The acid lability of the glycosidic bonds of L-iduronic acid residues in glycosaminoglycans

H. Edward CONRAD

Department of Biochemistry, University of Illinois, Urbana, IL 61801, U.S.A.

(Received 10 March 1980/Accepted 12 May 1980)

Heparan sulphate, heparin and dermatan sulphate were hydrolysed in 0.5 M-H₂SO₄ at 100°C. At intervals portions of the hydrolysate were removed and treated with HNO₂ at pH 4.0 to cleave the glycosidic bonds of the *N*-unsubstituted hexosamine residues and to convert both free and combined hexosamines into anhydrohexoses. These hydrolysis/deamination mixtures were reduced with NaBH₄ and analysed by radiochromatography for α -L-iduronosylanhydrohexose, β -D-glucuronosylanhydrohexose, and the free uronic acids and anhydrohexose. These data gave a kinetic profile of the cleavage of the α -L-iduronosyl and the β -D-glucuronosyl bonds in these glycosaminoglycans. The β -D-glucuronosyl bonds showed the expected resistance to acid hydrolysis, but the α -L-iduronosyl bonds were found to be as labile to acid as some neutral sugar glycosides. This unusual lability of the α -L-iduronosyl bond was also demonstrated in a direct comparison of the rates of acid hydrolysis of α -L-iduronosyl-anhydromannitol and β -D-glucuronosylanhydromannitol. The procedures used to follow the kinetics of glycosaminoglycan hydrolysis can also be used to obtain quantitative analyses of L-iduronic acid, D-glucuronic acid and hexosamine in these polymers.

Animal tissues contain a number of glycosaminoglycans having monosaccharide sequences consisting of uronic acid residues that alternate with *N*-acetylated or *N*-sulphated hexosamine residues. In three of these, heparan sulphate, heparin and dermatan sulphate, both D-glucuronic acid and L-iduronic acid appear in various proportions in the uronic acid positions. Complete, non-destructive release of monosaccharides from these glycosaminoglycans by acid hydrolysis has not been possible because of the marked acid stability of the glycosidic bonds of both the uronic acids and the amino sugars. This is one of the factors that has impeded progress on the determination of the structures of these polymers and that has made it very difficult to assay their concentrations in biological samples.

The marked acid resistance of the glycosides of D-glucuronic acid and D-galacturonic acid is well known (BeMiller, 1967), and has been considered a general property of uronic acid glycosides. However, L-iduronic acid glycosides have been reported to be more labile than D-glucuronic acid glycosides. Thus, when dermatan sulphate was hydrolysed with 1 M-H₂SO₄ at 100°C (Fransson *et al.*, 1968) or when heparin or heparan sulphate was hydrolysed with 2 M-trifluoroacetic acid at 100°C (Höök *et al.*, 1974a), a relatively high percentage of the L-

iduronosyl bonds was cleaved. These reports gave no quantitative comparison of the rates or extents of hydrolysis of D-glucuronosyl and L-iduronosyl bonds, and left open the question of whether the relative lability of the L-iduronosyl bonds might be due to the hydrolysis conditions, which were different from those used in the large body of work leading to the concept of the acid stability of uronic acid glycosides.

A quantitative comparison of the rates of hydrolysis of D-glucuronosyl and L-iduronosyl bonds in heparan sulphate, heparin and dermatan sulphate in 0.5 M-H₂SO₄ at 100°C is described in the present paper. The results show that the β -D-glucuronosyl bonds in these glycosaminoglycans exhibit the well-known acid resistance, whereas the α -L-iduronosyl bonds are actually more labile under these hydrolysis conditions than the glycosidic bonds of some neutral sugars. Thus, almost quantitative yields of free L-iduronic acid can be obtained when acid hydrolysates of these glycosaminoglycans are treated with HNO₂ to cleave the remaining hexosaminyll linkages. The procedures used to demonstrate the relative acid lability of the uronic acid glycosides can be adapted for use in the quantification of the amounts of D-glucuronic acid, L-iduronic acid and hexosamine in these polymers,

even though complete hydrolysis of all of the glycuronosyl bonds in the polymers may not be possible.

Experimental

Materials

Heparan sulphate, heparin and dermatan sulphate were obtained from Professor Martin B. Mathews (Department of Pediatrics, University of Chicago, Chicago, IL, U.S.A.). The heparan sulphate and heparin were prepared from beef lung; the dermatan sulphate was from hog mucosa. L-[³H]Idonic acid was isolated from a NaB³H₄-reduced heparin hydrolysis/deamination mixture by preparative paper chromatography in system II (see below).

Paper chromatography and quantification of ³H-labelled products

Paper chromatograms of NaB³H₄-reduced samples were run in two different descending chromatography systems. In system I, samples were spotted on strips (2.5 cm × 57 cm) of Whatman no. 3 chromatography paper and the chromatograms were developed in ethyl acetate/acetic acid/formic acid/water (18:3:1:4, by vol.). In system II, chromatographic separations were run on cellulose phosphate strips (Whatman Inc., Clifton, NJ, U.S.A.) developed in ethyl acetate/pyridine/5 mm-boric acid (3:2:1, by vol.). After development, the dried strips were cut into 1.25 cm segments for scintillation counting of radioactivity.

The hydrolysis/deamination reactions and the radiochromatographic analysis of the products

Portions (40 μl) of glycosaminoglycan solutions (10 mg/ml) were mixed with 20 μl of a D-[¹⁴C]glucose solution (0.045 μCi/μl, sp. radioactivity 250 Ci/mol; New England Nuclear, Boston, MA, U.S.A.) and 12 μl of 3 M-H₂SO₄ in a 6 mm × 50 mm test tube. The solution of D-[¹⁴C]glucose, added as an internal standard, gave 49 600 c.p.m./μl (50% counting efficiency, ¹⁴C-above-³H) when counted for radioactivity on paper chromatograms. The hydrolysis mixture was covered with a layer of mineral oil (white oil, no. 31-U.S.P.; American Oil Co., Chicago, IL, U.S.A.) and heated in unstoppered tubes at 100°C in a sand bath. At intervals, 8 μl portions of the hydrolysates were transferred with a Hamilton syringe to 6 mm × 50 mm test tubes and mixed with 20 μl of 5.5 M-NaNO₂ at room temperature to cleave the remaining glycosidic bonds of the *N*-unsubstituted hexosamine residues. After 10 min, 8 μl of 1 M-Na₂CO₃ and then 5 μl of 0.25 M-NaB³H₄ (sp. radioactivity 277 Ci/mol; New England Nuclear) were added, and the solution was corked and heated for 30 min in a 50°C sand bath to convert all of the

reducing carbohydrates into glycitols containing 1 equiv. of ³H/mol. The reduction mixture was cooled to room temperature and the excess NaB³H₄ was destroyed by careful addition of 20 μl of 0.5 M-H₂SO₄. After gas evolution had ceased, the solution was made slightly alkaline by addition of 1 M-NaOH, and 20 μl portions were spotted on chromatography strips (see above) for analysis. All operations involving NaB³H₄ were carried out in a fume hood to vent the ³H₂ that is formed. Strips were developed in system I for 15 h to assay for [³H]anhydrohexitol, or for 40 h to assay for the [³H]glycuronosylanhydrohexitols. The system II chromatograms were developed for 15 h for assay of the [³H]glyconic acids. The details of the counting and the calculations of the number of μmol of each separated component in the original hydrolysis/deamination mixture have been described (Conrad *et al.*, 1973). The NaB³H₄ used in the present work was standardized by reduction and chromatography of a portion of a standard solution of D-glucose. The reduced D-glucose gave 6.25 × 10⁶ c.p.m./μmol of D-[³H]glucitol on the chromatography strips.

Results

Radiochromatography of hydrolysis/deamination mixtures in chromatography system I

For a quantitative comparison of the relative rates of hydrolysis of D-glucuronosyl and L-iduronosyl bonds it was necessary to develop methodology for separation and quantification of all of the products formed when the glycosaminoglycans were subjected to hydrolysis/deamination reactions. For this purpose, solutions of heparan sulphate, heparin and dermatan sulphate containing a D-[¹⁴C]glucose internal standard were hydrolysed at 100°C in 0.5 M-H₂SO₄ for 4 h and the hydrolysates were deaminated with HNO₂ to cleave the hexosaminyl bonds and then aldehyde-reduced with NaB³H₄. The hydrolysis/deamination procedure yields mixtures containing uronic acids, anhydrohexose (2,5-anhydromannose from the glucosamine residues in heparan sulphate and heparin; 2,5-anhydrotalose from the galactosamine residues in dermatan sulphate), and uronosylanhydrohexoses. When these products are reduced with NaB³H₄, the free and the combined anhydrohexose residues are converted into [³H]anhydrohexitol residues, whereas the free D-glucuronic acid and L-iduronic acid are converted into L-[³H]gulonic acid and L-[³H]idonic acid respectively. These products were analysed by the radiochromatographic procedures.

Fig. 1 shows the paper-chromatographic profiles of hydrolysis/deamination mixtures prepared from heparan sulphate, heparin and dermatan sulphate. The chromatograms represented by Figs. 1(a)–1(c) were developed for only 15 h so that peak 5,

Fig. 1. Radiochromatograms of hydrolysis/deamination products of heparan sulphate, heparin and dermatan sulphate. Samples were hydrolysed in 0.5M-H₂SO₄ at 100°C, deaminated with HNO₂, reduced with NaB³H₄ and chromatographed in system I for 15 h (a–c) or 40 h (d–f) as described in the Experimental section. (a) and (d), heparan sulphate; (b) and (e), heparin; (c) and (f), dermatan sulphate.

identified previously (Shively & Conrad, 1970) as the free [³H]anhydrohexitol, would not run off the strip. By comparison with standard NaB³H₄-reduced uronic acids, peak 3 on each chromatogram represents free uronic acid. In this chromatography system the L-[³H]gulonic acid and L-[³H]idonic acid do not separate, even after the longer development times shown in Figs. 1(d)–1(f). This precludes the use of chromatography system I for quantification of the individual uronic acids. In previous work (Shively & Conrad, 1970, 1976a) peak 4 was recognized as a component of these mixtures that was derived from the uronic acids (primarily from L-iduronic acid), and, for purposes of quantification of the total uronic acid, peaks 3 and 4 were added together. During the present work it was observed that, in samples containing low concentrations of salt, most of the uronic acid appeared in peak 4. However, as the concentration of salt in the sample spotted on the chromatogram was increased, peak 3

increased and peak 4 decreased, but at all salt concentrations the sum of peaks 3 and 4 remained constant. The behaviour, observed for both L-gulonic acid and L-idonic acid from the glycosaminoglycans, and for standard L-[³H]gulonic acid (NaB³H₄-reduced standard D-glucuronic acid), is consistent with the identification of peak 4 as a mixture of L-[³H]gulonolactone and L-[³H]idonolactone. The previous study dealt only with heparin. As shown below, in the hydrolysis/deamination products of heparin approx. 90% of the free uronic acid formed is L-iduronic acid. Consequently, most of the lactone found in peak 4 in heparin reaction mixtures must be derived from L-iduronic acid. This is consistent with the previous conclusion that peak 4 is derived primarily from L-iduronic acid 2-sulphate, since most of the L-iduronic acid residues in heparin are 2-O-sulphated (Jacobsson *et al.*, 1979). However, the previous identification of peak 4 as an anhydrouronic acid,

based on the mass spectrum of its trimethylsilyl derivative and on its paper-chromatographic behaviour, appears to be ruled out by the present findings. It may be noted that the molecular ions of the trimethylsilyl derivatives of the proposed anhydrouronic acid and the lactone would have identical masses.

In the 15h development of chromatograms in system I, peaks 1, 2 and 3 are poorly resolved. However, when the chromatograms are developed for 40h in system I, peaks 4 and 5 run off the strip, but peaks 1, 2 and 3 are well separated and can be quantified individually. Peaks 1 and 2 are identified as D-glucuronosyl[^3H]anhydrohexitol and L-iduronosyl[^3H]anhydrohexitol respectively, by the following observations. (a) Peaks 1 and 2 migrate as disaccharides in system I, but are not observed on chromatograms of hydrolysates that have not been treated with HNO_2 (Shively & Conrad, 1970); therefore, peaks 1 and 2 must be disaccharides having anhydrohexitols at their reducing ends. (b) The paper-chromatographic migrations of peaks 1 and 2 from heparin and heparan sulphate in ethyl acetate/acetic acid/water (3:1:1, by vol.) were identical with those reported by Jacobsson *et al.* (1979) for D-glucuronosylanhydromannitol and L-iduronosylanhydromannitol respectively. (c) The acid hydrolyses of peaks 1 and 2 (see below) yield [^3H]anhydromannitol at rates that are consistent with the relative acid labilities of D-glucuronosyl and L-iduronosyl disaccharides noted previously (Jacobsson *et al.*, 1979). (d) Chromatographic profiles of hydrolysis/deamination products as glycosaminoglycan hydrolysis progresses (see below) show that peak 2 disappears rapidly in the later stages of hydrolysis with a corresponding rapid increase in free L-iduronic acid and anhydrohexose; in contrast, peak 1 disappears slowly, with a correspondingly low rate of D-glucuronic acid appearance.

Radiochromatographic separation of [^3H]glyconic acids in chromatography system II

Chromatography system II, developed previously for use in the quantification of *N*-acetylneuraminic acid (Roll & Conrad, 1977), offered two advantages for radiochromatographic analysis of the NaB^3H_4 -reduced uronic acids. First, the chromatography of the [^3H]glyconic acids in the basic solvent prevented the lactone formation that occurs during chromatography of glyconic acids in system I and in other acidic solvents. Secondly, by using cellulose phosphate paper as a support, very low ^3H background counts were found along the strip, in contrast with the high ^3H c.p.m. obtained when Whatman no. 1 or DEAE-cellulose strips were run in pyridine chromatography solvents (Conrad

et al., 1973). Consequently, system II gave single sharp peaks of the acidic forms of NaB^3H_4 -reduced uronic acids. In Fig. 2 the peak positions of the [^3H]glyconic acids are plotted relative to an internal D-[^{14}C]glucitol standard. L-[^3H]Gulonic acid and L-[^3H]idonic acid migrate as single peaks with R_{glucitol} values of 0.55 and 0.33 respectively. Baseline separations are obtained when mixtures of the two acids are run in system II, and no lactone forms of the acids are obtained, provided the reduced samples are adjusted to an alkaline pH before spotting the sample on the chromatogram. In hydrolysates containing mixtures of the two acids, the sum of the two peaks in system II is equal to the sum of peaks 3 and 4 in system I (see above).

An unusual feature of system II is that uronosyl-anhydrohexitols migrate more rapidly than L-gulonic

Fig. 2. Radiochromatograms of NaB^3H_4 -reduced uronic acids

D-Glucuronic acid (a) and L-iduronic acid (b) were reduced with NaB^3H_4 and chromatographed in system II as described in the Experimental section.

Table 1. *Chromatographic migration of uronosylanhydrohexitols in chromatography system II*

Glycosaminoglycan solutions containing [^{14}C]glucose as an internal standard were hydrolysed for 4 h, deaminated and analysed by radiochromatography in chromatography system II as described in the Experimental section. The mono- or disaccharides listed appeared as major peaks on radiochromatograms prepared from their respective glycosaminoglycan sources.

Mono- or di-saccharide*	Glycosaminoglycan source	$R_{\text{glucitol}}^\dagger$
IdoA($\alpha 1 \rightarrow 4$)AMan _{red.}	Heparin	1.15
IdoA($\alpha 1 \rightarrow 4$)AMan _{red.}	Heparan sulphate	1.18
GlcA($\beta 1 \rightarrow 4$)AMan _{red.}	Heparan sulphate	1.00
IdoA($\alpha 1 \rightarrow 3$)ATal _{red.}	Dermatan sulphate	0.64
GlcA($\beta 1 \rightarrow 3$)AMan _{red.}	Hyaluronic acid	1.00
GlcA($\beta 1 \rightarrow 3$)ATal _{red.}	Chondroitin 4-sulphate	0.48
IdoA _{red.}	Heparin, dermatan sulphate	0.33
GlcA _{red.}	Heparin sulphate	0.55

* Abbreviations used: IdoA, L-iduronic acid; GlcA, D-glucuronic acid; IdoA_{red.}, L-idonic acid; GlcA_{red.}, L-gulonic acid; AMan_{red.}, anhydromannitol; ATal_{red.}, anhydrotalitol.

† The ratio of the distance travelled by the mono- or di-saccharide to the distance travelled by the reduced ^{14}C internal standard.

Fig. 3. Rates of hydrolysis of β -D-glucuronosyl- ^3H -anhydromannitol and α -L-iduronosyl- ^3H -anhydromannitol

Solutions containing 96000 c.p.m. each of ^3H -labelled disaccharide and D- ^{14}C]glucose in $48\ \mu\text{l}$ of $0.5\ \text{M-H}_2\text{SO}_4$ were heated at 100°C . At intervals, $5\ \mu\text{l}$ portions were withdrawn from the hydrolysates, neutralized with $5\ \mu\text{l}$ of $1\ \text{M-Na}_2\text{CO}_3$ and analysed by radiochromatography in chromatography system I. The ^3H (c.p.m.) remaining in the disaccharide peak is plotted against hydrolysis time. (a) Glucuronosyl-anhydromannitol; (b) iduronosylanhydromannitol.

acids from each other and from the disaccharides and the anhydrohexitols.

Acid lability of the uronosylanhydromannitols

L-Iduronosyl- ^3H -anhydromannitol and D-glucuronosyl- ^3H -anhydromannitol, purified from heparin hydrolysis/deamination mixtures, were used to obtain a direct comparison of the acid susceptibility of the two uronic acid glycosides. Fig. 3 shows the hydrolysis of the two disaccharides in $0.5\ \text{M-H}_2\text{SO}_4$ at 100°C . The β -D-glucuronosyl bond exhibits the usual acid resistance, but the α -L-iduronosyl bond is hydrolysed much more rapidly. Since the hydrolysis conditions used here are the same as those used in previous studies (Conrad *et al.*, 1966; Gahan *et al.*, 1967; Shively & Conrad, 1970) to obtain rate constants for the acid hydrolysis of a variety of disaccharides and methyl glycosides, it is of interest to compare the rate constants for the hydrolysis of the uronosyl-anhydromannitols with those of other glycosides. These comparisons, presented in Table 2, show that the rate constant for hydrolysis of the α -L-iduronosyl bond falls in the mid-range of rate constants obtained for the several neutral sugar glycosides, whereas the rate constant for the β -D-glucuronosyl bond is of a magnitude similar to those obtained previously for glucuronosyl bond hydrolysis (BeMiller, 1967).

Hydrolysis of iduronic acid-containing glycosaminoglycans

In $0.5\ \text{M-H}_2\text{SO}_4$ at 100°C the N-sulphate groups of glycosaminoglycans are hydrolysed completely in less than 30 min (Cifonelli, 1966), whereas complete removal of the N-acetyl groups requires 4–5 h

acid or L-idonic acid, as shown in Table 1. The physical basis for the unusual chromatographic behaviour of the disaccharides has not been investigated further. However, chromatography system II offers a real advantage for the present study since it gives a clear separation of the free

Table 2. Rate constants for the hydrolysis of glycosidic bonds in 0.5 M-H₂SO₄ at 100°C

Substrate*	10 ³ × Hydrolysis rate constant, <i>k</i> (s ⁻¹)	Reference
Uronic acid glycosides		
GlcA(α1→3)Man	0.018	Gahan <i>et al.</i> (1967)
GlcA(α1→3)Fuc	0.004	Conrad <i>et al.</i> (1966)
GlcA(β1→4)AMan _{red.}	0.015	The present paper
IdoA(α1→4)AMan _{red.}	0.214	The present paper
Neutral sugar glycosides		
Glc(β1→4)Man	0.36	Gahan <i>et al.</i> (1967)
Glc(β1→3)Glc	0.29	Gahan <i>et al.</i> (1967)
Glc(β1→4)Glc	0.13	Conrad <i>et al.</i> (1966)
Man(β1→4)Glc	0.12	Gahan <i>et al.</i> (1967)
Glc(β1→4)GlcA	0.050	Conrad <i>et al.</i> (1966)
N-Acetylglucosamine glycosides		
GlcNAc(β1→Methyl)	2.99	Shively & Conrad (1970)
GlcNAc(β1→4)GlcNAc	0.64	Shively & Conrad (1970)
GlcNAc(α1→Methyl)	0.19	Shively & Conrad (1970)

* Abbreviations used: GlcA, D-glucuronic acid; IdoA, L-iduronic acid; Man, D-mannose; Fuc, L-fucose; AMan_{red.}, anhydromannitol; Glc, D-glucose; GlcNAc, N-acetyl-D-glucosamine.

(Shively & Conrad, 1970). Under these conditions the glycosidic bonds of the *N*-acetylated amino sugars are hydrolysed in 4–6 h, whereas those of the uronic acids and the *N*-unsubstituted amino sugars are hydrolysed much more slowly (Shively & Conrad, 1970). Thus, glycosaminoglycan hydrolysis results in a mixture of mono- and oligosaccharides in which the proportion of the hexosamine residues that is *N*-unsubstituted increases as the hydrolysis proceeds. Treatment of such hydrolysates with HNO₂ converts the free hexosamines into anhydrohexoses and cleaves the remaining glycosidic bonds of the *N*-unsubstituted amino sugars to oligosaccharides having reducing-terminal anhydrohexose residues. When all of the amino sugar glycosides have been cleaved, the only disaccharides that remain in the hydrolysis/deamination mixture are those containing the acid-resistant uronosyl bonds. The rates of loss of these disaccharides from the hydrolysis/deamination mixtures in the later stages of hydrolysis are a reflection of the acid resistance of the glycosidic linkages of the respective uronic acids to the hexosamine residues in the polymers. The amounts of the uronosylhydrohexoses and the free uronic acids and anhydrohexose in hydrolysis/deamination mixtures at progressive stages of hydrolysis can be determined by radiochromatographic analysis of NaB³H₄-reduced mixtures as shown in Figs. 1 and 2. The products observed in hydrolysis/deamination mixtures at progressive stages of hydrolysis of heparan sulphate, heparin and dermatan sulphate are shown in Fig. 4. Heparan sulphate (Fig. 4a), which contains a relatively high proportion of D-glucuronic acid, yields large amounts of D-

glucuronosylhydromannose that remain at a high level after 8 h of hydrolysis, a reflection of the acid stability of the β-D-glucuronosyl bond. In contrast, the amount of L-iduronosylhydromannose reaches a maximum at 4 h and then falls as the α-L-iduronosyl bonds are hydrolysed. At the end of the 8 h hydrolysis period about 80% of the L-iduronic acid in the polymer is recovered as the free uronic acid, whereas only 30% of the D-glucuronic acid is free. The same effect is seen in the heparin hydrolysis (Fig. 4b). In this case L-iduronic acid represents approx. 90% of the total uronic acid, and more than 80% of the L-iduronic acid appears as free uronic acid after 8 h of hydrolysis. Hydrolysis of dermatan sulphate (Fig. 4c), which also contains a very high percentage of L-iduronic acid, proceeds with similar kinetics, giving a high yield of free L-iduronic acid.

Analysis of the monosaccharide content of glycosaminoglycans

After 4–5 h of hydrolysis, when all of the hexosamine residues are *N*-unsubstituted, deamination with HNO₂ converts glycosaminoglycans quantitatively into mixtures containing only uronosylhydrohexoses, free uronic acids and free anhydrohexoses, products that can be quantified by radiochromatography as shown in Figs. 1 and 2. From the radiochromatographic data, the complete monosaccharide composition can be obtained as follows. The total μmol of L-iduronic acid in the sample is equal to the sum (μmol) of L-iduronosylhydrohexose and free L-iduronic acid; the total (μmol) of D-glucuronic acid is the sum (μmol) of D-glucuronosylhydrohexose and free D-glucuronic acid.

Fig. 4. Hydrolysis/deamination profiles of glycosaminoglycans
Solutions of heparan sulphate (a), heparin (b) and dermatan sulphate (c) were hydrolysed in 0.5 M-H₂SO₄ at 100°C and, at intervals, portions were withdrawn from the hydrolysates, deaminated with HNO₂, and analysed by radiochromatography as described in the Experimental section. Hydrolysis products are indicated as follows: ▲, D-glucuronic acid; ■, L-iduronic acid; ○, anhydrohexose; △, β-D-glucuronosylanhydrohexose; □, α-L-iduronosylanhydrohexose.

Table 3. Composition of glycosaminoglycans calculated from analysis of hydrolysis/deamination products at different stages of hydrolysis

Glycosaminoglycan were hydrolysed in 0.5 M-H₂SO₄ at 100°C and analysed by radiochromatography as described in the text. Values in parentheses are averages ± s.d.

Glycosaminoglycan	Hydrolysis time (h)	Monosaccharide composition* (μ mol/mg of glycosaminoglycan)					Percent IdoA $\ddagger$
		Uronic acids			Anhydrohexose	UA/AHex $\dagger$	
		IdoA	GlcA	Total			
Heparan sulphate	4	0.32	0.91	1.23	1.19	1.03	26
	6	0.41	0.95	1.36	1.30	1.05	30
	8	0.41	1.05	1.46	1.38	1.06	28
						(1.05 $\pm$ 0.02)	(28.0 $\pm$ 2.0)
Heparin	4	0.53	0.09	0.62	0.61	1.02	85
	6	0.59	0.09	0.68	0.61	1.11	87
	8	0.67	0.09	0.76	0.72	1.10	88
						(1.08 $\pm$ 0.05)	(86.7 $\pm$ 1.7)
Dermatan sulphate	4	1.75	0.18	1.92	1.64	1.17	91
	6	1.66	0.16	1.82	1.69	1.08	91
	8	1.62	0.19	1.81	1.59	1.14	90
						(1.13 $\pm$ 0.05)	(90.7 $\pm$ 0.6)

* Abbreviations used: IdoA, L-iduronic acid; GlcA, D-glucuronic acid; IdoA (μmol) = free IdoA (μmol) + iduronosylanhydrohexose (μmol); GlcA (μmol) = free GlcA (μmol) + glucuronosylanhydrohexose (μmol); anhydrohexose (μmol) = free anhydrohexose (μmol) + iduronosylanhydrohexose (μmol) + glucuronosylanhydrohexose (μmol).

† Ratio of total uronic acid to total anhydrohexose.

‡ Percentage of total uronic acid.

ronic acid; and the total (μmol) of hexosamine is the sum (μmol) of L-iduronosylhydrohexose, D-glucuronosylhydrohexose and free anhydrohexose. Table 3 shows the analytical values obtained in this manner for the glycosaminoglycan samples used in the present study. Compositional data obtained from analyses of hydrolysis/deamination mixtures after 4, 6 and 8 h of acid hydrolysis are shown. During the more extended hydrolysis periods, the only changes observed in the amounts of the hydrolysis/deamination products are those resulting from the hydrolysis of the uronosyl bonds (see Fig. 4). As Table 3 shows, these changes do not alter the values calculated for the amounts of D-glucuronic acid, L-iduronic acid and anhydrohexose (hexosamine) in these polymers. From the compositional data the percentage of each of the uronic acids and the ratio of total uronic acid to total hexosamine can be calculated.

Discussion

The initial step in the structural analysis of any polymer is the determination of its monomer composition. This primary requirement has presented a difficult challenge in the study of glycosaminoglycans containing both D-glucuronic acid and L-iduronic acid. This is because the available analytical methods have required a non-destructive conversion of these polymers into their monosaccharide constituents to determine the ratios of D-glucuronic acid to L-iduronic acid and to demonstrate the expected 1:1 molar ratio of hexosamine to total uronic acid. Because of the acid stability of the glycosides of both uronic acids and *N*-unsubstituted amino sugars, acid hydrolysis, the usual procedure for converting polysaccharides into monosaccharides, gives only partial depolymerization. Consequently, it has been necessary to develop alternative procedures for conversion of these glycosaminoglycans into monosaccharides.

The acid-resistant glycosidic bonds of *N*-unsubstituted amino sugars, generated rapidly from *N*-sulphated and *N*-acetylated amino sugars during acid hydrolysis, are cleaved rapidly, and essentially stoichiometrically, by treatment of glycosaminoglycan hydrolysates with HNO_2 (Shively & Conrad, 1976a). This reaction converts D-glucosamine and D-galactosamine residues into anhydrohexose residues, which are readily quantified by the radiochromatographic procedures developed in this laboratory (Shively & Conrad, 1976a,b). Thus, the primary problem that has remained in the complete conversion of glycosaminoglycans into monosaccharides has been the resistance of uronic acid glycosides to acid. The idea of the acid stability of uronosyl bonds is well established (BeMiller, 1967),

and is based primarily on studies with the common D-glucuronic acid and D-galacturonic acid. The glycosides of L-iduronic acid, a relative newcomer to the family of naturally occurring uronic acids (Hoffman *et al.*, 1956; Cifonelli *et al.*, 1958; Cifonelli & Dorfman, 1962), were perceived to have acid stability comparable with those of other uronic acid glycosides. This perception was reinforced by the failure to obtain free L-iduronic acid in heparin hydrolysates (Cifonelli & Dorfman, 1962), an effect that is actually attributable to the stability of the glycosidic bonds of the rapidly *N*-desulphated D-glucosamine residues to which the L-iduronic acid residues are linked. The difficulty in obtaining sufficient amounts of L-iduronic acid discouraged further attempts to characterize L-iduronic acid glycosides. More recent reports have noted that the L-iduronosyl bonds in dermatan sulphate (Fransson *et al.*, 1968) and in heparin (Höök *et al.*, 1974a,b; Jacobsson *et al.*, 1979) are more labile than the D-glucuronosyl bonds in these polymers, but no quantitative statement of the relative stabilities has appeared to suggest that L-iduronosyl bonds are atypical uronosyl bonds. Thus, the problem of uronosyl bond hydrolysis has remained.

One solution to the uronic acid hydrolysis problem has been to reduce the uronic acids in these polymers to neutral sugars before hydrolysis to render their glycosidic bonds more labile (Danishefsky *et al.*, 1963; Wolfrom *et al.*, 1964; Perlin & Sanderson, 1970; Taylor & Conrad, 1972). An alternative solution to the problem that is more direct than the carboxy reduction approach is described here. The present data show that the α -L-iduronosyl linkage is as acid labile as the glycosyl linkages of some neutral sugars. This lability is observed when the aglycone of the L-iduronic acid is either anhydromannitol (Fig. 3), D-glucosamine (Figs. 4a and 4b) or D-galactosamine (Fig. 4c). The β -D-glucuronosyl linkages to the same aglycones are much more stable. There is no indication that L-iduronic acid is destroyed more rapidly than any of the other monosaccharides under the hydrolytic conditions used in the present paper (Table 3). The almost quantitative release of free L-iduronic acid by treatment of heparin hydrolysates with HNO_2 suggests that heparin, one of the most readily available glycosaminoglycans, could serve as a relatively inexpensive source of L-iduronic acid.

The direct radiochromatographic analysis of hydrolysis/deamination mixtures offers a refinement in the approach to the analysis of both the amino sugar and the uronic acid content of L-iduronic acid-containing glycosaminoglycans. The hydrolysis, deamination and $[^3\text{H}]$ borohydride-reduction reactions are carried out with a minimum of sample manipulations. A ^{14}C -labelled internal

standard is added to each sample before hydrolysis so that the fraction of the original sample on each chromatography strip can be calculated from the amount of ^{14}C recovered on the strip. The amount of ^3H per μmol is identical for each product analysed. Finally, the inevitable failure to obtain complete hydrolysis is not a problem, since the uronosyl-anhydrohexitols can be separated and quantified readily, and their concentrations can then be used, along with those of the monosaccharides, to calculate the total (μmol) of each monosaccharide per mg of the original glycosaminoglycan sample. This approach is preferable to the procedure involving carboxy reduction with NaB^3H_4 (Taylor *et al.*, 1973; Shively & Conrad, 1976a,b), since much less NaB^3H_4 is required and since the sample manipulations are both fewer and less cumbersome.

A procedure similar to the one described in the present paper has been used by Höök *et al.* (1974a) to determine the relative, but not the absolute, amounts of D-glucuronic acid and L-iduronic acid in heparin. In the latter procedure 70% of the total uronic acid in heparin samples was released as free uronic acids by hydrolysis, deamination and re-hydrolysis of the deaminated sample. In this multi-step assay the ratios of free D-glucuronic acid to free L-iduronic acid are determined by g.l.c. of the borohydride-reduced trimethylsilyl derivatives of the uronic acid lactones. The unhydrolysed portions of the samples were not analysed but were assumed to contain D-glucuronic acid and L-iduronic acid in the same ratio that was found in the free uronic acid fraction. This assumption, which requires that the β -D-glucuronosylanhidromannose and the α -L-iduronosylanhidromannose bonds are hydrolysed at the same rate, may be a reasonable one since the unreduced β -D-glucuronosylanhidromannose is apparently hydrolysed more rapidly than β -D-glucuronosylanhidromannitol (Höök *et al.*, 1974b). However, any uncertainty concerning the relative amounts of D-glucuronic acid and L-iduronic acid in the unhydrolysed material is eliminated in the present approach by direct analysis of the disaccharide fraction. The latter analysis is also necessary to obtain a value for the total anhydrohexose.

This work was supported by U.S. Public Health Service Grant HL 21763.

References

- BeMiller, J. N. (1967) *Adv. Carbohydr. Chem.* **22**, 25–108
- Cifonelli, J. A. (1966) *Carbohydr. Res.* **2**, 150–161
- Cifonelli, J. A. & Dorfman, A. (1962) *Biochem. Biophys. Res. Commun.* **91**, 1032–1037
- Cifonelli, J. A., Ludowieg, J. & Dorfman, A. (1958) *J. Biol. Chem.* **233**, 541–545
- Conrad, H. E., Bamburg, J. R., Epley, J. D. & Kindt, T. J. (1966) *Biochemistry* **5**, 2808–2817
- Conrad, H. E., Varboncoeur, E. & James, M. E. (1973) *Anal. Biochem.* **51**, 486–500
- Danishefsky, I., Eiber, H. B. & Williams, A. H. (1963) *J. Biol. Chem.* **238**, 2895–2898
- Fransson, L.-Å., Rodén, L. & Spach, M. L. (1968) *Anal. Biochem.* **21**, 317–330
- Gahan, L. C., Sandford, P. A. & Conrad, H. E. (1967) *Biochemistry* **6**, 2755–2769
- Hoffman, P., Linker, A. & Meyer, K. (1956) *Science* **124**, 1252
- Höök, M., Lindahl, U. & Iverius, P.-H. (1974a) *Biochem. J.* **137**, 33–43
- Höök, M., Lindahl, U., Bäckström, G., Malmström, A. & Fransson, L.-Å. (1974b) *J. Biol. Chem.* **249**, 3908–3915
- Jacobsson, I., Höök, M., Pettersson, I., Lindahl, U., Larm, O., Wirén, E. & von Figura, K. (1979) *Biochem. J.* **179**, 77–87
- Perlin, A. S. & Sanderson, G. R. (1970) *Carbohydr. Res.* **12**, 183–192
- Roll, D. E. & Conrad, H. E. (1977) *Anal. Biochem.* **77**, 397–412
- Shively, J. E. & Conrad, H. E. (1970) *Biochemistry* **9**, 33–43
- Shively, J. E. & Conrad, H. E. (1976a) *Biochemistry* **15**, 3932–3942
- Shively, J. E. & Conrad, H. E. (1976b) *Biochemistry* **15**, 3943–3950
- Taylor, R. L. & Conrad, H. E. (1972) *Biochemistry* **11**, 1383–1388
- Taylor, R. L., Shively, J. E., Conrad, H. E. & Cifonelli, J. A. (1973) *Biochemistry* **12**, 3633–3637
- Wolfman, M. L., Vercellotti, J. R. & Thomas, G. H. S. (1964) *J. Org. Chem.* **29**, 536–539