Identification of *N*-Sulphated Disaccharide Units in Heparin-Like Polysaccharides

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1. Preparations of heparin and heparan sulphate were degraded with HNO_2 . The resulting disaccharides were isolated by gel chromatography, reduced with either NaBH_4 or NaB^3H_4 and were then fractionated into non-sulphated, monosulphated and disulphated species by ion-exchange chromatography or by paper electrophoresis. The non-sulphated disaccharides were separated into two, and the monosulphated disaccharides into three, components by paper chromatography. 2. The uronic acid moieties of the various non- and mono-sulphated disaccharides were identified by means of radioactive labels selectively introduced into uronic acid residues (^3H and ^{14}C in *D*-glucuronic acid, ^{14}C only in *L*-iduronic acid units) during biosynthesis of the polysaccharide starting material. Labelled uronic acids were also identified by paper chromatography, after liberation from disaccharides by acid hydrolysis or by glucuronidase digestion. Similar procedures, applied to disaccharides treated with NaB^3H_4 , indicated 2,5-anhydro-*D*-mannitol as reducing terminal unit. On the basis of these results, and the known positions and configurations of the glycosidic linkages in heparin, the two non-sulphated disaccharides were identified as 4-*O*-(β -*D*-glucopyranosyluronic acid)-2,5-anhydro-*D*-mannitol and 4-*O*-(α -*L*-idopyranosyluronic acid)-2,5-anhydro-*D*-mannitol. 3. The three monosulphated [$1\text{-}^3\text{H}$]anhydromannitol-labelled disaccharides were subjected to Smith degradation or to digestion with homogenates of human skin fibroblasts, and the products were analysed by paper electrophoresis. The results, along with the ^1H n.m.r. spectra of the corresponding unlabelled disaccharides, permitted the allocation of *O*-sulphate groups to various positions in the disaccharides. These were thus identified as 4-*O*-(β -*D*-glucopyranosyluronic acid)-2,5-anhydro-*D*-mannitol 6-sulphate, 4-*O*-(α -*L*-idopyranosyluronic acid)-2,5-anhydro-*D*-mannitol 6-sulphate and 4-*O*-(α -*L*-idopyranosyluronic acid 2-sulphate)-2,5-anhydro-*D*-mannitol. The last-mentioned disaccharide was found to be a poor substrate for the iduronate sulphotase of human skin fibroblasts, as compared with the disulphated species, 4-*O*-(α -*L*-idopyranosyluronic acid 2-sulphate)-2,5-anhydro-*D*-mannitol 6-sulphate. 4. The identified [$1\text{-}^3\text{H}$]anhydromannitol-labelled disaccharides were used as reference standards in a study of the disaccharide composition of heparins and heparan sulphates. Low *N*-sulphate contents, most pronounced in the heparan sulphates, were associated with high ratios of mono-*O*-sulphated/di-*O*-sulphated (*N*-sulphated) disaccharide units, and in addition, with relatively large amounts of 2-sulphated *L*-iduronic acid residues bound to C-4 of *N*-sulpho-*D*-glucosamine units lacking *O*-sulphate substituents.

The family of heparin-like glycosaminoglycans constitutes a spectrum of related polymers extending from typical heparin at one end (high contents of *N*- and *O*-sulphate groups and of *L*-iduronic acid units) to heparan sulphates at the other (low-sulphated, with high contents of *N*-acetyl groups and *D*-glucuronic acid units). The various possible com-

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binations of sugar units and sulphate residues allow for the occurrence of an exceedingly large number of different polymer sequences [see review by Lindahl & Höök (1978) for references to previous structural studies].

A multitude of biological functions have been ascribed to the heparin-like polysaccharides [Lindahl & Höök, 1978; see also *Fed. Proc. Fed. Am. Soc. Exp.*

Biol. 36, no. 1 (1977) for several reviews on the subject]. Whereas a number of these effects are probably due to relatively non-specific polyelectrolyte properties, other phenomena may be critically dependent on the fine structure of the polysaccharide chains. For instance, the binding of heparin to antithrombin, a prerequisite to the blood anticoagulant effect, requires one or more specific sequences of variously substituted sugar residues (Hopwood *et al.*, 1976; Rosenberg *et al.*, 1978). It is essential that improved methods for the elucidation of polymer sequences become available. Such methods would also greatly facilitate studies on the biosynthesis of the polysaccharides.

A chemical depolymerization method of particular value to structural studies involves deaminative cleavage of the polysaccharides by HNO_2 . Under certain conditions D-glucosamine residues with sulphated amino groups are converted into 2,5-anhydro-D-mannose units, with subsequent cleavage of the glucosaminidic linkages, whereas *N*-acetylated or *N*-unsubstituted glucosamine residues do not react (Shively & Conrad, 1976a). Heparin is thus largely degraded to a mixture of uronosyl-2,5-anhydromannose disaccharides. Of the various disaccharides expected to be present in a reduced mixture of heparin-deamination products the di-*O*-sulphated species, L-iduronosyl 2-sulphate-2,5-anhydro-D-mannitol 6-sulphate, and its carboxyl-reduced analogue, have been characterized (Lindahl & Axelsson, 1971; Shively & Conrad, 1976b). The mono-*O*-sulphated disaccharides present a more complex situation. Three different mono-*O*-sulphated disaccharides would be expected, namely D-glucuronosyl-2,5-anhydro-D-mannitol 6-sulphate, L-iduronosyl-2,5-anhydro-D-mannitol 6-sulphate and L-iduronosyl 2-sulphate-2,5-anhydro-D-mannitol. The present paper describes the separation and identification of these, and also of the two non-sulphated disaccharides, D-glucuronosyl-2,5-anhydro-D-mannitol and L-iduronosyl-2,5-anhydro-D-mannitol. The methods developed have been applied to the quantitative estimation of the six different (one di-*O*-sulphated, three mono-*O*-sulphated and two non-*O*-sulphated) *N*-sulphated disaccharide units in preparations of heparin and heparan sulphate. Kosakai *et al.* (1978) presented an alternative method for the separation of the same sulphated, but non-reduced, disaccharides. Similar results have also been obtained by B. Weissmann (personal communication) and by Hopwood (1978).

Experimental

Materials

The polysaccharide preparations used were as follows. Preparation I was a pig mucosal heparin (stage 14) that was purchased from Inolex Pharmaceutical Division, Park Forest South, IL, U.S.A., and purified by repeated precipitation with cetylpyridi-

nium chloride from 1.0M-NaCl, essentially as described (Lindahl *et al.*, 1965). The product had a sulphate/hexosamine molar ratio of 1.87. Preparation II was prepared from mouse mastocytoma tissue, after labelling *in vitro* with D-[1- ^3H]galactose. The procedure described by Höök *et al.* (1974a) was followed, except that 2mCi of [1- ^3H]galactose was substituted for [^{14}C]glucose in the incubation medium; also, unlabelled galactose (0.1%, w/v) rather than glucose was used to minimize co-precipitation of unincorporated label during purification of the polysaccharide. The product was digested with chondroitinase ABC and was then purified by ion-exchange chromatography on DEAE-cellulose (Höök *et al.*, 1975), yielding heparin with a specific radioactivity of 14×10^3 c.p.m./ μg of uronic acid. Degradation of the polysaccharide to monosaccharides (by a combination of chemical and enzymic procedures described below) showed that greater than 90% of the total ^3H label resided in the uronic acid components. Preparation III was obtained by incubating a mouse mastocytoma microsomal fraction with UDP-D-[^{14}C]glucuronic acid, UDP-D-[5- ^3H]glucuronic acid, UDP-*N*-acetyl-D-glucosamine and adenosine 3'-phosphate 5'-sulphatophosphate, as described elsewhere (Jacobsson *et al.*, 1979). The *N*- and *O*-sulphated components [denoted PS-N/O-SO $_3^-$ in the paper by Jacobsson *et al.* (1979), preparation III in the present paper] were isolated by ion-exchange chromatography. The $^3\text{H}/^{14}\text{C}$ ratio of this material was approx. 5. The uronic acid residues were uniformly ^{14}C -labelled. Preparation IV (sulphate/hexosamine molar ratio, 0.78) and preparation V (sulphate/hexosamine molar ratio, 0.69) were heparan sulphates isolated from whale lung and from bovine lung respectively; they were kindly donated by Dr. J. A. Cifonelli, University of Chicago, Chicago, IL, U.S.A.

Uronosyl-2,5-anhydromannose disaccharides, separated into three subgroups corresponding to non-sulphated, mono-*O*-sulphated and di-*O*-sulphated species, were prepared as described (Höök *et al.*, 1974a), after degradation of heparin with HNO_2 . D-Glucuronic acid and L-iduronic acid were obtained as reported previously (Höök *et al.*, 1974a). A sample of 2,5-anhydro-D-[1- ^3H]mannitol ($1.44 \mu\text{Ci}/\mu\text{mol}$) was kindly donated by Dr. B. Weissman, University of Illinois, Chicago, IL, U.S.A. 2,5-Anhydro-D-mannitol 6-sulphate (should really be 1-sulphate, in view of the symmetrical structure of the non-sulphated anhydro-alditol) was obtained by preparing the anhydro-alditol (Horton & Philips, 1976), which was then sulphated in the primary position as described for D-glucose (Whistler *et al.*, 1963); the product was identified by n.m.r. spectroscopy.

D-[1- ^3H]Galactose ($9.7 \text{mCi}/\mu\text{mol}$) and NaB^3H_4 ($247 \mu\text{Ci}/\mu\text{mol}$) were purchased from The Radiochemical Centre (Amersham, Bucks., U.K.). β -D-

Glucuronidase (type B10) isolated from bovine liver was purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. The preparation also contained α -L-iduronidase activity (see Matalon *et al.*, 1971).

Fibroblasts were grown in plastic flasks with Eagle's (1959) minimal essential medium supplemented with 10% foetal calf serum (LS-Labor Service, München, Germany) as described by Cantz *et al.* (1972). The cells were derived from skin of healthy individuals or from patients affected with mucopolysaccharidosis I (Hurler's syndrome, α -L-iduronidase deficiency), mucopolysaccharidosis II (Hunter's syndrome, iduronate sulphatase deficiency) or mucopolysaccharidosis VII (β -D-glucuronidase deficiency). Fibroblast homogenates were prepared as follows. Cells were harvested by treatment for 10 min with 0.1% tissue culture trypsin (Flow Laboratories, Bonn, Germany) and collected by centrifugation at 800g for 3 min. They were then suspended in 0.15M-NaCl, subjected to ten cycles of freezing and thawing and finally dialysed for 24 h against 0.15M-NaCl.

Analytical methods

Methods for the determination of uronic acid and radioactivity have been described (Lindahl *et al.*, 1976). Hexosamine analyses were performed by a modification (Gardell, 1953) of the Elson-Morgan reaction, after hydrolysis of saccharides in 4M-HCl at 100°C for 14 h. Sulphate was determined by the method of Terho & Hartiala (1971). Anhydromannose was estimated by the indole reaction (Dische & Borenfreund, 1950).

Paper electrophoresis was conducted on Whatman 3 MM paper in 1.6M-formic acid, pH 1.7 (40 V/cm) or in 0.08M-pyridine/0.046M-acetic acid, pH 5.3 (70 V/cm). Unlabelled sugar components were detected by staining with a silver-dip reagent (Smith, 1960). Preparative experiments were conducted with water-washed paper strips.

Paper chromatography was carried out on water-washed Whatman 3 MM paper in ethyl acetate/acetic acid/water (3:1:1, by vol.; solvent A), butan-1-ol/acetic acid/aq. 12.5% NH₃ (2:2:1, by vol.; solvent B), or 95% ethanol/1M-ammonium acetate (4:1, v/v; solvent C).

The ¹H n.m.r. spectra of disaccharides, dissolved in ²H₂O, were recorded at 100.0 MHz and 75°C on a Varian-HA-100D instrument, equipped with a Varian VFT-100 Fourier-transform system.

Smith degradation of disaccharides

Uronosyl-2,5-anhydro[1-³H]mannitol disaccharides (40×10^3 – 50×10^3 c.p.m.; 0.3 μ g or less of uronic acid) were dissolved in 50 μ l of 0.05M-sodium phosphate buffer, pH 7.0, containing 0.02M-NaIO₄ and 0.2M-NaClO₄. The mixtures were left at 37°C in

the dark. After 24 h the remaining periodate was destroyed by the addition of 5 μ l of 10% (w/v) D-mannitol. After another 2 h period the mixtures were transferred *in toto* to Whatman 3 MM papers and subjected to high-voltage electrophoresis at pH 1.7 for 1 h. The deionized radioactive components were eluted from the paper strips, evaporated to dryness and were then reduced by treatment for 3 h at room temperature with a mixture of 50 μ l of 0.5M-NaBH₄ in 0.5M-KOH and 0.55 ml of 0.1M-glycine buffer, pH 7. After destruction of excess borohydride by acidification with 4M-acetic acid, the samples were passed through small columns of Dowex 50 (H⁺), and the effluents were repeatedly evaporated to dryness in the presence of methanol. Smith degradation was finally completed by hydrolysing the oxidized and reduced disaccharides in 20 μ l of 0.025M-H₂SO₄ at 80°C for 60 min. The hydrolysates were directly applied to strips of Whatman 3 MM paper and analysed by high-voltage electrophoresis at pH 1.7 for 1 h.

Enzymic digestion of disaccharides

For digestion with the commercially available liver glucuronidases, a solution of disaccharide (generally less than 10 μ g of uronic acid) in 0.2 ml of 0.05M-sodium acetate buffer, pH 5.0, was mixed with 50 μ l of an enzyme suspension containing 2 mg of enzyme (23 200 units) and 10 mg of bovine serum albumin/ml of the same acetate buffer. After incubation for 10 h at 37°C another 50 μ l portion of enzyme was added, and incubation was allowed to continue for a total period of 20 h. Digestion was interrupted by heating the mixtures at 100°C for 3 min and the products were separated by high-voltage electrophoresis at pH 5.3.

Incubation of disaccharides with fibroblast homogenates was carried out as follows. To a solution of disaccharide (10×10^3 – 15×10^3 c.p.m. of ³H label in the anhydromannitol residue) in 5 μ l of 1.0M-sodium acetate buffer, pH 4.5, containing 0.02% (w/v) NaN₃, was added 30 μ l of fibroblast homogenate, containing 60–180 μ g of protein. Control incubations contained homogenates from normal cells heated for 10 min at 100°C. After incubation for 72 h, with twice-repeated addition of fibroblast homogenate the digests were heated at 100°C for 10 min and centrifuged at 8000g for 10 min. The supernatants were evaporated to dryness, dissolved in 10 μ l of water and analysed by high-voltage electrophoresis at pH 1.7.

Isolation of disaccharides

The principles of the preparation of disaccharides from heparin-like polysaccharides are outlined in Scheme 1. Unlabelled disaccharides, or disaccharides ³H-labelled at C-1 of the anhydromannitol units, were isolated from polysaccharide preparations I and IV, whereas disaccharides labelled in the uronic acid residues were obtained from preparations II and III.

Scheme 1. Flow scheme for preparation of disaccharides from heparin-like polysaccharides

The non-sulphated disaccharides used in the identification studies were obtained by the combined acid hydrolysis/deamination (pH 3.9) procedure; however, such disaccharides may also be liberated on direct deamination of some polysaccharides (see Table 2) at pH 1.5.

Degradation of polysaccharides. All the sulphated disaccharides were isolated after cleavage of the polysaccharides with HNO_2 at pH 1.5 according to the low-temperature procedure of Cifonelli & King (1972), or by the room-temperature modification of Shively & Conrad (1976a); the two procedures give identical results (Shively & Conrad, 1976a). The polysaccharide concentration was 1.5 mg/ml or less.

Non-sulphated disaccharides were obtained by subjecting polysaccharides to a combination of acid hydrolysis and deamination with HNO_2 . The procedure described by Höök *et al.* (1974a) was scaled-up, with omission of the second hydrolytic step. The initial hydrolysis in 2M-trifluoroacetic acid (100°C for 3 h) effects essentially complete desulphation and *N*-deacetylation with limited cleavage of glycosidic

linkages. Deamination of the products (at pH 3.9, leading to attack of glucosamine residues with unsubstituted amino groups (Lindahl *et al.*, 1973; Shively & Conrad, 1976a) affords non-sulphated disaccharides in about 60% yield.

Gel chromatography. Disaccharides were separated from larger oligosaccharides by gel chromatography on columns (1.0 cm or 2.2 cm $\times$ 190 cm) of Sephadex G-25, eluted with 0.2 M-NaCl, and were subsequently desalted by passage through columns of Sephadex G-15 equilibrated with aq. 10% ethanol. In later experiments the Sephadex G-25 columns were eluted with 0.2 M-NH₄HCO₃; the salt was eliminated from recovered fractions by freeze-drying.

Reduction of disaccharides. Reduction with unlabelled borohydride was carried out by the procedure described under 'Smith degradation of disaccharides' (4 mg or less of disaccharide per ml of reaction mixture).

Reduction with NaB³H₄ was carried out as follows. To a solution of 12 mg of disaccharide in 0.5 ml of 0.5 M-sodium borate buffer, pH 7.0, was added 0.2 ml of freshly made NaB³H₄ (25 mCi/ml; 95 mCi/mmol of borohydride). After 30 min at room temperature another 0.2 ml portion of NaB³H₄ was added. When the anhydromannose content (as determined by the indole reaction, which is negative for anhydromannitol) had been decreased to less than 1% of the initial value, the reaction was stopped by the addition (in a fume hood) of 1 g of Dowex 50 (H⁺). When bubbling had ceased the solution was filtered and the resin was washed thoroughly with water. The pH of the combined filtrate and wash was adjusted to 5 with aq. 0.1 M-NH₃, and the solution was evaporated to dryness in the presence of methanol. After purification by preparative paper chromatography in solvent C, the reduced disaccharides had a specific radioactivity of about 150×10^3 c.p.m./ μ g of uronic acid.

Reductions were generally carried out after isolation of the disaccharides by gel chromatography. Recently a simplified procedure was used, involving reduction immediately after the deaminative degradation of polysaccharides. After termination of the deamination reactions the pH was adjusted to 8.5–9.0 by the addition of 1 M-Tris solution, and borohydride, labelled or unlabelled, was added. When reduction was complete, the pH was adjusted, first to about 4 with acetic acid and then to neutrality with NaOH; the reduced di- and/or oligo-saccharides were recovered by gel chromatography on a column of Sephadex G-15, eluted with 0.2 M-NH₄HCO₃.

Fractionation of disaccharides. The methods used to separate disaccharide fractions into non-sulphated, monosulphated and disulphated species varied with the amount of sample. Large samples (more than 10 mg of material) were separated by ion-exchange chromatography as described by Höök *et al.* (1974a), except that DEAE-cellulose (Whatman DE-52) was

used instead of ECTEOLA-cellulose. Smaller samples were fractionated by preparative paper electrophoresis at pH 1.7 (Höök *et al.*, 1974b). Non-sulphated disaccharides were separated from free uronic acids [formed during degradation of polysaccharides by the combined acid hydrolysis/deamination (pH 3.9) procedure] by paper electrophoresis at pH 5.3. Labelled components on paper were located by means of guide-strips (scintillation counting) and were eluted with water.

Further separation of the disaccharides was accomplished by paper chromatography. The non-sulphated disaccharides were separated into two components by paper chromatography in solvent A for 30 h. Fractionation of the monosulphated disaccharides involved the combined use of two solvents, A and B, as outlined in the Results and Discussion section, each run extending over a 4-day period.

Partial desulphation of disaccharide. Disulphated disaccharide was partially desulphated by hydrolysis in 0.5 M-trifluoroacetic acid (1.0 mg of disaccharide/ml or less) at 100°C for 20 min. After evaporation of the hydrolysates to dryness the resulting non-sulphated, monosulphated and disulphated (unchanged) disaccharides were separated as described above.

Results and Discussion

Isolation of disaccharides

The isolation of uronosyl-anhydromannitol disaccharides (Scheme 1) is conventional up to the point where the disaccharides have been recovered in fractions of disulphated, monosulphated and non-sulphated species [see e.g. Höök *et al.* (1974a,b) for similar separations]. Such fractions, as obtained by either ion-exchange chromatography or paper electrophoresis, will be referred to by the letters *a* (di-*O*-sulphated), *b* (mono-*O*-sulphated) and *c* (non-sulphated). Subsequent separations were performed by paper chromatography. Solvent A separated the non-sulphated disaccharides into a faster-migrating (*c-1*) and a slower-migrating (*c-2*) component (Fig. 1). In the same solvent the monosulphated disaccharides were resolved into two peaks. The faster-migrating component (*b-1*) was further separated into two components, *b-1- α* and *b-1- β* , by paper chromatography in solvent B (Fig. 4). The more retarded component in solvent A, *b-2*, practically co-migrated with *b-1- β* in solvent B. Monosulphated disaccharides were also obtained by partial hydrolytic desulphation of disulphated disaccharide, as described in the Experimental section. About 50% of the hydrolysis products were recovered as monosulphated disaccharides; these were separated further by paper chromatography in solvent B into components *b-1- α* (10–20%) and *b-1- β* (80–90%).

Identification of non-sulphated disaccharides

In the biosynthesis of heparin, L-iduronic acid residues are formed by C-5 epimerization of D-glucuronic acid units at the polymer level; the process involves exchange of the C-5 hydrogen with hydrogens of the medium (Lindahl *et al.*, 1976; Jacobsson *et al.*, 1979). By supplementing the appropriate cell-free biosynthetic system with a mixture of UDP-D-[^{14}C]glucuronic acid and UDP-D-[5- ^3H]glucuronic acid, it is possible to obtain a polysaccharide (prepara-

tion III) in which the glucuronic acid residues are both ^{14}C - and ^3H -labelled, whereas the iduronic acid residues are ^{14}C -labelled only. Separation by paper chromatography of the non-sulphated disaccharides derived from preparation III (Fig. 1) showed that the slower-migrating component (*c-2*) was doubly labelled and should therefore contain glucuronic acid, whereas the faster-migrating component (*c-1*) lacked ^3H label, indicating iduronic acid.

These results were corroborated by analysis of the corresponding disaccharides, derived from heparin (preparation II) obtained by incubating mastocytoma slices with [^3H]galactose. The disaccharides were hydrolysed with 2M-trifluoroacetic acid at 100°C for 4h and were then subjected to paper chromatography in solvent A (Fig. 2). Disaccharide *c-1* yielded two major components which migrated like iduronic acid and iduronolactone. Disaccharide *c-2* produced small amounts of glucuronic acid and glucuronolactone; however, most of the label remained at the position of the unhydrolysed disaccharide, suggesting a high degree of resistance towards acid hydrolysis for glucuronosyl-anhydromannitol (but not for iduronosyl-anhydromannitol; see also Foot-

Fig. 1. Separation of the non-sulphated disaccharides *c-1* (^{14}C -labelled only) and *c-2* (doubly labelled) from polysaccharide preparation III by paper chromatography in solvent A

Separation time was 30h. For additional details, see the text. ●, ^{14}C ; ○, ^3H .

Fig. 2. Paper chromatography (solvent A) of [^3H]glucuronosyl-labelled disaccharides (a) *c-2* and (b) *c-1* after acid hydrolysis

The standards indicated below the tracings are: I, disaccharide *c-2* (intact); II, D-glucuronic acid; III, L-iduronic acid; IV, D-glucuronolactone; V, L-iduronolactone. For additional details, see the text.

Fig. 3. Paper electrophoresis at pH 5.3 of [^3H]glycuronosyl-labelled disaccharides *c-1* (●) and *c-2* (○) after digestion with β -D-glucuronidase/ α -L-iduronidase mixture. The standards indicated below the tracings are: I, intact disaccharides (*c-1* or *c-2*); II, D-glucuronic acid and L-iduronic acid. For additional details, see the text.

note 3 in Höök *et al.*, 1974b). Alternatively, digestion with commercial β -D-glucuronidase was employed; since this preparation also contains α -L-iduronidase activity, degradation of both disaccharides could be expected. Disaccharide *c-2* was almost completely cleaved, as shown by the presence of free uronic acid as the only labelled digestion product present in significant amounts (Fig. 3). In contrast, under identical conditions only about one-third of component *c-1* was cleaved. Repeated incubation released another one-third of the label as uronic acid monosaccharide, suggesting that, although disaccharide *c-1* was degraded slowly, it was completely susceptible to the enzyme preparation used. Analysis of the liberated uronic acids by paper chromatography in solvent A showed that the material from component *c-2* was [^3H]glucuronic acid only, whereas that from component *c-1* was [^3H]iduronic acid.

Enzymic degradation, as described above, of either non-sulphated disaccharide reduced with NaB^3H_4 released an uncharged labelled component that migrated like 2,5-anhydro-D- $[\text{H}]$ mannitol on paper chromatography in solvent A. Taken together with the uronic acid identification, these findings indicate that of the two non-sulphated disaccharides separated

by paper chromatography in solvent A the faster-migrating species (*c-1*) is 4-O-(α -L-idopyranosyluronic acid)-2,5-anhydro-D-mannitol, whereas the slower-migrating species (*c-2*) is 4-O-(β -D-glucopyranosyluronic acid)-2,5-anhydro-D-mannitol (Fig. 6).

Identification of monosulphated disaccharides; sugar moieties

Monosulphated disaccharides, isolated from the doubly labelled microsomal polysaccharide (preparation III), showed ^3H in the slower-migrating peak only (Fig. 4a). This material (disaccharide *b-2*) thus contains glucuronic acid. The faster-migrating ^{14}C -, but not ^3H -, labelled peak (*b-1*) representing iduronic acid-containing species was further resolved into two components (*b-1- α* and *b-1- β*) by paper chromatography in solvent B (Fig. 4b).

Uronic acid monosaccharides were prepared from [$1\text{-}^3\text{H}$]glucuronosyl-labelled disaccharides by digestion with a mixture of β -D-glucuronidase and α -L-iduronidase followed by preparative paper electrophoresis, as described in the Experimental section.

Fig. 4. Separation of monosulphated disaccharides from polysaccharide preparation III by paper chromatography. The glucuronic acid-containing component, *b-2* (doubly labelled), is separated from the iduronic acid-containing fraction, *b-1* (^{14}C -labelled only), by paper chromatography in solvent A for 90h (a). The latter fraction is then separated into components *b-1- α* (faster migrating) and *b-1- β* (slower migrating) by a second chromatography, in solvent B for 90h (b). For additional details, including explanation of the radioactive labels, see the text. ●, ^{14}C ; ○, ^3H .

Fig. 5. Paper electrophoresis at pH 1.7 of [$1\text{-}^3\text{H}$]anhydro-mannitol-labelled disaccharides (a) *b-1- β* (●) and *b-2* (○); (b) disaccharide *b-1- α* , after digestion with β -D-glucuronidase/ α -L-iduronidase mixture. The standards indicated below the tracings are: I, intact disaccharides (*b-1- α* , *b-1- β* or *b-2*); II, anhydro-mannitol sulphate. For additional details, see the text.

The uronic acids released from disaccharides *b-2* and *b-1-β* were identified by paper chromatography in solvent A as glucuronic acid and iduronic acid respectively. Disaccharide *b-1-α* was resistant to glucuronidase digestion. Instead, free uronic acid was obtained from this species by acid hydrolysis (2M-trifluoroacetic acid, 100°C, 4h) and was subsequently identified as iduronic acid by paper chromatography. Acid hydrolysis (same conditions) yielded iduronic acid also from disaccharide *b-1-β* whereas disaccharide *b-2* was converted into a non-sulphated disaccharide, presumably glucuronosyl-anhydromannitol (see above and Fig. 2).

Disaccharides *b-1-α*, *b-1-β* and *b-2*, reduced with NaB³H₄, were subjected to acid hydrolysis (2M-trifluoroacetic acid, 100°C, 3h) followed by digestion with a mixture of β-D-glucuronidase and α-L-iduronidase. Analysis of the products by paper electrophoresis at pH 5.3 showed an uncharged labelled component which co-migrated with standard 2,5-

anhydro[³H]mannitol on paper chromatography in solvent A.

Identification of monosulphated disaccharides; location of sulphate groups

(a) *Enzyme digestion.* The 2,5-anhydro[³H]mannitol-labelled disaccharides, *b-1-α*, *b-1-β* and *b-2* were digested with a mixture of β-D-glucuronidase and α-L-iduronidase, and the products were analysed by paper electrophoresis at pH 1.7 (Fig. 5). Whereas disaccharides *b-1-β* and *b-2* both released a labelled component that migrated like 2,5-anhydromannitol 6-sulphate, disaccharide *b-1-α* was resistant towards degradation. The iduronic acid residue would thus be non-sulphated in disaccharide *b-1-β* but sulphated in disaccharide *b-1-α*.

The same disaccharides were digested with homogenates of normal and pathological fibroblasts (Table 1). Digestion of disaccharides *b-1-β* and *b-2*

Table 1. *Digestion of disaccharides with fibroblast homogenates*

Disaccharides, labelled with ³H in the anhydromannitol units, were digested with homogenates of normal cells, α-L-iduronidase-deficient (Hurler) cells, iduronate sulphatase-deficient (Hunter) cells, or β-D-glucuronidase-deficient cells, as described in detail in the Experimental section. The digestion products were separated into non-sulphated components (invariably less than 5% of the total radioactivity), monosulphated disaccharides, monosulphated anhydromannitol, and disulphated disaccharide, by high-voltage paper electrophoresis at pH 1.7. The separations obtained are illustrated by the related experiment shown in Fig. 5; the migration rate of the disulphated disaccharide (not shown in Fig. 5) is about 1.8 times that of the monosulphated disaccharide. For each of the two monosulphated iduronic acid-containing disaccharides (*b-1-α* and *b-1-β*) two sets of values are given; the values to the left refer to the disaccharides formed on reduction of the initial deamination products of polysaccharides, whereas those to the right refer to the products isolated after partial hydrolytic desulphation of the disulphated disaccharide (a). Values corresponding to less than 5% of the total radioactivity are indicated by a dash. Abbreviation: n.d., not determined.

Substrate	Fibroblast homogenate	Products (% of total ³ H radioactivity)		
		Monosulphated disaccharide	Sulphated anhydromannitol	Disulphated disaccharide
<i>a</i>	Boiled homogenate	5	—	89
	Normal cells	—	50	46
	α-L-Iduronidase-deficient	52	6	41
	Iduronate sulphatase-deficient	—	7	88
	β-D-Glucuronidase-deficient	—	58	35
<i>b-1-α*</i>	Boiled homogenate	93; 91	—; —	—; —
	Normal cells	92; 89	—; —	—; —
	α-L-Iduronidase-deficient	94; 91	—; —	—; —
	Iduronate sulphatase-deficient	92; 87	—; —	—; —
<i>b-1-β</i>	Boiled homogenate	98; 73	—; 18	—; —
	Normal cells	n.d.; 6	n.d.; 90	n.d.; —
	α-L-Iduronidase-deficient	82; 78	13; 12	—; —
	Iduronate sulphatase-deficient	7; 4	89; 93	—; —
	β-D-Glucuronidase-deficient	4; n.d.	92; n.d.	—; n.d.
<i>b-2</i>	Boiled homogenate	96	—	—
	Normal cells	19	76	—
	α-L-Iduronidase-deficient	17	79	—
	Iduronate sulphatase-deficient	24	74	—
	β-D-Glucuronidase deficient	84	14	—

* Disaccharide *b-1-α* was also incubated with fibroblast homogenate at pH 3.8; no significant degradation was observed.

with homogenates of normal fibroblasts converted both disaccharides into a labelled component that migrated similarly to 2,5-anhydromannitol 6-sulphate. The same result was obtained on digesting disaccharide *b-2* with α -L-iduronidase-deficient or with iduronate sulphatase-deficient homogenates, but not on digestion with a β -D-glucuronidase-deficient homogenate. Conversely, disaccharide *b-1- β* was degraded by either the iduronate sulphatase-deficient or the β -D-glucuronidase-deficient homogenate, but not by the α -L-iduronidase-deficient homogenate. These results are compatible with the structures assigned to the disaccharides in Fig. 6. Disaccharide *b-1- α* was expected to yield labelled anhydromannitol or non-sulphated disaccharide on digestion with homogenates of normal or α -L-iduronidase-deficient cells respectively. Instead, it was virtually resistant towards degradation by any cell homogenate, under conditions where the disulphated analogue, disaccharide *a*, was largely converted into sulphated anhydromannitol (by a homogenate of normal cells) or into monosulphated disaccharide (by α -L-iduronidase-deficient homogenate). The same

results were obtained irrespective of whether the putative disaccharide substrate was formed as a primary deamination product or by partial hydrolytic desulphation of the disulphated disaccharide *a*. Obviously, these results cannot be taken as evidence for the structure proposed. Should this structure be confirmed by other methods, the action of iduronate sulphatase on iduronosyl-anhydromannitol disaccharides must be highly dependent on the presence of a sulphate group, not only on the iduronosyl moiety but also on the anhydromannitol unit (see also Hopwood, 1979).

(b) *Smith degradation*. Glycol groups are present in the glycuronosyl but not in the anhydromannitol moieties of the disaccharides investigated (see Fig. 6). Smith degradation should therefore release anhydromannitol units as intact monosaccharides, sulphated or non-sulphated, depending on the location of sulphate groups in the parent disaccharide. The degradation products from disaccharides *b-1- β* and *b-2* contained a major component that migrated similarly to anhydromannitol 6-sulphate on high-voltage paper electrophoresis at pH 1.7. This component was essen-

Fig. 6. Structures of disaccharides isolated from deamination products of heparin or heparan sulphate. The notations in parentheses are those used for reference throughout the text.

tially absent from the derivatives of disaccharide *b-1-α*, which, instead, yielded large amounts of non-sulphated labelled material in addition to some unchanged disaccharide. These results support the conclusion that sulphated anhydromannitol occurred in disaccharides *b-1-β* and *b-2* but not in disaccharide *b-1-α*.

(c) *Proton n.m.r.* Additional evidence for the position of sulphate groups in the two monosulphated, iduronic acid-containing disaccharides, *b-1-α* and *b-1-β*, was obtained by proton n.m.r. spectroscopy. The spectra resembled closely those of the corresponding non-reduced disaccharides (Kosakai *et al.*, 1978). In the ^1H n.m.r. spectrum of heparin, signals ascribable to H-1 and H-5 of α -L-idopyranosyluronic acid residues, sulphated at C-2, appear at 5.13 and 4.70 p.p.m. respectively (Perlin *et al.*, 1970). With disaccharide *b-1-α* the corresponding signals appeared at 5.01 and 4.75 p.p.m. respectively. The spectrum of disaccharide *b-1-β* showed a similar signal from H-5 of the α -L-idopyranosyluronic acid residue, at 4.73 p.p.m., but a significant upfield shift of the H-1 signal, to 4.72 p.p.m. An upfield shift of the same magnitude, about 0.3 p.p.m., was noted for the signal from H-1 of the non-sulphated α -L-idopyranosyluronic acid residue in the disaccharide *c-1*. Relating the H-1 signals of the two monosulphated disaccharides to those of the 2-*O*-sulphated iduronic acid unit in heparin and the non-sulphated iduronic acid unit in the disaccharide *c-1*, thus clearly suggests that the iduronic acid residue in disaccharide *b-1-α* is sulphated, at C-2, whereas that in disaccharide *b-1-β* is non-sulphated. Both disaccharides showed coupling constants for the H-1 and H-2 signals of about 2 Hz, consistent with the α -L-*ido* configuration

and $^1\text{C}_4$ conformation for the uronic acid residues (Perlin, 1977).

The third monosulphated disaccharide, *b-2*, produced signals ascribable to H-1 and H-5 of the uronic acid residue, at 4.82 and 4.52 p.p.m. The coupling constants for H-1 and H-2, 9.0 and 8.5 Hz respectively, are in accordance with the β -D-*gluco* configuration and $^4\text{C}_1$ conformation for the uronic acid residue. No significant difference in chemical shift was observed between the uronosyl H-1 and H-5 signals for disaccharide *b-2* and the corresponding signals for the sulphate-free analogue *c-2*. This finding, and the relatively upfield location of the H-1 signal [a chemical shift to about 5.1 p.p.m. would be expected, had the uronic acid residue been sulphated at C-2 (Pasika & Cragg, 1963)], both corroborate the evidence of other experiments (see above), showing that the sulphate group of disaccharide *b-2* is located on the anhydromannitol and not on the uronic acid moiety.

The ^1H n.m.r. spectra together with the other results of the present investigation permit an assignment of structures to the three mono-*O*-sulphated disaccharides (Fig. 6). These are 4-*O*-(α -L-idopyranosyluronic acid 2-sulphate)-2,5-anhydro-D-mannitol for disaccharide *b-1-α*, 4-*O*-(α -L-idopyranosyluronic acid)-2,5-anhydro-D-mannitol 6-sulphate for disaccharide *b-1-β* and 4-*O*-(β -D-glucopyranosyluronic acid)-2,5-anhydro-D-mannitol 6-sulphate for disaccharide *b-2*.

Composition of N-sulphated disaccharide units in polysaccharide preparations

Attempts were made to determine the disaccharide composition of heparin and heparan sulphate

Table 2. *Analysis of disaccharides in deamination products of heparin-like polysaccharides*

Polysaccharides were deaminated at pH 1.5; the resulting glycuronosyl-anhydromannose disaccharides were isolated by gel chromatography on Sephadex G-25, reduced with NaB^3H_4 [except the products of preparation II (labelled with ^3H]galactose during biosynthesis) that were reduced with NaBH_4] and fractionated by paper electrophoresis at pH 1.7. Finally, the non- and mono-sulphated disaccharides were separated by paper chromatography in solvents A and B. Preparation I was subjected to the modified (and more simple) procedure in which the reduction with NaB^3H_4 is carried out directly after the deamination reaction. For additional experimental details and description of the polysaccharide preparations, see the Experimental section.

Polysaccharide preparation	Yield of disaccharides* (% of total polysaccharide)	Distribution of disaccharides (% of total disaccharides)					
		<i>a</i>	<i>b-1-α</i>	<i>b-1-β</i>	<i>b-2</i>	<i>c-1</i>	<i>c-2</i>
I	62	74	10	6	11	<1	<1
II	50	51	12	18	18	<1	<1
IV	24	42	35	6	12	<1	5.2
V	12	13	68	5.4	8.2	1.9	3.9

* Values calculated from the elution patterns obtained on gel chromatography (Sephadex G-25) of deamination products. These values may underestimate the proportion of *N*-sulphated disaccharide units in the intact polysaccharides, because of (a) the deaminative ring-contraction reaction that converts *N*-sulphated D-glucosamine residues to 2-*aldehydo*-D-pentofuranoside units without cleavage of glycosidic linkages (Shively & Conrad, 1976a,b), and (b) the presence in the polysaccharides of -GlcNAc-uronic acid-GlcNSO₃⁻ sequences, in which the *N*-acetylglucosaminidic linkage is resistant towards deaminative cleavage.

preparations, by using the six identified glycuronosyl- $^{[3]}\text{H}$ anhydromannitol disaccharides as reference standards in the analysis of deamination products. The yield of disaccharides obtained on deamination of heparin and heparan sulphate preparations varied from about 10 to 60% of the total materials (Table 2; however, see note regarding anomalously low values). This variation reflects the difference in *N*-sulphate contents. Apparently, a decrease in *N*-sulphate is associated with a relative increase in mono-*O*-sulphated (versus di-*O*-sulphated) disaccharide units; in the two heparan sulphate preparations the di-*O*-sulphated disaccharide units thus account for less than half of the total *N*-sulphated disaccharide units. The heparan sulphates also differ from the heparins by having relatively large proportions of *N*-sulphated disaccharide units that contain *O*-sulphated iduronic acid residues but lack *O*-sulphate on the glucosamine moiety (see also Hovingh & Linker, 1974; Cifonelli & King, 1977). Interestingly, the same disaccharide units appear to prevail in so-called ω -heparin from whale tissues (Kosakai *et al.*, 1978).

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References

- Cantz, M., Kresse, H., Barton, R. W. & Neufeld, E. F. (1972) *Methods Enzymol.* **28**, 884–897
- Cifonelli, J. A. & King, J. A. (1972) *Carbohydr. Res.* **21**, 173–186
- Cifonelli, J. A. & King, J. A. (1977) *Biochemistry* **16**, 2137–2141
- Dische, Z. & Borenfreund, E. (1950) *J. Biol. Chem.* **184**, 517–522
- Eagle, H. (1959) *Science* **130**, 432–437
- Gardell, S. (1953) *Acta Chem. Scand.* **7**, 207–215
- 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
- Höök, M., Lindahl, U., Hallén, A. & Bäckström, G. (1975) *J. Biol. Chem.* **250**, 6065–6071
- Hopwood, J. J. (1979) *Carbohydr. Res.* in the press
- Hopwood, J. J., Höök, M., Linker, A. & Lindahl, U. (1976) *FEBS Lett.* **69**, 51–54
- Horton, D. & Philips, K. (1976) *Methods Carbohydr. Chem.* **7**, 68–70
- Hovingh, P. & Linker, A. (1974) *Carbohydr. Res.* **37**, 181–192
- Jacobsson, I., Bäckström, G., Höök, M., Lindahl, U., Feingold, D. S., Malmström, A. & Rodén, L. (1979) *J. Biol. Chem.* in the press
- Kosakai, M., Yamauchi, F. & Yosizawa, Z. (1978) *J. Biochem. (Tokyo)* **83**, 1567–1575
- Lindahl, U. & Axelsson, O. (1971) *J. Biol. Chem.* **246**, 74–82
- Lindahl, U., Bäckström, G., Jansson, L. & Hallén, A. (1973) *J. Biol. Chem.* **248**, 7234–7241
- Lindahl, U., Cifonelli, J. A., Lindahl, B. & Rodén, L. (1965) *J. Biol. Chem.* **240**, 2817–2820
- Lindahl, U. & Höök, M. (1978) *Annu. Rev. Biochem.* **47**, 385–417
- Lindahl, U., Jacobsson, I., Höök, M., Bäckström, G. & Feingold, D. (1976) *Biochem. Biophys. Res. Commun.* **70**, 492–499
- Matalon, R., Cifonelli, J. A. & Dorfman, A. (1971) *Biochem. Biophys. Res. Commun.* **42**, 340–345
- Pasika, W. W. & Cragg, L. H. (1963) *Can. J. Chem.* **41**, 777–782
- Perlin, A. S. (1977) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **36**, 106–109
- Perlin, A. S., Casu, B. & Sanderson, G. R. (1970) *Can. J. Chem.* **48**, 2260–2268
- Rosenberg, R. D., Armand, G. & Lam, L. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 3065–3069
- Shively, J. E. & Conrad, H. E. (1976a) *Biochemistry* **15**, 3932–3942
- Shively, J. E. & Conrad, H. E. (1976b) *Biochemistry* **15**, 3943–3950
- Smith, I. (1960) in *Chromatographic and Electrophoretic Techniques* (Smith, I., ed.), vol. 1, pp. 246–260, Interscience, New York
- Terho, T. T. & Hartiala, K. (1971) *Anal. Biochem.* **41**, 471–476
- Whistler, R. L., Spencer, W. W. & BeMiller, J. N. (1963) *Methods Carbohydr. Chem.* **2**, 301–302