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Specificity of Flavobacterial Glycuronidases Acting on Disaccharides Derived from Glycosaminoglycans

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The specificity of the unusual flavobacterial glycuronidases that act on disaccharides containing $\Delta^{4,5}$-unsaturated uronic acids was reinvestigated. The results show that the enzyme that hydrolyses the uronidic bond in disaccharides from hyaluronic acid and the chondroitin sulphates appears to be mainly specific for β-D-(1→3)-derived linkages. The enzyme that hydrolyses the uronidic bond in a variety of disaccharides obtained from heparan sulphate and heparin appears to be specific for β-D-(1→4)- and α-L-(1→4)-derived linkages. Thus the glycuronidases seem to be specific for linkage position rather than anomeric configuration, as had been thought previously. In addition, the data confirm other evidence that the major glucuronidic linkages in heparan sulphate and heparin have the β-D-configuration, and the iduronidic linkages the α-L-configuration.

Flavobacteria produce a variety of constitutive enzymes that degrade glycosaminoglycans (Linker *et al.*, 1960). The organisms can be induced to produce 10-fold higher amounts of these same enzymes and to synthesize additional enzymes (Hoffman *et al.*, 1960; Linker & Hovingh, 1965) not normally detectable. Unusual types of glycuronidases have also been isolated from the induced organisms (Warnick & Linker, 1972; Dietrich, 1969; Yamagata *et al.*, 1968). These enzymes are specific for glycuronides containing α,β-unsaturated uronic acids, products of the eliminases that degrade heparin, heparan sulphate, hyaluronic acid and the chondroitin sulphates. The glycuronidases do not appear to act on saturated uronides. Two types of glycuronidases have been described previously (Warnick & Linker, 1972), one of which hydrolyses unsaturated disaccharides obtained from hyaluronic acid and the chondroitin sulphates and the other degraded disaccharides obtained from heparin and heparan sulphate. As the unsaturated uronic acid in all of these is identical, the major difference at the time was thought to be the original linkage type, namely a β-linkage in hyaluronate and chondroitin sulphate disaccharides and an α-linkage in the heparin and heparan sulphate uronides. Therefore the enzyme acting on the disaccharides from heparin and heparan sulphate was thought to be an α-D-glycuronidase (Warnick & Linker, 1972). However, some evidence has indicated (Perlin *et al.*, 1971; Helting & Lindahl, 1971; Linker, 1975) that the major uronidic linkages in heparin and heparan sulphate are β-D and α-L, rather than α-D as previously believed (Wolfrom *et al.*, 1964*b*). This is unresolved, as other data still support α-D-glycuronidic linkages for heparan sulphate (Silva *et al.*, 1976). As linkage type has important structural and biological implications, we have reinvestigated the specificity of these interesting glycuronidases to determine what structural features are responsible for the differential activity.

Materials and Methods

Materials

Ox liver heparin was obtained from ICN (Irvine, CA, U.S.A.). Chondroitin 6-sulphate isolated from shark cartilage was obtained from Calbiochem (La Jolla, CA, U.S.A.) and heparan (heparitin) sulphate (0.9M-NaCl fraction) was isolated from ox lung (Linker & Hovingh, 1973). Chondroitin 4-sulphate was prepared from ox tracheal cartilage (Meyer *et al.*, 1956). Disaccharides containing $\Delta^{4,5}$-unsaturated uronic acid as the non-reducing unit were obtained by the action of flavobacterial heparinase (EC 4.2.2.7) and heparanase (EC 4.2.2.8) (Hovingh & Linker, 1970) (the name has been changed from heparitinase to conform with recent nomenclature), chondroitinase (EC 4.2.2.4) (Hoffman *et al.*, 1960) and pneumococcal hyaluronidase (EC 4.2.2.1) (Linker *et al.*, 1956). All disaccharides that served as substrates were isolated and well-characterized as described previously [see above and Linker *et al.* (1960) and Hovingh & Linker, 1974)]. Their structures are shown in Fig. 1 to avoid repetition of lengthy and somewhat confusing nomenclature. [As the asymmetry on C-5 is lost owing to eliminase action, the same compound is obtained from D-glucuronic acid and L-iduronic acid. The nomenclature of the linkage, originally either β-D or α-L and α-D or β-L

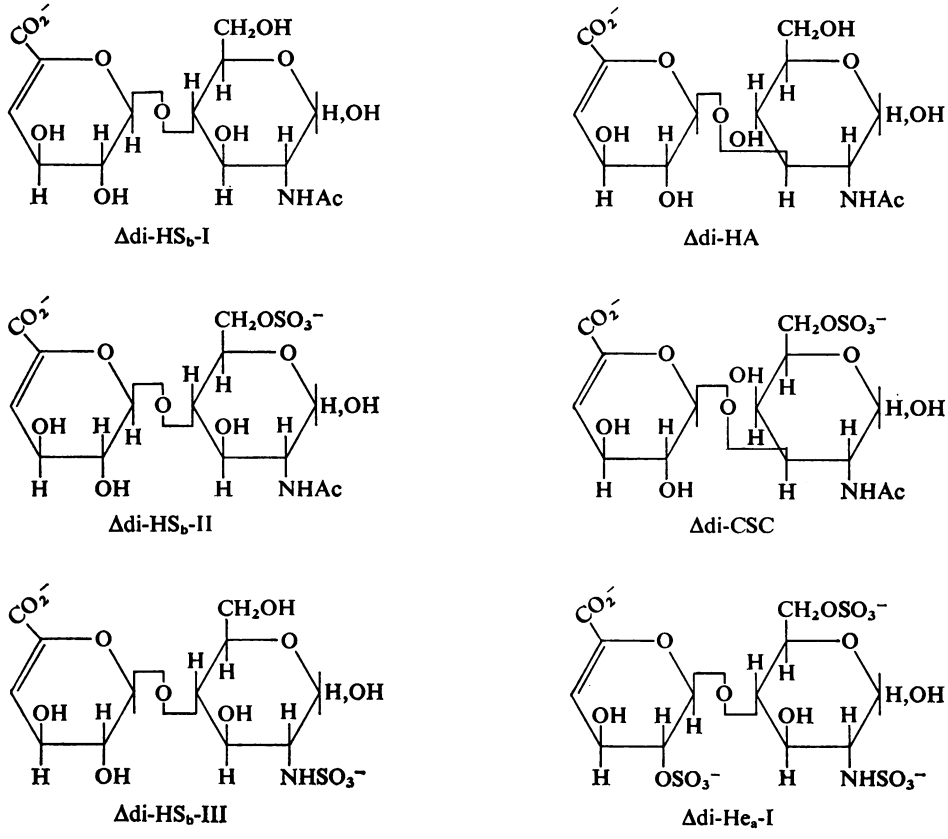

Fig. 1. *Structures of* $\Delta^{4,5}$*-unsaturated disaccharides*

The structures are based on data referred to in the text and on the optical rotations of the reduced compounds shown in Table 1. The following abbreviations taken from a previous publication (Hovingh & Linker, 1974) are used: Δdi-HS_b-I, Δdi-HS_b-II, Δdi-HS_b-III, disaccharides obtained from heparan sulphate; Δdi-HA, disaccharide obtained from hyaluronic acid, Δdi-CSC, disaccharide obtained from chondroitin 6-sulphate; Δdi-He_a-I, disaccharide obtained from heparin.

respectively, becomes difficult. The correct name for the unsaturated uronic acid is 4-deoxy-(α or β)-L-threohex-4-enopyranosyl uronic acid. The linkage could be named accordingly, but as the emphasis here relates to the original linkage in the polymer, which obviously has not been altered, we will use 'β-D', 'α-L', etc. for the disaccharides, indicating the original bond.] Strontium Δ-digalacturonate was obtained as a much appreciated gift from Dr. Charles Nagel, Washington State University, WA, U.S.A. To convert this disaccharide into the sodium salt, it was dissolved in water and passed through a small column of AG-50(X4; H^+ form) containing 1g of resin. The eluate was evaporated to dryness in the presence of sodium acetate, redissolved and desalted on a column (90cm×1.1cm) of Sephadex G-10. *Flavobacterium heparinum* was grown, induced on the specific substrate required, the cells were collected and sonicated, and the crude enzyme extract was prepared as described previously (Linker & Hovingh, 1972*a*).

Methods

Optical rotations. These were obtained with a Zeiss polarimeter in a 1.0dm cell at the sodium D-line wavelength at a sample concentration of 1% (w/v) in water.

Paper chromatography. Whatman no. 1 paper was used with butanol/acetic acid/water (10:3:7, by vol.) as solvent system by using downward irrigation. Compounds were located by u.v. absorption by using a short-wavelength lamp or by spraying the chromatograms with an alkaline $AgNO_3$ reagent (Trevelyan *et al.*, 1950).

Column chromatography. Enzyme purifications were carried out on the following columns: Bio-Rad hydroxyapatite (Bio-Gel HPT, 6.0cm×2.1cm) (Bio-Rad Laboratories, Richmond, CA, U.S.A.); Whatman cellulose phosphate (P11, 7.0cm×1.1cm) (Whatman, Clifton, NJ, U.S.A.). The columns were prepared by suspending the materials in a beaker in the buffer to be used for elution. The material was left to settle and fines were removed by washing with water. The columns were then packed by gravity flow.

Chemical methods. Borohydride reduction was carried out as described previously (Warnick & Linker, 1972).

Enzyme assays. Heparinase activity was assayed by measuring increase in A_{232} with heparin as substrate (Linker & Hovingh, 1965); chondroitinase was measured the same way with chondroitin 6-sulphate as substrate, and heparanase with heparan (heparitin) sulphate-0.9 as substrate. The glycuronidases were assayed by measuring the decrease in A_{232} with one of the $\Delta^{4,5}$-unsaturated disaccharides as substrate, as indicated below. Reactions were carried out in 0.1M-sodium acetate, pH7.0, with 2mg of substrate/ml at 30°C. The presence of idurono sulphatase (EC 3.1.6.–) (Dietrich *et al.*, 1973) was detected by incubating the trisulphated disaccharide from heparin (Δdi-He$_a$-I) with enzyme and checking for hydrolysis by the appearance of disulphated disaccharide by paper chromatography.

Results

Optical rotation of reduced disaccharides

To eliminate the contribution of the anomeric atom at the reducing end, unsaturated disaccharides obtained from heparin, heparan sulphate, chondroitin 6-sulphate and poly(galacturonic acid) were reduced with $NaBH_4$. Owing to eliminase action, all the uronic acids are $\Delta^{4,5}$-unsaturated (see Fig. 1), though the disaccharides retain the uronidic linkages of the original polymer (derived from D-glucuronic acid, L-iduronic acid and D-galacturonic acid). The molecular rotations of the disaccharides before and after reduction of the reducing end are shown in Table 1.

In terms of molecular rotation, all the reduced disaccharides from heparin and heparan sulphate, as well as the 1→3-linked disaccharide from chondroitin 6-sulphate (known to have a β-D linkage) fall into the group of β-D-linked compounds (see also Wolfrom *et al.*, 1964*a*).

Enzyme fractionation

The glycuronidases were first prepared from the crude extract of *Flavobacterium* induced to heparin, as follows. The extract, consisting of about 80ml of solution (Linker & Hovingh, 1972*a*) was added to a hydroxyapatite column and eluted with a phosphate/NaCl gradient (formed by adding 250ml of 0.25M-sodium phosphate buffer, pH6.8, containing 0.5M-NaCl from one vessel to a mixing vessel containing 250ml of 0.01M-sodium phosphate buffer, pH6.8); fractions (about 6ml) were collected and assayed for enzyme activity. The elution pattern is shown in Fig. 2. The fractions within the major part of the peak showing activity on heparan sulphate disaccharide were combined and dialysed against water for 8h. The enzyme solution was then added to a cellulose phosphate column to remove the chondroitin sulphate eliminase (EC 4.2.2.4) (Fig. 2). The cellulose phosphate column was eluted with the same buffer and gradient as the hydroxyapatite. The glycuronidase was usually not adsorbed and appeared in the void volume, but the eliminase was retained. Occasionally the glycuronidase was adsorbed, but was eluted early in the gradient before the contaminating eliminase. The eluate containing the 'heparan glycuronidase' was dialysed against water for 8h and freeze-dried. Starting with 80ml of crude extract, 45mg of enzyme was obtained. The specific activity

Table 1. *Optical rotation of reduced disaccharides*

Source	Abbreviation	Linkage	Molecular rotation ($[\alpha]_D^{25}$) Before reduction	After reduction
Heparan sulphate	Δdi-HS$_b$-I	(1→4)	−7200	−17400
	Δdi-HS$_b$-II	(1→4)	+2500	−1500
	Δdi-HS$_b$-III	(1→4)	+4200	−6000
Heparin	ΔdiHe$_a$-I	(1→4)	+4000	−13200
Chondroitin sulphate	Δdi-CS-6	β-D-(1→3)	0	−25700
Cellobiose		β-D-(1→4)	+12400	−3000
Maltose		α-D-(1→4)	+45000	+35000
Pectin	Δ-di-galU	α-D-(1→4)	+72500	+49000

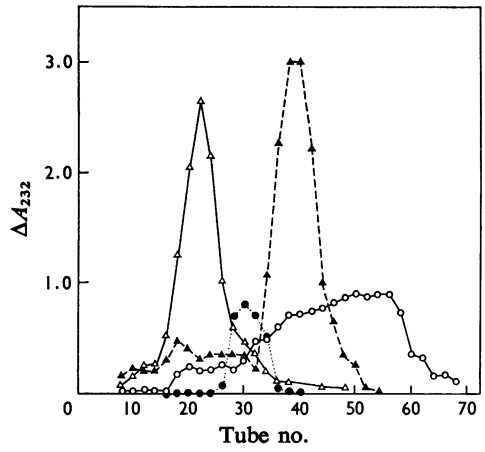

Fig. 2. *Fractionation of crude enzymes from heparin-induced flavobacteria on a hydroxyapatite column*
The activity of the eliminases was measured by the increase in A_{232}. The activity of the glycuronidases was measured by the decrease of this absorption. The following substrates were used to assay enzyme activities: △, heparin; ▲, chondroitin 6-sulphate; ○, Δdi-HS_b-I; ●, Δdi-CSC. From each tube 0.1 ml was withdrawn and added to a test tube containing 0.1 ml of substrate solution (2 mg/ml in 0.1 M-sodium acetate, pH 7.0, for disaccharides, 20 mg/ml in 0.1 M-sodium acetate, pH 7.0, for polymers). After 2 h at 30°C for the eliminases and 3 h for the glycuronidases 1.0 ml of 0.03 M-HCl was added and the solutions were transferred to a cuvette and the A_{232} was read in a spectrophotometer.

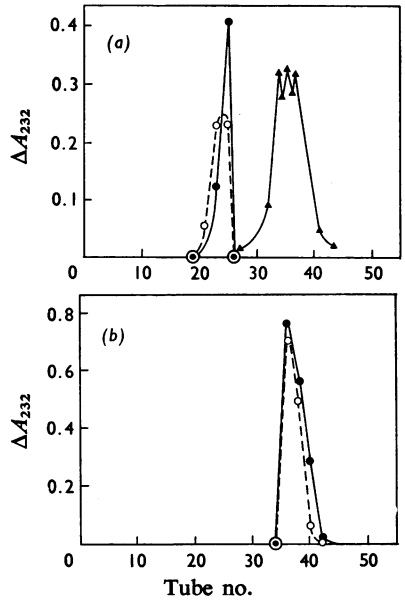

Fig. 3. *Fractionation of crude enzyme extract obtained from chondroitin 6-sulphate-induced flavobacteria*
(*a*) Phosphocellulose column; (*b*) hydroxyapatite column. Solutions in the tubes were assayed as in Fig. 2, except that for substrate Δdi-HS_b-I the incubation time was extended to 18 h to obtain sufficient sensitivity. The following substrates were used: ▲, chondroitin 6-sulphate; ○, Δdi-HS_b-I; ●, Δdi-CSC.

was 21 units/mg (a unit represents 1 μmol of disaccharide Δdi-HS_b-I hydrolysed/h) of enzyme protein (Lowry *et al.*, 1951), which represents a 25-fold purification over the preparation described previously (Warnick & Linker, 1972). The 'heparan glycuronidase' contained no eliminases, sulphatase or chondroitin sulphate glycuronidase activities.

As shown in Fig. 2, a relatively small amount of a glycuronidase which acts on chondroitin sulphate disaccharide is also present in the crude extract. Fractions in this peak of activity were combined and the 'chondroitin sulphate glycuronidase' was isolated as described for the heparan sulphate enzyme. Approx. 4 mg of enzyme was obtained with a specific activity of 6.5 units/mg of enzyme protein (a unit represents 1 μmol of chondroitin 6-sulphate disaccharide hydrolysed/h). This represents a 20-fold purification over the crude extracts. Small amounts of heparin eliminase were present.

Neither enzyme was highly purified, but attempts to purify them further by chromatography with DEAE-cellulose, concanavalin A–Sepharose or CM-cellulose were not successful. $(NH_4)_2SO_4$ fractionation led to considerable losses in activity.

Alternatively, the chondroitin sulphate glycuronidase was prepared on a small scale from chondroitin 6-sulphate-induced flavobacteria to avoid potential contamination by the 'heparan sulphate glycuronidase' (a heparin-induced enzyme). The crude enzyme was prepared as described above by using chondroitin 6-sulphate for induction. The freeze-dried crude extract (about 90 mg) was fractionated on a phosphocellulose column (4.8 cm × 1.1 cm) by gradient elution (100 ml of 0.05 M-sodium phosphate buffer, pH 6.8, containing 0.2 M-NaCl was run into a mixing vessel holding 100 ml of 0.01 M-sodium phosphate buffer, pH 6.8). The elution pattern is shown in Fig. 3(*a*). Peak fractions containing the glycuronidase were combined, dialysed against water and placed on a column (4.5 cm × 1.1 cm) of hydroxyapatite. The column was eluted with a buffer gradient similar to that above (but containing 0.5 M-NaCl rather than 0.2 M-NaCl). The elution pattern is shown in Fig. 3(*b*). The glycuronidase peak tubes contained a total of 0.1 mg of protein (specific activity about 50 units/mg

of protein). The enzyme was not isolated but used in solution. When the disaccharide from hyaluronic acid was used as substrate for assay, the elution pattern was identical with that for chondroitin sulphate disaccharide. The enzyme shows activity toward Δdi-HS_b-I (heparan sulphate disaccharide): this is discussed below.

Hydrolysis of disaccharides by the glycuronidases

The compounds shown in Fig. 1 were incubated with the enzymes under the following conditions: substrates were dissolved at 3mg/ml in 0.1M-sodium acetate, pH7.0; enzyme was added to give a final concentration of 3 units/ml for the heparan sulphate enzyme and 0.5 unit/ml for the chondroitin sulphate enzyme; and the solution incubated at 30°C. At suitable time intervals, a 0.05ml sample was withdrawn, added to 2ml of 0.03M-HCl and the A_{232} was measured. The time course of hydrolysis as measured by decrease in u.v. absorbance is shown in Figs. 4(*a*) and 4(*b*).

The 'heparan sulphate glycuronidase' hydrolyses the unsaturated disaccharides obtained from heparan sulphate (e.g. Δdi-HS_b-I, Δdi-HS_b-II, and Δdi-HS_b-III) very well regardless of the presence or position of sulphate on the glucosamine. The trisulphated disaccharide from heparin is degraded very poorly, if at all, owing to the presence of a sulphate group in the uronic acid moiety (Dietrich *et al.*, 1973) (also see below). The disaccharides from hyaluronic acid, chondroitin 6-sulphate and pectic acid are not hydrolysed. The enzyme therefore appears to be a 1→4-glycuronidase. On the other hand, the 'chondroitin sulphate glycuronidase', whether obtained from heparin sulphate- or chondroitin sulphate-induced organisms, acts well on the disaccharides from hyaluronic acid and chondroitin 6-sulphate and also has considerable activity on the non-

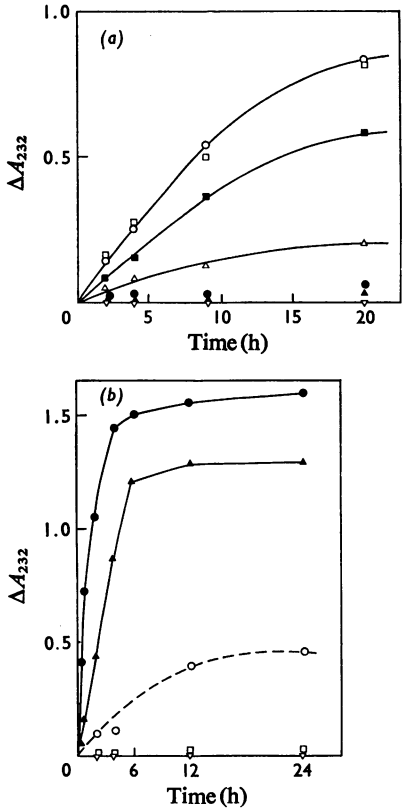

Fig. 4. *Hydrolysis of disaccharides by the heparan sulphate glycuronidase (a) and chondroitin sulphate glycuronidase (b)*

The following substrates were used: ○, Δdi-HS_b-I; □, Δdi-HS_b-II; ■, Δdi-HS_b-III; ▲, Δdi-HA; △, Δdi-He_a-I; ●, Δdi-CSC; ▽, Δdi-galacturonate. Enzyme activity was measured by the loss of A_{232} owing to the liberation of the $\Delta^{4,5}$-unsaturated uronic acid which rearranges to an α-oxo acid (Warnick & Linker, 1972).

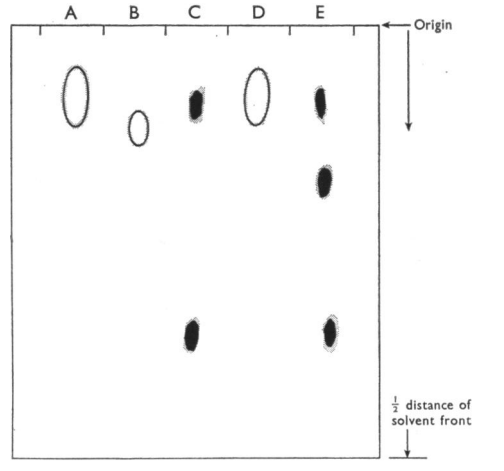

Fig. 5. *Tracing of paper chromatogram showing degradation of the trisulphated disaccharide from heparin (Δdi-He_a-I)*

A, Δdi-He_a-I; B, disulphated disaccharide obtained by the action of crude chondroitinase on Δdi-He_a-I; C, hydrolysate of the disulphated disaccharide by heparan sulphate glycuronidase; D, Δdi-He_a-I incubated with the heparan sulphate glycuronidase; E, hydrolysate of Δdi-He_a-I by crude heparinase; the fastest spot is α-oxo acid, the next glucosamine *N*-sulphate and the slowest glucosamine *NO*-disulphate. The open areas show u.v. absorption and react with the $AgNO_3$ spray reagent; the filled areas react with the $AgNO_3$ reagent only.

sulphated disaccharide (Δdi-HS_b-I) from heparan sulphate. In agreement with earlier data (Yamagata *et al.*, 1968), the enzyme did not act on disaccharide obtained from chondroitin 4-sulphate owing to the presence of sulphate on the 4-position of the galactosamine unit. This enzyme therefore appears to be essentially a 1→3-glycuronidase.

Neither the heparan sulphate (1→4)- nor the chondroitin sulphate (1→3)-glycuronidase showed any activity when the reduced disaccharides (see Table 1) were used as substrates.

Trisulphated disaccharide from heparin (Δdi-He_a-I) was incubated with a crude enzyme extract from chondroitin 6-sulphate-induced flavobacteria (under the conditions described for purified 1→4-glycuronidase). As shown in Fig. 5, the trisulphated disaccharide is indeed converted into a disulphated disaccharide which moves more rapidly than the trisulphated disaccharide and retains u.v. absorption. The disulphated disaccharide was separated from the trisulphated compound by chromatography on Dowex 1 (X10) (Linker & Hovingh, 1972*b*). Both the disulphated and trisulphated disaccharides were incubated with the 1→4-glycuronidase. The disulphated disaccharide was rapidly hydrolysed to glucosamine *NO*-disulphate and α-oxo acid (Fig. 5, and Linker & Hovingh, 1965), but no product was formed from the trisulphated disaccharide (although Fig. 4*a* does show some activity when the u.v. assay is used).

Discussion

The determination of the detailed structure of heparin has presented one of the most difficult problems in the chemistry of polysaccharides. Although the main structure appeared to have been determined chemically in 1964 (Wolfrom *et al.*, 1964*a*), it is considerably more complicated than simply a polymer of alternating α-D-(1→4)-linked glucuronic acid and D-glucosamine residues. The presence of L-iduronic acid had been missed, for reasons made apparent later (Wolfrom *et al.*, 1969), but discrepancies concerning the α-D-glucuronidic linkages are still not explained. The α-D-linked disaccharides isolated by Wolfrom and co-workers (Wolfrom *et al.*, 1964*b*) although obtained in a low yield, were adequate to represent a substantial part of the molecule. In our studies (Linker & Hovingh, 1972*b*), the repeating disaccharide unit of heparin was obtained in a much better yield (40%) by enzymic degradation. We did not verify the type of uronidic linkage, but assumed it to be α-D-configuration as accepted at the time. Heparan sulphate, which closely resembles heparin in composition, optical rotation and enzymic degradation (Linker & Hovingh, 1973), was thought to have the same types of glycosidic linkages. Therefore when we isolated an unusual glycuronidase from heparin-induced flavobacteria, which degraded disaccharides from heparin and heparan sulphate, but not from hyaluronic acid or the chondroitin sulphates, we postulated that it was specific for linkages derived from α-D-(or β-L-) uronic acids (Warnick & Linker, 1972). A similar glycuronidase isolated earlier from the organisms induced to chondroitin sulphate (Linker *et al.*, 1960) hydrolysed disaccharides from hyaluronic acid and chondroitin sulphate, but not those from heparin or heparan sulphate. It was thought to be specific for linkages derived from β-D-uronic acids.

The presence of α-D linkages only in heparin was first questioned by Perlin (Perlin & Sanderson, 1970). The same author showed (Perlin *et al.*, 1971) that an α-L-iduronic linkage was the major uronidic linkage. The isolation of an α-L-iduronidase from mammalian liver (Weissman & Santiago, 1972) and the demonstration that an α-L-iduronidase deficiency was the major defect in Hurler's syndrome (Matalon & Dorfman, 1972; Bach *et al.*, 1972) supported the presence of this linkage in heparan sulphate as well as in heparin. Helting demonstrated the presence of some β-D-glucuronidic linkages in heparin (Helting & Lindahl, 1971). We were able to show the presence of β-D-glucuronidic as well as α-L-iduronidic linkages in heparan sulphate (Linker, 1975). Evidence presented here on the optical rotations of the reduced disaccharides from heparan sulphate and heparin (Table 1) also supports the presence of β-D-(or α-L-) uronidic linkages. It seems that the difference in specificity between the assumed 'α' and 'β' glycuronidases is not due to anomeric configuration but another factor.

The 'heparan sulphate glycuronidase' (see Fig. 4*a*) hydrolyses all disaccharides obtained from heparan sulphate. The presence or absence of sulphate groups on the glucosamine moiety seems to have little effect on activity. However, trisulphated disaccharide from heparin, which contains a sulphate group on the uronic acid residue, is not appreciably degraded until this group is removed. There is a report, however, of a glycuronidase capable of hydrolysing the uronic acid, which still contains sulphate (Ototani *et al.*, 1974).

The chondroitin sulphate (1→3)-glycuronidase (see Fig. 4*b*) acts well on disaccharides from hyaluronic acid and chondroitin 6-sulphate, but not on the disaccharide from chondroitin 4-sulphate, owing to the presence of the sulphate group on the hexosamine (Yamagata *et al.*, 1968). The enzyme also shows considerable activity, though at a low rate, toward the non-sulphated disaccharide (Δdi-HS_b-I) from heparan sulphate, but essentially no activity toward disaccharide Δdi-HS_b-II, which has a sulphate group on the 6-position of the acetylglucosamine unit. As the 1→4-glycuronidase degrades Δdi-HS_b-II very well (Fig 4*a*) it is unlikely that the activity toward Δdi-HS_b-I is due to contamination by this enzyme.

The present data show that the heparan sulphate glycuronidase is specific for unsaturated uronidic linkages derived from β-D-(or α-L-) (1→4)-linked uronic acids. The chondroitin sulphate glycuronidase, on the other hand, is mainly specific for linkages derived from β-D- (or α-L-) (1→3)-linked uronic acids. The α-D-(1→4)-linked unsaturated digalacturonate is not degraded, indicating the absence of an enzyme acting on α-D-uronidic linkages.

That specificity toward linkage position is involved becomes most apparent when disaccharide ΔdiHS$_b$-I is compared with the disaccharide from hyaluronic acid. There are no sulphate groups nor different amino sugars to confuse the issue. The only difference between the two disaccharides is the position of the linkage, 1→4 versus 1→3. Other examples of this interesting type of specificity are known (Nelson *et al.*, 1969). It should also be pointed out here that a β-D-endoglucuronidase of leeches which acts on hyaluronic acid does not degrade heparan sulphate (Linker, 1975), indicating specificity for 1→3 linkages.

The disaccharide substrates used here, which are completely degraded by the enzymes, had been obtained in good yield from heparan sulphates (Hovingh & Linker, 1974) and heparin (Linker & Hovingh, 1972*b*) and are well characterized. Therefore the data support the presence of β-D-glucuronidic and α-L-iduronidic linkages as the major uronidic bonds in these polysaccharides and make the presence of a significant number of α-D-glucuronidic linkages in heparan sulphate (Silva *et al.*, 1976) unlikely.

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