Hydrazinolysis of heparin and other glycosaminoglycans

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Heparin, carboxy-group-reduced heparin, several sulphated monosaccharides and disaccharides formed from heparin, and a tetrasaccharide prepared from chondroitin sulphate were treated at 100°C with hydrazine containing 1% hydrazine sulphate for periods sufficient to cause complete *N*-deacetylation of the *N*-acetylhexosamine residues. Under these hydrazinolysis conditions both the *N*-sulphate and the *O*-sulphate substituents on these compounds were completely stable. However, the uronic acid residues were converted into their hydrazide derivatives at rates that depended on the uronic acid structures. Unsubstituted L-iduronic acid residues reacted much more slowly than did unsubstituted D-glucuronic acid or 2-*O*-sulphated L-iduronic acid residues. The chemical modification of the carboxy groups resulted in a low rate of C-5 epimerization of the uronic acid residues. The hydrazinolysis reaction also caused a partial depolymerization of heparin but not of carboxy-group-reduced heparin. Treatment of the hydrazinolysis products with HNO₂ at either pH 4 or pH 1.5 or with HIO₃ converted the uronic acid hydrazides back into uronic acid residues. The use of the hydrazinolysis reaction in studies of the structures of uronic acid-containing polymers and the implications of the uronic acid hydrazide formation are discussed.

Polymer-bound hexosamines occur primarily as *N*-acetylated derivatives. It was observed early that, when the 2-amino groups of amino sugar residues in biopolymers were unsubstituted, the hexosaminyl bonds could be cleaved with HNO₂ at pH 4 (Foster *et al.*, 1953). This suggested that a reaction sequence involving *N*-deacetylation of hexosamine-containing polymers, followed by treatment with HNO₂, could be developed for the selective cleavage of such polymers. Initial attempts to obtain *N*-deacetylation of polymer-bound hexosamines by the use of hydrazine or hydrazine hydrate (Akabori *et al.*, 1952) gave only partial *N*-deacetylation and caused degradation of the carbohydrate moieties of glycoproteins (Matsushima & Fujii, 1957; Wolfrom & Juliano, 1960; Yosizawa & Sato, 1962*a,b*). Later, it was found that the *N*-acetylhexosamine residues could be quantitatively deacetylated with a mixture of hydrazine and hydrazine sulphate (Bradbury, 1956) without degradation of the carbohydrate (Yo-

sizawa *et al.*, 1966). This improved hydrazinolysis procedure was then coupled to the HNO₂ deamination procedure to obtain a reaction sequence for selective cleavage of *N*-acetylhexosaminyl bonds (Dmitriev *et al.*, 1973*a,b*, 1975). Since the early attempts to use the hydrazinolysis/deamination reaction sequence for the structural analysis of glycosaminoglycans (Matsushima & Fujii, 1957; Wolfrom & Juliano, 1960), there has been no systematic study of the hydrazinolysis of glycosaminoglycans, and the hydrazinolysis reaction has had only limited use in the structural analysis of glycosaminoglycans (Thunberg *et al.*, 1982), and in the preparation of labelled glycosaminoglycans (Höök *et al.*, 1982).

The present work was undertaken to develop the hydrazinolysis/deamination procedure for use in the structural analysis of heparin, a polymer composed of a sequence of sulphated disaccharides that contain a uronic acid residue (either D-glucuronic acid or its C-5 epimer, L-iduronic acid) and a D-glucosamine residue. The disaccharide units vary in their degrees of *N*-sulphation and *O*-sulphation. Most of the D-glucosamine residues in heparin are *N*-sulphated, but some are *N*-acetylated. When the polymer is treated with HNO₂ at

Abbreviations used (in sequences): AMan_R, 2,5-anhydro-D-mannitol; ATal_R, 2,5-anhydro-D-talitol; GlcA, D-glucuronic acid; IdoA, L-iduronic acid; GalNAc, *N*-acetyl-D-galactosamine; GalNAc_R, *N*-acetyl-D-galactosaminitol.

pH 1.5, it is specifically cleaved at the *N*-sulpho-D-glucosamine residues without loss of *O*-sulphate substituents to yield a mixture of oligosaccharides having 2,5-anhydro-D-mannose residues, formed from the *N*-sulpho-D-glucosamine residues, at their reducing terminals (Delaney & Conrad, 1983). When this mixture is reduced with NaB³H₄ and separated by high-pressure liquid chromatography on an anion-exchange column, approximately 20 ³H-labelled oligosaccharide peaks are separated (Delaney & Conrad, 1983). Among the components of this oligosaccharide mixture are two unsulphated disaccharides, namely GlcA→AMan_R and IdoA→AMan_R, three monosulphated disaccharides, namely GlcA→AMan_R(6-SO₄), IdoA→AMan_R(6-SO₄) and Ido(2-SO₄)→AMan_R, and a disulphated disaccharide, namely IdoA(2-SO₄)→AMan_R(6-SO₄) (Delaney *et al.*, 1980). The remaining peaks are tetra- or higher oligosaccharides containing the *N*-acetyl-D-glucosamine residues, which do not react with HNO₂. For use of the hydrazinolysis/deamination reaction sequence in characterizing these latter oligosaccharides, the hydrazinolysis reaction would have to give stoichiometric *N*-deacetylation without loss of the *O*-sulphate constituents, since the latter are the primary features that distinguish the different disaccharide components that might be obtained from an unidentified oligosaccharide. If, in addition, the *N*-sulphate substituents were stable under the hydrazinolysis conditions, hydrazinolysis followed by HNO₂ deamination at pH 4 would permit specific cleavage of heparin at the *N*-acetyl-D-glucosamine residues to yield a previously uncharacterized group of oligosaccharide fragments. The present work shows that both the *N*-sulphate and the *O*-sulphate substituents of heparin are retained in the hydrazinolysis reaction. However, under the hydrazinolysis reaction conditions, the carboxy groups of the uronic residues are converted into hydrazides, which then undergo a slow epimerization at C-5 of the uronic acid residues. The implications of these previously undescribed side reactions for polysaccharide structural analyses are described in the present paper.

Experimental

Materials

The heparin fraction used in the present work was a product from the commercial manufacture of pig mucosa heparin, kindly supplied by Mr. Erwin Coyne, Cohelfred Laboratories, Chicago, IL, U.S.A. The material had an anticoagulant activity of 136 i.u./mg and contained no detectable dermatan sulphate or chondroitin sulphate. Carboxy-group-reduced heparin, prepared from ox lung heparin, has been described previously (Shively &

Conrad, 1976b). The model oligosaccharides used in the present study were prepared from heparin or chondroitin sulphate. For preparation of the heparin model compounds, heparin was treated with HNO₂ at pH 1.5 (Shively & Conrad, 1976a), and the resulting oligosaccharide mixture was reduced with NaB³H₄ (sp. radioactivity 450 Ci/mol; Amersham Corp., Arlington Heights, IL, U.S.A.) and fractionated by paper chromatography (Conrad, 1980; Delaney & Conrad, 1983) and by high-pressure liquid chromatography (Delaney *et al.*, 1980) to obtain ³H-labelled GlcA→AMan_R, GlcA→AMan_R(6-SO₄), IdoA→AMan_R(6-SO₄), IdoA(2-SO₄)→AMan_R and IdoA(2-SO₄)→AMan_R(6-SO₄). Anhydromannitol and carboxy-group-reduced IdoA(2-SO₄)→AMan_R(6-SO₄) were prepared in their ³H-labelled forms from carboxy-group-reduced heparin as described previously (Shively & Conrad, 1976b). ³H-labelled GlcA→ATal_R was isolated from chondroitin sulphate after acid hydrolysis, HNO₂ treatment and NaB³H₄ reduction (Conrad, 1980). *N*-Sulphated D-[³H]glucosaminitol was prepared by *N*-sulphation (Levy & Petrcek, 1962) of NaB³H₄-reduced D-glucosaminitol. NaB³H₄-reduced GlcA→GalNAc→GlcA→GalNAc was prepared as previously described (Glaser & Conrad, 1980).

Chromatography and electrophoresis

³H-labelled reaction products were analysed by paper chromatography on 2.5 cm × 57 cm Whatman no. 3 strips in ethyl acetate/acetic acid/formic acid/water (18:3:1:4, by vol.) (chromatography system I) or in butan-1-ol/acetic acid/1 M-NH₃ (3:2:1, by vol.) (chromatography system II), or by paper electrophoresis in pyridine/acetic acid/water (1:10:400, by vol., pH 4) (electrophoresis system I) or in formic acid/acetic acid/water (2:7:70, by vol. pH 1.7) (electrophoresis system II). After development, the dried strips were cut into 1.25 cm segments, which were analysed for labelled components by scintillation counting of radioactivity. Alternatively, reaction mixtures were analysed by high-pressure liquid chromatography on a 4.5 mm × 25 cm Whatman SAX column (Delaney & Conrad, 1980). Effluent fractions (0.5 ml) were collected and their radioactivities counted as before.

The stability of heparin and carboxy-group-reduced heparin under the hydrazinolysis reaction conditions was determined by comparison of their Bio-Gel P-30 (Bio-Rad Laboratories, Richmond, CA, U.S.A.) elution profiles before and after reaction. For each analysis a portion of the reaction mixture was chromatographed on a 1.1 cm × 114 cm column of Bio-Gel P-30 (100–200 mesh) packed in 0.5 M-NaCl. The column was eluted with 0.5 M-NaCl, and 1 ml fractions were

collected and analysed for their carbohydrate content by the carbazole assay (Bitter & Muir, 1962) (heparin fractions) or the indole assay (Lagunoff & Warren, 1962) (carboxy-group-reduced heparin fractions).

Hydrazinolysis of model compounds

Anhydrous hydrazine (95%) was redistilled and stored over molecular sieves. For hydrazinolysis of ^3H -labelled model compounds, the compound (approx. 2000000 ^3H d.p.m.) and D-[^{14}C]glucitol (sp. radioactivity 267 Ci/mol, 100000 ^{14}C d.p.m.), added as an internal standard, were evaporated to dryness at room temperature in a stream of air in a 6 mm $\times$ 50 mm tube, and 50 μl of hydrazine containing 1% (w/v) hydrazine sulphate was added. The tube was sealed and heated at 100°C for the desired interval. The cooled tube was opened, and the hydrazine was evaporated in a stream of air at room temperature. The dried sample was dissolved in 50 μl of water and a portion was analysed by paper chromatography or paper electrophoresis. When the effect of temperature or incubation time on the extent of hydrazinolysis was studied, portions (15 μl) from a single reaction mixture, prepared as above, were heated in sealed capillary tubes under the desired conditions and prepared for analysis in the same manner.

Uronic acid hydrazide residues of the reaction products were converted back into uronic acid residues by treatment of the hydrazinolysis products with HIO_3 . A 10 μl portion of the hydrazinolysis reaction mixture was placed in the bottom of a 6.5 ml Shevy-Stafford centrifuge tube (A. H. Thomas Co., Philadelphia, PA, U.S.A., catalogue no. 2598-G15) and the sample was frozen by placing the tip of the tube in a solid- CO_2 /propan-2-ol bath. Then nine 5 μl portions of 6M- HIO_3 were added successively to the tube. The reaction, which converted the hydrazine, the hydrazine sulphate and the uronic acid hydrazide into $\text{N}_2 + \text{I}_2$, was very vigorous, and it was necessary to keep the tube immersed in the solid- CO_2 bath during the HIO_3 additions to prevent hydrolysis of glycoside and ester bonds. After the last addition, the I_2 was extracted from the aqueous solution with two 5 ml portions of diethyl ether. Excess HIO_3 was then destroyed by addition of 47% HI and extracted with diethyl ether until no further I_2 was formed. Usually 40–50 μl of the HI was required. The sample was then made basic by addition of 1M-NaOH and analysed.

Hydrazinolysis of polymers

Approx. 35 mg of heparin or carboxy-group-reduced heparin was dissolved in 600 μl of anhydrous hydrazine containing 1% hydrazine sulphate. Portions (200 μl) of the solution were sealed

in 6 mm $\times$ 50 mm test tubes and heated at 100°C for 3 h or 6 h. The reaction mixtures were freeze-dried and the dried samples were dissolved in 0.5 ml of 0.25M- HIO_3 . The small amount of I_2 that was formed was extracted with ether and the sample was chromatographed on a Bio-Gel P-30 column as above. Under these milder HIO_3 oxidation conditions, the *N*-sulphate and *O*-sulphate substituents and the glycosidic bonds were completely stable.

Results

Kinetics of N-deacetylation

Fig. 1 shows the kinetics of the *N*-deacetylation of *N*-acetyl-D-glucosaminitol by hydrazine in the presence and in the absence of hydrazine sulphate at 100°C. Fig. 1(b) shows that both reactions are pseudo-first-order with respect to *N*-acetyl-D-glucosaminitol. When hydrazine sulphate was present the reaction was essentially complete in 5 h, whereas 14 h was required for complete *N*-deacetylation in the absence of the catalyst. The t_1 for the catalysed reaction was 53 min ($k = 0.0130 \text{ min}^{-1}$), whereas that for the uncatalysed reaction was 146 min ($k = 0.0047 \text{ min}^{-1}$). Analysis of the reaction kinetics at 50°C, 65°C, 80°C and 100°C yielded an activation energy of 153.6 kJ/mol (36.7 kcal/mol) for the catalysed reaction.

Reactions of heparin-derived model compounds with hydrazine

The stability of *O*-sulphate substituents in the hydrazinolysis reaction was tested with several well-characterized mono- and di-saccharides containing primary and/or secondary sulphate esters. These were treated with hydrazine/hydrazine sulphate at 100°C for 10 h, and the products were examined by paper electrophoresis and paper chromatography. Initially it was shown that the paper electrophoretic and paper chromatographic migrations of $\text{AMan}_R(6\text{-SO}_4)$, carboxy-group-reduced $\text{IdoA}(2\text{-SO}_4) \rightarrow \text{AMan}_R(6\text{-SO}_4)$ and *N*-sulpho-D-glucosamine were unaffected by the *N*-deacetylation reaction conditions. Recovery was quantitative for all substrates. Thus the primary and secondary *O*-sulphates as well as the *N*-sulphate substituents on these structures were completely stable under the conditions required for complete *N*-deacetylation of amino sugars.

When model compounds that contained uronic acid residues were treated in the same manner, however, products were formed that migrated on paper electrophoretograms more slowly than did the starting disaccharides. This is illustrated in Fig. 2, where Figs. 2(a)–2(c) show the profiles of the products electrophoresed at pH 4 and Figs. 2(d)–2(f) show the products electrophoresed at

Fig. 1. Kinetics of *N*-deacetylation of *N*-acetyl-D-glucosaminitol

A mixture of NaB^3H_4 -reduced *N*-acetyl-D-glucosamine and $\text{D}-[^{14}\text{C}]$ glucitol, added as an internal standard, was dissolved in hydrazine alone (●) or hydrazine containing 1% hydrazine sulphate (○), and several portions of each solution were transferred to capillary tubes, which were sealed and heated at 100°C for different timed intervals. The reaction mixtures were analysed for *N*-acetyl-D- $[^3\text{H}]$ glucosaminitol and D- $[^3\text{H}]$ glucosaminitol by using paper chromatography system I. Total ^3H radioactivity (c.p.m.) on each strip was normalized to the same D- $[^{14}\text{C}]$ glucitol radioactivity (c.p.m.), and the percentage of total ^3H radioactivity (c.p.m.) on the strip that was found in D- $[^3\text{H}]$ glucosaminitol was plotted versus hydrazinolysis reaction time in (a). The same data were used in the first-order rate plot in (b), where a is the amount of *N*-acetyl-D- $[^3\text{H}]$ glucosaminitol present at time zero and x is the amount of D- $[^3\text{H}]$ glucosaminitol present at each time interval.

pH 1.7. At pH 4 $\text{IdoA}(2\text{-SO}_4) \rightarrow \text{AMan}_\text{R}(6\text{-SO}_4)$, $\text{IdoA}(2\text{-SO}_4) \rightarrow \text{AMan}_\text{R}$ and $\text{IdoA} \rightarrow \text{AMan}_\text{R}$ have net charges of -3 , -2 and -1 respectively, whereas at pH 1.7 the same compounds have charges of -2 , -1 and 0 . The positions of migration of the original disaccharides, shown by the arrows, reflect these charge differences. After reaction with hydrazine/hydrazine sulphate, each disaccharide was converted into a product that had

lost one negative charge. This loss could not be due to loss of a sulphate ester because both primary and secondary sulphate esters were stable under the reaction conditions (above). Furthermore, $\text{IdoA} \rightarrow \text{AMan}_\text{R}$, which has no sulphate substituent, also lost a negative charge. Thus the results indicate that the charge that was lost was that of the carboxy group of the uronic acid residue, and suggest that the uronic acid residue was converted into a hydrazide. The electrophoretic migrations of the products were consistent with this conclusion. Acyl hydrazides, which have a pK of approx. 3.2 (Paulsen & Stoye, 1970), would carry very little charge at pH 4 but would be positively charged at pH 1.7. The electrophoretic behaviour of the hydrazinolysis reaction product of $\text{IdoA} \rightarrow \text{AMan}_\text{R}$ indicates that this product is essentially uncharged at pH 4 (Fig. 3c) but positively charged at pH 1.7 (Fig. 3f), as expected for the hydrazide. Similarly, at pH 4 the $\text{IdoA}(2\text{-SO}_4) \rightarrow \text{AMan}_\text{R}$ reaction product carries a negative charge due to the sulphate group, but at pH 1.7 it has a net charge of 0 resulting from the negatively charged sulphate group and the positively charged hydrazide. The same argument explains the electrophoretic behaviour of the product formed from $\text{Ido}(2\text{-SO}_4) \rightarrow \text{AMan}_\text{R}(6\text{-SO}_4)$. Analogous results have been obtained with $\text{GlcA} \rightarrow \text{AMan}_\text{R}(6\text{-SO}_4)$ and $\text{GlcA} \rightarrow \text{AMan}_\text{R}$ (results not shown). Thus these findings are consistent with the conclusion that the uronic acid-containing disaccharides are converted into hydrazides in the hydrazinolysis reactions. Further evidence for hydrazide formation is presented below.

Rates of hydrazide formation

The results in Fig. 3 show that $\text{IdoA}(2\text{-SO}_4) \rightarrow \text{AMan}_\text{R}(6\text{-SO}_4)$ and $\text{IdoA}(2\text{-SO}_4) \rightarrow \text{AMan}_\text{R}$ were converted almost completely into hydrazides, whereas $\text{IdoA} \rightarrow \text{AMan}_\text{R}$ was only partially converted. This indicates that the rates of hydrazide formation are different for different model disaccharides. Rates of formation of hydrazides of the model disaccharides were therefore compared. Fig. 3 shows the rate of conversion of $\text{IdoA}(2\text{-SO}_4) \rightarrow \text{AMan}_\text{R}(6\text{-SO}_4)$ into its hydrazide at 100°C in the presence and in the absence of hydrazine sulphate. The hydrazine sulphate markedly stimulated the rate of hydrazide formation just as it did the *N*-deacetylation reaction (Fig. 1). Rates for conversion of the other model disaccharides into their hydrazides were also determined. As shown in Table 1, significant differences in rates were observed. The D-glucuronic acid-containing disaccharides were converted into hydrazides at relatively high rates. However, for the L-iduronic acid-containing disaccharides the rate was high when the L-iduronic acid residue was substituted

Fig. 2. Paper electrophoresis of uronic acid-containing model compounds after hydrazinolysis IdoA(2-SO₄)→AMan_R(6-SO₄) (a and d), IdoA(2-SO₄)→AMan_R (b and e) and IdoA→AMan_R (c and f) were heated at 100°C in hydrazine/hydrazine sulphate for 10h and analysed by paper electrophoresis for 2h in system I (a, b and c) and system II (d, e and f). The arrows indicate the migration positions of the starting materials.

Table 1. Conversion of model disaccharides into hydrazides in hydrazine containing 1% hydrazine sulphate
For experimental details see the text.

Disaccharide	Rate of hydrazide formation		Total recovery* (%)
	10 ⁴ k (s ⁻¹)	t _i (min)	
GlcA→AMan _R (6-SO ₄)	0.79	147	—
IdoA(2-SO ₄)→AMan _R (6-SO ₄)	0.51	228	99
IdoA(2-SO ₄)→AMan _R	0.50	231	99
GlcA→AMan _R	0.43	264	104
GlcA→ATal _R	0.39	294	98
IdoA→AMan _R (6-SO ₄)	0.23	495	102
IdoA→AMan _R	0.13	894	103

* % of starting material recovered in the hydrazide plus the starting material after hydrazinolysis at 100°C for 14h.

with sulphate at C-2 but much lower when the 2-O-sulphate substituent was absent. Sulphation on the anhydromannose residue appeared to increase the rate of hydrazide formation. Measurement of the rates of conversion of Ido(2-SO₄)→AMan_R(6-SO₄) into its hydrazide at 50°C, 65°C, 80°C and 100°C yielded an activation energy of 171.5 kJ/mol (41.0 kcal/mol) for the catalysed reaction.

Table 1 also shows that in all cases the total

recovery of hydrazide plus unreacted disaccharide, obtained after a 14h hydrazinolysis period, was quantitative. Thus there was no apparent destruction of disaccharides during the reaction period.

Epimerization of uronic acid hydrazides

Portions of 6h reaction mixtures obtained in the hydrazinolysis of standard disaccharides were analysed by high-pressure liquid chromatography

Fig. 3. Kinetics of formation of the hydrazone of IdoA(2-SO₄)→AMan_R(6-SO₄)

Solutions of IdoA(2-SO₄)→AMan_R(6-SO₄) plus D-[¹⁴C]glucitol dissolved in hydrazine (●) or hydrazine containing 1% hydrazine sulphate (O) were heated at 100°C for different time intervals. The starting material in each sample was separated from the hydrazone product by paper electrophoresis, and the percentage of the total sample appearing in the hydrazone peak was plotted for each time interval.

on an anion-exchange column as shown in Fig. 4. Figs. 4(a), 4(c) and 4(e) show the profiles of the reaction mixtures obtained with IdoA→AMan_R(6-SO₄), IdoA(2-SO₄)→AMan_R and IdoA(2-SO₄)→AMan_R(6-SO₄) respectively. In each case the peak of the hydrazone derivative emerged from the column early whereas the unmodified material (Delaney *et al.*, 1980) emerged in the position of the original disaccharide (arrow). A second portion of each reaction mixture was treated with HIO₃, a reagent that will oxidize acyl hydrazides to carboxylic acids (Bray & Cuy, 1924; Kolthoff, 1924). The HIO₃-treated hydrazinolysis mixtures were analysed by high-pressure liquid chromatography on the same anion-exchange column, as shown in Figs. 4(b), 4(d) and 4(f). In each case the products of the HIO₃ treatment were recovered quantitatively in two peaks. The major peaks chromatographed in the positions of the original disaccharides. The conversion of the hydrazinolysis reaction products of these disaccharides back

Fig. 4. High-pressure liquid chromatography of the hydrazinolysis products of model compounds before and after treatment with HIO₃

IdoA→AMan_R(6-SO₄), IdoA(2-SO₄)→AMan_R and IdoA(2-SO₄)→AMan_R(6-SO₄) were heated at 100°C in hydrazine/hydrazine sulphate for 6 h. One-half of each reaction mixture was analysed directly by high-pressure liquid chromatography on a Whatman SAX column. The remaining sample was treated with HIO₃ and then analysed in the same manner. (a) and (b) Hydrazinolysis reaction products from IdoA→AMan_R(6-SO₄) before (a) and after (b) HIO₃ treatment. (c) and (d) Reaction products from IdoA(2-SO₄)→AMan_R before (c) and after (d) HIO₃ treatment. (e) and (f) Reaction products from IdoA(2-SO₄)→AMan_R(6-SO₄) before (e) and after (f) HIO₃ treatment. Samples shown in (a), (b), (c) and (d) were eluted isocratically in 40 mM-potassium phosphate buffer, pH 4.6. Samples shown in (e) and (f) were eluted isocratically in 185 mM-KH₂PO₄, pH 4.6. The arrows indicate the peak positions of the compounds before the hydrazinolysis treatment.

into the original disaccharides by HIO₃, a reagent that oxidizes hydrazides, is a further indication that the hydrazinolysis reaction products were uronic acid hydrazone derivatives.

The small peaks recovered in the HIO₃-treated samples were formed from the hydrazone derivatives, since they were not present in the hydrazinolysis reaction mixtures and were not formed by direct HIO₃ treatment of the original di-

saccharides (results not shown). These small peaks appeared to be isomers of the original disaccharides formed by epimerization of the L-iduronic acid residues to D-glucuronic acid residues. The following observations support this conclusion: (a) on paper electrophoretograms the small peaks all co-migrated with the large peaks of the original disaccharides in the mixture, an indication that in each case the charge per unit mass was the same for the starting disaccharide and the new product; (b) when the mixtures of products were eluted from paper electrophoretograms and hydrolysed with 0.5M-H₂SO₄ at 100°C for 4h, the acid-resistant GlcA→AMan_R disaccharide (Conrad, 1980) was recovered in each hydrolysate in an amount expected on the basis of the amount of epimer peak found in each reaction mixture; (c) the small peak formed by HIO₃ oxidation of the IdoA→AMan_R(6-SO₄) hydrazinolysis product (Fig. 5b) was eluted from the anion-exchange column in the position of a GlcA→AMan_R(6-SO₄) standard (Delaney *et al.*, 1980). Thus the uronic acid hydrazides appear to undergo a slow epimerization at C-5 of the uronic acid residue. As shown in Table 2, the percentage of total products recovered in the epimer peak increases with increasing duration of the hydrazine/hydrazine sulphate treatment. Furthermore, the amount of epimerization differed for the different disaccharides and was lowest for the D-glucuronic acid-containing disaccharide.

β-Elimination

The demonstration that uronic acid hydrazides undergo slow epimerization under the hydrazinolysis reaction conditions is an indication that the proton on C-5 of the uronic acid hydrazides is labile to base. This raises the possibility that, when the hydrazinolysis reaction is used in the structural analysis of glycosaminoglycans or oligosaccharides derived from them, the internal uronic acid hydrazide residues may undergo a β-elimination reaction in addition to, or in preference to, the epimerization reaction. Such a reaction, which would result in cleavage of linkages to C-4 of the uronic acids and conversion of the uronic acids

into Δ^{4,5}-unsaturated residues, would not be observed for the model disaccharides used above, since these disaccharides do not have internal uronic acid residues. The possibility that a β-elimination reaction might occur was tested with ³H-labelled GlcA→GalNAc→GlcA→GalNAc_R, prepared from chondroitin sulphate. Like the uronic acid residues in heparin, the internal D-glucuronic acid residue in this tetrasaccharide is linked in the 4-position. Cleavage of this compound by β-elimination would release from the reducing end of the tetrasaccharide a ³H-labelled aldehyde-reduced disaccharide having an unsaturated uronic acid. To determine whether this cleavage occurred during hydrazinolysis, the tetrasaccharide was heated with hydrazine/hydrazine sulphate to remove its N-acetyl groups and convert its uronic residues into hydrazides. The product formed after hydrazinolysis for 6h at 100°C was treated with HIO₃ and analysed by paper chromatography as shown in Fig. 5. Two products were formed in this reaction sequence. The N-deacetylated product (Fig. 5c), which was recovered in 95% yield, migrated more slowly than did the original tetrasaccharide (Fig. 5a). After N-re-acetylation the hydrazinolysis product co-migrated with the original tetrasaccharide (not shown). Treatment of both the N-re-acetylated hydrazinolysis product (not shown) and the original tetrasaccharide (Fig. 5b) with chondroitinase AC released the ³H-labelled unsaturated disaccharide from the reducing terminals of these tetrasaccharides. The N-deacetylated tetrasaccharide, however, was not attacked by chondroitinase AC (Fig. 5d). The complete cleavage of the N-re-acetylated compound by chondroitinase AC, which will not attack such oligosaccharides at L-iduronic acid residues, indicates that no significant epimerization occurred at the internal uronic acid residue.

The second product of the hydrazinolysis/deamination reaction, which migrated as a small peak at segment 36 (Fig. 5c), was identified by paper chromatography and paper electrophoresis as D-galactosaminitol. In a series of experiments not described here, it was found that the reducing

Table 2. Epimerization of model disaccharides at 100°C in hydrazine containing 1% hydrazine sulphate
For experimental details see the text.

Disaccharide	Conversion into epimer (%)	
	After 3h	After 6h
IdoA→AMan _R (6-SO ₄)	5.9	14.6
IdoA(2-SO ₄)→AMan _R (6-SO ₄)		12.1
IdoA(2-SO ₄)→AMan _R	2.6	8.8
GlcA→AMan _R (6-SO ₄)		3.6

Fig. 5. Hydrazinolysis of $\text{GlcA} \rightarrow \text{GalNAc} \rightarrow \text{GlcA} \rightarrow \text{GalNAc}_R$

Tetrasaccharide was heated at 100°C with hydrazine/hydrazine sulphate for 6h, then treated with HIO_3 and analysed by paper chromatography in system II. (a) Tetrasaccharide. (b) Tetrasaccharide after treatment with chondroitinase AC. (c) Tetrasaccharide after hydrazinolysis and HIO_3 treatment. (d) Tetrasaccharide after hydrazinolysis, HIO_3 treatment and then chondroitinase AC treatment.

terminal D-galactosaminitol residue was released in yields that increased with increasing time of hydrazinolysis, that the release of the D-galactosaminitol did not occur when the internal D-glucuronic acid residue of the tetrasaccharide substrate was reduced to a D-glucose residue, and that the D-galactosaminitol release occurred, not in the hydrazinolysis step, but in the treatment of the hydrazinolysis product with HIO_3 . Thus the D-galactosaminitol release appeared to result from HIO_3 -catalysed hydrolysis of a small amount of acid-labile 2-acetamido-2-deoxy-3-O-(β -D-glucopyranosyluronic acid)-D-galactitol that was formed by β -elimination at the uronic acid hydrazide residue formed during the hydrazinolysis step.

Hydrazinolysis of heparin

The above results indicated that, during the 6h hydrazinolysis reaction period required for complete N-deacetylation of a substrate, at least 5% of the chondroitin tetrasaccharide might undergo a β -elimination reaction. Although this amount of degradation would not be a problem in the determination of the structure of a pure oligosaccharide, it could result in a significant decrease in the molecular mass of a polymeric glycosaminoglycan substrate. This possibility was tested by studying the effect of hydrazinolysis on heparin. Fig. 6 compares Bio-Gel P-30 elution profiles of heparin and a heparin sample that was treated at

Fig. 6. Size-exclusion chromatography of heparin and carboxy-group-reduced heparin before and after hydrazinolysis. Heparin and carboxy-group-reduced heparin were dissolved in hydrazine/hydrazine sulphate together with a portion of D-[^{14}C]glucitol. A portion of each solution was treated directly with HIO_3 and analysed by chromatography on Bio-Gel P-30. A second portion was heated at 100°C for 6 h and then treated with HIO_3 and analysed by Bio-Gel P-30 chromatography. (a) Heparin starting material. (b) Heparin after hydrazinolysis for 6 h. (c) Carboxy-group-reduced heparin starting material. (d) Carboxy-group-reduced heparin after hydrazinolysis for 6 h. Arrows indicate the elution positions of the D-[^{14}C]glucitol.

100°C with hydrazine/hydrazine sulphate for 6 h. The increase in K_{av} and polydispersity show that the heparin undergoes a slow depolymerization as a result of the hydrazinolysis treatment. Uronic acid analysis (Conrad, 1980) of the hydrazine-treated heparin samples showed that the percentage of total uronic acids recovered as L-iduronic acid fell from an initial value of 77% to 75% at 3 h and 71% at 6 h. When carboxy-group-reduced heparin was subjected to the same treatment, no depolymerization occurred (Figs. 6c and 6d). This suggests that the polymer degradation will occur only in uronic acid-containing polymers that can undergo hydrazide formation and subsequent β -elimination.

Reaction of uronic acid hydrazides with HNO_2

The utility of the hydrazinolysis reaction in the structural analysis of uronic acid-containing poly-

mers requires that the hydrazinolysis step be followed by a cleavage of the polymer at the *N*-deacetylated amino sugar residues by treatment with HNO_2 at pH 4. To test the effect of direct treatment of the hydrazinolysis reaction product with HNO_2 on the fate of the uronic acid hydrazide residues, $\text{IdoA}(2\text{-SO}_4) \rightarrow \text{AMan}_R(6\text{-SO}_4)$ was treated with hydrazine/hydrazine sulphate at 100°C for 6 h and the isolated hydrazide derivative was analysed before and after treatment with HNO_2 at pH 4. The results (not shown) were identical with those shown in Figs. 4(e) and 4(f). Thus the reaction of the hydrazide with HNO_2 at pH 4 converts the uronic acid hydrazide residues back into the corresponding uronic acid residues. Similar results were obtained with several of the other model disaccharides. Furthermore, the pH 1.5 HNO_2 treatment also resulted in a quantitative conversion of uronic acid hydrazide residues into their corresponding uronic acid residues.

Discussion

Both heparin and related glycosaminoglycans are shown here to be quantitatively *N*-deacetylated without loss of either *N*- or *O*-sulphate substituents. Since *O*-sulphate substituents are also retained in the HNO_2 reaction (Shively & Conrad, 1976a), the requirements originally set forth for the selective cleavage of glycosaminoglycans by the hydrazinolysis/deamination procedure are met. However, the observation that uronic acids are converted into hydrazides in the hydrazinolysis reaction is important when one considers several further conversions that the hydrazides may undergo in the initial or subsequent steps in the hydrazinolysis/deamination reaction sequence.

First, hydrazide formation labilizes the hydrogen atom on C-5 of the uronic acid, resulting in C-5 epimerization of the uronic acid residues. Since the epimerization is a relatively slow reaction, it does not cause serious difficulty in the analysis of polysaccharides known to contain a single type of uronic acid, or in the characterization of oligosaccharides for which the uronic acid composition and/or position are established by some independent means. However, for polymers such as heparin, heparan sulphate and dermatan sulphate, which contain both D-glucuronic acid and L-iduronic acid, the chemical epimerization that is introduced as a part of the present structural approach may lead to recoveries of these uronic acids in proportions that are somewhat different from those in the original polysaccharide.

Secondly, the labilization of the C-5 hydrogen atom in the uronic acid hydrazide residue resulted in a slow β -elimination of substituents linked to C-4 of the uronic acid residues, both in a model tetrasaccharide prepared from chondroitin sulphate and in heparin. Since internal uronic acid residues in carbohydrate polymers are commonly linked at C-4, β -elimination may occur during hydrazinolysis to an extent that may vary with the structure of the polymers in which uronic acids are found. Carboxy-group reduction of glycosaminoglycans appears to eliminate the degradation that occurs in the hydrazinolysis of these polymers, an effect noted earlier by Wolfrom & Juliano (1960).

A third problem anticipated in the hydrazinolysis/deamination procedure was the possible conversion of the uronic acid hydrazides to azides by reaction with HNO_2 . The azides could then undergo several different rearrangements, which, depending on the reaction conditions, could lead to different products. However, the present results show that the uronic acid azides formed by treatment with HNO_2 at either pH 4 or pH 1.5 break down spontaneously to regenerate the free acid, a reaction reported previously for aldonic acid

azides (Thompson & Wolfrom, 1946). Thus, after the hydrazinolysis step, three options may be considered in the structural analysis of heparin and other glycosaminoglycans: (a) direct oxidation of the polymer with HIO_3 to regenerate the *N*-deacetylated polysaccharide; (b) treatment of the polymer with HNO_2 at pH 4 to obtain simultaneous deaminative cleavage at the D-glucosamine residues and regeneration of the uronic acid residues; or (c) treatment of the polymer with HNO_2 at pH 1.5 to obtain simultaneous deaminative cleavage at the *N*-sulpho-D-glucosamine residues and regeneration of the uronic acid residues.

A final problem that might result from hydrazide formation is the diminished susceptibility of the altered polymer to enzymes. Thus, in the widely applicable method for labelling amino sugar residues described by Höök *et al.* (1982), chondroitin sulphate and other glycosaminoglycans were partially *N*-deacetylated by hydrazinolysis and then re-acetylated with [^{14}C]acetic anhydride. The labelled chondroitin sulphate was partially, but not completely, degraded to disaccharides by treatment with chondroitinase. A possible explanation for the incomplete digestion by chondroitinase is that the enzyme could not attack the polymer at the uronic acid hydrazides. It is clear from the present work that this labelling procedure should be modified to include a step for re-oxidation of the hydrazides to the uronic acids. If this step were included, the glycosaminoglycans could be completely *N*-deacetylated and *N*-re-acetylated with labelled acetic anhydride to regenerate the original polymer uniformly labelled at a very high specific radioactivity. Furthermore, since the *N*-sulphate substituents on heparin and heparan sulphate polymers are stable under the hydrazinolysis conditions, these polymers could also be labelled by the *N*-deacetylation/hydrazide oxidation/re-acetylation procedure with the only apparent structural alterations being those resulting from the limited degree of β -elimination and uronic acid epimerization that would occur.

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