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Note

Maintenance of heparan sulfate structure throughout evolution: Chemical and enzymic degradation, and ^{13}C-n.m.r.-spectral evidence

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Heparan sulfate proteoglycans are complex macromolecules that consist of a protein backbone to which heparan sulfate chains are covalently linked[1]. They are ubiquitous compounds found in a wide variety of vertebrate tissues[2,3], and are actively synthesized by cells in culture[4,5]. These proteoglycans have been found to be present on the plasma membrane and in the extracellular matrix[6,7], and, they exhibit a peculiar structural variability according to the tissue and species of origin[2]. Despite their wide occurrence, little is known of their biological function. They are thought to be implicated in several biological processes, such as cell–cell recognition and control of cell growth[3], tissue differentiation[8], organization of extracellular matrices[9], and cell–matrix and cell–substrate adhesion[10].

TABLE I

DISTINCTION OF HEPARAN SULFATE FROM OTHER SULFATED GLYCOSAMINOGLYCANS

Parameter	*Heparin*	*Heparan sulfate*	*Dermatan sulfate*	*Chondroitin sulfate*
Potassium acetate precipitation	+	–	–	–
Anticoagulant activity (USP)	+	–	–	–
Enzymic degradation				
Heparinase	+	–	–	–
Heparitinase I	–	+	–	–
Heparitinase II	±	+	–	–
Chondroitinase ABC	–	–	+	+
Electrophoretic mobility (agarose)				
Phosphate buffer	1.0	0.7	0.85	0.85
1,3-Diaminopropane buffer	1.0	1.0	1.3	1.6
Barium–1,3-diaminopropane buffer	1.0	2.0	2.5	3.0
Metachromatic activity (nm)	505	525	535	535
Sulfate: hexosamine ratio	2.4–2.8	0.8–1.8	0.9–1.1	0.9–1.1

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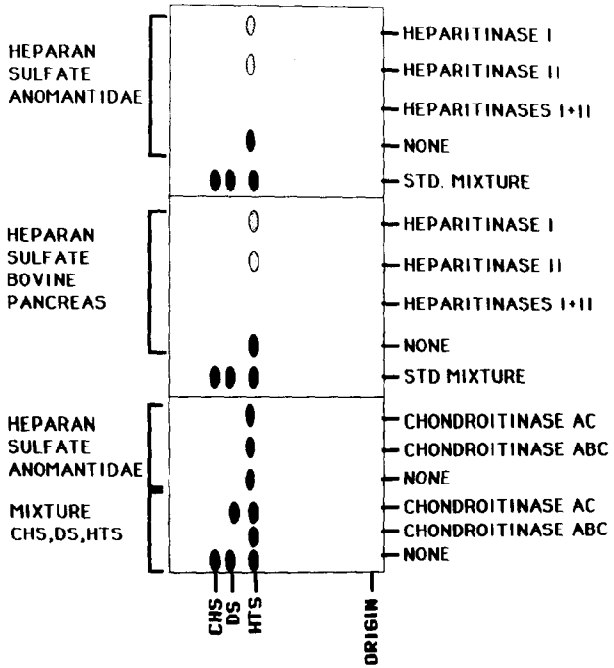

Fig. 1. Degradation of mollusc and mammalian heparan sulfate by different enzymes. [About 100 μg of bovine pancreas and *Anomantidae* heparan sulfate, mixture of 20 μg of chondroitin sulfate (CHS), dermatan sulfate (DS), and heparan sulfate (HTS) were incubated with 0.01 unit of chondroitinase AC, chondroitinase ABC, heparitinase I, heparitinase II, and a mixture of heparitinases I and II in 0.05M ethylenediamine–acetate buffer, pH 7.0, in a final volume of 20 μL for 6 h. After incubation, 5-μL aliquots were subjected to electrophoresis in diaminopropane–acetate buffer as described in Methods. The Figure shows the tracings of the compounds made visible after fixation with cetyltrimethylammonium bromide and Toluidine Blue staining. The disaccharide products formed by the action of the enzymes were not precipitated by cetyltrimethylammonium bromide, and thus were not stained by Toluidine Blue.]

Heparan sulfate-like compounds having structural characteristics similar to those described for heparan sulfates from mammalian tissues have also been isolated from invertebrates[1]. Due to the widespread occurrence of these compounds in the animal kingdom, it became extremely important to ascertain if the heparan sulfate-like compounds present in invertebrates are indistinguishable from the ones found in mammalian and other vertebrate tissues. For this reason a comparative study between heparan sulfates from vertebrate and invertebrate tissues has now been undertaken.

The distinction between heparan sulfates from the other sulfated glycosaminoglycans used in the present studies is summarized in Table I. The heparan sulfates from the five species of molluscs, as well as the heparan sulfates of mammalian origin, are degraded by a combination of heparitinases I and II, and are resistant to chondroitinase ABC and heparinase. They exhibit a typical electro-

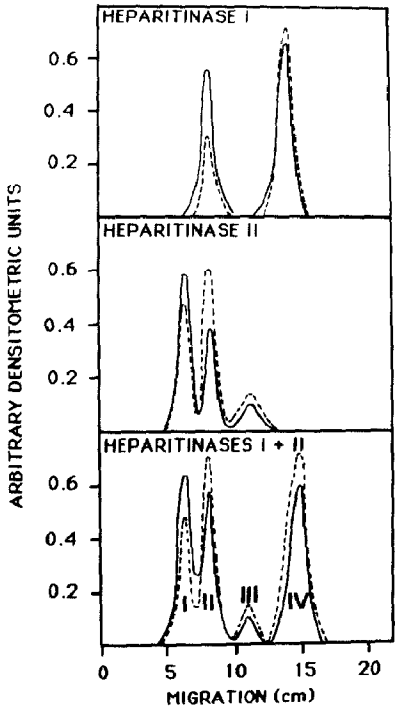

Fig. 2. Disaccharide products formed from mollusc and mammalian heparan sulfate by action of heparitinases. [The incubation mixtures, prepared as in Fig. 1, were subjected to paper chromatography as described in Methods. The Figure shows the densitometric measurements on the disaccharide products made visible by silver nitrate staining. Key: ——, disaccharide products formed from heparan sulfate from *Anomantidae*; and -----, disaccharide products formed from heparan sulfate from bovine pancreas. I, ΔGlcA-GlcNS, 6S, *O*-(4-deoxyhex-4-enopyranosyluronic acid)-(1→4)-2-deoxy-2-sulfamino-D-glucose-6-sulfate; II, ΔGlcA-GlcNS, α-(4-deoxyhex-4-enopyranosyluronic acid)-(1→4)-2-deoxy-2-sulfamino-D-glucose; III, ΔGlcA-GlcNAc, 6S, *O*-(4-deoxyhex-4-enopyranosyluronic acid)-(1→4)-2-acetamido-2-deoxy-D-glucose 6-sulfate; IV, ΔGlcA-GlcNAc, *O*-(4-deoxyhex-4-enopyranosyluronic acid)-(1→4)-2-acetamido-2-deoxy-D-glucose; Hep, heparin; HS, heparan sulfate; and CHS, chondroitin sulfate.]

TABLE II

ANALYTICAL DATA OF THE DISACCHARIDE PRODUCTS OBTAINED FROM *Anomantidae* sp. BY ACTION OF HEPARITINASES I AND II

Disaccharide[a]	*Molar proportions*			
	Hexosamine	*Sulfate*	*Uronic acid*	*Acetyl groups*
ΔGlcA-GlcNS, 6S	1.0	1.95	1.1	0.0
ΔGlcA-GlcNS	1.0	0.85	0.9	0.0
ΔGlcA-GlcNAc, 6S	1.0	1.10	1.2	0.93
ΔGlcA-GlcNAc	1.0	0.0	1.1	0.92

[a]Abbreviations as in Fig. 2.

TABLE III

MOLECULAR WEIGHT AND DISACCHARIDE COMPOSITION OF MOLLUSC HEPARAN SULFATES

Species	$\times 10^{-3}$	*Disaccharides*[a] *(molar ratios)*			
		ΔGlcA-GlcNS, 6S	*ΔGlcA-GlcNS*	*ΔGlcA-GlcNAc, 6S*	*ΔGlcA-GlcNAc*
Anomantidae	12.5	1.0	1.6	1.0	1.8
Mytella	10.5	2.5	1.2	1.0	2.8
Pomacea	33.0	6.2	4.2	1.0	2.6
Tagelus	24.0	3.5	2.7	1.0	0.5
Anomalocardia	18.0	6.1	1.5	1.0	0.4
Bovine pancreas	26.0	1.4	2.2	1.0	4.0

[a]Abbreviations as in Fig. 2.

phoretic migration in agarose-gel electrophoresis with three different buffer systems. The action of the different enzymes upon a heparan sulfate from mollusc compared to a mammalian heparan sulfate is shown in Fig. 1. Both heparan sulfates are susceptible to heparitinase I and heparitinase II, and are similarly resistant to the action of chondroitinases AC and ABC. The products formed from mollusc and mammal heparan sulfates by the action of heparitinase I, heparitinase II, and a combination of the two enzymes are shown in Fig. 2. Four main disaccharides are formed from both heparan sulfates. They were prepared on a large scale by paper chromatography followed by electrophoresis, and analyzed. Table II shows the results of these analyses. Two *N*-acetylated disaccharides, one of them 6-sulfated, and two *N*-sulfated disaccharides, one of them 6-sulfated, were formed by the action of the two enzymes. Table III shows the relative amounts of these disaccharides formed from heparan sulfates of the five species of mollusc. All of them contained the same four main disaccharide units as the mammalian heparan sulfates.

The ^{13}C-n.m.r. spectrum of the heparan sulfate from *Anomantidae* sp. compared to those of heparan sulfate and heparin from mammals is shown in Fig. 3. Both the mollusc and mammal heparan sulfate show two major peaks, at 104 and 97 p.p.m., which correspond to the anomeric carbon atoms of glucuronic acid and glucosamine, respectively. Also, the peak corresponding to CH_3 of the acetyl groups is present in the spectra of both of the heparans, and almost absent from the spectrum of heparin. The absence of the signal at 101 p.p.m., which is prominent in the heparin spectrum, is indicative of the fact that sulfated iduronic acid residues are represented to little, if any, extent in the mollusc heparan sulfate.

Conclusions. — The chemical, enzymic, and ^{13}C-n.m.r.-spectral data reported herein strongly suggest that the main structural features of heparan sulfates have been maintained throughout evolution. The differences observed for the five heparan sulfates reside only in the proportions of the disaccharide units, which vary according to the species of origin. This variation was also observed for more than fifteen heparan sulfates from different mammalian tissues and species[2].

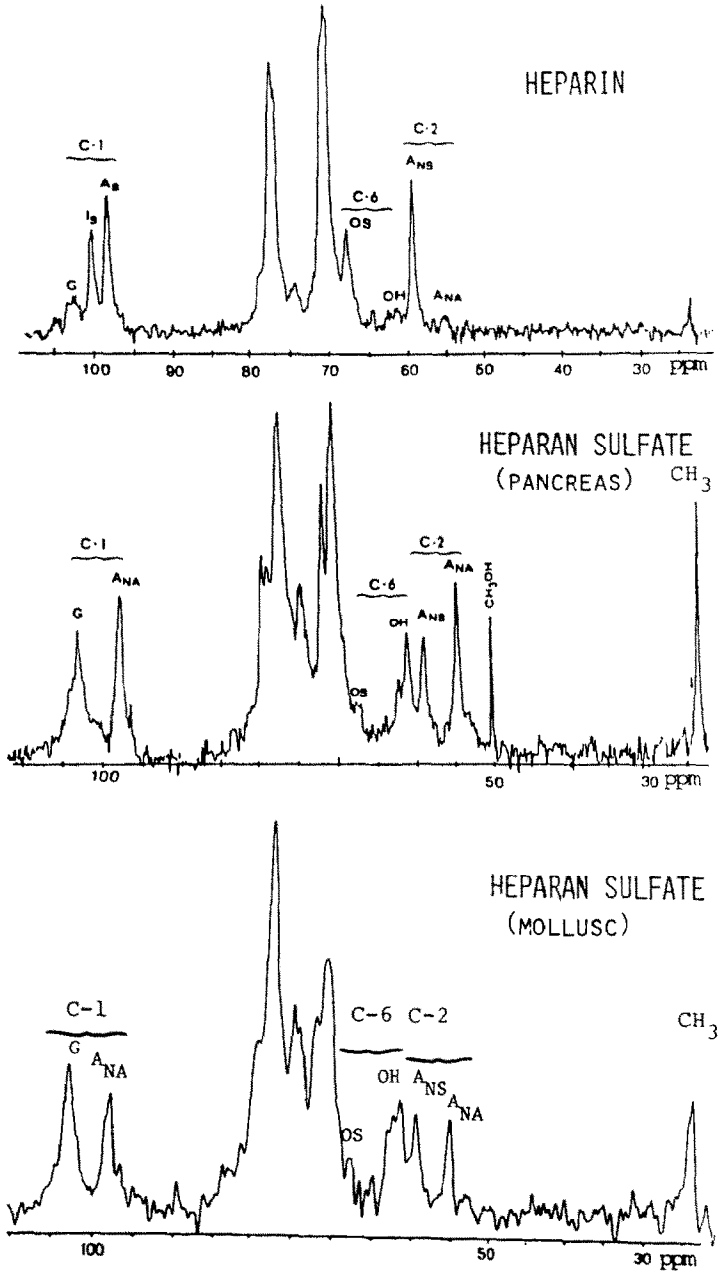

Fig. 3. ^{13}C-N.m.r. spectra of heparin and of mollusc and mammalian heparan sulfates. [The signals of iduronate (I), glucuronate (G), hexosamine (A), and acetyl groups (CH_3) are indicated.]

TABLE IV

DISTRIBUTION OF SULFATED GLYCOSAMINOGLYCANS IN INVERTEBRATES

Species	*Sulfated glycosaminoglycans (μg/g of dry tissue)*				
	Heparan sulfate	*Chondroitin sulfate*	*Dermatan sulfate*	*Heparin*	*Others (unknown)*
Insecta					
Cornitermes cumulans	11	6.7			1.9
Camponotus rufipes	0.2	3.2			
Periplaneta americana	38	7.8			
Crustacea					
Penaeus brasiliensis	95	185			
Scyllarides brasiliense	498	102			
Callinectes sapidus	125	94			
Macrobrachium acanthurus	183	61	26		166
Penaeus sp.	335	851	64		51
Arachnida					
Nephila clavipes	21	13			
Mollusca					
Aulocombia ater	112	58			
Perna perna	153	21			
Mesodesma donacium	67	59		716	
Anomalocardia brasiliana	130	89		2,010	
Tagelus gibbus	405	132	19		82
Mytella guyanensis	660	634	634		679
Anomatidae	122	2.5			
Tivela mactroides	73	110		1,650	
Donnax striatus	161	46		2,090	
Oxystila phlogera	244	12	17		308
Pomacea sp.	337	310			32
Biomphalaria glabrata	139	155	269		252
Fasciolaria aurantica	710		391		1,346
Tegula viridula	358	973	1,076		256
Strombus goliath	751	177	530		751
Cassis tuberosa	1,895	842	1,263		1,316
Lolligo brasiliense	27	279			
Octopus sp.	229	142	719		
Annelida					
Pheretima hawayana	181				
Coelenterata					
Actiniaria 1	47				
Actiniaria 2	30				
Tunicata					
Ascidia nigra			303		1,592
Styella plicata					8,698
Echinodermata					
Holothuria grisea					2,374
Lythechinus variegatus					985
Porifera					
Spongiaria 1					212
Spongiaria 2			338		367

The presence of heparan sulfate-like compounds in invertebrates has been described by several authors (for a review, see ref. 11). From the data shown in Table IV, which summarizes a systematic study conducted in our laboratory, these compounds are present in the main classes of invertebrates.

We have, so far, been unable to detect heparan sulfate-like compounds in Tunicata, Echinodermata, and Porifera. This does not necessarily mean that these classes do not contain heparan sulfate. The presence of high proportions of sulfated polysaccharides having some of the physicochemical properties of heparan sulfate (Unknown, Table IV) might have hampered the isolation of these compounds in the species analyzed. In conclusion, any proposal for the eventual biological function of heparan sulfate has to take into account its widespread occurrence throughout the animal kingdom.

EXPERIMENTAL

Substrates, enzymes, and materials. — Heparin from bovine intestinal mucosa, and heparan sulfate from bovine pancreas were gifts from Dr. P. Bianchini (Opocrin Research Laboratories, Modena, Italy). Chondroitin 4- and 6-sulfates, and chondroitinase AC and ABC, were purchased from Miles Laboratories (Elkhart, IN). Heparinase and heparitinases were prepared from induced *Flavobacterium heparinum* cells by methods previously described[12,13]. Ethylenediamine (1,2-diaminoethane) was purchased from Aldrich Co. (Milwauke, WI).

Extraction and purification of mollusc heparan sulfates. — *Anomantidae* sp., *Anomalocardia brasiliana. Mytella guyanensis, Pomacea* sp., and *Tagelus gibbus* were collected in different regions of Rio Grande do Norte, Brazil, at different time-intervals during a period of four years. Immediately after each collection, the molluscs were heated in water for 15 min at 100° for removal of the shells. The meat (15–20 kg) was ground with 2 vol. of 0.6M NaCl in a Waring Blendor. The pH of the mixture was adjusted to 8.0 with NaOH, and 10 g of Superase (Pfizer Laboratory, New York, NY) were added. After incubation for 24 h at 60°, with agitation and periodic adjustment of the pH, the mixture was filtered through cheese cloth. To the filtrate was added 500 g of Amberlite IRA-900, ion-exchange resin (Rohm and Haas, São Paulo, Brazil), and the mixture was agitated for 24 h at room temperature under a layer of toluene. The suspension was then filtered through the same cheese cloth. The resin retained on the cloth was successively washed with 10 L of water at 50° and 10 L of 0.6M NaCl at room temperature. The washed resin was suspended in 1 L of M NaCl, agitated for 4 h, and the suspension filtered; this operation was repeated twice. The filtrates were combined, and maintained for 48 h at 5° after addition of 2 vol of methanol. The precipitate formed was collected by centrifugation at 5000 r.p.m. for 30 min, washed with 80% methanol, and dried. The precipitate (5 g) was suspended in 100 mL of 2.0M potassium acetate, and the pH was adjusted to 5.7. The solution was kept for 24 h

in the cold, and the precipitate formed was removed by centrifugation; this step removes heparin present in *Anomalocardia brasiliana*[11]. To the supernatant liquor was added methanol (2 vol.), and the precipitate formed after 2 h at −20° was collected by centrifugation, washed twice with methanol (2 vol.), and dried. The precipitate was suspended in 100 mL of 0.05M ethylenediamine–acetate buffer, pH 7.0, containing 5 units of chondroitinase ABC, and incubated for 24 h at 37°. The purified heparan sulfate was then collected by precipitation with two vol. of methanol, washed with 80% methanol, and dried.

Enzymic degradation of heparan sulfate. — A typical incubation mixture contained 0.1 U of enzymes, 100 μg of heparan sulfate, and other additions as indicated, in 0.05M ethylenediamine–acetate buffer, pH 7.0, in a final volume of 30 μL. The incubation mixtures were spotted on Whatman No. 1 paper, and subjected to chromatography in 5:3 (v/v) isobutanoic acid–M NH_4OH for 24 h. Electrophoresis of the degradation products was performed on Whatman No. 3MM paper in 0.25M $(NH_4)HCO_3$ buffer, pH 8.5. The unsaturated products formed were detected by means of a short-wave u.v. lamp. The disaccharides formed were quantitated and characterized as previously described[11–13].

^{13}C-N.m.r. spectroscopy. — The spectra were recorded at 35° with a Varian CFT-20 Fourier-transform spectrometer (at 20 MHz) equipped with a 5-mm probe. Heparan sulfate and heparin samples (50–60 mg) were dissolved in 0.4 mL of ^2H_2O (99.7% ^2H).

Other methods. — Hexosamine was determined, after hydrolysis with 4M HCl for 6 h at 100°, by the Rondle–Morgan procedure[14], and uronic acid by the carbazole reaction[15]. Total sulfate was measured by a method previously described[16]. The structures of the disaccharides obtained from the mollusc heparan sulfate by action of the heparitinases were determined by methods previously described[17,18]. Agarose-gel electrophoresis was performed in three different buffer systems, as previously described[11]. Molecular weight was estimated by poly(acrylamide) gel electrophoresis, and acetyl groups were determined by gas–liquid chromatography as described elsewhere[19]. Metachromatic activity of the compounds was performed as described by Jaques[20]. Anticoagulant activity was determined according to the United States Pharmacopea assay.

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