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Free radical induced oxidative depolymerisation of chondroitin sulphate and dermatan sulphate

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The glycosaminoglycan (GAG) dermatan sulphate (DS) is a potentially useful therapeutic for antithrombotic applications. Natural DS is isolated as a polymer of a molecular weight of around 25 000. The predominant disaccharide unit is IdoA-GalNAc4SO_3 but the antithrombotic activity is mediated by the binding of heparin cofactor II (HCII) to repeats of IdoA2SO_3-GalNAc4SO_3. We hoped, in parallel with other bioactive GAGs, such as heparin, to improve the phamacological properties of DS by lowering the molecular weight using oxidative free radical depolymerisation. We found indeed that the method gives a smooth and repeatable reduction in molecular weight using chondroitin sulphate as a model system but it appears to selectively attack the disaccharide unit IdoA2SO_3-GalNAc4SO_3 in DS, severely reducing its activity towards HCII. In the course of these investigations we found that, in contrast to prior work on heparin, uncharged polysaccharides cannot be used as standards for the determination of molecular weight of DS and chondroitin sulphate by high performance size exclusion chromatography with high ionic strength buffers. © 1997 Elsevier Science Ltd

INTRODUCTION

Glycosaminoglycans (GAGs) are ubiquitous polysaccharide components of vertebrate connective tissue. They are classified into seven structural groups, keratan sulphate (**1**), hyaluronate (**2**), chondroitin 4- and 6-sulphates (**3**), dermatan sulphate (**4**), heparin (**5**) and heparan sulphate (**5**) (Fig. 1). All are linear polymers of a repeating disaccharide unit composed of a hexosamine and either a uronic acid moiety or, in the case of (**1**), a galactosamine moiety. The classes are differentiated on the basis of the stereochemistry and sulphation pattern of the disaccharide units. However, there may be substantial variation in the disaccharide units within any one polymer chain. This gives rise to a very diverse group of compounds the biological roles of which are only just being delineated (Fransson, 1985).

GAGs are widely incorporated as active ingredients in a variety of healthcare and pharmaceutical products such as cosmetics, dietary supplements, wound dressings, surgical fluid replacements and anticoagulants. Of particular interest is the use of dermatan sulphate (**4**) as an antithrombotic agent in place of heparin (**5**) (Linhardt and Hileman, 1995). Heparin (**5**) is the most commonly used anticoagulant (Linhardt, 1991) and it is also widely used as an antithrombotic agent (Barrow *et al.*, 1994), where its high anticoagulant activity causes problems with uncontrolled bleeding. Dermatan sulphate (**4**), on the other hand, has a relatively low anticoagulant activity compared to its antithrombotic effects (Cohen *et al.*, 1994).

The antithrombotic properties of dermatan sulphate are largely due to the ability of the GAG to bind heparin cofactor II (HC II) and potentiate its inhibition of thrombin (Sheehan *et al.*, 1994). The binding site of dermatan sulphate to HC II consists of at least three repeats of the disulphated disaccharide IdoA2SO_3-GalNAc4SO_3. The antithrombotic activity of dermatan sulphate preparations can be correlated with their IdoA2SO_3 content, as determined by nuclear magnetic

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Abbreviations:—GAG, glycosaminoglycan; GalNAc, *N*-acetylgalactosamine; GalNAc4SO_3, *N*-acetylgalactosamine 4-sulphate; GalNAc6SO_3, *N*-acetylgalactosamine 6-sulphate; GlcA, D-glucuronic acid; HPSAX, high performance strong anion exchange chromatography; GlcNAc, *N*-acetyl glucosamine; HPSEC, high performance size exclusion chromatography; IdoA, L-iduronic acid; IdoA2SO_3, L-iduronic acid 2-sulphate; LMW, low molecular weight; NMR, nuclear magnetic resonance; UA, uronic acid; UA2SO_3, uronic acid 2-sulphate.

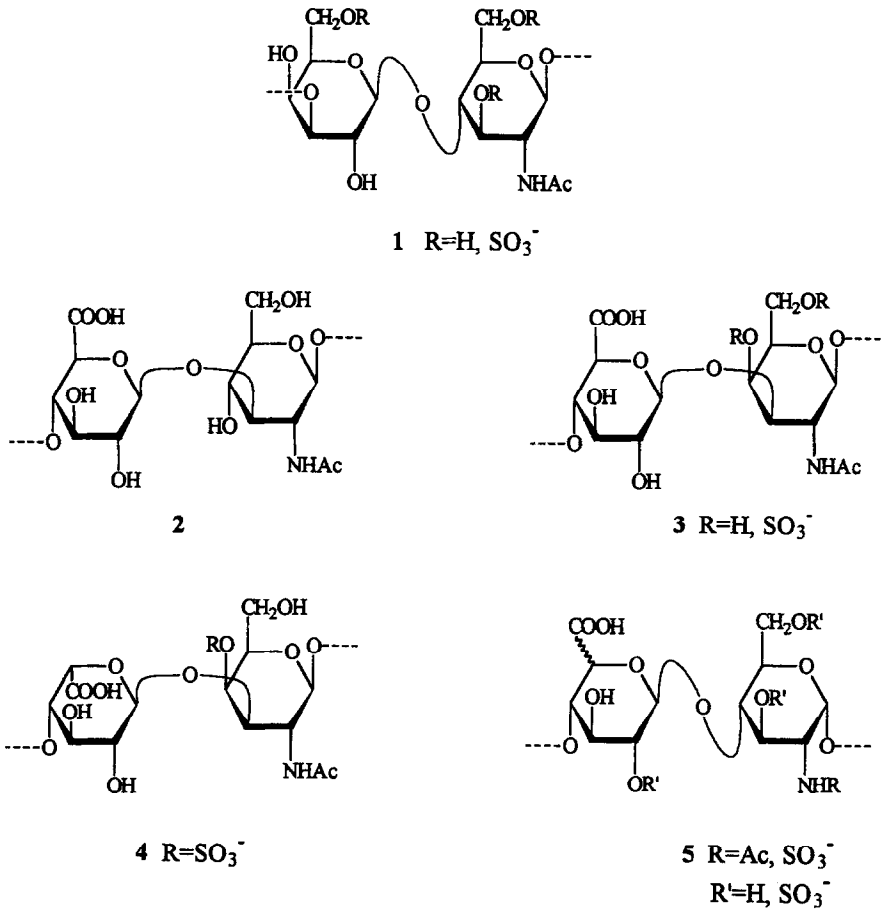

Fig. 1. Predominant disaccharide units in GAGs.

		Predominant sulphated positions
1	Keratan sulphate	Hexosamine C-6
2	Hyaluronic acid	None
3	Chondroitin 4-sulphate	Hexosamine C-4
3	Chondroitin 6-sulphate	Hexosamine C-6
4	Dermatan sulphate	Hexosamine C-4
5	Heparin and heparan sulphate	Very varied

resonance spectroscopy (NMR) and analysis of the disaccharides present after enzyme degradation (Mascellani *et al.*, 1994).

A major problem in the use of GAGs such as heparin and dermatan sulphate as clinical antithrombotics is their poor bioavailability. In the case of heparin the bioavailability can be improved by depolymerisation of the full length material to give low molecular weight (LMW) products (Hirsh and Levine, 1992; Barrowcliffe, 1995). LMW dermatan sulphates have also been shown to have increased bioavailability without loss of bioactivity (Dol *et al.*, 1990; Legnani *et al.*, 1994).

Dermatan sulphate can be depolymerised by a number of different methods that cleave at different regions of the polymer and give rise to different end groups. Treatment with enzymes, such as chondroitinase ABC or testicular hyaluronidase (Slim *et al.*, 1994), or base hydrolysis (Kiss, 1974), cleaves the α-1,4 link between the galactosamine and the uronic acid at random positions in the chain. Both enzyme hydrolysis and base catalysed elimination leave a galactosamine residue at the reducing end and a 4,5-unsaturated uronic acid at the non-reducing end of the polymer. Periodate oxidation of the polymer followed by reduction and hydrolysis, on the other hand, cleaves only at uronic acid residues with unsubstituted vicinal diols, which leaves the $IdoA2SO_3$ residues required for HC II binding intact (Mascellani *et al.*, 1994). This method of cleavage leaves a GalNAc at the non-reducing end and the tetronic acid resulting from cleavage of the uronic acid at the reducing end of the depolymerised material.

Dermatan sulphate can also be oxidative–reductively depolymerised by free radicals generated from hydrogen peroxide with a metal ion catalyst (Volpi, 1994). In this case the structure of the products and the specificity for the cleavage site are unclear. However, this reaction has considerable promise for the industrial production of LMW dermatan sulphates. The conditions of the reac-

tion are mild and it can be quenched at any point by removal of the metal ions by a chelating agent. The reagents are inexpensive and suitable for use on a large scale (Bianchini and Mascellani, 1988). The reaction also has potential to achieve some selectivity of cleavage by the appropriate choice of metal ion catalyst (Liu and Perlin, 1994).

A number of studies of free radical depolymerisation have been conducted on other GAG species. The cleavage of hyaluronate (**1**) by ferrous ions in air has been reported to give fragments with 4,5-unsaturated uronic acids at the non-reducing ends (Kennedy and Cho Tun, 1972). Later work on the same depolymerisation in oxygen reported GlcNAc at both reducing and non-reducing ends, indicating that the GlcA residue is much more susceptible to degradation (Uchiyama *et al.*, 1990). Free radical cleavage of heparin on the other hand has been reported to result in fragments with *N*-sulphated glucosamine and iduronic acid residues at both the reducing and non-reducing ends, without 4,5-unsaturation of the uronic acids at the non-reducing termini (Casu, 1994). It has also been reported that the oxidative–reductive depolymerisation of heparin with oxygen and an iron (II) catalyst preferentially attacks the non-sulphated D-glucuronic acid residues rather than the sulphated L-iduronic acid moieties (Nagasawa *et al.*, 1992).

In the light of the suitability of the reaction for large-scale use and the uncertainty surrounding the products and specificity of the oxidative–reductive free radical cleavage of dermatan sulphate, it was decided to investigate the reaction further. We have examined the rates of depolymerisation and structural changes of dermatan sulphate and chondroitin sulphate on reaction with hydrogen peroxide and copper ions under different conditions.

In order to follow the course of the depolymerisation reaction we required a means of determining the molecular weight of the products. We chose to use size exclusion chromatography (SEC) (Melrose and Ghosh, 1993) because of the ready availability of the equipment and also because the speed of the technique and the lack of sample preparation required allowed us to monitor the depolymerisation in real time. The major drawback of SEC is that it measures molecular size rather than molecular weight, and factors such as the shape of the molecule and degree of solvation are known to affect the apparent molecular weight (Dubin, 1994). This is particularly true of GAGs, which are linear molecules with a high negative charge density. In order to measure the absolute molecular weight of a compound it is generally necessary to calibrate the SEC column by use of standard compounds of known molecular weight that are very similar, or identical, in structure to the compounds of interest. In the absence of commercially available, well defined GAG standards with a narrow size distribution it was necessary to prepare our own and to verify their molecular weight by another method. The molecular weights of GAG samples have been determined by a variety of different methods including viscosity measurements (Rodén *et al.*, 1972), laser light scattering (Komatsu *et al.*, 1993), electrophoresis (Volpi, 1994), mass spectroscopy (Juhasz and Biemann, 1995), end group derivatisation (Ahsan *et al.*, 1993) and NMR (Desai and Linhardt, 1995).

Bergman *et al.* (1993) have reported that the differences in hydrodynamic volume between heparin and commercially available polysaccharide standards can be reduced by manipulation of the experimental conditions during SEC. This allows the calibration of SEC systems to determine heparin molecular weights with non-identical standards. We have used dermatan sulphate and chondroitin sulphate molecular weight standards to investigate whether these results could be extended to other GAG species.

EXPERIMENTAL

HPSEC

Size exclusion chromatography was performed on a Gilson HPLC System (John Morris Scientific Ltd, New Zealand) comprising 305/306 series pumps with associated manometric module and dynamic mixer for high pressure mixing, 231 XL sampling auto-injector and 160 Diode Array Detector, under the control of Gilson UniPoint software. The columns used were a Superdex 75 HR 10/30 (Pharmacia) and Biosep SEC 4000 (Phenomenex) in series. The mobile phase was sodium chloride at the appropriate concentration in 10mM potassium phosphate buffer (pH 7.2) at a flow rate of 1 ml/min. Data was collected at 214 nm and analyzed by UniPoint. Pullulan standards from Showa Denko KK were supplied by Chromspec Distributors Ltd, New Zealand.

NMR Spectroscopy

^{13}C and ^{1}H NMR spectroscopy was performed on a Varian Unity 500 spectrometer operating at 125.7 MHz for the collection of ^{13}C spectra and 500 Mhz for ^{1}H spectra. 100mg samples were lyophilized three times from 99.8 atom% D_2O (Acros) and dissolved in 0.8 ml of the same solvent in a 5 mm NMR tube for analysis. The carbon spectra were acquired at 80°C, an acquisition time of 1.3 s, with a delay of 1.0 s between pulses and 10 000–20 000 transients were acquired until a satisfactory signal to noise ratio was achieved. The spectra were referenced to internal methanol. Proton spectra were also collected at 80°C with an acquisition time of 4.1 s and a delay between pulses of 5.0 s. Sixteen transients were collected. The HMQC-SE carbon proton corelation spectrum was collected at 60°C.

Viscometry

Viscosity measurements were made on dilute solutions of each sample in NaCl (0.2M) at 25°C, using an Ubbe-

lohde viscometer (capillary length 130 mm, capillary internal diameter 0.5 mm, bulb volume 1.0 ml). The viscometer gave an average efflux time of 120 seconds when calibrated with NaCl (0.2M) solution.

Each sample (200 mg of freeze dried material) was dissolved in NaCl (0.2M, 6 ml) solution. The solution was stirred for 1 h to completely dissolve the sample and then centrifuged. The top 4.0 ml was transferred to the viscometer. Efflux times were measured at 25°C. The solution was diluted progressively by the addition of NaCl (0.2M) solution to the viscometer and the efflux times were determined at each concentration. For each measurement, the viscosity of the solution (μ) was calculated from the efflux time (t) using the equation

$$\mu = kt$$

where k is the viscometer constant.

The reduced specific viscosity (μ_{red}) was then calculated from the equation

$$\mu_{red} = \frac{1}{c}\frac{\mu - \mu_o}{\mu_o}$$

where μ_o is the viscosity of the solvent (NaCl, 0.2M), and c is the sample concentration (g/100 ml).

The intrinsic viscosity [μ] was then deduced by plotting the reduced specific viscosity (μ_{red}) against solution concentration and taking the intercept on the ordinate axis as the intrinsic viscosity [μ],

$$[\mu] = \lim_{c \to 0} \mu_{red}$$

The molecular weight was obtained using the following expression:

$$[\mu] = K(M)^a$$

where the Mark Houwink and Sakurada constants (K and a) for chondroitin sulphate and dermatan sulphate were $K = 5 \times 10^{-6}$ and $a = 1.14$ as determined by Wasteson (1971).

Disaccharide compositional analyses

Constitutive disaccharide quantitation was carried out using digestion with chondroitinase ABC (Seikagaku), as described by Volpi (1994). HPSAX was conducted using a Spectraphysics Model SP8700 HPLC pump, a Spectraphysics SP8750 solvent delivery system, a Spectraphysics SP8440 UV/VIS detector (232 nm), a Waters Millennium 2010 chromatography manager, and a Spherisorb 5, SAX, 250 × 4.6 mm column. The flow rate was 1 ml/min. From 0 to 8 min, 0.15M NaCl in a pH 6.00, 0.001M NaH_2PO_4 buffer was eluted isocratically. From 8 to 30 min the NaCl concentration was linearly increased to 0.47M (keeping the pH and phosphate concentration constant), and then from 30 to 35 min the NaCl concentration was linearly increased to 2.0M. The column was then eluted isocratically for 5 min with 2.0M NaCl in a pH 6.00, 0.001M NaH_2PO_4 buffer.

The disaccharides were identified by their retention times using standards from Seikagaku (H-mix Cat. Number 400576), and Sigma Chemical Company [α-UA2SO_3(1-4)GlcNAc, α-UA(1-4)GlcNAc4SO_3 and α-UA(1-4)GlcNAc6SO_3]. Quantitation was achieved by comparison of response of the unknowns with known amounts of the Seikagaku disaccharides.

Depolymerisation of GAG samples

GAG starting material (2.0 g) was dissolved in 100 ml of a buffer containing $Cu(OAc)_2$ (0.001M) and NaOAc (1.5M). Hydrogen peroxide (100 volume, 8 ml) was added to the reaction solution and the mixture was kept at room temperature (20°C) for 2 h with stirring. The reaction was quenched with an equivalent volume of EDTA (0.1M, pH 6.5). The solution was reduced in volume by 75% and desalted on a column of Biogel P2 (Biorad). The desalted sample, dissolved in water, was loaded onto a column of Purolite A860 strong anion exhange resin in the hydroxide form (ChemColour Industries) and the column washed with four volumes of water. The GAG was eluted with 2.0M NaCl solution, desalted on Biogel P2 as before and lyophilised.

During the course of the reaction, 100 µl samples were taken, quenched with an equivalent volume of EDTA (0.1M, pH 6.5) and analysed by HPSEC.

RESULTS AND DISCUSSION

Samples of chondroitin sulphate and dermatan sulphate were obtained from New Zealand Pharmaceuticals Ltd. The chondroitin sulphate was shown to be a 40:60 mixture of GlcA-GalNAc6SO_3 and of GlcA-GalNAc4SO_3 by assignment of the ^{13}C NMR spectrum (Fig. 2 (a), Table 1) by comparison with published data (Bociek *et al.*, 1980) and integration of the well resolved signals for the U1 (106.8 ppm, GlcA-GalNAc6SO_3, 106.3 ppm GlcA-GalNAc4SO_3), A1 (103.6 ppm, GlcA-GalNAc6SO_3, 104.0 ppm GlcA-GalNAc4SO_3) and A2 (53.8 ppm, GlcA-GalNAc6SO_3, 54.5 ppm GlcA-GalNAc4SO_3) carbons. The ^1H NMR spectrum is shown in Fig. 3.

The dermatan sulphate was analysed by enzyme digestion and HPLC analysis of the disaccharides produced, and by ^{13}C and ^1H NMR spectroscopy (Mascellani *et al.*, 1994). Disaccharide analysis showed that it consisted of 85% UA-GalNAc4SO_3, 5% UA2SO_3-GalNAc4SO_3, 5% UA-GalNAc6SO_3 and 4% UA-GalNAc4,6SO_3. Unfortunately the stereochemistry at C-5 of the uronic acid is lost on enzyme digestion. Assignment of the ^{13}C NMR spectrum (Fig. 4 (a), Table 1) by comparison to published data (Ludwig-Baxter and Perlin, 1991) showed that the major disaccharide unit present was IdoA-GalNAc4SO_3 but there were a number of minor peaks

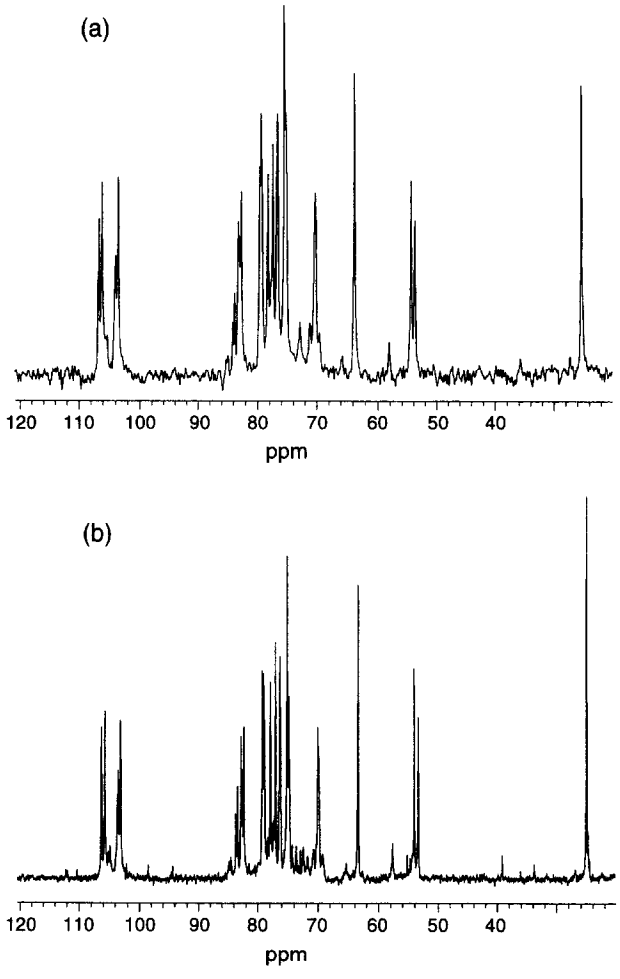

Fig. 2. ^{13}C NMRs of (a) full length and (b) depolymerised chondroitin sulphate.

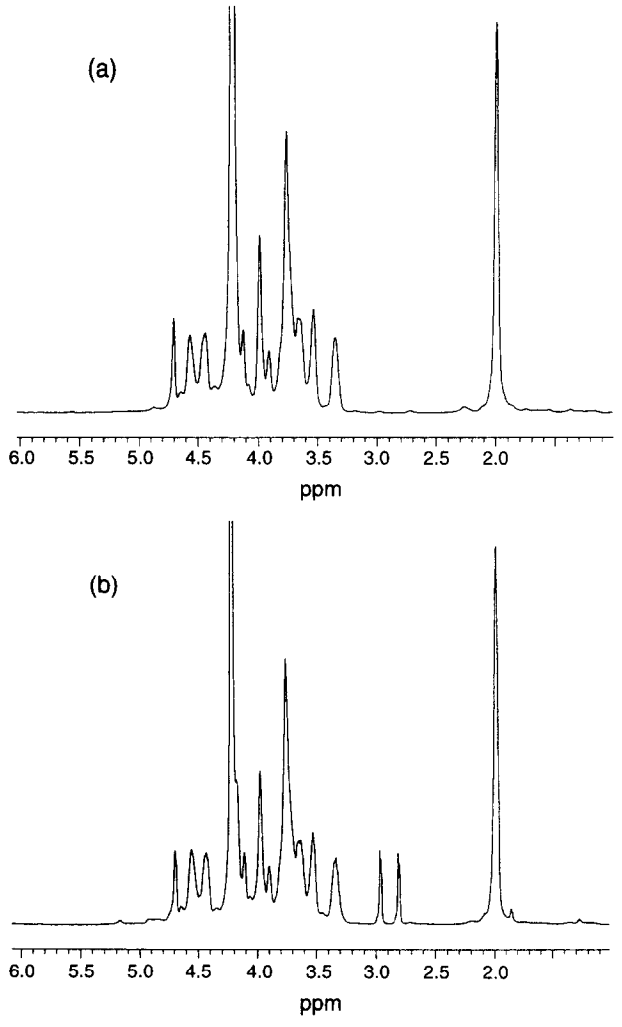

Fig. 3. ^{1}H NMRs of (a) full length and (b) depolymerised chondroitin sulphate.

visible that it was not possible to assign. The identity of the major disaccharide was confirmed by ^{1}H NMR (Fig. 5 (a)) (Mascellani *et al.*, 1994). Integration of the minor signals at 5.11 ppm (U1) and 4.42 ppm (U5) also showed the presence of 5% IdoA2SO_3 as assigned by Mascellani *et al.* (1994), in agreement with the disaccharide analysis. There were a number of other minor signals in the spectra but they could not be positively assigned to protons from the

Table 1. NMR assignments for predominant chondroitin sulphate and dermatan sulphate disaccharides

Assignment	GlcA-GalNAc6SO_3		GlcA-GalNAc4SO_3		IdoA-GalNAc4SO_3	
	$\delta^{1}H$ (ppm)	$\delta^{13}C$ (ppm)	$\delta^{1}H$ (ppm)	$\delta^{13}C$ (ppm)	$\delta^{1}H$ (ppm)	$\delta^{13}C$ (ppm)
U1	4.45	106.8	4.45	106.8	4.82	105.8
U2	3.36	75.5	3.36	75.5	3.46	72.0
U3	3.54	76.7	3.54	76.7	3.85	73.7
U4	3.65	83.8	3.67	83.3	4.01	82.7
U5	3.77	79.5	3.77	79.5	4.67	72.1
U6		177.0		177.0		177.0
A1	4.58	104.0	4.58	103.6	4.61	104.8
A2	4.00	54.5	4.00	53.8	3.95	54.9
A3	4.00	82.7	3.77	78.3	3.95	78.2
A4	4.45	70.3	4.71	79.4	4.59	78.9
A5	3.92	75.5	3.77	77.5	3.75	77.4
A6	4.21	70.3	3.77	63.8	3.72	63.8
NAc CH_3	2.00	25.5	2.00	25.5	2.00	25.5
NAc CO		177.7		177.7		177.7

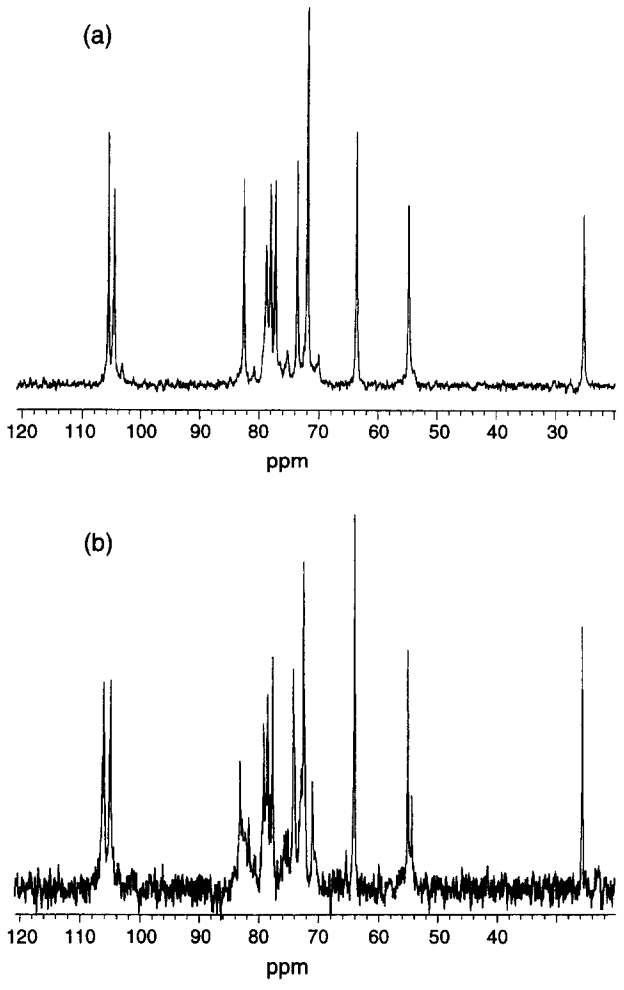

Fig. 4. ^{13}C NMRs of (a) full length and (b) depolymerised dermatan sulphate.

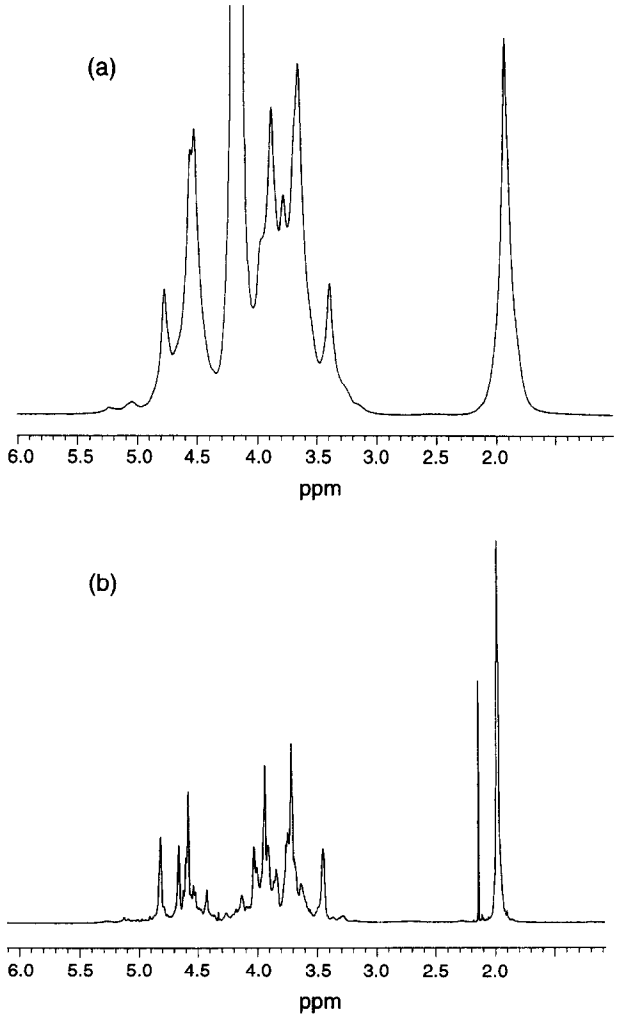

Fig. 5. ^{1}H NMRs of (a) full length and (b) depolymerised dermatan sulphate.

remaining disaccharide units shown to be present by the disaccharide analysis.

The samples of dermatan sulphate and chondroitin sulphate were treated with hydrogen peroxide with a catalyic amount of copper (II) acetate in a 1.5M sodium acetate buffer at room temperature (Bianchini and Mascellani, 1988). No attempt was made to alter the pH of the resulting solutions, which was around 9.6 and remained stable during the reaction. An initial trial without the sodium acetate buffer showed that the rate of depolymerisation was very slow but that there was some loss of sulphate groups from *O*-6 of chondroitin 6-sulphate as shown by ^{13}C NMR spectroscopy.

At regular intervals samples were taken and quenched with EDTA. These were injected directly on to the SEC column, to monitor the rate of depolymerisation. The SEC column was standardised using samples whose molecular weight was determined by viscosity measurements (see below). Under the conditions described the molecular weight of the GAG samples fell rapidly with a $t_{1/2}$ of about 60 min. The rates of depolymerisation of dermatan sulphate and chondroitin sulphate were the same.

To prepare samples for NMR analysis the reaction was stopped at the required point by addition of EDTA and the solution passed down a desalting column. The GAG was isolated by ion-exchange chromatography on Purolite A860, desalted again and lyophilised. Chondroitin sulphate that had been reduced from a molecular weight of 26000 to around 6000 gave a ^{13}C NMR spectrum (Fig. 2 (b)) that did not differ significantly from that of the starting material (Fig. 2 (a)). Small peaks at 99.2 ppm and 94.8 ppm in the depolymerised material are due to the anomeric carbons of the reducing sugars at the end of the chain and the area under the peaks is consistent with the chain length determined by HPSEC.

Comparison of the NMR spectra of depolymerised dermatan sulphate (M_w 5300) with those of the starting material, on the other hand, shows that at least one other significant disaccharide unit is now visible, besides the predominant IdoA-GalNAc4SO_3 of the full length material.

In the ^{13}C spectrum of the depolymerised material (Fig. 4 (b)) there is a new U1 peak at slightly lower field (106.2 ppm) than the IdoA-GalNAc4SO_3 U1 (105.8 ppm) and similarly the A1 peak at 104.8 ppm has

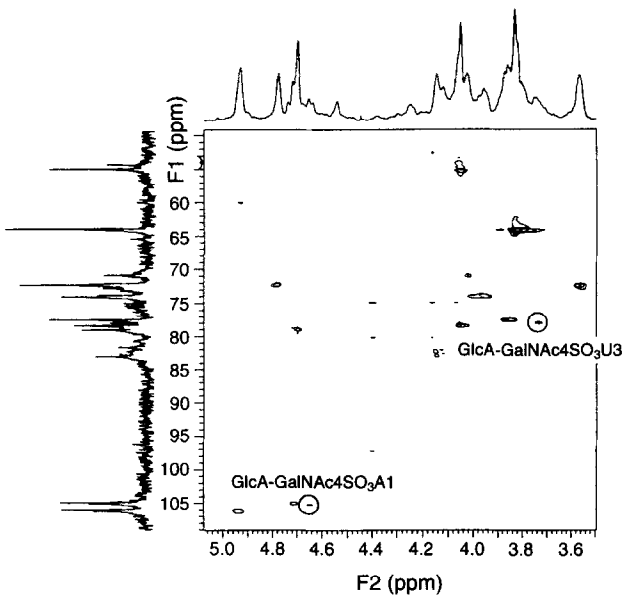

Fig. 6. ^{13}C,^{1}H correlation NMR spectrum of depolymerised dermatan sulphate.

a new companion at 105.0 ppm. The IdoA-GalNAc4SO_3 A2 peak at 54.9 ppm is also doubled with a new peak at 54.4 ppm. These signals are all consistent with the presence of GlcA-GalNAc4SO_3 units in the depolymerised material (Bociek *et al.*, 1980). New peaks in the depolymerised dermatan sulphate spectrum at 79.5 ppm and 76.8 ppm are due to the U5 and U3 carbons of GlcA-GalNAc4SO_3. A proton–carbon correlation spectrum (Fig. 6) shows that the GlcA-GalNAc4SO_3 A1 and U3 ^{13}C peaks are linked to new ^{1}H signals at 4.64 ppm and 3.77 ppm which also have the right chemical shift values for their respective GlcA-GalNAc4SO_3 protons (Table 1) (Sugahara *et al.*, 1994).

It is not clear whether the GlcA-GalNAc4SO_3 signals showing in the NMR of the depolymerised material are due to C5 inversion of the predominant IdoA-GalNAc4SO_3 residues in the starting material or to preferential degradation of the IdoA by the free radical process, as seen in heparin (Nagasawa *et al.*, 1992), allowing GlcA signals to be seen that were too small to be visible in the starting material.

The ^{1}H spectrum (Fig. 5 (b)) also shows that the IdoA2SO_3 residues present in the starting material (U1 peak at 5.11 ppm) have been degraded by the free radical depolymerisation.

The ^{13}C spectrum did not show any peaks due to anomeric carbons of the reducing terminus of the chain (90–100 ppm) but they would be expected to be around 5% of the major signals, calculated from the chain length as determined by HPSEC, and so are likely to be lost in the noise in this case. There were no peaks in the ^{1}H or ^{13}C spectra of the depolymerised dermatan sulphate due to double bonds (^{1}H, 6.0 ppm; ^{13}C, 110–120 ppm).

Elemental analysis showed that the depolymerised material had a lower sulphur/nitrogen ratio than the starting material (Table 2). This could be due to preferential attack of the free radicals on oversulphated residues leading to fewer oversulphated disaccharides or to simple removal of sulphate groups. Attempts to determine the carboxylate to sulphate ratio by potentiometric titration (Volpi, 1994) did not give clear results. To test the possibility that the loss of sulphates could be due to the high pH, the pH of the sodium acetate buffer was adjusted to 6.5 with acetic acid and the depolymerisation of dermatan sulphate repeated. Analysis of the samples by HPSEC and microanalysis to determine sulphur content showed that the depolymerisation was slower at the lower pH (Table 2) but that the sulphur/nitrogen ratio was the same for material with the same molecular weight. There was no significant depolymerisation of dermatan sulphate when dissolved in sodium acetate at pH 9.6 without the addition of hydrogen peroxide so the faster rate of the reaction at high pH was not due to simple base catalysed hydrolysis. This is confirmed by the absence of signals due to double bonds in the NMR spectra of the material depolymerised at high pH.

Attempts to analyse the composition of depolymerised dermatan sulphate by degradation with chondroitinase and HPLC analysis of the resulting disaccharides showed that there was a considerable amount of undegraded material. Volpi has also shown that free radical treatment makes the depolymerised dermatan sulphate (Volpi *et al.*, 1995a) and chondroitin sulphate (Volpi *et al.*, 1995b) resistant to degradation by chondroitinase ABC. The reasons for the resistance of the depolymerised dermatan sulphate to chondroitinase degradation are not clear. Neither loss of sulphate groups nor inversion of the IdoA residues would produce disaccharide units not present in the natural substrates of the enzyme. It is possible that loss of the sulphate from IdoA2SO_3 residues is accompanied by 2,3-epoxide formation, as has been observed in heparin degradation under alkaline conditions (Hricovini *et al.*, 1995), and regions containing the epoxide are not accepted by the enzyme. Alternatively the epoxides may be irreversible inhibitors of the enzyme.

The depolymerised dermatan sulphate had less than

Table 2. Change in molecular weight and sulphur/nitrogen ratio during depolymerisation of dermatan sulphate at differing pH

Depolymerisation conditions	Sulphur/nitrogen	Molecular weight[a]
Starting material	1.20	18 000
pH 9.6, 2 h	0.75	4 500
pH 9.6, 4 h	0.78	4 000
pH 6.5, 2 h	0.89	6 500
pH 6.5, 4 h	0.78	4 200

[a]Molecular weight determined by HPSEC.

Table 3. HPSEC retention times of standards in buffers of differing salt concentration

Standard	Molecular weight	Retention time (minutes)		
		0.2M NaCl	0.5M NaCl	1.4M NaCl
Pullulan 1	5800	28.4	—	—
Pullulan 2	23700	24.3	—	—
Pullulan 3	48000	21.7	—	—
Pullulan 4	100000	18.7	—	—
Pullulan 5	186000	16.8	—	—
Pullulan 6	380000	16.1	—	—
Pullulan 7	853000	15.3	—	—
Chondroitin 1	3900	30.8	29.2	28.7
Chondroitin 2	5200	26.4	26.9	26.9
Chondroitin 3	5900	25.1	25.5	25.4
Chondroitin 4	8900	22.2	22.5	22.9
Chondroitin 5	14000	20.7	21.0	21.2
Dermatan 1	9400	22.6	22.8	23.6
Dermatan 2	18000	21.0	21.2	21.4

20% of the HCII binding activity of standard dermatan sulphate as determined by the chromogenic assay described by Ferrari *et al.* (1994).

Samples of chondroitin sulphate and dermatan sulphate for the determination of molecular weight were prepared by preparative HPSEC of the untreated and depolymerised GAGs. Narrow bands were collected from the HPSEC column and desalted. The average molecular weight of each sample was determined by measuring the inherent viscosity of the GAG solution at a range of concentrations and extrapolating to find the intrinsic viscosity (Flory, 1953). The Mark Houwink equation was used to calculate the average molecular weight from this data (Wasteson, 1971) (Table 3). These standard GAG samples were used to calibrate an HPSEC system consisting of a Biosep 4000 column and a Superdex HR 75 column in series. A standard calibration curve for pullulan (Dubin, 1994) molecular weight standards obtained commercially was also prepared on the same system. The standard GAG samples were also subjected to SEC in buffers of various NaCl concentrations. The results are shown in Table 3 and Fig. 7.

It is clear that the ionic strength of the buffer has very little effect on the molecular volume of chondroitin sulphate under these HPSEC conditions, in contrast to the findings of Bergman *et al.* (1993) with heparin. The molecular volume of the chondroitin sulphate samples does not approach that of pullulan standards. From these results we can see that this approach cannot be used to standardise this SEC system for GAG samples. The challenge remains that in order to appropriately calibrate such an SEC system GAG standards will need to be prepared and their molecular weight determined by alternative means. In this study we chose to use viscometry because of the availability of the equipment and its wide applicability. However, the method is time consuming and there is uncertainty in the quantification

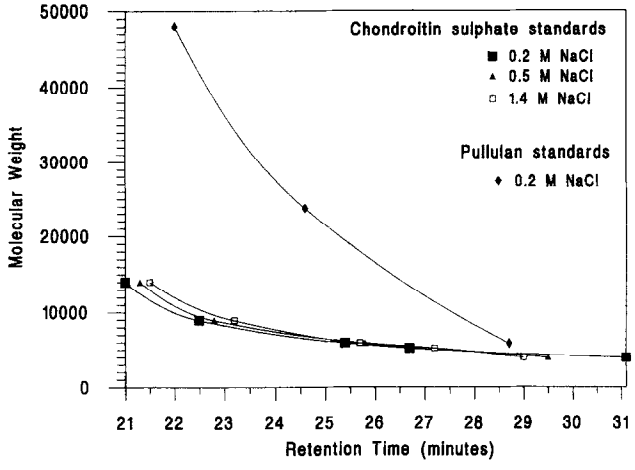

Fig. 7. HPSEC of pullulan standards and chondroitin sulphate standards in buffers of differing NaCl concentration.

of the two constants in the Mark Houwink equation relating the molecular weight to the intrinsic viscosity of different biopolymers (Walter and Jacon, 1994; Rodén *et al.*, 1972; Flory, 1953).

CONCLUSION

Depolymerisation of chondroitin sulphate and dermatan sulphate with hydrogen peroxide and a copper (II) catalyst leads to a smooth reduction in molecular weight leaving the majority of the polymer structurally unaltered. However, there is sufficient, as yet undefined, change in the structure to render the depolymerised material more resistant to chondroitinase degradation. In the case of dermatan sulphate the depolymerisation leads to a reduction in the amount of $IdoA2SO_3$, which is important for HCII binding activity, and an increase in the amount of GlcA, which has a detrimental effect on bioactivity. This makes the method unsuitable for

the production of LMW dermatan sulphate for use as an antithrombotic agent.

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