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Characterization of a low-sulfated chondroitin sulfate from the body of *Viviparus ater* (mollusca gastropoda). Modification of its structure by lead pollution

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A chondroitin sulfate was purified from the body of *Viviparus ater* (Mollusca gastropoda) and analyzed for molecular mass, constituent disaccharides, and structure by ^{1}H NMR and ^{1}H 2D NMR. A quite unique glycosaminoglycan species was isolated having a high molecular mass (greater than 45,000) and low charge density, about 0.60, due to the presence of 42% non-sulfated disaccharide, 5% 6-sulfated disaccharide, 48% 4-sulfated disaccharide, and 5% 4,6-disulfated disaccharide. Specimens of Mollusca were also submitted to lead exposure for different times, and the effect on chondroitin sulfate structure was studied. After 96 h treatment a strong decrease in chondroitin sulfate content was observed with a significant modification of its structure producing a more desulfated polymer, in particular in position 4 of the galactosamine unit. Simultaneously, the amount of unsaturated non-sulfated disaccharide increased with an overall decrease of the charge density.

Abbreviations: **CS, chondroitin sulfate. DQS, Double-Quantum correlation Spectroscopy. UH = GlcA, glucuronate. NH = GalNAc, N-acetyl-galactosamine.**

Introduction

Glycosaminoglycans (GAGs) (hyaluronic acid, keratan sulfate, chondroitin sulfates (CSs), heparan sulfate and heparin) are linear, complex, polydisperse polysaccharides extracted and purified from different animal tissues [1–3]. They are very heterogeneous polysaccharides in terms of relative molecular mass, charge density, physico-chemical properties, biological and pharmacological activities [3]. These anionic polymers are widespread in nature, occurring in a great variety of organisms typically isolated as proteoglycans linked to a protein core having several biological functions, including the regulation of cell growth and development, mainly by interactions of their glycosaminoglycans chains with proteins [for review see 4–6]. In the animal kingdom, sulfated glycosaminoglycans abound in vertebrate tissues, and invertebrate species are also a rich source of polysaccharides with novel structures [7–9]. GAGs have also been isolated and characterized from different families of mollusca [10–12]. In particular, CSs with different structures were purified from one Mollusca gastropoda (*Pomacea sp.*) and two Mollusca bivalve with the former constituted of a large amount of non-sulfated disaccharide [10]. Currently, a low-sulfated CS has been purified and characterized only from one species of Mollusca gastropoda [10] and we do not know if this kind of polysaccharide is peculiar for this species or for different species of Molluscs belonging to the same class (in particular gastropoda). On the other hand, mucopolysaccharides and glycoproteins are produced in considerable amounts by gastropods and serve a wide range of functions including mechanical or protective support and lubrication, and as components of egg gels and capsules [for review see 13–15]. This promped us to study the presence and the structure of GAGs isolated from the body of another Mollusca gastropoda (*Viviparus ater*). Furthermore, it is known that several Molluscs accumulate heavy metals [16], and though their effect on several enzyme activities, cell components and intermediate metabolism [16,17] has been studied, no data are available on the possible modification of GAG structure. Lead is a toxic element and is considered an environmental hazard by the European Economic Community (EEC) countries. For these reasons, lead was cho-

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sen for this study to evaluate the effect of pollution by heavy metals on CS structure and properties isolated from *Viviparus ater.*

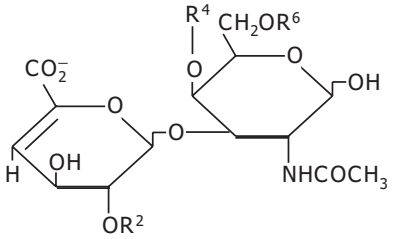
Materials and methods

Ecteola-cellulose resin (condensation product of epichlorohydrin, triethanolamine and cellulose) was from Serva, Heidelberg, Germany. Chondroitinase ABC from *Proteus vulgaris* [E.C. 4.2.2.4], chondroitinase AC II Arthro from *Arthrobacter aurescens* [E.C. 4.2.2.5], and chondroitinase B from *Flavobacterium heparinum* [unclassified] were obtained from Sigma. Papain [E.C.3.4.22.2], trypsin [E.C.3.4.21.4] and chymotrypsin [E.C. 3.4.21.1] were from Sigma. Heparin from beef intestinal mucosa, dermatan sulfate from beef intestinal mucosa and CS from beef trachea were extracted and purified as reported elsewhere [18] and as essentially reported below. CS A (CS 4-sulfate) was from Sigma. Unsaturated chondroitin sulfate/dermatan sulfate disaccharides were obtained from Seikagaku Corporation (see Table 1). All reagents were analytical grade.

Purification of the glycosaminoglycan component

Specimens of *Viviparus ater* (De Cristofori and Ian, 1832) (Mollusca gastropoda prosobranchia) were collected in the spring from a canal near Modena (Italy) [19]. After each collection, the Molluscs were killed and the shell, the mucus and embryos removed. The body was defatted by chloroform/methanol 2 : 1 and acetone. After centrifugation at 10,000 *g* for 10 min and drying at 60 °C for 24 h, the pellet was solubilized (100 mg/5 ml) in 50 mM tris pH 7.0. 100 µl of papain (1 mg/ml) was added to 10 ml and the solution incubated for 24 h at 60 °C in a stirrer. After boiling for 10 min, trypsin and chymotrypsin (50 µg) were sequentially

Table 1. Percentage of unsaturated disaccharides (%weight) and the sulfate to carboxyl ratio of chondroitin sulfate purified from the body of *Viviparus ater* at different times of lead exposure. 25 µg CS quantified by uronic acid assay were used for constituent disaccharides and charge density analyses for polysaccharides purified from the body of Mollusca at different times of lead pollution. The percentage of each unsaturated disaccharide and of the amount of GAGs are averages of three different analyses with coefficient of variations lower than about 13%.



	R^2	R^4	R^6	*Control*	*48 h*	*72 h*	*96 h*	*168 h*
ΔDi-0s	H	H	H	41.9	49.8	47.8	68.5	75.0
ΔDi-6s	H	H	SO_3^-	5.0	2.8	1.9	3.6	3.3
ΔDi-4s	H	SO_3^-	H	48.2	43.1	45.7	24.4	17.7
ΔDi-2,6dis	SO_3^-	H	SO_3^-	0.0	0.0	0.0	0.0	0.0
ΔDi-4,6dis	H	SO_3^-	SO_3^-	4.9	4.3	4.6	3.5	4.0
ΔDi-2,4dis	SO_3^-	SO_3^-	H	0.0	0.0	0.0	0.0	0.0
ΔDi-2,4,6tris	SO_3^-	SO_3^-	SO_3^-	0.0	0.0	0.0	0.0	0.0
SO_3^-/COO^-				0.60	0.46	0.57	0.35	0.29
µg GAGs/g tissue				12.8	11.7	12.2	6.9	4.7

ΔDi-0S [2 - acetamido - 2 - deoxy - 3 - 0 - (4 - deoxy - α - L - *threo* - hex - 4 - enepyranosyluronic acid) - D - galactose]
ΔDi-4S [2 - acetamido - 2 - deoxy - 3 - 0 - (4 - deoxy - α - L - *threo* - hex - 4 - enepyranosyluronic acid) - D - galactose 4 - sulfate]
ΔDi-6S [2 - acetamido - 2 - deoxy - 3 - 0 - (4 - deoxy - α - L - *threo* - hex - 4 - enepyranosyluronic acid) - D - galactose 6 - sulfate]
ΔDi-2,6diS [2 - acetamido - 2 - deoxy - 3 - 0 - (4 - deoxy - α - L - *threo* - hex - 4 - enepyranosyluronic acid 2 - sulfate) - D - galactose 6 - sulfate]
ΔDi-4,6diS [2 - acetamido - 2 - deoxy - 3 - 0 - (4 - deoxy - α - L - *threo* - hex - 4 - enepyranosyluronic acid) - D - galactose 4,6 - disulfate]
ΔDi-2,4diS [2 - acetamido - 2 - deoxy - 3 - 0 - (4 - deoxy - α - L - *threo* - hex - 4 - enepyranosyluronic acid 2 - sulfate) - D - galactose 4 - sulfate]
ΔDi-2,4,6triS [2 - acetamido - 2 - deoxy - 3 - 0 - (4 - deoxy - α - L - *threo* - hex - 4 - enepyranosyluronic acid 2 - sulfate) - D - galactose 4,6 - disulfate]

added at 24 h intervals at 37 °C. The sample was boiled at the end of each incubation. The mixture was brought to pH 9.0 by adding 2 N NaOH. After 24 h at 40 °C, the product (brought to pH 6.0 with 2 N acetic acid) was centrifuged at 5,000 g for 15 min, and the pellet washed two times with 50 mM tris pH 7.0. Three volumes of ethanol saturated with sodium acetate were added to the pooled supernatants and stored at +4 °C for 24 h. The precipitate was recovered by centrifugation at 5,000 g for 15 min and dried at 60 °C for 6 h. The dried precipitate was dissolved in 1 ml of 0.05 M sodium chloride by prolonged mixing. After centrifugation at 5,000 *g* for 15 min, the supernatant was applied to a column (1 cm × 8 cm for a single specimen, and 2 × 20 cm for a pool) packed with ecteola-cellulosa equilibrated with 0.05 M NaCl. After washing the resin with 2 volumes of 0.05 M NaCl, 1 volume of 3 M NaCl was added. Two volumes of ethanol saturated with sodium acetate were added to the eluate and stored at +4 °C for 24 h. After centrifugation at 5,000 g for 15 min, the pellet was dried at 60 °C for 6 h.

Effect of lead exposure

For this experiment, about 100 specimens of *Viviparus ater* of the same size (30–35 × 20–25 mm) were collected in the canal free of lead at the time of collection (0.04 mg/l). The snails were acclimatized for a week in aquarium tanks in the same canal water with a biological filtering system. For the *in vivo* experiments, the snails were transferred to tanks containing 5 ppm (0.5 g/l) of lead nitrate [$Pb(NO_3)_2$]. The lead concentrations were chosen on the basis of high value of lethal concentration in our experimental conditions. The 96 h LC_{50} was previously determined and gave a value of 1400 mg/l $Pb(NO_3)_2$ (true dissolved lead dosed by atomic absorbance spectroscopy was 117 ± 4 mg/l [17]. The water temperature and pH were kept constant throughout the experiment. The animals were extracted from the polluted aquarium 48, 72, 96, and 168 hours later, dissected, weighed and treated as described above.

Analytical methods

Quantitative analysis of GAGs was performed with the carbazole assay for uronic acids according to Bitter and Muir [20] against a calibration curve constructed with increasing amounts of CS A (from 0.5 to 10 μg).

Agarose-gel electrophoresis of GAGs was performed as reported elsewhere [18,19]. Densitometric scanning was performed with a Macintosh llsi computer interfaced with a Microtek Color Scanner from Microtek International Inc., Hsinchu, Taiwan. Image processing and analysis program, Ver. 1.41 was from Jet Propulsion Lab., NASA, Florida, U.S.A..

The peak molecular mass of purified CS species was determined by high-performance size-exclusion chromatography [21] using an HPLC supplied by Jasco. Columns were Protein Pak 125 (7.8 mm × 30 cm) and 300 (7.5 mm × 30 cm) assembled in series (Waters, cod. 84601 and cod. T72711). The mobile phase was composed of a 125 mM Na_2SO_4 and 2 mM NaH_2PO_4 adjusted to pH 6.0 with 0.1 N NaOH. Flow rate was 0.9 ml/min with a back pressure of 25–30 Kg/cm^2. The peak molecular mass of *Viviparus ater* CS was determined by a calibration curve plotted with glycosaminoglycan standards having molecular mass from 45,000 to 2,000 (see Figure 2) produced as previously reported [21] and evaluated by means of analytical ultracentrifugation, according to Nieduszinski [22] and by high-performance size-exclusion chromatography utilizing a calibration curve calculated by glycosaminoglycans with known molecular mass [21].

Qualitative and quantitative evaluation of CS constituent disaccharides and sulfate to carboxyl ratio were obtained by degradation of polysaccharide (25 μg quantified by carbazole test for CS purified at various times of lead exposure) with chondroitinase ABC and separation of unsaturated disaccharides by HPLC at 232 nm using a 5-μm Spherisorb SAX column (250 × 4.6 mm from Phase Separations, Deeside Industrial Park, Deeside Clwyd, UK). Isocratic separation was run from 0 to 10 min with 0.2 M NaCl pH 4.00 and linear gradient separation was from 10 to 90 min with 100% 0.2 M NaCl pH 4.00 to 100% 1.2 M NaCl pH 4.00, at a flow of 1.4 ml/min. The non-sulfated and variously sulfated disaccharides were identified according to the standards and retention times by Seikagaku Kogyo Co. [23].

The glucuronate to iduronate ratio was determined by comparing the percentage of unsaturated disaccharides after digestion of purified CS with chondoitinase ABC and ACll. Furthermore, the possible production of oligosaccharides after chondroitinase B treatment of *Viviparus ater* CS was evaluated by polyacrylamide-gel electrophoresis separation according to Rice *et al.* [24]. Finally, the presence of iduronic acid inside *Viviparus ater* CS was also evaluated by ^{1}H NMR spectra (see below).

The ^{1}H NMR spectra of CS were recorded with a Bruker AMX400 Wb spectrometer operating at 400.13 MHz. The CS sample was previously lyophilized three times with D_2O. The ^{1}H chemical shifts (δ) were quoted with respect to external sodium 4,4-dimethyl-4-silapentene-1-sulfonate (0.0 ppm). DQS [25] parameters: spectral width (f2) = 5 ppm, 2048 complex point; spectral width (f1) = 10 ppm, 512 t1 increments with 32 scans per t1 value; relaxation delay = 0.2s, 18–70 ms evolution time, 135 degree read pulse.

Results

Quantitative analysis of sulfated polysaccharides purified from a single specimen of *Viviparus ater* by uronic acid assay gave 12.8±1.1 μg of GAGs/g of tissue (0.00128% w/w). CS preparation was extracted from ten specimen (about 40 g) of *Viviparus ater* for further analysis. Agarose-gel electrophoresis showed about 95% CS and 5% of der-

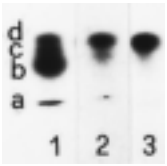


Figure 1A. Agarose-gel electrophoresis of 1. GAGs standard (a, slow moving heparin; b, fast moving heparin or heparan sulfate; c, dermatan sulfate; d, beef trachea chondroitin sulfate), 2. total GAGs isolated from Mollusca, and 3. *Viviparus ater* chondroitin sulfate after further purification (5 μg calculated by the uronic acid assay [20]).

matan sulfate (Figure 1A band 2, and 1B panel A) evaluated by densitometric scanning, excluding the presence of heparin or heparan sulfate. The presence of hyaluronic acid was excluded on the basis of agarose-gel electrophoresis stained with Stains-All (3,3'-dimethyl-9-methyl-4,5,4',5'-dibenzothiacarbocyanine) [26] (not shown). CS was further purified by specific degradation of dermatan sulfate (about 5% as above reported) by chondroitinase B treatment and reprecipitation with ethanol saturated with sodium acetate. 100% CS was obtained (Figure 1A band 3, and 1B panel B).

A molecular mass greater than about 45,000 was found by high-performance size-exclusion chromatography [21] (Figure 2). The digestion of purified polysaccharide by chondroitinase ABC produces about 42% non-sulfated disaccharide, 5% 6-sulfated disaccharide, 48% 4-sulfated disaccharide, and about 5% disulfated disaccharide in position 4 and 6 of N-acetyl-galactosamine, with a sulfate to carboxyl ratio of 0.60 (Table 1). Degradation of CS chains by chondroitinase ACII produced about 100% unsaturated disaccharides, and no oligosaccharides were observed after treatment with chondroitinase B and polyacrylamide-gel electrophoresis separation [24] (not shown) confirming that *Viviparus ater* CS was totally constituted of glucuronic acid. The absence of iduronic acid was also confirmed by ^{1}H NMR analysis (see below).

^{1}H NMR analysis of *Viviparus ater* CS (Figure 3a) identified glycogen as contaminant, due to the great amount of this polysaccharide in Mollusca [13]. Nevertheless, glycogen did not interfere with the identification of the two components of CS, non-sulfated and 4-sulfate. The anomeric protons of GalNAc and GlcA units were present at 4.6–4.5 ppm. Lowering the sample temperature from 300 to 290 K the water signal moves to lower fields and a signal at 4.79 ppm due to GalNAc H-4 can be detected. Homonuclear double quantum (DQS, 18 ms evolution time) experiment was performed in order to confirm previous assignments. The DQS spectrum (Figure 3b) showed two NH-1/NH-2 and two UH-1/UH-2 correlations, and it is apparent that a NH-1 and a UH-1 signals were partially overlapped. Two different NH-2/NH-3 correlations, one from CS 4-sulfate and one from non-sulfated CS were distinguished. The ratio between the methyl integral and the integral of the anomeric signals of CS at 4.6–4.5 ppm was near 3 : 2, indicating that the CS was completely acetylated. The ^{1}H NMR and ^{1}H 2D NMR analysis confirmed the presence of non-sulfated and 4-sulfated regions of CS chains in a ratio of about 1 : 1, composed of 100% glucuronic acid. In fact, according to Bossennec *et al.* [27], no UH-1 signal is present at 4.9 ppm related to iduronic acid. The shape of the ^{1}H NMR spectrum was also compatible with a polysaccharide having a high molecular mass and with a small amount of GalNAc sulfated in position 6, for which signals in the region 4.4–4.2 (which appears broad and unresolved) are expected for H-6 protons.

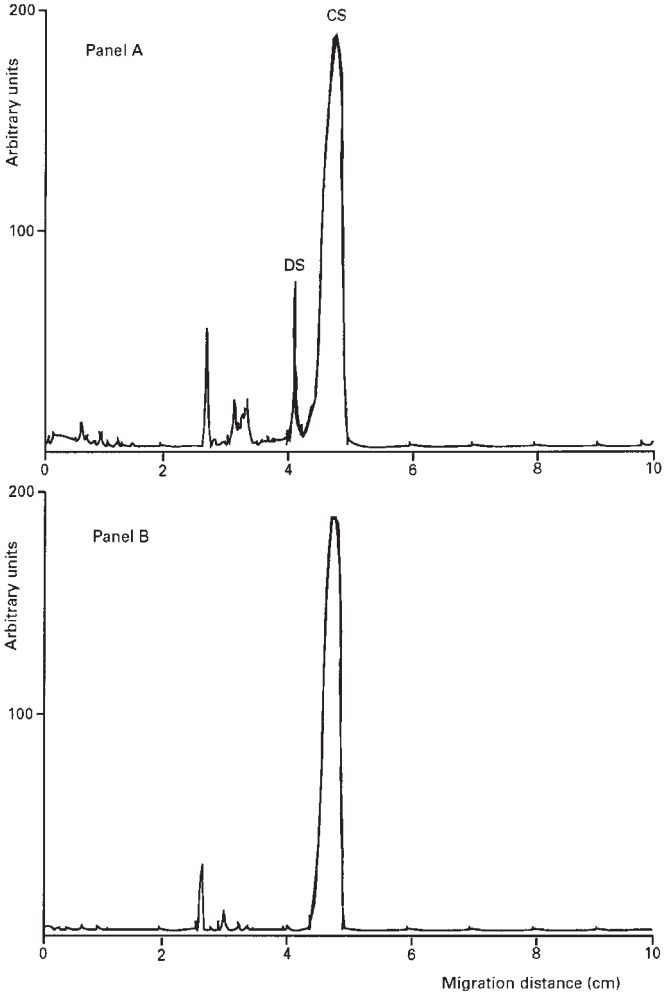


Figure 1B. Densitometric scanning of (panel A) total GAGs isolated from Mollusca (track 2 of Figure 1A) (DS: dermatan sulfate; CS: chondroitin sulfate), and (panel B) after further purification (track 3 of Figure 1A).

Table 1 reports the effect of lead exposure on the total content of GAGs and on the percentage of unsaturated disaccharides. After 96 h a large decrease of the amount of GAGs is evident with a significant reduction in the percent-

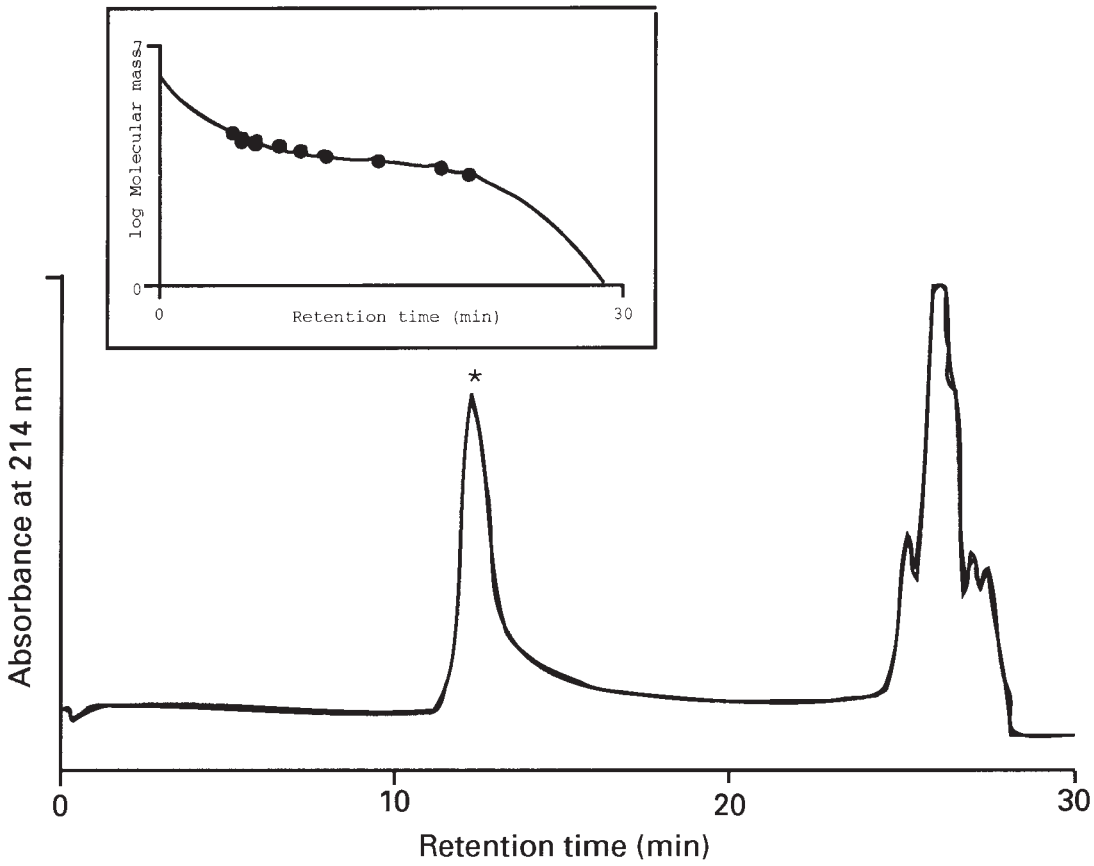


Figure 2. High-performance size-exclusion chromatography of chondroitin sulfate purified from *Viviparus ater.* The asterisk indicates the high-molecular mass chondroitin sulfate. Low-molecular mass species at the end of the chromatografic profile are due to presence of salts. The third-degree grade polynomial curve determined by using glycosaminoglycan standards of molecular mass from 45,000 to 2,000, and utilized to calculate the *Viviparus ater* chondroitin sulfate molecular mass is also reported. For structure and properties of standard see [21].

age of unsaturated disaccharide 4-sulfate and in the charge density. Simultaneously, the amount of unsaturated non-sulfated disaccharide increases. No appreciable variation of the disulfated disaccharide percentage was observed. High-performance size-exclusion chromatography of CS extracted at different times of lead exposure did not show modification of the molecular mass after 168 h treatment (not shown).

Discussion

The extraction and purification methods adopted in this study are commonly used to purify GAGs from various tissues and sources [1,9,10,13]. This excludes the possibility that the CS from *Viviparus ater* could be desulfated during the purification process. This polysaccharide shows peculiar properties, such as a high molecular mass, low charge density due to the presence of about 42% non-sulfated disaccharide, and sulfate groups nearly exclusively located in position 4 of the N-acetyl-galactosamine. It is noteworthy the presence of about 5% 4,6-disulfated disaccharide, since the more sulfated polysaccharide regions are generally responsable for several biological functions [3].

Up till now, a low-sulfated CS has been purified and characterized only from another species of Mollusca gastropoda [10] and we do not know if this kind of polysaccharide is peculiar to this species or to different species of molluscs belonging to the same class (in particular gastropoda). Nader *et al.* [10] described a CS from the body of a gastropoda *Pomacea* sp. showing about 43% non-sulfated disaccharide and 57% monosulfated disaccharide in position 4 [10] with a structure very similar to that purified from *Viviparus ater.* The structure of CSs of two Mollusca gastropoda (*Viviparus ater* and *Pomacea* sp. [10]) differ from those purified from the body of two Mollusca bivalve in the amount (about 30% greater) of disaccharide sulfated in the 6 position of N-acetyl-galactosamine [10].

The structure of CS purified from the body of *Viviparus ater* is different from that extracted from mucus surrounding embryos in the developmental pouch, as the latter has

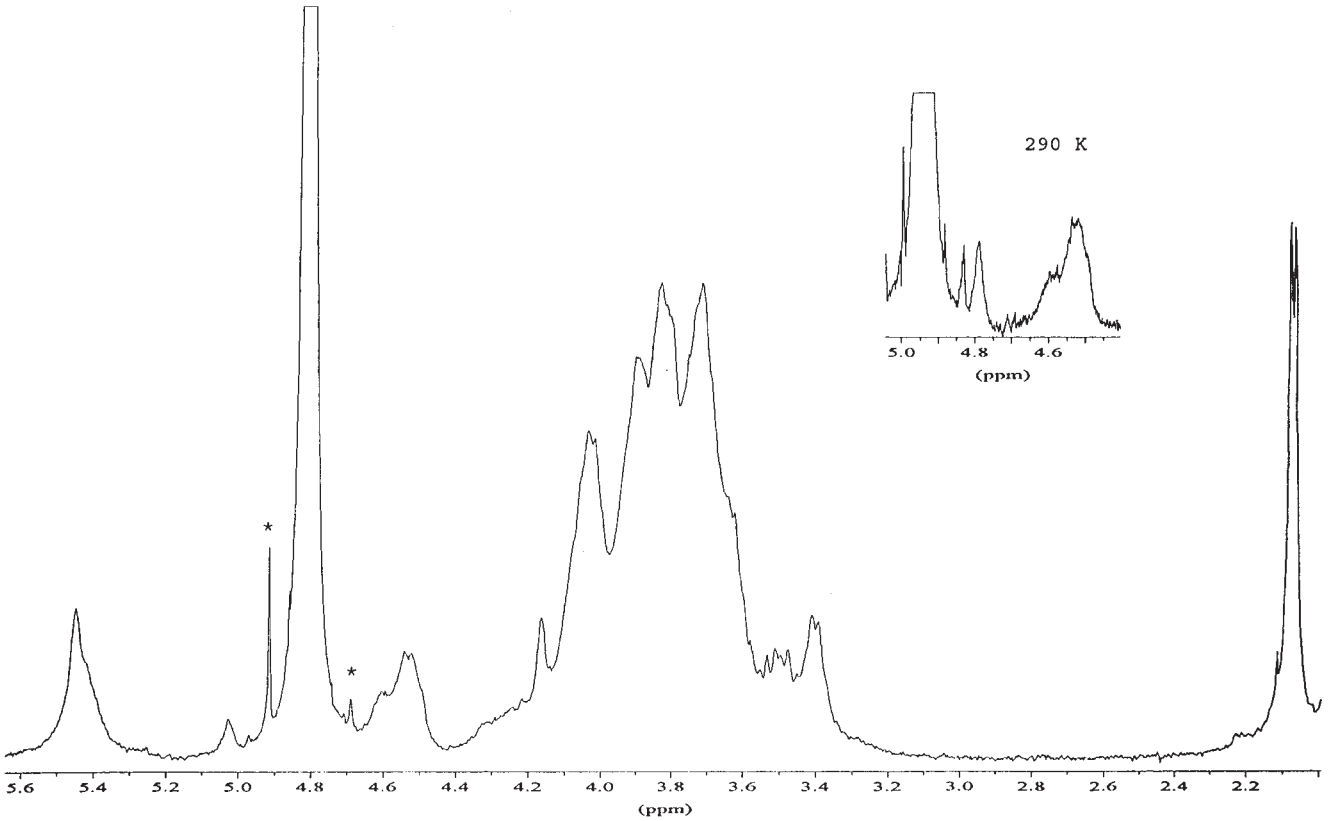


Figure 3a. ^{1}H NMR spectrum of 2 mg of *Viviparus ater* chondroitin sulfate in 0.8 ml D_2O at 300 °K. The partial ^{1}H NMR spectrum acquired at 290 ° K shows the presence of NH-4 signal of GalNAc-4S at 4.79 ppm. Asterisks indicate artefacts.

a lower molecular mass and a greater sulfate to carboxyl ratio [19] probabily related to different biological functions.

Lead is toxic substance, and it is considered to be an environmental hazard by the EEC countries. Furthermore, lead is constantly increasing in the environment due to industrial usage, gasoline additives and other sources. Shellfish among the invertebrates are well known for their ability to selectively accumulate trace metals from the environment, and metal pollution in bivalves and gastropods has long been studied [16,28]. The consequences of lead pollution on several enzymes in the foot, mantle, digestive gland [29], and nervous system [17] have already reported, and heavy metals have been extensively studied in order to explain their effects on enzymatic activities, cell components and intermediate metabolism [16,17]. However, no information is available regarding the effect of lead on GAG structure and properties. A decrease in total GAG content accompanied by slight alteration of polysaccharide pattern was reported by Olczyk *et al.* [30] after treatment of rats with mercuric chloride, whilst modification of heparan sulfate proteoglycans in cultured vascular endothelial cells was observed after exposure to lead [31]. The concentration of 0.5 g/l of lead nitrate was chosen on the basis of dose response treatment. At this concentration, evident cell modifications were observed after 48 h, whereas at the lower amounts cells remained histologically unchanged [17]. After 96 h of lead exposure we observed a strong decrease in CS content with a significant modification of its structure producing a more desulfated polymer, with a specific desulfatation in position 4 of the galactosamine unit. No modification of the dermatan sulfate percentage (about 5%) was noted (not shown) confirming that the quantitative decrease of glycosaminoglycans is quite totally related to the amount of CS. Modification of CS structure could be related to alteration of several enzyme activities, in particular those of polysaccharide metabolism or to a homeostatic mechanism recruited in animals in response to stress.

Acknowledgments

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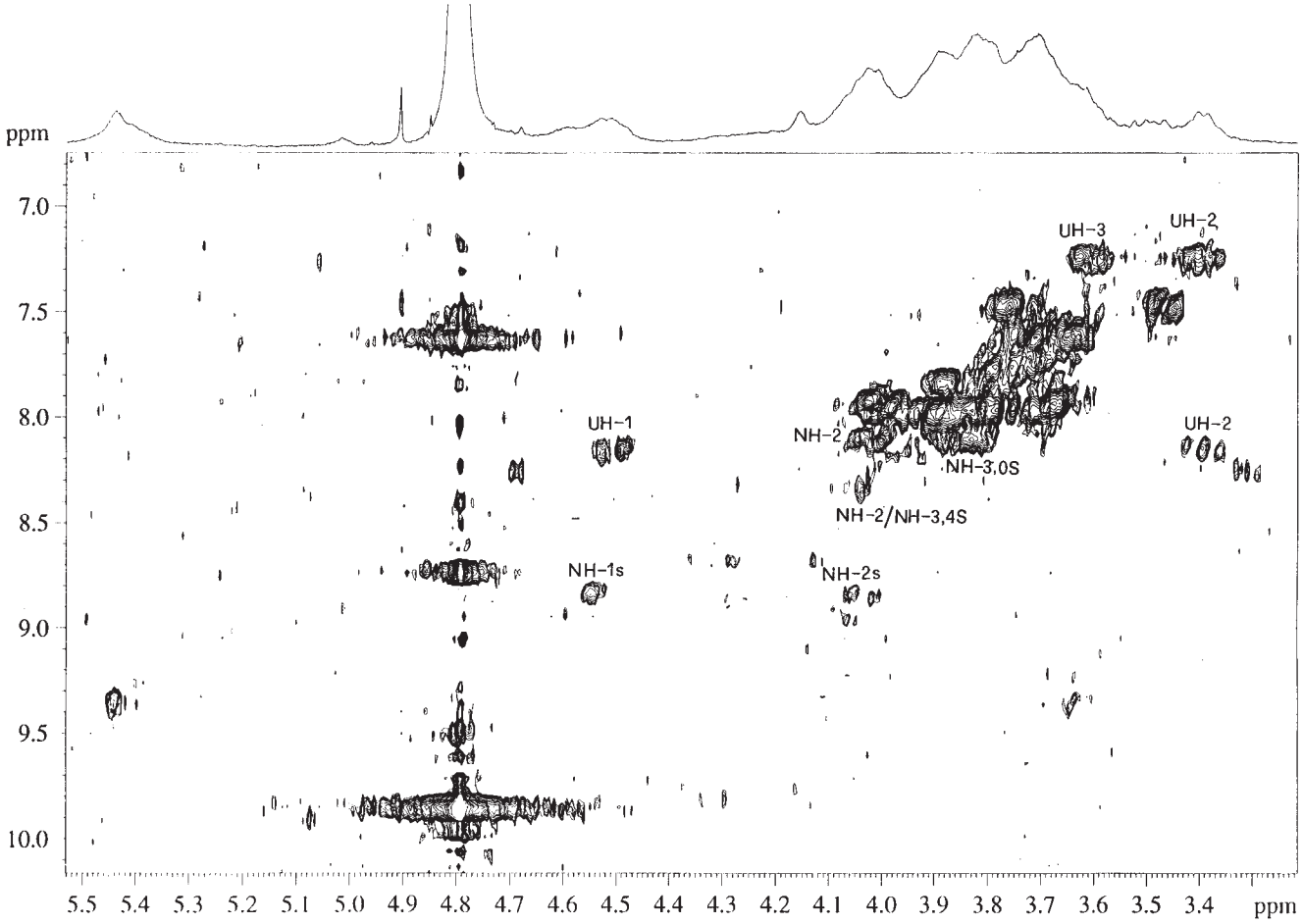

Figure 3b. DQS spectrum of *Viviparus ater* chondroitin sulfate at 300 °K. Only chondroitin sulfate correlations are labelled. UH = GlcA, glucuronate. NH = GalNAc, N-acetyl-galactosamine. 0S, non-sulfated chondroitin. 4S, chondroitin 4-sulfate.

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