄, 5:3, v/v (Solvent A), and stained with silver nitrate reagent or toluidine blue.

Other Methods - Amino sugars were measured after acid hydrolysis (4 m HCl) for 6 h at 100° by a modified Elson Morgan reaction (21). Uronic acid was measured by the Dische’s carbazole reaction (22). Sulfate was measured by a recently described procedure (23). Anticoagulant activity was measured by the USP assay. Sugars released after acid hydrolysis were identified by chromatography in Solvent A and in butanol:pyridine:water, 6:4:3, by volume (Solvent B).

RESULTS

Electrophoresis of Sulfated Mucopolysaccharides - The agarose gel electrophoresis of sulfated mucopolysaccharides extracted from 22 species of invertebrates is shown in Fig. 1. The sulfated mucopolysaccharides from all the species analyzed show one to four components in electrophoresis, most of them with electrophoretic migrations similar to the sulfated mucopolysaccharides standards and all of them staining metachromatically with toluidine blue (purple to bright red).

Sulfated Mucopolysaccharides of Insecta - Fig. 1, II shows the agarose gel electrophoresis of sulfated mucopolysaccharides obtained from ant, cockroach, and termite. The sulfated mucopolysaccharides from termite contain at least two components, whereas the materials from ant and cockroach show only one band with an intermediate migration between heparitin sulfate and chondroitin sulfate B standards. The sulfated mucopolysaccharides obtained from the three organisms were incubated with chondroitinase ABC and extracts from heparin-induced Flavobacterium heparinum and subjected to agarose gel electrophoresis and paper chromatography. The chondroitinase ABC degraded partially the sulfated mucopolysaccharides present in the cockroach, almost completely the sulfated mucopolysaccharides present in the ant, and the upper band of the sulfated mucopolysaccharides present in termite, indicating the presence of chondroitin sulfate in these organisms (Fig. 1). The extracts of F. heparinum degraded completely all the sulfated mucopolysaccharides from ant and cockroach and degraded partially the sulfated mucopolysaccharides present in termites. The degradation products formed from the sulfated mucopolysaccharides upon the action of the enzymes are shown in Fig. 1. The sulfated mucopolysaccharides from termite and cockroach formed as main degradation products glucosamine 2,6-bisulfate and N-acetylgalactosamine by the action of F. heparinum extracts. These compounds are also the main products formed from heparitin sulfate by the action of these extracts (Fig. 2).

The main disaccharides formed from the sulfated mucopolysaccharides of termite when incubated with chondroitinase ABC are ΔDi-6S and small amounts of ΔDi-4S, whereas ΔDi-4S is the main disaccharide formed from the sulfated mucopolysaccharides of cockroach when incubated with the same enzyme, indicating the presence of chondroitin and chondroitin sulfate A or B, or both, respectively, in these species.

Sulfated Mucopolysaccharides of Crustacea - The agarose gel electrophoresis of sulfated mucopolysaccharides extracted from shrimp, lobster, and crab is shown in Fig. 1, VI. The sulfated mucopolysaccharides from the shrimp and crab show the presence of at least two compounds with different electrophoretic migrations. Lobster sulfated mucopolysaccharides shows the presence of only one component.

The results on the degradation of these compounds with the specific mucopolysaccharidases are shown in Fig. 1. The sulfated mucopolysaccharides from the three organisms analyzed are completely degraded by the F. heparinum extracts. The products formed by the action of these enzymes differ from each other. Whereas the sulfated mucopolysaccharides from shrimp and crab form mainly glucosamine 2,6-bisulfate and N-acetylgalactosamine, the lobster sulfated mucopolysaccharides form glucosamine 2,6-bisulfate and glucosamine N-sulfate as the main products (Fig. 3). This is compatible with the suggestion that the sulfated mucopolysaccharides from shrimp and crab are heparitin sulfates. The finding that no N-acetylgalactosamine is formed from lobster sulfated mucopolysaccharides suggests that this sulfated mucopolysaccharide is a heparin-like compound.

The sulfated mucopolysaccharides that migrate as chondroitin sulfate A/C from crab is degraded by both chondroitinase AC and chondroitinase ABC producing ΔDi-6S and ΔDi-4S as the main disaccharide products (Fig. 3). This strongly suggests that chondroitin sulfates A and C are present in this organism. Lobster sulfated mucopolysaccharides also shows the presence of small amounts of another band with migration similar to chondroitin sulfate B, which disappears from the stained agarose gel only after chondroitinase ABC treatment, but not by the action of chondroitinase AC (Fig. 1, VIII).

Similarly a band present in sulfated mucopolysaccharides from shrimp disappears after chondroitinases AC and ABC treatment (Fig. 1, VII). The product formed by the action of these enzymes has a higher chromatographic migration than the known chondroitin sulfate disaccharides and remain to be identified (Fig. 3).

Sulfated Mucopolysaccharides in Arachnida - Fig. 1, X shows an agarose gel electrophoresis of sulfated mucopolysaccharides extracted from one species of spider as well as the action of the mucopolysaccharidases upon the sulfated mucopolysaccharides. Two compounds are present, one with electrophoretic migration of chondroitin sulfate A/C that disappears after treatment with chondroitinases AC and ABC, and another with the migration of heparitin sulfate that disappears only after treatment with crude F. heparinum extracts. This result suggests the presence of chondroitin sulfate A or C, or both, and heparitin sulfate in Arachnida.

Sulfated Mucopolysaccharides of Mollusca - The agarose gel electrophoresis of sulfated mucopolysaccharides extracted from the six species analyzed is shown in Fig. 1, XI. As it can be observed, the electrophoretic pattern of the two species of the order Filobanchia are very similar. Both species show the presence of sulfated mucopolysaccharides with electrophoretic migrations similar to chondroitin sulfate A and heparin/ heparitin sulfate. In the order Eullamelibranchia, both species show only a sulfated mucopolysaccharide with the migration

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FIG. 1. Agarose gel electrophoresis of sulfated mucopolysaccharides. About 100 μg of sulfated mucopolysaccharides were incubated with 0.05 units of chondroitinase AC, chondroitinase ABC, heparitinase, and 100 μg of crude Flavobacterium heparinum extracts in 0.1 M ethylenediamine/acetate buffer, pH 7.0, for heparitinase I and F. heparinum extracts and 0.01 M Tris/HCl buffer, pH 8.0, for the chondroitinases in a final volume of 20 μl. After 12 h incubation, 3-μl aliquots of the incubation mixtures were applied in agarose gel slabs and subjected to electrophoresis for 1 h at 100 V. The sulfated mucopolysaccharides were then stained as described under "Materials and Methods." 1, Sulfated mucopolysaccharides standard mixture of chondroitin sulfate A, chondroitin sulfate B, heparitin sulfate, and heparin incubated with the enzymes: a, F. heparinum extracts; b, chondroitinase ABC and heparitinase I; c, chondroitinase AC; d, chondroitinase ABC; e, H2O; f, standards. 11, Sulfated mucopolysaccharides from Insecta: a, cockroach; b, ant; c, termite; s, sulfated mucopolysaccharides standard. 11, Sulfated mucopolysaccharides from cockroach, termite, and ant, respectively, incubated with the enzymes: a, F. heparinum extracts; b, chondroitinase ABC; c, H2O; s, standards. 11, Sulfated mucopolysaccharides from Crustacea; a, shrimp; b, lobster; c, crab; s, standards. 111, Sulfated mucopolysaccharides from Cnidaria: a, Gymnopolis; b, Anthomedusa; c, Medusa; d, Hydromedusa; e, Stelletta plicata; f, Ascidia nigra; s, standards.
formed higher amounts from the species of Filobranchia, whereas glucosamine 2,6-bisulfate and free glucosamine (products characteristic of heparin) are the main products formed from the species of the order Eulamellibranchia. These combined results suggest that heparitin sulfate and chondroitin sulfate AC are the main sulfated mucopolysaccharides formed in higher amounts from the species of Filobranchia, whereas glucosamine 2,6-bisulfate and free glucosamine (products characteristic of heparin) are the main products formed from the species of the order Eulamellibranchia. Anticoagulant activities by the USP assay confirmed these results. The sulfated mucopolysaccharides from the order Eulamellibranchia had an anticoagulant activity of 150 IU/mg similar to mammalian heparin.

The sulfated mucopolysaccharides present in species from the order Cephalopoda were incubated with Flavobacterium heparinum extracts upon the sulfated mucopolysaccharides in the organism. The sulfated mucopolysaccharides of both species. The products formed by the action of these enzymes are shown in Fig. 7. The chondroitinase AC and ABC formed a disaccharide from the sulfated mucopolysaccharides obtained from Lolligo with chromatographic migration similar to the disaccharide obtained from chondroitin sulfate E extracted from the cartilagenous tissue of squid (24). It is important to note that the cartilagenous tissue was removed from this organism prior to extraction of the sulfated mucopolysaccharides in the present study. Besides this disaccharide, smaller amounts of disaccharides characteristic of chondroitin sulfates A and C are formed by the action of both enzymes. The sulfated mucopolysaccharides of Octopus are degraded to ADi-OS and ADi-4S. Also glucosamine 2,6-bisulfate is formed in small amounts by the action of F. heparinum extracts upon the sulfated mucopolysaccharides of both species. The sulfated mucopolysaccharides of Annelida—Only a single band of sulfated mucopolysaccharides with an intermediate migration between heparitin sulfate and chondroitin sulfate B was observed in agarose gel electrophoresis. The compound was resistant to the action of chondroitinase AC and ABC, but was completely degraded by F. heparinum extracts (Fig. 1, XII). This result suggests the presence of heparin sulfate in the organism.

Sulfated Mucopolysaccharides of Coelenterata—The agarose gel electrophoresis of the sulfated mucopolysaccharides extracted from the two species of Coelenterata is shown in Fig. 1, XIII and XIV. One of the species (A) shows the presence of only one band of sulfated mucopolysaccharides with migration of heparitin sulfate standard, while the other (B) besides this sulfated mucopolysaccharide shows the presence of a slow migrating compound. All these sulfated mucopolysaccharides were resistant to the action of chondroitinase AC and ABC, but were completely degraded by F. heparinum extracts (Fig. 1, XIII and XIV). These results suggest the presence of heparin-like or heparitin sulfate-like compounds in these organisms.

Sulfated Mucopolysaccharides of Porifera, Echinodermata, and Tunicata—Several sulfated mucopolysaccharides bands were present in all of the six species analyzed (Fig. 1, XV). All these compounds were incubated with chondroitinase AC, chondroitinase ABC, and F. heparinum extracts. Only the bands with the same electrophoretic migrations of chondroitin sulfate B from Porifera and Ascidia nigra were completely degraded only by chondroitinase ABC (not shown).

This result suggests the presence of chondroitin sulfate B in these two organisms. The other compounds were only partially degraded by F. heparinum extracts. Nevertheless no identifiable products could be detected by chromatography. Since these compounds were only partially degraded by the enzyme mixture, hexosamine, sulfate and uronic acid determinations were performed to ascertain their mucopolysaccharide nature. The results of these determinations are shown in Table I. The ratios of hexosamine/uronic acid/sulfate obtained are indicative that these compounds are indeed sulfated mucopolysaccharides. Besides glucosamine and galactosamine small
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Fig. 5. Products formed from sulfated mucopolysaccharides of Mollusca by the action of Flavobacterium heparinum extracts. The experiment was performed as described in Fig. 2 except that sulfated mucopolysaccharides from different organisms were used: 1, Perna perna; 2, Mesodesma donacium; 3, Anomalocardia brasiliense. See legend to Fig. 2 for definition of abbreviations.

Fig. 6. Densitometry of sulfated mucopolysaccharides from Mollusca after degradation with mucopolysaccharidases and electrophoresis. The experiment was performed as described in Fig. 4 except that sulfated mucopolysaccharides from different organisms were used as indicated. After electrophoresis and toluidine blue staining the sulfated mucopolysaccharides remaining after the action of the different enzymes were quantitated by densitometry. Absorbance, arbitrary densitometric units at 650 nm.

Fig. 7. Products formed from sulfated mucopolysaccharides of Mollusca by the action of the different mucopolysaccharidases. The experiment was performed as described in Fig. 2 except that sulfated mucopolysaccharides from Lolligo (1) and Octopus (2) were used. A, Flavobacterium heparinum extracts; B, chondroitinase ABC; C, chondroitinase AC; D, water. See legend to Fig. 2 for definition of abbreviations.

The total amounts of sulfated mucopolysaccharides extracted from the invertebrates and the ratios of hexosamine, uronic acid, and sulfate of these sulfated mucopolysaccharides are shown in Table I. Large variations in the amounts of sulfated mucopolysaccharides were observed among the species analyzed. Most of the marine organisms contain much higher amounts of sulfated mucopolysaccharides when compared to the terrestrial ones. The ratios of hexosamine, uronic acid, and sulfate in the sulfated mucopolysaccharides of the invertebrates are within the same order of magnitude of the ones found in the different types of mammalian sulfated mucopolysaccharides already reported. The relative amounts of the types of sulfated mucopolysaccharides found in the different organisms are also shown in Table I.

DISCUSSION

The results shown in this paper indicate that all organisms analyzed contain one or more types of sulfated mucopolysaccharides. It shows also that the total amount and type of sulfated mucopolysaccharides varies from one species to an-
## Analytical data of sulfated mucopolysaccharides from invertebrates

<table>
<thead>
<tr>
<th>Species</th>
<th>Amount (mg)</th>
<th>Molar Ratios to Hexosamine</th>
<th>Sulfated Muco polysaccharides</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Uronic acid</td>
<td>Sulfate</td>
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<td></td>
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<tr>
<td>Insecta</td>
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<td>1.2</td>
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* The abbreviations used are: ChSA, chondroitin 4-sulfate; ChSB, dermatan sulfate; ChSC, chondroitin 6-sulfate; HTS, heparan sulfate; Hep, heparin; SMPS, sulfated mucopolysaccharides.

The presence of heparin in some bivalves is already well documented. These sulfated mucopolysaccharides have already been isolated from Spisula solidissima and Cyprina islandica (6) as well as from Anodonta sp. (10). In Anodonta besides heparin, the presence of chondroitin, hyaluronic acid, and highly sulfated chondroitin have also been described (10). The present paper shows the presence of heparin or heparin-like compounds (besides other sulfated mucopolysaccharides) in six other species of Mollusca. Furthermore, it provides evidence that a large variation of sulfated mucopolysaccharides composition occurs when different orders of Mollusca are compared. On the other hand, in species of the same order, a similar sulfated mucopolysaccharide composition is observed.

An important observation derived from the present study is that large amounts of heparin are present in Eulamellibranchia in about 1 order of magnitude higher than beef lung tissue (the usual source of commercial heparin).

Another important finding was that most of the species analyzed contain heparin-like or heparitin sulfate-like compounds which were not previously reported in invertebrates previously studied, except for bivalves (6, 10).

All the sulfated mucopolysaccharides analyzed seem to differ somewhat from the mammalian sulfated mucopolysaccharides, as well as from each other in regard to electrophoretic migration and the types of products formed by the action of the mucopolysaccharidases. This is not surprising since these variations were also found among sulfated mucopolysaccharides obtained from different tissues of a same mammalian species (4).

The distribution of the acidic mucopolysaccharides in the animal kingdom (based on the present studies as well as on the ones referred to) is shown in Fig. 8 (modified from Hunt (5)). It is clear from these studies that all species that exhibit some cell organization contain one or more types of sulfated mucopolysaccharides. In contradistinction, except for hyaluronic acid, no other mucopolysaccharides have been reported in bacteria. We were also unable to detect sulfated mucopoly-
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FIG. 8. The distribution of acidic mucopolysaccharides in the major groups of the animal kingdom.

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REFERENCES

Saccharides in Flavobacterium heparinum and Staphylococcus aureus, in fungi Blastocladiella emersonii and Pythium sp., and in protozoans Trypanosoma cruzi, Acantamoeba sp., and Tetrahymena pyriformis using the methods described in this paper. These combined results suggest that the emergence of sulfated mucopolysaccharides corresponds to the emergence of tissue-organized life forms and are in accordance with the earlier suggestion that the sulfated mucopolysaccharides might be involved in the process of cell differentiation, conferring to the cells some of their particular characteristics such as adhesiveness and recognition, and possibly their morphology. In agreement with this are the studies of Humphreys (25), Moscona (26), and MacLennan and Dodds (27) who studied aggregation of cells of Spongilla and characterized an aggregation factor composed of sulfate, uronic acid, hexosamines, and other sugars. This compound has a similar composition to the ones isolated by ourselves from Porifera. An important observation made by those authors was that the cell aggregation factor had some specificity. This observation would reinforce the present findings on the heterogeneity of the sulfated mucopolysaccharides found in Porifera and Tunicata. If these compounds are involved in cell recognition, we would expect an incredible variety of molecular structures. Our results, although preliminary, and based upon a relatively small number of randomly selected species, suggest that these compounds have indeed different structures and occur in different proportions depending on the species analyzed.

2 C. P. Dietrich, unpublished observations.
**Distribution of sulfated mucopolysaccharides in invertebrates.**

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