Biochem. J. (1986) 235, 225–236 (Printed in Great Britain)

The disaccharides formed by deaminative cleavage of *N*-deacetylated glycosaminoglycans

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Chondroitin 4-sulphate, chondroitin 6-sulphate, dermatan sulphate and keratan sulphate were *N*-deacetylated by treatment with hydrazine and then cleaved with HNO_2 at pH 4.0, and the resulting products were reduced with NaB^3H_4. This reaction sequence cleaved the glycosaminoglycans at their *N*-acetyl-D-glucosamine or *N*-acetyl-D-galactosamine residues, which were converted into ^3H-labelled 2,5-anhydro-D-mannitol ($AMan_R$) or 2,5-anhydro-D-talitol ($ATal_R$) residues respectively. The end-labelled disaccharides, composed of D-glucuronic acid (GlcA), L-iduronic acid (IdoA) or D-galactose (Gal) and one of the anhydrohexitols, were identified as follows: both chondroitin 4-sulphate and chondroitin 6-sulphate gave GlcA→$ATal_R$(4-SO_4), GlcA→$ATal_R$(6-SO_4), IdoA→$ATal_R$ (4-SO_4) and GlcA(2-SO_4)→$ATal_R$(6-SO_4); dermatan sulphate gave IdoA→$ATal_R$(4-SO_4), GlcA→$ATal_R$(4-SO_4), GlcA→$ATal_R$(6-SO_4)→IdoA(2-SO_4)$ATal_R$(4-SO_4) and IdoA→$ATal_R$ (4,6-diSO_4); keratan sulphate gave Gal(6-SO_4)→$AMan_R$(6-SO_4), Gal→$AMan_R$(6-SO_4), Gal(6-SO_4)→$AMan_R$ and Gal→$AMan_R$. Several additional disaccharides were generated by treatment of the uronic acid-containing disaccharides with hydrazine to epimerize their uronic acid residues at C-5. A number of these disaccharides were found to be substrates for lysosomal sulphatases and glycuronidases. Methods were developed for the separation of all of the disaccharide products by h.p.l.c. The rate of *N*-deacetylation of chondroitin 4-sulphate by hydrazinolysis was significantly lower than the rate of *N*-deacetylation of chondroitin 6-sulphate or chondroitin. Dermatan sulphate was *N*-deacetylated at an intermediate rate. The relative amounts of disaccharides obtained from chondroitin 4-sulphate, chondroitin 6-sulphate and dermatan sulphate under optimum hydrazinolysis/deamination conditions were comparable with the amounts of the corresponding products released from the polymers by chondroitinase treatment.

INTRODUCTION

Glycosaminoglycans, including CS, DS, KS, heparin and HS, are composed of a repeating disaccharide backbone consisting of a hexosamine and a uronic acid (or, in the case of KS, a Gal residue) (Rodén, 1980). In heparin and HS, the GlcN residues are either *N*-sulphated or *N*-acetylated. These polymers can be specifically cleaved at *N*-sulpho-D-GlcN residues by HNO_2 at pH 1.5 (Shively & Conrad, 1976*a*), but the GlcNAc residues do not react with HNO_2. In CS, DS and KS, all of the hexosamine residues are *N*-acetylated. Consequently, these polymers are not cleaved by HNO_2. However, if these polymers can be *N*-deacetylated, they can then be cleaved at the *N*-unsubstituted hexosamine residues by treatment with HNO_2 at pH 4, with the formation of oligosaccharides having reducing terminal anhydromannose residues from GlcN residues, or anhydrotalose residues from GalN residues (Conrad, 1980). Thus a reaction scheme involving sequential *N*-deacetylation and HNO_2 treatment has been useful in the sequencing of heparin tetrasaccharides (Bienkowski & Conrad, 1985) and in the preparation of disaccharides from CS, DS and KS (Hopwood & Elliot, 1983; Hopwood & Muller, 1983).

Recent studies from this laboratory have shown that, when glycosaminoglycans are subjected to hydrazinolysis, the uronic acid residues are converted into hydrazides, and that these hydrazides, once formed, can undergo both epimerization and β-elimination reactions (Shaklee & Conrad, 1984). The hydrazides can be converted back into uronic acids by treatment with either HIO_3 or HNO_2 (Shaklee & Conrad, 1984). The value of the hydrazinolysis/deamination reaction procedure in the structural analysis of heparin and heparin sulphate (Bienkowski & Conrad, 1985) has been established. The present work was undertaken to assess the value of this reaction sequence for the structural analysis of C4S, C6S, DS and KS.

EXPERIMENTAL

Materials

Reference standards of C4S, C6S, DS, KS-I (corneal KS) and KS-II (skeletal KS) were obtained from Dr.

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Abbreviations used: CS, chondroitin sulphate; C4S, chondroitin 4-sulphate: C6S, chondroitin 6-sulphate; DS, dermatan sulphate; KS, keratan sulphate; UA, uronic acid; GlcA, D-glucuronic acid; IdoA, L-iduronic acid; $ATal_R$, 2,5-anhydro-D-talitol; $AMan_R$, 2,5-anhydro-D-mannitol; ΔDi-0S_R, 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-D-galactitol; ΔDi-4S_R, 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-4-*O*-sulpho-D-galactitol; ΔDi-6S_R, 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-6-*O*-sulpho-D-galactitol; ΔUA, β-D-gluco-4-enepyranosyluronic acid; ΔDi-diS_{BR}, 2-acetamido-2-deoxy-3-*O*-(2-*O*-sulpho-β-D-gluco-4-enepyranosyluronic acid)-4-*O*-sulpho-D-galactitol; ΔDi-diS_{DR}, 2-acetamido-2-deoxy-3-*O*-(2-*O*-sulpho-β-D-gluco-4-enepyranosyluronic acid)-6-*O*-sulpho-D-galactitol; ΔDi-diS_{ER}, 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-4,6-di-*O*-sulpho-D-galactitol.

Mathews and Dr. Cifonellli of the University of Chicago (Chicago, IL, U.S.A.). Samples of DS (CS Type B, lot no. 102F-0057), C6S (CS type C, lot no. 70F-0606), chondroitinase ABC, chondroitinase AC and β-glucuronidase (Type B-3) were obtained from Sigma Chemical Co. NaB^3H_4 (450 Ci/mol) was obtained from Amersham. ^{3}H-labelled IdoA→$AMan_R$, GlcA→$AMan_R$, IdoA(2-SO_4)→$AMan_R$(6-SO_4) and $AMan_R$(6-SO_4) were prepared from heparin (Shively & Conrad, 1976*b*), whereas IdoA→$ATal_R$, GlcA→$ATal_R$, $ATal_R$(4-SO_4), $ATal_R$(6-SO_4) and $ATal_R$ were prepared from DS (Conrad, 1980). [^{14}C]Glucitol was prepared by $NaBH_4$ reduction of [U-^{14}C]glucose (269 Ci/mol; New England Nuclear). 'ΔDi-diS_E' (Suzuki *et al.*, 1968) was a gift from Dr. Osami Habuchi.

Chromatography and electrophoresis

Samples of NaB^3H_4-reduced disaccharides were spotted on strips (2.5 cm × 57 cm) of chromatography paper and run in two different descending paper-chromatography systems. In system 1, samples were run on Whatman no. 3 paper and developed in butan-1-ol/acetic acid/1 M-NH_3 (3:2:1, by vol.). In system 2, chromatographic samples were run on cellulose phosphate strips (Whatman) developed in ethyl acetate/pyridine/5 mM-boric acid (3:2:1 by vol.). Paper electrophoresis was carried out on Whatman no. 3 strips in pyridine/acetic acid/water (1:10:400, by vol., pH 4) (electrophoresis solvent A) or in formic acid/acetic acid/water (2:7:70, by vol., pH 1.7) (electrophoresis solvent B). After development, the dried strips were cut into 1.25 cm segments, which were analysed for radioactivity by liquid scintillation counting in a fluid containing 4 g of diphenyloxazole/l of toluene. H.p.l.c. was run on a 4.5 mm × 25 cm Whatman SAX column (Delaney *et al.*, 1980*a*) or on a 4 mm × 30 cm Varian Micro-Pak AX-5 column developed in aqueous KH_2PO_4 solutions at the concentrations described in the Results section. Effluent fractions (0.5 ml) were collected and radioactivities were determined as previously described (Delaney *et al.*, 1980*a*).

Polymer analysis

Stock glycosaminoglycan/[^{14}C]glucitol solutions were prepared by mixing 200 μl of 25 mg/ml glycosaminoglycan solution with 50 μl of [^{14}C]glucitol (100000 c.p.m./μl). A 15 μl portion of the stock solution was placed in a 100 μl Reacti-Vial (Pierce Chemical Co.), the sample was dried in an air stream and finally dissolved in 20 μl of anhydrous hydrazine containing 0.2 mg of hydrazine sulphate. The sample was then capped and placed in a 100 °C sand bath for 10 h. The cooled sample was dried in a stream of air and freeze-dried to remove most of the hydrazine, and 5–10 μl of 3 M-H_2SO_4 was added to the sample to adjust the pH to 4.0. HNO_2 (20 μl), pH 4, was then added to the sample. After 30 min at room temperature, the sample was chilled on ice, adjusted to pH 8.5 with 5–10 μl of 1 M-Na_2CO_3, mixed with 10 μl of 0.5 M-NaB^3H_4 in 0.2 M-Na_2CO_3, pH 10.2, and reduced for 2 h at 0 °C. Excess NaB^3H_4 was destroyed by addition of 5 μl of 3 M-H_2SO_4, and the sample was dried in a stream of air, redissolved in water and re-dried. Finally, the sample was redissolved in 60 μl of water and portions were analysed by paper chromatography or h.p.l.c. In studies involving the rates of release of disaccharides from reference glycosaminoglycans, a similar set-up was used. For these studies, a 10 mg/ml reference glycosaminoglycan solution was used to prepare the stock solution, and 50 μl of the solution was dried in a 6 mm × 50 mm test tube. The sample was redissolved in 100 μl of hydrazine containing 1 mg of hydrazine sulphate, and 15 μl portions were sealed into capillary tubes and heated at 100 °C for the times desired. After reaction, each sample was removed from the capillary tube, dried in a 6 mm × 50 mm tube, and worked up and analysed as above.

For analysis of the disaccharides released from reference polymers by the action of chondroitinase ABC or chondroitinase AC, 15 μl of the glycosaminoglycan/[^{14}C]glucitol solution was placed in a 1.5 ml Eppendorf tube. A 10 ml portion of enriched Tris buffer (Saito *et al.*, 1968), pH 7.3, and 40 μl (0.4 unit) of chondroitinase AC or chondroitinase ABC dissolved in a 1:10 dilution of the same buffer were added to the sample. After 3 h at 37 °C, the sample was dried in an air stream, chilled on ice, and reduced with NaB^3H_4 at 0 °C as previously described (Glaser & Conrad, 1979). After acidification, the sample was dried twice in an air stream and redissolved in 60 μl of water, and portions were analysed by paper chromatography and h.p.l.c.

Disaccharide characterization

The disaccharides released following hydrazinolysis/deamination/NaB^3H_4 treatment of reference glycosaminoglycans were purified as described in the Results section. Elution from paper chromatograms or paper electrophoretograms was carried out by using the spin thimbles (Reeve Angel). The kinetics and the products of acid hydrolysis of the major disaccharides were used in the structural analysis of these disaccharides. For these studies, approx. 500000 c.p.m. of ^{3}H-labelled unknown was placed in a 6 mm × 50 mm tube, dried in a stream of air, dissolved in 60 μl of 0.5 M-H_2SO_4, covered with mineral oil and heated at 100 °C. Portions were removed and analysed directly by paper chromatography in system 1. When only limited amounts of starting material were available, samples were taken only at 45 min and 6 h. Further identification of unknown hydrolysis products was carried out by eluting the compounds and comparing them with reference standards or previously identified compounds by paper electrophoresis or h.p.l.c. β-Glucuronidase digestion of isolated disaccharides was carried out according to the procedure of Jacobsson *et al.* (1979). Products were separated from any residual substrate by paper chromatography in system 1. Purified uronic acid-containing disaccharides were epimerized as described previously (Shaklee & Conrad, 1984).

RESULTS

Disaccharides were formed by cleavage of CS, DS and KS with acid, enzymes or hydrazinolysis/deamination, and were purified and identified by chromatographic or electrophoretic comparisons with previously characterized standards (Conrad, 1980; Bienkowski & Conrad, 1985). Table 1 lists the properties of the standards from CS and DS that were used in these comparisons as well as those of the products characterized in the present study. In most cases the identification of a new compound was based, in part, on whether it migrated like an unsulphated, a monosulphated or a disulphated compound on chromatograms or electrophoretograms.

Table 1. Chromatographic and electrophoretic properties of disaccharides

The paper-chromatographic and paper-electrophoretic systems are described in the Experimental section. Paper-chromatographic values are $R_{glucitol}$ values. Paper-electrophoretic values are relative to IdoA(2-SO_4)→$AMan_R$(6-SO_4). H.p.l.c. separations were all run at a flow rate of 1 ml/min under the isocratic conditions indicated.

Compound	Paper chromatography		Paper electrophoresis		H.p.l.c. Whatman SAX		H.p.l.c. Varian AX	
	1	2	A	B	[KH_2PO_4] (mM)	Elution time (min)	[KH_2PO_4] (mM)	Elution time (min)
Standards								
GlcA→$ATal_R$	0.63	0.46	0.45	0.19	5	17.0	5	25.0
IdoA→$ATal_R$	0.77	0.61	0.41	0.18	5	16.5	5	25.0
$ATal_R$(4-SO_4)	0.68	3.29	0.70	0.76	5	19.0	—	—
$ATal_R$(6-SO_4)	0.61	2.38	0.67	0.80	5	22.5	—	—
$AMan_R$(6-SO_4)	0.69	2.38	0.72	0.81	5	22.0	—	—
New compounds								
I. GlcA→$ATal_R$(6-SO_4)	0.23	0.42	0.78	0.62	40	24.5	25	32.5
IdoA→$ATal_R$(6-SO_4)	0.31	0.58	0.74	0.64	40	25.0	25	34.5
II. GlcA→$ATal_R$(4-SO_4)	0.32	0.67	0.72	0.58	40	26.5	25	38.5
III. IdoA→$ATal_R$(4-SO_4)	0.38	0.67	0.69	0.62	40	27.0	25	43.5
VI. IdoA→$ATal_R$(4,6-diSO_4)	0.15	0.36	0.93	0.92	180	24.0	135	27.5
							115	35.5
GlcA→$ATal_R$(4,6-diSO_4)	—	0.36	—	—	—	—	135	23.5
							115	30.5
$ATal_R$(4,6-diSO_4)	0.27	1.64	1.15	1.23	—	—	—	—
VII. IdoA(2-SO_4)→$ATal_R$(4-SO_4)	0.15	0.61	0.96	1.0	180	19.5	135	30.0
							115	40.0
GlcA(2-SO_4)→$ATal_R$(4-SO_4)	—	0.61	—	—	—	—	135	25.0
							115	30.5
VIII. GlcA(2-SO_4)→$ATal_R$(6-SO_4)	0.11	0.33	1.03	0.93	180	10.0	135	17.5
							115	23.5
IdoA(2-SO_4)→$ATal_R$(6-SO_4)	—	0.33	—	—	—	—	135	21.0
							115	27.5
IV. Gal(6-SO_4)→$AMan_R$(6-SO_4)	0.11	1.0	0.86	1.04	70	12.5	—	—
Va. Gal(6-SO_4)→$AMan_R$	0.38	1.54	0.52	0.62	5	17.0	5	21.0
Vb. Gal→$AMan_R$(6-SO_4)	0.38	1.50	0.54	0.63	5	20.0	5	22.5
Vc. Gal_R(6-SO_4)	0.42	1.46	0.64	0.71	5	26.0	—	—
Gal→$AMan_R$	0.84	1.24	0.11	0.13	—	—	—	—

Disaccharides were prepared from C6S, C4S, DS and KS by *N*-deacetylation with hydrazine and cleavage of the *N*-deacetylated polymers with HNO_2. The resulting mixtures of disaccharides were reduced with NaB^3H_4 and separated by paper chromatography. Fig. 1 shows profiles obtained for six different reference glycosaminoglycans. Each polymer gave a characteristic profile of radiolabelled peaks, with one or two peaks usually predominating. The individual peaks from each chromatogram were eluted from the paper segments and purified further by preparative paper electrophoresis and paper chromatography. The products formed by hydrazinolysis/deamination cleavage are designated with Roman numerals in Fig. 1 and Table 1. Several additional new products were obtained by enzymic cleavage or by epimerization of the original disaccharides. These secondary products are shown in Table 1 beneath the disaccharides from which they were derived.

Identification of products from CS and DS: the major disaccharides

The identifications of peaks I, II and III were based on several obervations. On paper electrophoresis and paper chromatography compounds I, II, and III migrated like monosulphated disaccharides, with migration rates similar to those of previously identified monosulphated disaccharides from heparin (Bienkowski & Conrad, 1985). Each of the disaccharides was subjected to graded acid hydrolysis in 0.5 M-H_2SO_4 at 100 °C, and the ^3H-labelled hydrolysis products were identified by their migration rates on chromatograms or electrophoretograms and quantified by the amount of ^3H in each. Fig. 2 shows the kinetics of hydrolysis of each disaccharide. Fig. 2(*a*) shows that compound I, the major compound obtained from C6S, was hydrolysed slowly ($t_{\frac{1}{2}}$ = 1 h) to give GlcA→$ATal_R$ (● symbols) as the major acid-resistant labelled product remaining after a 6 h hydrolysis period. The latter compound was hydrolysed very slowly to yield $ATal_R$ (■ symbols), consistent with the known acid resistance of D-glucuronosyl bonds (Conrad, 1980). As shown in Fig. 2(*b*), compound II, the major compound from C4S, was hydrolysed more rapidly ($t_{\frac{1}{2}}$ = 15 min), and again gave GlcA→$ATal_R$ as the primary labelled product. Both compounds I and II were cleaved by β-glucuronidase, yielding labelled monosulphated monosaccharides that were identified as $ATal_R$(6-SO_4) and $ATal_R$(4-SO_4) respectively by paper-chromatographic, paper-electrophoretic and h.p.l.c.

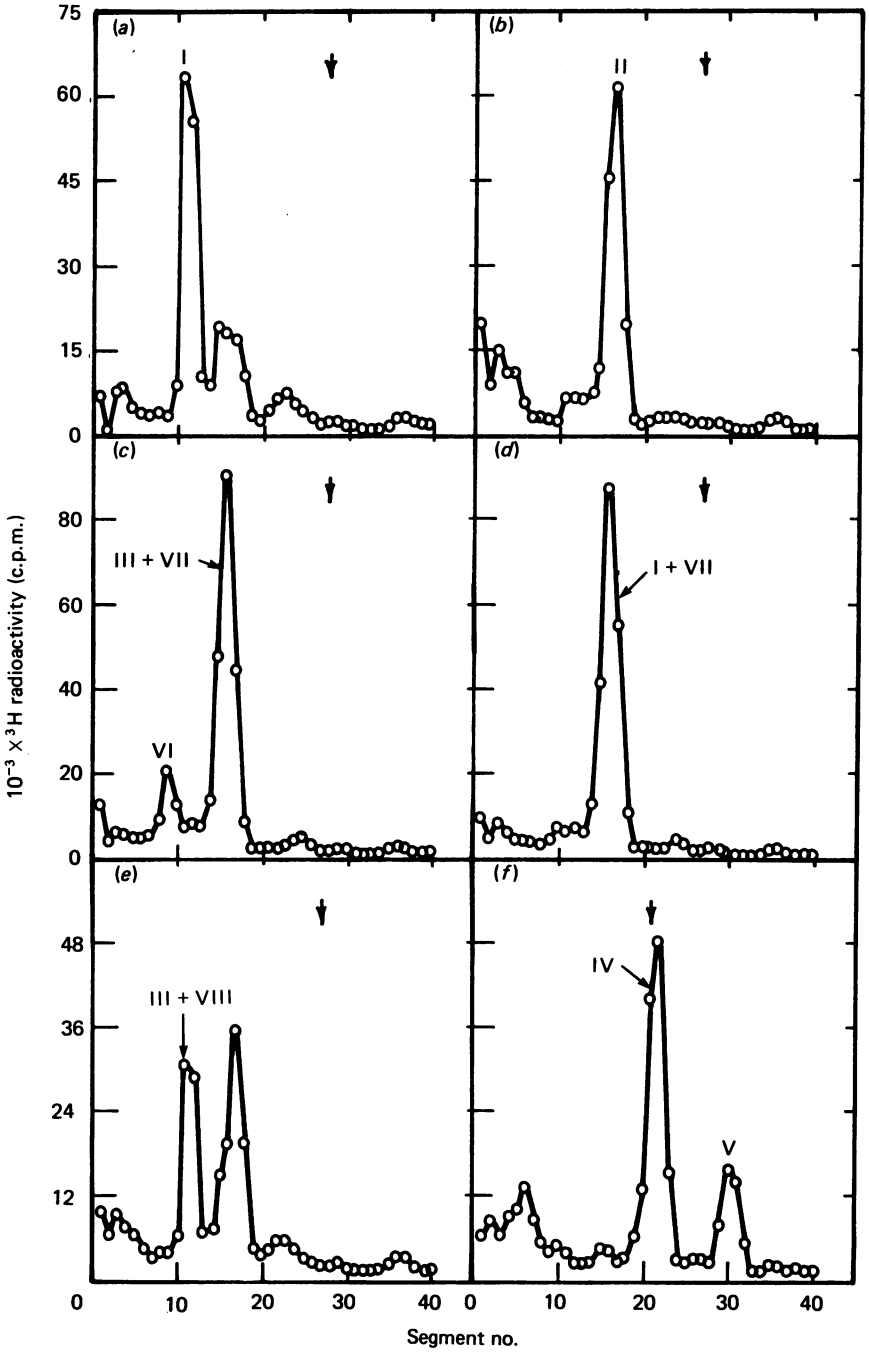

Fig. 1. Paper chromatography of products formed by reactions of glycosaminoglycans in the hydrazinolysis/HNO_2 reaction sequence

Reference glycosaminoglycan/[^{14}C]glucitol stock solutions were treated successively with hydrazine/hydrazine sulphate, HNO_2 and NaB^3H_4 as described in the Experimental section and the reaction mixtures were analysed by paper chromatography in system 2. The glycosaminoglycans shown are C6S from Mathews and Cifonelli (*a*), C4S from Mathews and Cifonelli (*b*), DS from Mathews and Cifonelli (*c*), DS from Sigma (*d*), C6S from Sigma (*e*) and KS-II from Mathews and Cifonelli (*f*). The arrows indicate the positions of migration of the D-[^{14}C]glucitol internal standard. The KS chromatogram (*f*) was run for 15 h whereas the other chromatograms were developed for 24 h.

comparison with standards. These monosulphated monosaccharides were obtained as minor intermediates during the course of acid hydrolysis because of the relative acid-lability of their sulphate ester groups compared with the lability of their glucuronosyl bonds. On the basis of the known structures of C6S and C4S, the chromatographic and electrophoretic properties of the disaccharides, the identification of the acid and enzymic hydrolysis products, the hydrolysis rates and the fact that primary sulphate esters (6-sulphates) are more acid-resistant than are secondary sulphate esters (4-sulphates) (Turvey, 1965), compounds I and II were identified as GlcA→$ATal_R(6\text{-}SO_4)$ and GlcA→$ATal_R(4\text{-}SO_4)$ respectively.

Compound III, the major product obtained from DS,

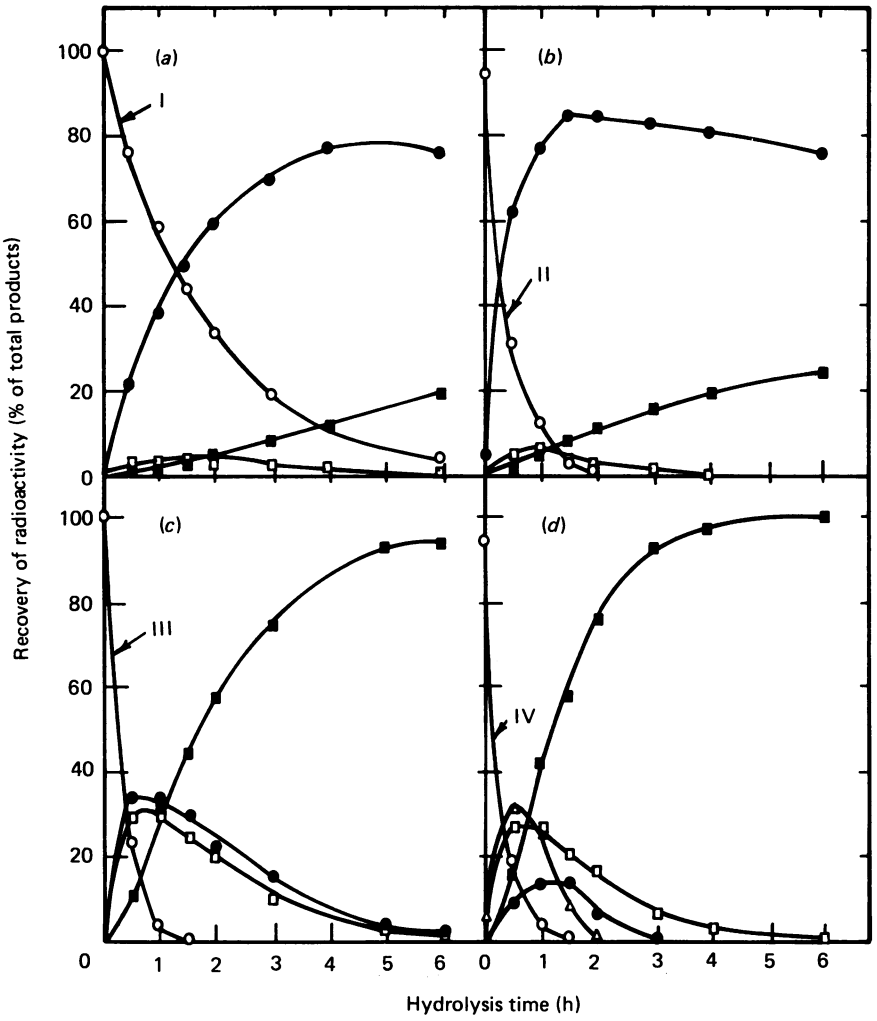

Fig. 2. Kinetics of acid hydrolysis of the major disaccharides formed by the hydrazinolysis/deamination/NaB^{3}H$_4$ treatment of reference glycosaminoglycans

Compounds I (*a*), II (*b*) III (*c*) and IV (*d*) from Fig. 1 were purified by paper chromatography in system 2 and paper electrophoresis in solvent A and were dissolved in 0.5 M-H_2SO_4, covered with mineral oil and heated at 100 °C. At the intervals shown, portions of the hydrolysis mixture were analysed by paper chromatography in system 1. The percentages of the total radioactivity recovered in starting material (○), unsulphated disaccharide (●), monosulphated monosaccharide (□), unsulphated monosaccharide (■) and unsulphated disaccharide (△, panel *d*) were calculated and plotted versus hydrolysis time. See the text for specific identification of hydrolysis products in each panel.

was hydrolysed rapidly ($t_{\frac{1}{2}}$ = 15 min), yielding $ATal_R$ as the major hydrolysis product after 6 h (Fig. 2*c*). The mixture of labelled hydrolysis products obtained at early time points was separated by paper electrophoresis into two components, identified as IdoA→$ATal_R$ (□ symbols) (Conrad, 1980) and $ATal_R$(4-SO_4) (● symbols). The hydrolysis results, the chromatographic and electrophoretic migrations of the compound and the known structure of DS allowed identification of compound III as IdoA→$ATal_R$(4-SO_4).

Identification of products from CS and DS: the minor disaccharides

Three additional compounds, peaks VI and VII from DS (Figs. 1*c* and 1*d*) and peak VIII from CS (Fig. 1*e*), were identified as disulphated disaccharides on the basis of their paper-chromatographic and paper-electrophoretic migrations. Complete acid hydrolysis of compound VI gave a single labelled product (Fig. 3*b*), which migrated with $ATal_R$. This indicated that compound VI had the acid-labile IdoA→$ATal_R$ bond. Partial hydrolysis of compound VI (Fig. 3*a*) gave, in addition to $ATal_R$, a mixture of hydrolysis intermediates. The peaks indicated by the horizontal bar in Fig. 3(*a*) were eluted together, resolved by paper electrophoresis and paper chromatography, and shown to be a mixture of IdoA→$ATal_R$, $ATal_R$(4-SO_4) and $ATal_R$(6-SO_4). The hydrolysis intermediate found at segment 8 in Fig. 3(*a*) was eluted and characterized by paper electrophoresis as a disulphated monosaccharide. It was identical with a product obtained by incubation of compound VI with a lysosomal preparation from cultured chondrocytes, shown previously to be rich in α-iduronidase (Shaklee *et al.*, 1985). These results indicate that compound VI is IdoA→$ATal_R$(4,6-diSO_4) and that the disulphated monosaccharide released

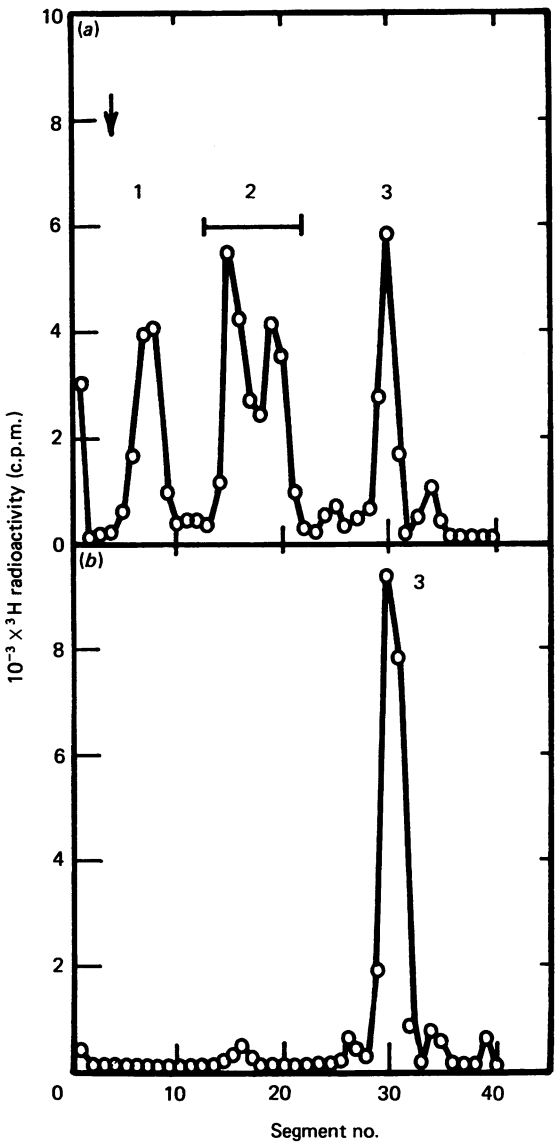

Fig. 3. Paper chromatography of the partial (*a*) and total (*b*) acid hydrolysis products formed from Compound VI, which was derived from DS

Compound VI was isolated from paper chromatograms (Fig. 1*c*) and purified by paper chromatography in system 1 and paper electrophoresis in solvent A. The purified compound was dissolved in 0.5 M-H_2SO_4 (60 μl total volume), covered with mineral oil and heated at 100 °C. At 45 min (*a*) and 6 h (*b*) portions of the sample were removed and analysed by paper chromatography in system 1. The arrow in panel (*a*) indicates the migration position of the starting material. As described in the text, peaks were identified as $ATal_R(4,6\text{-}diSO_4)$ (peak 1), a mixture of IdoA→$ATal_R$, $ATal_R(4\text{-}SO_4)$, and $ATal_R(SO_4)$ (peak 2) and $ATal_R$ (peak 3).

by partial acid hydrolysis or α-iduronidase digestion was $ATal_R(4,6\text{-}diSO_4)$.

Compound VII was obtained from both DS samples (Figs. 1*c* and 1*d*). It was separated from compound III (above) by paper chromatography in system 1. Paper chromatography and paper electrophoresis indicated that it was a disulphated disaccharide (Table 1). Compound VII was identified by analysis of the products released on total and partial acid hydrolysis and enzymic hydrolysis (detailed results not shown). Total acid hydrolysis of the compound gave $ATal_R$ as the only labelled product, indicating a backbone structure of IdoA→$ATal_R$ for compound VII. Partial acid hydrolysis yielded IdoA→$ATal_R$ and $ATal_R(4\text{-}SO_4)$, as well as $ATal_R$. Enzymic hydrolysis of compound VII with a chondrocyte lysosomal preparation, which contained α-idurono-2-sulphatase and α-L-iduronidase (Shaklee *et al.*, 1985), gave $ATal_R(4\text{-}SO_4)$ as the major digestion product. These results indicate that the structure of compound VII is IdoA$(2\text{-}SO_4)$→$ATal_R(4\text{-}SO_4)$.

Compound VIII, from the Sigma C6S (Fig. 1*e*), was separated from GlcA→$ATal_R(6\text{-}SO_4)$ by paper chromatography in system 1. Paper electrophoresis of compound VIII indicated that it was a disulphated disaccharide (Table 1). Total hydrolysis of compound VIII gave GlcA→$ATal_R$ as the major product, whereas partial hydrolysis of the compound gave GlcA→$ATal_R(6\text{-}SO_4)$, GlcA→$ATal_R$ and a small amount of $ATal_R(6\text{-}SO_4)$. These results indicated that compound VIII was GlcA$(2\text{-}SO_4)$→$ATal_R(6\text{-}SO_4)$. It is of note that the sulphate located on the GlcA was quite acid-labile. Final confirmation of the structure of compound VIII as GlcA$(2\text{-}SO_4)$→$ATal_R(6\text{-}SO_4)$ was based on the demonstration that extracts of Hunter fibroblasts, which contain lysosomal β-glucurono-2-sulphatase and β-glucuronidase, but not α-idurono-2-sulphatase (Shaklee *et al.*, 1985), converted compound VIII into a mixture of GlcA→$ATal_R(6\text{-}SO_4)$ and $ATal_R(6\text{-}SO_4)$.

Identification of KS products: compounds IV, Va and Vb

Fig. 2(*d*) shows the kinetics of hydrolysis of compound IV, the major disaccharide obtained from KS (Fig. 1*f*). Total hydrolysis of this compound gave $AMan_R$ (■ symbols). The compounds obtained at early time points in hydrolysis of compound V were identified by chromatographic and electrophoretic comparison with standards as $AMan_R(6\text{-}SO_4)$ (Shively & Conrad, 1976*b*), Gal→$AMan_R$ and two monosulphated disaccharides, Gal$(6\text{-}SO_4)$→$AMan_R$ and Gal→$AMan_R(6\text{-}SO_4)$. The last-mentioned two compounds are identified below. On the basis of the hydrolysis results, the mobility of the compound on paper electrophoresis (Table 1) and the known structure of KS, compound IV was identified as Gal$(6\text{-}SO_4)$→$AMan_R(6\text{-}SO_4)$.

Fig. 1(*f*) shows that peak V was also found among the depolymerization products of KS. On paper chromatograms peak V gave a small amount of Gal→$AMan_R$ and one major peak that could not be further resolved. The major component separated into three monosulphated compounds on anion-exchange h.p.l.c. (detailed results not shown). These compounds, labelled Va, Vb and Vc, were obtained in the proportions 1:25:5. Compounds Va and Vb were identified as products that were chromatographically and electrophoretically identical with monosulphated disaccharide intermediates obtained by partial hydrolysis of Gal$(6\text{-}SO_4)$→$AMan_R(6\text{-}SO_4)$ (compound IV, above). Fig. 4(*a*) shows the paper-chromatographic profile of the products formed by partial hydrolysis of Gal$(6\text{-}SO_4)$→$AMan_R(6\text{-}SO_4)$. The peak containing compounds Va and Vb was eluted from the paper chromatogram and separated by anion-exchange h.p.l.c. into the two compounds Va and Vb. Both migrated like monosulphated disaccharides on paper chromatograms. Their

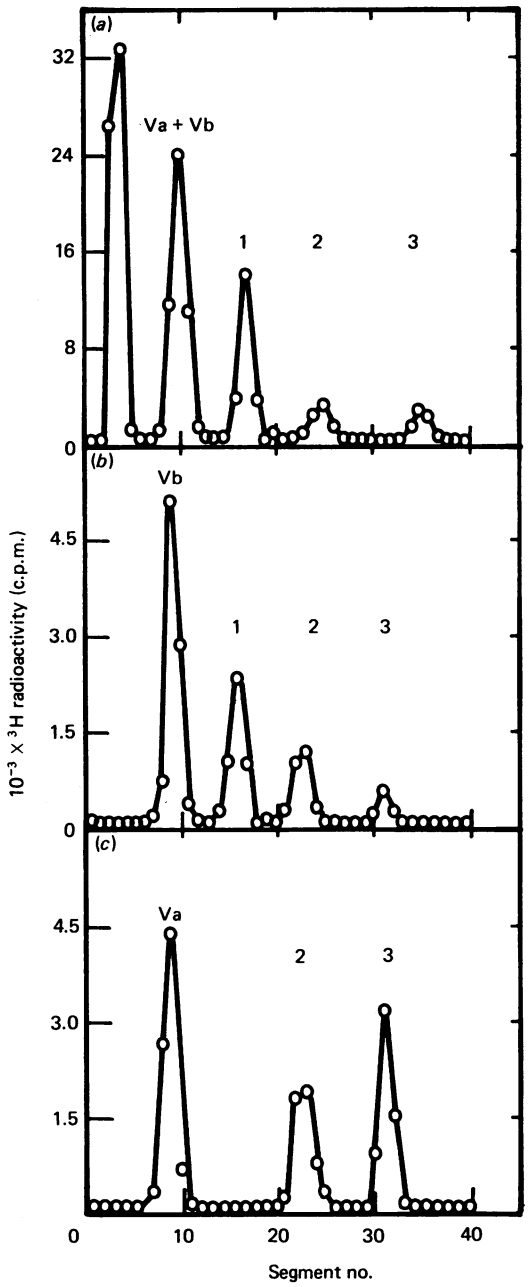

Fig. 4. Paper chromatography of the partial acid hydrolysis products of the sulphated disaccharides from KS

Gal(6-SO_4)→$AMan_R$(6-SO_4) (compound IV) was hydrolysed in 50 μl of 0.1 M-HCl at 100 °C for 2 h and analysed by paper chromatography in system 1 (*a*). The mixture of monosulphated disaccharides (Va+Vb) was eluted from the chromatogram and the two components were separated by h.p.l.c. Compounds Vb (*b*) and Va (*c*) were hydrolysed and analysed by paper chromatography as in (*a*). Peaks 1, 2 and 3, identified by comparison of their migration distances with those of standards, are $AMan_R$(6-SO_4), Gal→$AMan_R$ and $AMan_R$ respectively.

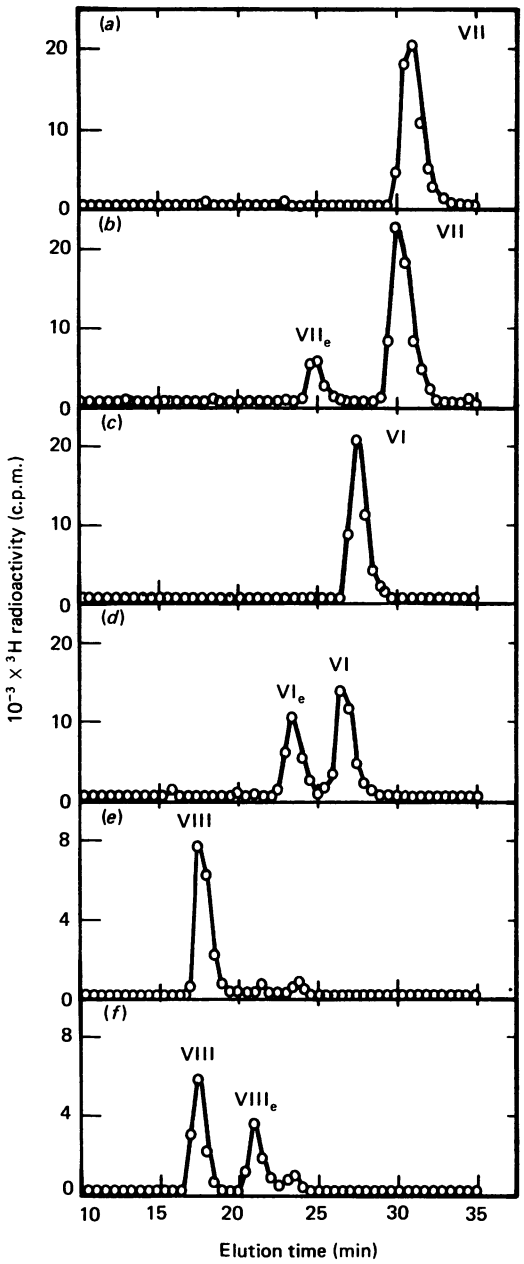

Fig. 5. H.p.l.c. of disulphated disaccharides from CS and DS before (*a*, *c* and *e*) and after (*b*, *d* and *f*) treatment with hydrazine/HIO_3

IdoA(2-SO_4)→$ATal_R$(4-SO_4) (*a* and *b*), IdoA→$ATal_R$(4,6-diSO_4) (*c* and *d*) and GlcA(2-SO_4)→$ATal_R$(6-SO_4) (*e* and *f*) were analysed by isocratic elution from a Varian AX-5 column with 135 mM-KH_2PO_4. Samples shown in panels (*b*) and (*d*) were obtained by treatment with hydrazine/hydrazine sulphate at 100 °C for 11 h, whereas, the sample shown in panel (*f*) was obtained by treatment for 24 h. The epimeric products are designated by a subscript 'e' following the number of the parent disaccharide.

identities were established by analysis of their partial acid hydrolysis products. Figs. 4(*b*) and 4(*c*) show the partial acid hydrolysis products of compounds Vb and Va respectively. Compound Vb yielded labelled products identified previously as $AMan_R$(6-SO_4) (peak 1), Gal→$AMan_R$ (peak 2) and $AMan_R$ (peak 3), indicating that the sulphate group on compound Vb was on the $AMan_R$ residue. Thus peak Vb was identified as Gal→$AMan_R$(6-SO_4). Compound Va gave the same labelled hydrolysis intermediates as compound Vb, except that the $AMan_R$(6-

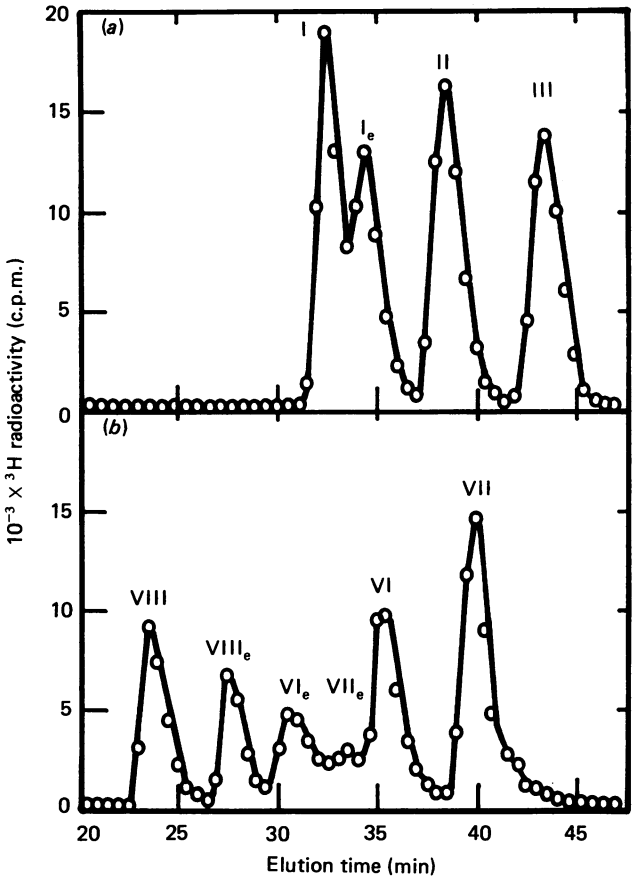

Fig. 6. H.p.l.c. separation of disaccharides formed by treatment of CS and DS with hydrazine/HNO_2/NaB^3H_4

CS and DS monosulphated disaccharides (*a*) and disulphated disaccharides (*b*), prepared either by hydrazine/HNO_2 treatment or by epimerization of isolated disaccharides with hydrazine, were analysed by h.p.l.c. on a Varian AX-5 column. The monosulphated disaccharides were eluted isocratically with 25 mM-KH_2PO_4. The disulphated disaccharides were eluted isocractically with 115 mM-KH_2PO_4. Peaks were identified by their chromatographic and electrophoretic properties shown in Table 1. Epimeric derivatives are designated by a subscript 'e' following the number of the parent disaccharide.

SO_4) peak at segment 16 in Fig. 4(*b*) was not found. The absence of $AMan_R(6\text{-}SO_4)$ indicated that the sulphate ester in this monosulphated monosaccharide must be on the Gal residue. Therefore compound Va was identified as $Gal(6\text{-}SO_4)\rightarrow AMan_R$. In experiments not presented here compound Vc was identified as $Gal_R(6\text{-}SO_4)$. Further work on the origin of this compound was not carried out.

Preparation of C-5 uronic acid epimers of the disaccharides obtained from CS and DS

When uronic acid-containing disaccharides are heated with hydrazine, they are converted into hydrazides that can undergo epimerization at C-5 of the uronic acid moiety (Shaklee & Conrad, 1984). Treatment of the hydrazides with HNO_2 or HIO_3 converts them back into a mixture containing the original and the epimerized disaccharides (Shaklee & Conrad, 1984). This reaction sequence permits the preparation of a series of new disaccharides, some of which may turn out to represent naturally occurring structures. Disaccharides VI, VII and VIII (above) were used as starting materials to prepare such a series of epimerized products, designated VI_e, VII_e and $VIII_e$ respectively. They were treated with hydrazine/HIO_3, and the products were separated by anion-exchange h.p.l.c. Of several h.p.l.c. columns tried, a Varian AX-5 column (weak anion-exchanger, 5 μm particle size) was found to give the best resolution of the disaccharides and their epimers.

Figs. 5(*a*), 5(*c*) and 5(*e*) show the h.p.l.c. profiles of $IdoA(2\text{-}SO_4)\rightarrow ATal_R(4\text{-}SO_4)$, $IdoA\rightarrow ATal_R(4,6\text{-}diSO_4)$ and $GlcA(2\text{-}SO_4)\rightarrow ATal_R(6\text{-}SO_4)$ respectively. Figs. 5(*b*), 5(*d*) and 5(*f*) show these same compounds after hydrazine/HIO_3 treatment. In each case, a new peak representing the C-5 uronic acid epimer of the starting material was observed. The components of the epimerization mixtures were not separable by paper chromatography or paper electrophoresis (Table 1).

Fig. 6(*a*) shows that a mixture of the monosulphated disaccharides obtained from CS and their epimerized products can be separated by h.p.l.c. with the use of isocratic elution from an Varian AX column with 25 mM-KH_2PO_4. Similarly, Fig. 6(*b*) shows the isocratic separation of the disulphated disaccharides with 115 μM-KH_2PO_4 on the same column. Thus all of these products can be separated from each other in a single run by using a step in the concentration of the eluent after the monosulphated disaccharides have emerged.

Glycosaminoglycan analysis: h.p.l.c. of chondroitinase products

The disaccharide composition of glycosaminoglycans is usually determined by analysis of the products released when the glycosaminoglycans are treated with bacterial enzymes (chondroitinases, keratanases, heparinases). The use of hydrazinolysis/deamination cleavage of the glycosaminoglycans offers an alternative method for such analyses, provided that the reaction products are recovered in good yields. To determine the feasibility of the use of the hydrazinolysis/deamination method, several of the glycosaminoglycan standards were first analysed by using the enzymic methods to obtain baseline values for their compositions. Fig. 7 shows the profiles of products obtained when the reference CS and DS standards were treated with chondroitinase and the resulting disaccharides were reduced with NaB^3H_4 and then analysed by anion-exchange h.p.l.c. As shown in Table 2, the Whatman SAX column gave better separation of the reduced chondroitinase products than did the Varian AX-5 column used for the separation of the hydrazinolysis products. The identities of the major peaks in the profiles of the chondroitinase digests were established on the basis of previous studies (Delaney *et al.*, 1980*b*). The identifications of the minor disulphated disaccharide peaks were based largely on the comparison of the retention times of these peaks and their yields with those of the minor peaks obtained below in the profiles of the hydrazinolysis products from the same standards.

Fig. 7(*a*) shows the chondroitinase ABC reduction products from the Sigma DS. The major peak in the profile at 31.5 min and the minor peak with a 44 min retention time have been identified previously as $\Delta Di\text{-}4S_R$ and $\Delta Di\text{-}6S_R$ respectively (Delaney *et al.*, 1980*b*). A single disulphated disaccharide, eluted at 61.5 min, was obtained from Sigma DS. This compound was identified as the

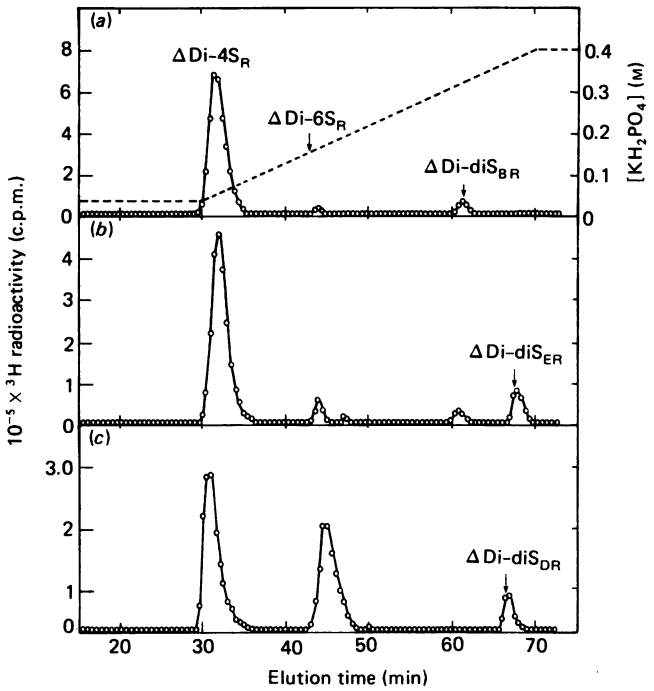

Fig. 7. Direct h.p.l.c. of chondroitinase-treated Sigma DS (*a*), Mathews and Cifonelli DS (*b*) and Sigma C6S (*c*)

The reference glycosaminoglycans were treated with chondroitinase ABC, reduced with NaB^3H_4 and analysed directly by h.p.l.c. on a Whatman SAX column with the gradient conditions shown (----) in panel (*a*). Peak identifications are made in the text.

aldehyde-reduced form of ΔUA(2-SO_4)→ATal(4-SO_4) derived from IdoA(2-SO_4)→GalNAc(6-SO_4) and referred to previously as 'ΔDi-diS_B' (Suzuki *et al.*, 1968). This identification was based on the following evidence: (1) the disaccharide was not obtained following chondroitinase AC digestion of the starting material, indicating an IdoA origin for the uronic acid residue of the compound; (2) the hydrazinolysis results presented below and those described by Hopwood & Muller (1983) show that IdoA(2-SO_4)→ATal$_R$(4-SO_4) is the only disulphated disaccharide present in this DS sample; (3) this material is also present in the DS from Mathews and Cifonelli, a result predicted on the basis of the hydrazinolysis data below.

Fig. 7(*b*) shows the chondroitinase ABC products obtained from the Mathews and Cifonelli DS. The profile is qualitatively similar to that of the Sigma DS, except for the presence of a second disulphated disaccharide, with a retention time of 68 min. This compound was identified as the aldehyde-reduced form of ΔUA→ATal(4,6-diSO_4), derived from IdoA→GalNAc(4,6-diSO_4) and referred to previously as ΔDi-diS_E (Suzuki *et al.*, 1968). The identification is based on the following evidence: (1) this compound was not obtained on chondroitinase AC treatment of the original polymer, indicating an IdoA origin for the uronic acid residue of the disaccharide; (2) the disaccharide was not present in Sigma DS, but was present in the sample to the same extent predicted by the hydrazinolysis data below; (3) the hydrazinolysis data showed IdoA→ATal(4,6-diSO_4) as a major disulphated disaccharide component from this sample; (4) this compound co-chromatographed with standard ΔDi-diS_E.

Fig. 7(*c*) shows the chondroitinase ABC products from Sigma C6S. As predicted from the hydrazinolysis data, significant amounts of both ΔDi-4S_R and ΔDi-6S_R were obtained, as well as a disulphated disaccharide with a retention time of 67 min. This compound was identified as the aldehyde-reduced form of ΔUA(2-SO_4)→GalNAc-(6-SO_4), derived from GlcA(2-SO_4)→GalNAc(6-SO_4) units of the polymer and referred to previously as ΔDi-diS_D (Suzuki *et al.*, 1968). The identification is based on two observations: (1) this disaccharide was obtained on both chondroitinase AC and chondroitinase ABC digestion, indicating a GlcA origin for the uronic acid residue of the compound; (2) GlcA(2-SO_4)→ATal$_R$(6-SO_4) was the only significant disulphated disaccharide obtained from this polymer following hydrazinolysis (see below). Table 2 summarizes the chromatographic properties of these unsaturated disaccharides.

Table 2. Chromatographic properties of chondroitinase-released disaccharides

Details are given in the Experimental section. The h.p.l.c. gradient for both columns consisted of a 30 min isocratic segment at 40 mM-KH_2PO_4 followed by a 40 min linear gradient to 400 mM-KH_2PO_4.

	Paper chromatography ($R_{glucitol}$)		H.p.l.c. elution time (min)	
Compound	System 1	System 2	Whatman SAX	Varian AX
ΔDi-4S_R	0.59	0.67	32.0	49.0
ΔDi-6S_R	0.56	0.82	44.0	49.0
ΔDi-diS_{DR}	0.30	—	66.5	66.5
ΔDi-diS_{ER}	0.26	0.41	68.0	70.0
ΔDi-diS_{BR}	0.26	0.46	61.0	67.0
ΔDi-0S_R	1.0	0.95	—	—

Glycosaminoglycan analysis: rates of hydrazinolysis

The rates of *N*-deacetylation of the reference glycosaminoglcyans by hydrazinolysis were determined by measuring the yields of disaccharides formed when portions of the reaction mixtures were treated with HNO_2 at different stages of the hydrazinolysis reaction and analysed for disaccharides. Fig. 8(*a*) shows significant differences in the rates of release of total monosulphated disaccharides from C6S (● symbols) and C4S (○ symbols), with the 6-sulphated polymer giving approximately 3-fold more disaccharides than did the 4-sulphated polymer at early time points. The rate of disaccharide release from chondroitin (results not shown) was nearly identical with the rate of release from C6S. Thus the difference between rates of *N*-deacetylation of C4S and C6S appeared to be due to an interference with the *N*-deacetylation reaction by the 4-sulphate substituents. The intermediate rate of release of disaccharides from DS (■ symbols), in which IdoA→GalNAc(4-SO_4) disaccharide units make up 80% of the polymer (Table 3), is consistent with this conclusion, but suggests that the type of uronic acid adjacent to the GalNAc residue can also affect the rate of *N*-deacetylation. The effect of the differences in rates of hydrazinolysis of C6S and C4S is seen in the intermediate rate of disaccharide release obtained for the

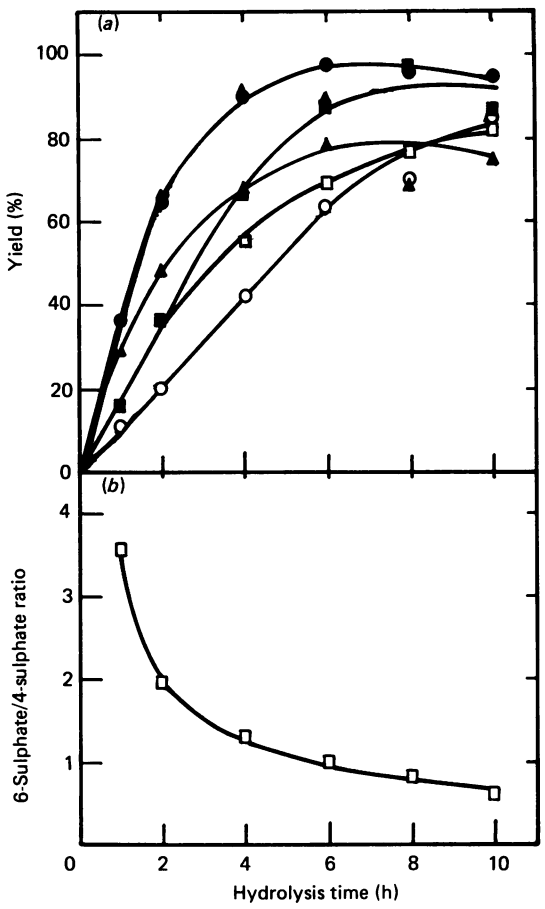

Fig. 8. Kinetics of disaccharide release following hydrazinolysis/HNO_2 treatment of Mathews and Cifonelli C6S (●), Mathews and Cifonelli C4S (○), Mathews and Cifonelli DS (■), Sigma C6S (□) and KS-II (▲)

Samples were treated with hydrazine/hydrazine sulphate at 100 °C and, at the indicated time intervals, portions were treated with HNO_2 and then NaB^3H_4. The products were analysed by h.p.l.c. on a Varian AX-5 column or by paper chromatography in system 2. The total yield of disaccharides (*a*) was determined and plotted as the percentage of the total yield of disaccharides obtained by treatment of these glycosaminoglycans with chondroitinase ABC. In the case of the KS sample, the yield of products was compared with the total amount (mol) of galactose present in the sample. The ratio of GlcA→ATalR(6-SO_4) to GlcA→$ATal_R$(4-SO_4) at each time point in the hydrazinolysis of Sigma C6S (□) was determined and is plotted in panel (*b*).

Sigma C6S (□ symbols), which has similar amounts of 4-sulphated and 6-sulphated disaccharide units (see Table 3). Analysis of the disaccharide mixtures obtained at different time points during the reaction gave the ratios of the 6-sulphated disaccharide [GlcA→$ATal_R$(6-SO_4)] to the 4-sulphated disaccharide [GlcA→$ATal_R$(4-SO_4)] shown in Fig. 8(*b*). These ratios declined rapidly in the early stages of the reaction and then asymptotically approached a final constant value between 0.65 and 0.75. This value was found to be nearly identical with 0.74, the ratio of ΔDi-6S to ΔDi-4S determined by chondroitinase ABC analysis of the same polymer (Table 3).

The rate of conversion of KS into disaccharides is shown in Fig. 8(*a*) (▲ symbols). The initial rate of disaccharide formation was similar to that found for C6S, but the reaction slowed in the later stages and gave final yields that were lower than those obtained for the polymers described above. The results indicate that maximal yields of disaccharides from most polymers are obtained after a hydrazinolysis time of 8–10 h. As shown below, the results are similar to those obtained by chondroitinase analysis.

Comparison of the yields of disaccharides obtained by chondroitinase and hydrazinolysis cleavage of glycosaminoglycans

Table 3 shows a quantitative comparison of the disaccharide yields obtained by enzymic and chemical depolymerization of the glycosaminoglycans under optimal conditions for cleavage and disaccharide separations. Several points are evident. First, these results show that the hydrazinolysis procedure for cleavage of glycosaminoglycans gave disaccharides in yields that were comparable with those obtained by standard chondroitinase procedures. For example, the ratio of 6-sulphated disaccharides to 4-sulphated disaccharides obtained by chondroitinase ABC analysis of Sigma C6S was 0.74, whereas the ratio obtained for the same polymer by the hydrazinolysis procedure was 0.71. Secondly, no significant epimerization of uronic acids occurred in the polymers during the hydrazinolysis period, even though the isolated disaccharides showed significant epimerization when treated under similar conditions. This was especially apparent in the analysis of the DS samples, since these contain primarily IdoA residues, which, in the disaccharide substrates, were found to epimerize more extensively than the GlcA residues (Shaklee & Conrad, 1984). The h.p.l.c. analysis of the IdoA-containing disulphated disaccharides from the DS samples showed little, if any, of the corresponding GlcA epimers. Finally, the yields of disaccharides that were obtained from these polymers were, for the most part, comparable with those obtained in the hydrazinolysis rate studies (Fig. 8). The yields for DS and KS were also comparable with the values obtained by Hopwood and co-workers (Hopwood & Elliot, 1983; Hopwood & Muller, 1983). The exceptions to this appear to be the CS samples, which showed lower yields than were obtained at the later time points of the rate studies presented above. Some possible reasons for the variability of the CS yields are discussed below.

DISCUSSION

The use of the hydrazinolysis/deamination reaction sequence for glycosaminoglycan cleavage circumvents some of the problems associated with other cleavage methods. First, the uronic acid structures are retained in the disaccharides formed in this reaction sequence, as are both *N*- and *O*-sulphate substituents (Shaklee & Conrad, 1984). Secondly, this approach can be used for all glycosaminoglycans, including heparin and HS (Shaklee & Conrad, 1984; Bienkowski & Conrad, 1985), so that multiple sets of reagents are not required when several different glycosaminoglycans are to be analysed. Thirdly, the disaccharides released from chondroitin sulphates by the hydrazinolysis/deamination procedure are obtained in proportions identical with those obtained by chondroitinase digestion (Yamagata *et al.*, 1968). Finally, the

Table 3. Composition of reference glycosaminoglycans formed by hydrazinolysis and chondroitinase treatment

Products	Composition (%)									
	Sample . . . KS-I*	KS-II*	C4S*		C6S*		DS*	DS†		C6S†	
Chondroitinase products											
ΔDi-4S$_R$			97	(71)	17	(15)	80	81	(4)	53	(47)
ΔDi-6S$_R$			3	(2)	83	(63)	6	3	(1)	39	(34)
ΔDi-diS$_{DR}$										8	(7)
ΔDi-diS$_{ER}$							10				
ΔDi-diS$_{BR}$							4	6			
Hydrazinolysis products§											
I. GlcA→ATal$_R$(6-SO$_4$)			3		70		4	2		37	
II. GlcA→ATal$_R$(4-SO$_4$)			85		20		9	17		49	
III. IdoA→ATal$_R$(4-SO$_4$)			4		3		71	72		3	
IV. Gal(6-SO$_4$)→AMan$_R$(6-SO$_4$)	46	71									
Va. Gal(6-SO$_4$)→AMan$_R$		1									
Gal→AMan$_R$	6	3									
Vb. Gal→AMan$_R$(6-SO$_4$)	48	22									
Vc. Gal$_R$(6-SO$_4$)	Trace	3									
VI. IdoA→ATal$_R$(4,6-diSO$_4$)							12				
VII. IdoA(2-SO$_4$)→ATal$_R$(4-SO$_4$)							4	9			
GlcA→ATal$_R$(4,6-diSO$_4$)										Trace	
VIII. GlcA(2-SO$_4$)→ATal$_R$(6-SO$_4$)			7‖		7‖					10‖	
Total§	74	73	67		67		90	76		80	

* Glycosaminoglycan reference samples obtained from Mathews and Cifonelli.

† Glycosaminoglycans reference samples obtained from Sigma Chemical Co.

‡ All values represent percentages of the total yield of disaccharides obtained in chondroitinase ABC digests. The values in parentheses show the disaccharide yields obtained in chondroitinase AC digests.

§ The reference glycosaminoglycans were treated with hydrazine/hydrazine sulphate at 100 °C for 10 h, followed by treatment with HNO_2 and NaB^3H_4. Portions were then analysed directly by h.p.l.c. on the Varian AX-5 column. All values, including the totals, represent percentages of the total yield of disaccharides obtained in chondroitinase ABC digests, except in the case of the KS samples, for which the values represent percentages of the total Gal in these samples.

‖ The direct h.p.l.c. analysis of hydrazine-treated C4S and C6S from Mathews and Cifonelli showed a peak corresponding to GlcA(2-SO$_4$)→ATal$_R$(6-SO$_4$), even though no corresponding ΔDi-diS$_{DR}$ was obtained following chondroitinase digestion of these samples. This anomalous peak causes an overestimation of the amount of this compound in Sigma C6S.

reagents required for this approach are inexpensive compared with the enzymes described above.

Some limitations of this chemical cleavage approach in structural analysis of glycosaminoglycan should also be considered. First, the yield of disaccharides obtained following hydrazinolysis/deamination treatment of glycosaminoglycans, although high, is not always quantitative and exhibits some variability, which can be controlled in part by choosing the optimal hydrazinolysis period for the glycosaminoglycan under consideration. Since the proportions in which the disaccharides are recovered by hydrazinolysis/deamination are the same as those obtained by chondroitinase digestion, the variable yield is not accompanied by selective losses. Secondly, uronic acid-containing disaccharides are known to undergo epimerization during hydrazinolysis under the reaction conditions used in the present study (Shaklee & Conrad, 1984), and such a reaction could alter the ratio of IdoA/GlcA disaccharides obtained from the polymers. The present study shows that uronic acid epimerization did not occur to any great extent following hydrazinolysis of CS and DS polymers since epimerized disaccharides were not obtained on HNO_2 treatment of the *N*-deacetylated polymers. For example, a comparison of the h.p.l.c. profiles of some of the uronic acid-containing disaccharides obtained by the hydrazinolysis/deamination procedure (Figs. 5*a*, 5*c* and 5*e*) with those of their epimerized products (Figs. 5*b*, 5*d* and 5*f*) shows that the epimerized forms were not present in the hydrazinolysis/deamination products from the polymers. Thus epimerization occurs in the disaccharide but not the polysaccharide reactions. Thirdly, the disaccharides obtained following hydrazinolysis/deamination treatment of CS and DS are 1,3-linked and thus subject to cleavage by β-elimination in alkaline solution. Therefore the NaB^3H_4 reduction conditions used must be carefully controlled (low alkalinity, low temperature, high NaB^3H_4 concentration) in order to obtain maximal yield of products (Glaser & Conrad, 1979). The β-elimination reactions that occur during polymer hydrazinolysis (Shaklee & Conrad, 1984) and disaccharide reduction (Glaser & Conrad, 1979) may be the source of some of the variable yields observed here.

The results presented clearly show the potential of the hydrazinolysis/deamination scheme for glycosaminoglycan structural analysis. This cleavage method will convert all disaccharide units of a glycosaminoglycan into disaccharides, regardless of their monosaccharide composition or sulphate substitution. Thus the selectivity obtained from the specificity of the enzymes, which often results in incomplete cleavage of the glycosidic bonds (Yamagata *et al.*, 1968; Blake & Conrad, 1979; Fukuda & Matsumura, 1976; Nakagawa *et al.*, 1980), is complemented by complete conversions obtained in the chemical cleavage. In addition, bond cleavage by hydrazinolysis/deamination, in contrast with the bacterial

eliminases, results in the preservation of the uronic acid structure and the ready identification of the structure of the disaccharide unit that was present in the original polymer. A specific example of the value of the hydrazinolysis/deamination approach for obtaining structural information is seen in the results obtained for KS, which indicate that sulphated Gal is almost always found glycosidically linked to sulphated GlcNAc [i.e. very little Gal(6-SO_4)→$AMan_R$ is found], but that sulphated GlcNAc can have either Gal or Gal(6-SO_4) attached to it [i.e. both Gal→$AMan_R$(6-SO_4) and Gal(6-SO_4)→$AMan_R$ (6-SO_4) are found]. This result may indicate that GlcNAc sulphation must precede Gal sulphation during KS biosynthesis. An additional observation made in the present study is that one of the C6S fractions yielded a small amount of GlcA→$ATal_R$(4,6-diSO_4), which was present in the untreated GlcA(2-SO_4)→$ATal_R$(6-SO_4) peak (Fig. 5*e*). This compound, which would not be distinguished from the corresponding IdoA-containing compound in chondroitinase ABC digests, was identified by comparison of its h.p.l.c. retention time with that of a synthetic standard prepared by epimerization of the IdoA→$ATal_R$(4,6-diSO_4) isolated as a native component of the Sigma C6S. Finally, the ability to cleave C6S glycosidic bonds at a significantly greater rate than C4S bonds by the hydrazinolysis/deamination treatment may aid in the preparation or structural analysis of larger oligosaccharides derived from these polymers. For the structural characterization of these polymers, the selectivity of this reaction complements that of the cleavage of these polymers by hyaluronidase, which cleaves the glycosidic bonds of 4-sulphated GalNAc residues faster than those of 6-sulphated GalNAc residues (Knudson *et al.*, 1984).

This work was supported by U.S. Public Health Services Grant AM28588.

Received 27 June 1985/27 September 1985; accepted 8 October 1985

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