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α-L-IDURONIDASE, β-D-GLUCURONIDASE, AND 2-SULFO-L-IDURONATE 2-SULFATASE: PREPARATION AND CHARACTERIZATION OF RADIOACTIVE SUBSTRATES FROM HEPARIN

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ABSTRACT

Radioactive disaccharide substrates for α-L-iduronidase, β-D-glucuronidase, and 2-sulfo-L-iduronate 2-sulfatase have been prepared from heparin by deaminative cleavage followed by reduction with $NaBT_4$. Six disaccharides were isolated from this reaction mixture and identified. Acid hydrolysis of the major disaccharide, *O*-(α-L-idopyranosyluronic acid 2-sulfate)-(1→4)-(2,5-anhydro-D-mannitol-*1-t* 6-sulfate (IdAs–Ms), produced 48% of *O*-(α-L-idopyranosyluronic acid)-(1→4)-(2,5-anhydro-D-mannitol-*1-t* 6-sulfate) (IdA–Ms) and 25% of *O*-(α-L-idopyranosyluronic acid)-(1→4)-2,5-anhydro-D-mannitol-*1-t*. The most-sensitive substrate for determining α-L-iduronidase activity was IdA–Ms which, when incubated with leucocyte and skin-fibroblast homogenates prepared from patients having a deficiency of α-L-iduronidase (Mucopolysaccharidosis Type I; MPS-I), was hydrolysed to yield 2,5-anhydro-D-mannitol-*1-t* 6-sulfate at a rate 50-times less than that found for normal control-preparations. Similarly, *O*-(β-D-glucopyranosyluronic acid)-(1→4)-(2,5-anhydro-D-mannitol-*1-t* 6-sulfate) was degraded by whole-cell homogenates prepared from β-D-glucuronidase-deficient (Mucopolysaccharidosis, Type VII) fibroblasts, to yield 2,5-anhydro-D-mannitol-*1-t* 6-sulfate at a rate 60-times less than that found for MPS-I and normal control-preparations. IdAs–Ms was degraded by 2-sulfo-L-iduronate 2-sulfatase at a rate more than 45-times greater than that found for *O*-(α-L-idopyranosyluronic acid 2-sulfate)-(1→4)-2,5-anhydro-D-mannitol-*1-t*. *C*-6 Sulfation of the anhydro-D-mannitol-*1-t* residue is an important structural determinant in the mechanism of action of both α-L-iduronidase and 2-sulfo-L-iduronate 2-sulfatase on disaccharide substrates.

INTRODUCTION

Heparan sulfate is an *N*-sulfated glycosaminoglycan which is synthesised by many, if not all, mammalian cell lines[1–4] and appears to be associated with the cell surface of all cultured mammalian cells[5–7]. Several genetic disorders of glycosaminoglycan catabolism are described where specific exoenzymes involved in the degrada-

tion of heparan sulfate have grossly reduced activities, leading to the accumulation of partially degraded heparan sulfate and the expression of clinical defects[8-10]. Heparan sulfate appears to be degraded by the concerted action of possibly two endoglycosidases[11,12], four exoglycosidases (α-L-iduronidase, β-D-glucuronidase, 2-amino-2-deoxy-α-D-glucosidase, and 2-acetamido-2-deoxy-α-D-glucosidase), and at least three sulfatases (2-sulfo-L-iduronate 2-sulfatase, sulfamidase, and 2-amino-2-deoxy-D-glucose 6-sulfate sulfatase)[8-10]. Experience with the determination of the activities of mutant enzymes produced in genetic disorders has underlined the need to use substrates that closely match the structures of the presumed, natural substrates[13,14]. In this laboratory, advantage has been taken of the fact that sulfoamino groups in heparin (a polysaccharide having a structure similar to that of heparan sulfate) react with nitrous acid to produce oligosaccharide (mostly disaccharide) fragments which, after reduction with sodium borotritide, yield useful substrates for enzymes thought to be involved in the degradation of heparan sulfate[15-17].

Methods for the preparation of *O*-(β-D-glucopyranosyluronic acid)-(1→4)-2,5-anhydro-D-mannitol-*1-t* (GlcA–M), *O*-(β-D-glucopyranosyluronic acid)-(1→4)-(2,5-anhydro-D-mannitol-*1-t* 6-sulfate) (GlcA–Ms), *O*-(α-L-idopyranosyluronic acid)-(1→4)-2,5-anhydro-D-mannitol-*1-t* (IdA–M), *O*-(α-L-idopyranosyluronic acid 2-sulfate)-(1→4)-2,5-anhydro-D-mannitol-*1-t* (IdAs–M), *O*-(α-L-idopyranosyluronic acid)-(1→4)-(2,5-anhydro-D-mannitol-*1-t* 6-sulfate) (IdA–Ms), *O*-(α-L-idopyranosyluronic acid 2-sulfate)-(1→4)-(2,5-anhydro-D-mannitol-*1-t* 6-sulfate) (IdAs–Ms) are described, and conditions for their enzymic degradation by homogenates of cultured human-skin fibroblasts are demonstrated. A preliminary communication reporting some of the results for α-L-iduronidase has been presented[36].

EXPERIMENTAL

Materials. — Heparin (Grade II, prepared from pig-intestinal mucosa Sigma Chemical Co.) was purified[18] by repeated precipitation with cetylpyridinium chloride from 1.2M NaCl. $NaBT_4$ (10 Ci/mmol) was obtained from The Radiochemical Centre (Amersham, Bucks, U.K.). Sephadex G-10 and G-25 (superfine) were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden) and Bio-Gel P-4, Dowex-50W (X4, 100–200 mesh), and Dowex-1 (X4, 100–200 mesh) resins from Bio-Rad Laboratories (Richmond, CA, U.S.A.).

Fibroblasts [(2-sulfo-L-iduronate 2-sulfatase)-deficient, GM-615–Mucopolysaccharidosis Type II (MPS-II), Hunter; α-L-iduronidase-deficient, GM-415–MPS-IH, Hurler and GM-1323–MPS-IS, Scheie; β-D-glucuronidase-deficient, GM-121–MPS-VII] were obtained from the Human Genetic Mutant Cell Repository (Institute for Medical Research, Camden, N.J., U.S.A.). Similar enzyme-deficient lines and normal fibroblasts were established from skin biopsies taken in this hospital[19]. All lines were maintained in culture as described by Carey and Pollard[19], and used for the preparation of cell homogenates 6 to 10 days after subculture.

General methods. — High-voltage electrophoresis (h.v.e.) was performed on

Whatman 3MM chromatography paper in 1.74M formic acid (pH 1.7) at 45 V/cm for 1 h, or in 0.05M sodium acetate (pH 5.1) at 45 V/cm for 50 min, using a Camag System. Paper chromatography (p.c.) was performed on Whatman 3MM paper with descending development in the following solvents: *1*, ethyl acetate–acetic acid–water (3:1:1, v/v); *2*, M aqueous ammonium acetate–ethanol (3.5:6.5); *3*, 1-butanol–acetic acid–7.4M aqueous ammonia (2:2:1); and *4*, isobutyric acid–2M aqueous ammonia (15:9).

Gel chromatography was conducted by using glass columns filled with Sephadex G-25 or G-10 equilibrated with 10% aqueous ethanol or 0.2M NaCl in 0.05M Tris HCl buffer (pH 7.6), as described in detail in the text or legends to Figures.

Uronic acid was determined by the carbazole method of Bitter and Muir[20], using an equimolar mixture of D-glucurono-6,3-lactone and 2,5-anhydro-D-mannitol as the standard. Protein was determined by the Folin method of Lowry *et al.*[35]. Anhydromannose was determined by the indole method described by Dische and Borenfreund[21]. The relative amounts of glucuronic acid and iduronic acid in 2M trifluoroacetic acid hydrolysates of the disaccharide fractions were determined by g.l.c. as described by Höök *et al.*[22]. Sulfate was determined by the method of Terho and Hartiala[23]. Oxidation with periodate was performed at pH 4.5 and 4° for 40 h in the dark[24].

Trace amounts of reducing substances in Tris solutions used in $NaBT_4$ reductions were removed as follows: Tris (12.1 g), $NaBH_4$ (100 mg), and water (100 ml) were mixed at 25° for 2 h, and then applied to a column (2 × 7 cm) of Dowex-1 (Cl^- form); the first 20 ml of eluent was discarded, and the next 60 ml of eluent used to buffer reductions with $NaBT_4$.

Radioactivity was measured with a Model 6868 ISOCAP/300 Ambient Temperature Liquid Scintillation Counter from Searle Analytic Inc. (Des Plaines, Ill., U.S.A.). For counting, aqueous samples (1.0 ml) were mixed with 2 ml of PCS Solubilizer (Amersham/Searle, Arlington Heights, Ill., U.S.A.). Under these conditions, tritium was determined at 30% efficiency.

Borotritide reduction of the nitrous acid deamination products of heparin. — Heparin (10 mg) in water (1 ml) was treated with a solution (10 ml) of nitrous acid in 0.25M H_2SO_4 at pH 1.5, prepared at −5° as described by Shively and Conrad[26]. After 10 min at 25°, the reaction mixture was neutralized with 2M Na_2CO_3, rotary-evaporated to ~3 ml, applied to a column (2.7 × 50 cm) of Bio-Gel P-4, and eluted with 10% aqueous ethanol. The desalted, uronic acid-containing material (96% recovery) was freeze-dried, and incubated for 3 days at 4° with $NaBT_4$ (100 mCi) and $NaBH_4$ (2 mg) in 5 ml of a Tris solution (prepared as described under General methods). Approximately 8% of the anhydromannose residues remained unreduced after this period. More $NaBH_4$ (3 mg) was added to the reaction mixture, which was incubated for a further day at 4° and then mixed with 30 g of Dowex-50 (H^+) resin in water (80 ml) for 1 h in a fume hood. Excess of borate was removed from the Dowex-free filtrate by three cycles of addition of 100 ml of methanol and rotary evaporation to dryness at less than 40°. The residue was dissolved in 2 ml of 0.2M

NaCl in 0.05M Tris–HCl buffer (pH 7.6), and chromatographed as described in the legend to Fig. 1.

Preparation of leucocytes and skin fibroblasts for enzyme assay. — Peripheral blood leucocytes were prepared from 10 ml of blood by sedimentation in 3% (w/v) aqueous dextran; the final wash[25] was with 0.15M NaCl. Skin fibroblasts, cultured in 25-cm^2 Falcon Flasks (Bio Quest, Cockeysville, MD, U.S.A.) to obtain approximately 5×10^5 cells at confluency were harvested by the trypsin/EDTA procedure 1 day after confluency and washed with 0.15M NaCl (3×10 ml) under the conditions described by Carey and Pollard[19]. Leucocytes and fibroblasts were suspended in 0.1% (v/v) aqueous Triton X-100 (0.5 ml), and disrupted by freezing (solid CO_2–ethanol) and thawing six times.

Enzyme-assay procedures. — Each of the disaccharide fractions isolated from the nitrous acid deamination products of heparin was desalted on Sephadex G-10 by elution with 10% (v/v) aqueous ethanol, evaporated to dryness and, based on radioactivity measurement, diluted to 400 to 500 μmol/l with water. The specific radioactivity of these disaccharide fractions was ~500 μCi/μmol of uronic acid.

Assays for α-L-iduronidase and β-D-glucuronidase were performed by incubating ~20 μg of leucocyte-homogenate protein for 2 h and 5 μg of fibroblast-homogenate protein for 1 h at 37° in 0.05M sodium formate buffer (pH 3.6) containing 460 pmol of tritiated disaccharide substrate in a final volume of 12 μl. These conditions yield the maximum rate of disaccharide-substrate degradation by α-L-iduronidase (J. J. Hopwood and V. Muller, unpublished observations). After incubation, the whole reaction mixture was subjected to h.v.e. at pH 1.7 or 5.1.

2-Sulfo-L-iduronate 2-sulfatase activity was determined by the method of Lim *et al.*[16] with some modifications. Both leucocyte and fibroblast homogenates (0.2-ml aliquots) were dialysed for 16 h at 4° in 2 litres of 0.15M NaCl. Dialysed leucocyte-homogenate protein (~40 μg) and dialysed fibroblast-homogenate protein (~30 μg) were incubated for 6 h at 37° in 0.1M sodium acetate buffer (pH 4.0) containing 0.2 μmol of NaN_3 and 1578 pmol of tritiated disaccharide substrate in a final volume of 36 μl. After incubation, the whole reaction mixture was subjected to h.v.e. at pH 1.7 or 5.1.

RESULTS AND DISCUSSION

Isolation of radiolabelled disaccharide fragments from the deamination products of heparin. — Selection of the Shively and Conrad[26] conditions for the nitrous acid deamination of heparin was based on the need for a maximum yield of disaccharides with a minimum contamination from products of side reactions. In this deamination reaction, *N*-sulfated glucosamine (2-amino-2-deoxy-D-glucose) residues were converted into 2,5-anhydromannose residues with concomitant cleavage of the corresponding glucosaminidic linkage. The anhydromannose residues were tritium-labelled by reduction with $NaBT_4$, and the labelled deamination products were fractionated by gel filtration on a column of Sephadex G-25 into three, major, uronic

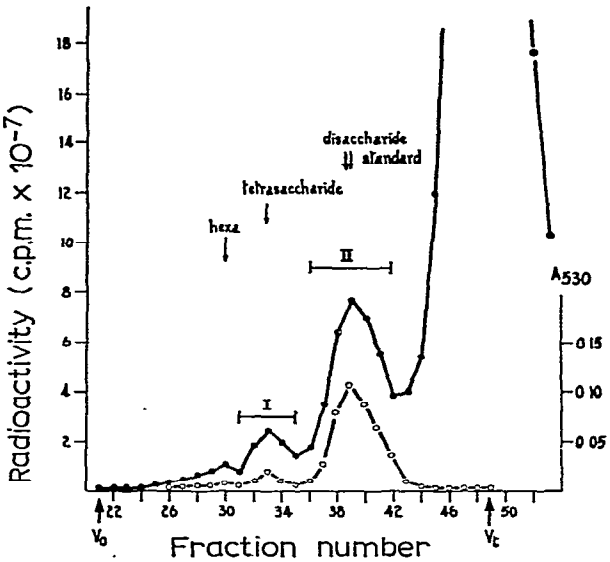

Fig. 1. *Gel chromatography of degradation products derived from heparin after treatment with nitrous acid and NaBT*$_4$. The reaction mixture (desalted on Bio-Gel P-4) was applied to a Sephadex G-25 column (1 × 200 cm), and eluted with 0.2M NaCl in 0.05M Tris HCl buffer (pH 7.6) at 7–8 ml/h. Fractions (3 ml) were analysed for radioactivity (●—●) and uronic acid-containing material (○—○; carbazole reaction, absorbance at 530 nm), and combined as indicated (I—I). The double arrow indicates the peak elution position of the sulfated disaccharide standard [2-acetamido-2-deoxy-3-*O*-(β-L-*threo*-hex-4-enopyranosyluronic acid)-6-*O*-sulfo-D-galactose; Miles Laboratories, Inc., Elkhart, Indiana, U.S.A.], which was quantitated by its absorbance[34] at 232 nm.

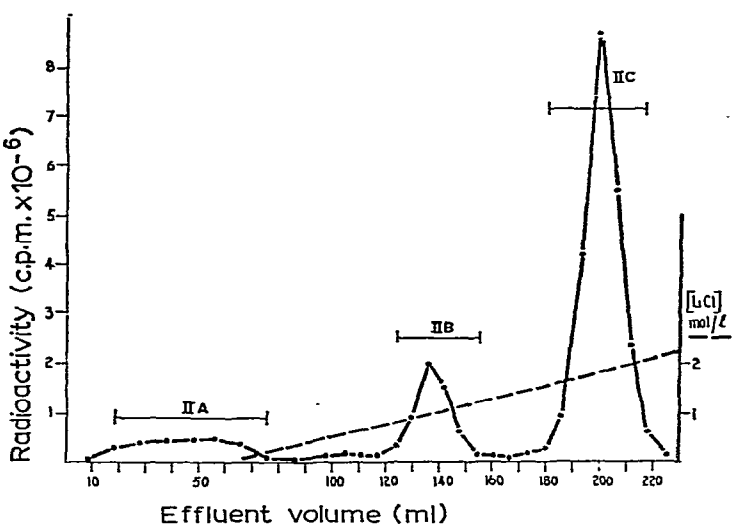

Fig. 2. *Ion-exchange chromatography on Dowex-1 of Fraction II from Fig. 1.* Fraction II was applied to a Dowex-1 (Cl^- form) column (2 × 7 cm), washed with 50 ml of 0.05M LiCl in 0.05M Tris HCl buffer (pH 7.6), and eluted at a rate of 37 ml per h at 25° with a linear gradient generated from 0.05M LiCl in 0.05M Tris HCl buffer (pH 7.6) and 3.0M LiCl in 0.05M Tris HCl buffer (pH 7.6) by using a LKB 11300 Ultrograd gradient mixer. Fractions of ~7 ml were collected, assayed for radioactivity (●—●), and combined as indicated (I—I). The combined fractions were desalted by passage through a Sephadex G-10 column (1 × 100 cm) eluted with 10% (v/v) aqueous ethanol.

TABLE I

ANALYSIS OF HEPARIN AND ITS BOROTRITIDE-REDUCED DEAMINATION PRODUCTS

Heparin reaction	*Yield (μg of uronic acid)*	*Iduronic acid/ uronic acid (molar ratio)*	*Sulfate/uronic acid (molar ratio)*	*[t]/uronic acid (d.p.m. × 10^{-6}/μg)*
Heparin	3300	0.76	2.45	—
I	600	0.68	1.32	3.98
II	2400	0.87	1.75	5.96
IIA	118	0.05	<0.03	4.70
IIAa	108	<0.02	[a]	4.47
IIAb	6	[a]	[a]	4.61
IIB	347	0.48	0.92	6.00
IIBa	17	[a]	[a]	5.81
IIBb	182	<0.02	0.90	5.77
IIBc	146	>0.98	0.87	5.81
IIBc1	37	[a]	[a]	6.10
IIBc2	73	[a]	[a]	5.53
IIC	1840	>0.98	1.93	6.04

[a]Insufficient material for analysis.

acid-containing fractions (Fig. 1). The largest and most-retarded peak of uronic acid-containing material had the same elution position as the disaccharide standard. Assuming that two consecutive peaks differ by a disaccharide unit[22], the less-retarded peaks would represent tetra- and hexa-saccharides, respectively, as indicated in Fig. 1. The eluate comprising fraction II was subjected to ion-exchange chromatography on Dowex-1 (Cl^- form), to give three radioactive and uronic acid-positive sub-fractions (IIA–C; Fig. 2). The recovery of uronic acid-containing material was >95% of that applied to the column.

Characterization of Fractions II and IIA–C. — Analytical data for these fractions are shown in Table I. Chromatography of IIA on paper (Solvent *1*) separated two distinct radioactive fractions (IIAa and IIAb), representing 95% and 5%, respectively, of the total radioactivity. Both IIAa and IIAb remained at the origin after h.v.e. at pH 1.7, but moved with the same mobility towards the anode after h.v.e. at pH 5.1. On the basis of these results and from the expected deamination products of heparin[22,26–29], IIAa and IIAb were assumed to be GlcA–AnMannitol-*t* (GlcA–M) and IdA–AnMannitol-*t* (IdA–M), respectively.

Fraction IIB migrated during h.v.e. at pH 1.7 and 5.1 as a single radioactive component having a mobility similar to that reported for monosulfated disaccharides derived from heparin[16,22,30], and gave (Fig. 3) three radioactive sub-fractions (IIBa, IIBb, and IIBc) following paper chromatography in Solvent *1*. Due to lack of material, further investigation of IIBa was not undertaken. The uronic acid component of IIBb and IIBc, assumed to be glucuronic and iduronic acid, respectively (Table I), was, as expected, destroyed by periodate treatment. In paper chromatography

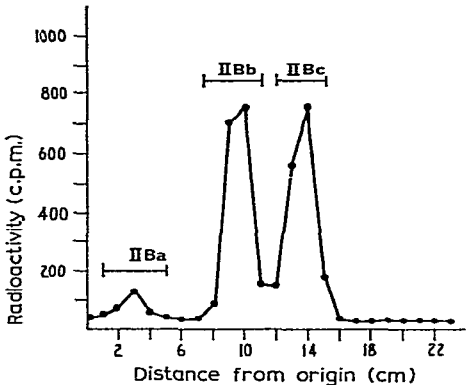

Fig. 3. *Paper chromatography (Solvent 1) of desalted Fraction IIB from Fig. 2.* For preparative chromatography, the paper was prewashed with Solvent *1*; each 1-cm segment was eluted with water, and the eluates were analysed for radioactivity (●—●), and combined as indicated (I—I).

(Solvent *3*), Fraction IIBb migrated as a single radioactive component and, on the basis of these results, was assumed to be GlcA–Ms. However, fraction IIBc gave two radioactive fractions (IIBc1 and IIBc2) following chromatography in Solvent *3* (Table I). The slower fraction IIBc1, co-chromatographed on paper in Solvent *3* with IdA–Ms (fraction IIC2Va, Fig. 8) and was degraded by cell homogenates from normal and (2-sulfo-L-iduronate 2-sulfatase)-deficient fibroblasts, to produce labelled material that migrated as a single component at the same rate as 2,5-anhydro-D-mannitol-*1-t* 6-sulfate when subjected to h.v.e. at pH 1.7. However, IIBc1 was not degraded by cell homogenates from α-L-iduronidase-deficient fibroblasts. These results suggest that the radiolabelled material in fraction IIBc1 is IdA–Ms. Fraction IIBc2 was degraded by both α-L-iduronidase-deficient and normal fibroblasts, to give labelled product(s) that remained at the origin when subjected to h.v.e. in acid at pH 1.7. More than 90% of this labelled material that failed to migrate at pH 1.7 moved during h.v.e. at pH 5.1 as a single peak of radioactivity at the same rate as IdA–M. Cell homogenates from (2-sulfo-L-iduronate 2-sulfatase)-deficient fibroblasts failed to degrade fraction IIBc2. These results suggest that the labelled material in fraction IIBc2 is IdAs–M.

Fraction IIC chromatographed as a single component in Solvents *1*, *2*, and *3*, and moved during h.v.e. at pH 1.7 and pH 5.1 at a rate similar to that reported for disulfated disaccharides[16,22,30]. These results and the uronic acid and sulfate data reported in Table I, plus the finding that the iduronic acid residue was destroyed by periodate treatment, strongly suggest that most of this major component isolated from the deamination degradation-products of heparin is IdAs–Ms.

On the basis of the foregoing results and from the expected deamination products of heparin[22,26–29,31,32], structures proposed for the labelled material in all six disaccharides isolated from fraction II are summarized in Table II.

Acid hydrolysis of fraction IIC. — The yield of IdAs–Ms (fraction IIC) from the deamination products of heparin was 56% (Table I). In contrast, the yield of

TABLE II

PROPOSED STRUCTURES FOR DISACCHARIDE FRACTIONS ISOLATED FROM BOROTRITIDE-REDUCED DEAMINATION PRODUCTS OF HEPARIN

Fraction[a]	*Proposed structure*	*Abbreviation*
IIAa	*O*-(β-D-glucopyranosyluronic acid)-(1→4)-2,5-anhydro-D-mannitol-*1-t*	GlcA–M
IIAb (IICb)	*O*-(α-L-idopyranosyluronic acid)-(1→4)-2,5-anhydro-D-mannitol-*1-t*	IdA–M
IIBb	*O*-(β-D-glucopyranosyluronic acid)-(1→4)-(2,5-anhydro-D-mannitol-*1-t* 6-sulfate)	GlcA–Ms
IIBc1 (IICc2Va)	*O*-(α-L-idopyranosyluronic acid)-(1→4)-(2,5-anhydro-D-mannitol-*1-t* 6-sulfate)	IdA–Ms
IIBc2	*O*-(α-L-idopyranosyluronic acid 2-sulfate)-(1→4)-2,5-anhydro-D-mannitol-*1-t*	IdAs–M
IIC (IICd)	*O*-(α-L-idopyranosyluronic acid 2-sulfate)-(1→4)-2,5-anhydro-D-mannitol-*1-t* 6-sulfate)	IdAs–Ms

[a]Fraction numbers refer to combined fractions in Fig. 2; numbers in parenthesis refer to combined fractions in Figs. 6 and 8.

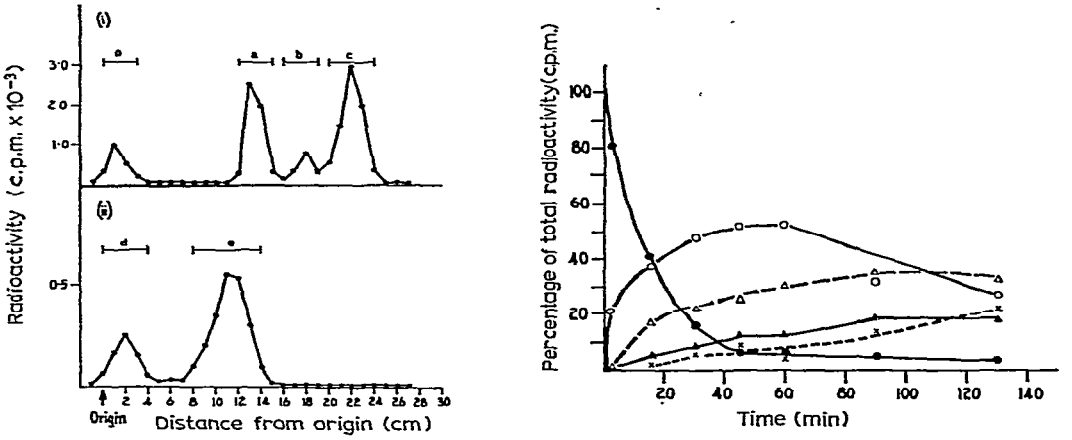

Fig. 4. (*i*) H.v.e. (at pH 1.7) of Fraction IIC after acid hydrolysis in 0.1M HCl at 100° for 30 min. The peaks were identified (see Results and Discussion) as IdA–Ms (a), 2,5-anhydro-D-mannitol-*1-t* 6-sulfate (b), and IdAs–Ms (c). (*ii*) Radioactive material recovered at the origin (o) after h.v.e. at pH 1.7 [(*i*)] was subjected to further h.v.e. at pH 5.1: d, 2,5-anhydro-D-mannitol-*1-t*, and IdA–M, respectively.

Fig. 5. *Progress of the acid hydrolysis of Fraction IIC.* Sealed capillary tubes containing IIC (50,000 d.p.m.) in 0.5 ml of 0.1M HCl were heated at 100°. At the times indicated, the acid in one of the tubes was neutralised with M KOH, and the hydrolysate was subjected to h.v.e. at pH 1.5 and 5.1, as described in Fig. 4 and in the Experimental section; ●—● shows the proportion of counts remaining as the starting-material. The other symbols show the distribution of counts in the identified products of the hydrolysis: IdA–Ms (○—○), IdA–M (△---△), 2,5-anhydro-D-mannitol-*1-t* 6-sulphate (▲—▲), and 2,5-anhydro-D-mannitol-*1-t* (×---×).

IdA–M (fraction IIAb) and IdA–Ms (fraction IIBc1) was 0.2% and 1.1%, respectively (Table I). From an investigation of the kinetics of the acid hydrolysis of fraction IIC (IdAs–Ms), it was anticipated that conditions for the optimum production of the potentially useful α-L-iduronidase substrates IdA–M and IdA–Ms would be found. When fraction IIC was hydrolysed, it was converted into the series of products shown in Fig. 4; the kinetics of hydrolysis of fraction IIC are shown in Fig. 5. The initial, radiolabelled products of hydrolysis are monosulfated IdA–M disaccharide and a small proportion of 2,5-anhydro-D-mannitol-*1-t* 6-sulfate. These products were further hydrolysed to produce IdA–M and 2,5-anhydro-D-mannitol-*1-t*. IdAs–M (equivalent to fraction IIBc2) was not detected in these hydrolysates, indicating the extreme lability of the sulfate residue on the C-2 position of iduronic acid compared to the 6-sulfate on anhydromannitol.

Ion-exchange chromatography on Dowex-1 of acid hydrolysates of fraction IIC gave four peaks of radiolabelled material (Fig. 6). Analytical data for these fractions are shown in Table III. The radioactive material in fraction IICa was identified as 2,5-anhydro-D-mannitol-*1-t* from its failure to migrate during h.v.e. at pH 5.1 and from its mobility in p.c. (Solvent *1*) compared to a sample prepared by deamination of 2-amino-2-deoxy-D-glucose[33] followed by reduction with $NaBT_4$. All of the radiolabelled material in IICb was identified as IdA–M from its mobilities in h.v.e. at pH 1.7 and 5.1 and in p.c. (Solvent *1*) compared to fraction IIAb, and from the loss of uronic acid-positive material on treatment with periodate. The third peak (IICc) in Fig. 6 was not symmetrical; the material in the first two tubes was

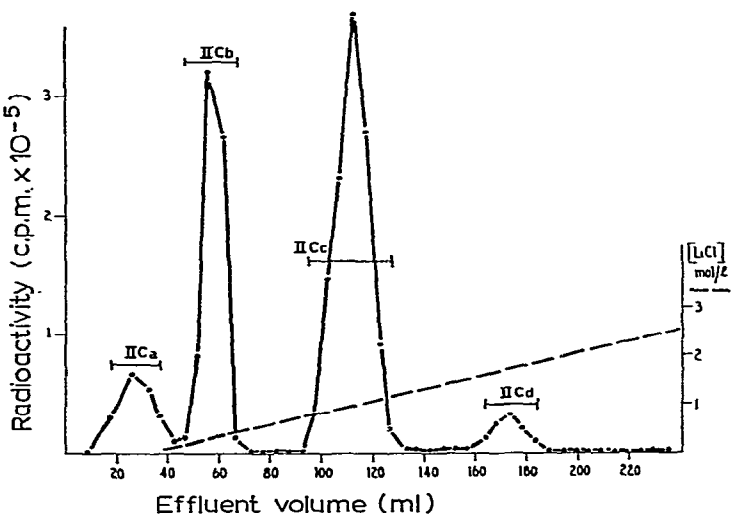

Fig. 6. *Ion-exchange chromatography on Dowex-1 of the products of acid hydrolysis of Fraction IIC.* Fraction IIC (6.04 × 10^9 d.p.m.; equivalent to 1 mg of uronic acid-positive material) was heated in a sealed tube with 4.1 ml of 0.1M HCl at 100° for 45 min, made neutral with M KOH, diluted to 38 ml with water, applied to a column (2 × 7 cm) of Dowex-1 (Cl^- form) resin, and eluted with a linear LiCl gradient as described in the legend to Fig. 2. Fractions (~5 ml) were assayed for radioactivity (●—●) and combined as indicated (I—I). The individual fractions under the peaks were desalted on Sephadex G-10, as described in the legend to Fig. 2.

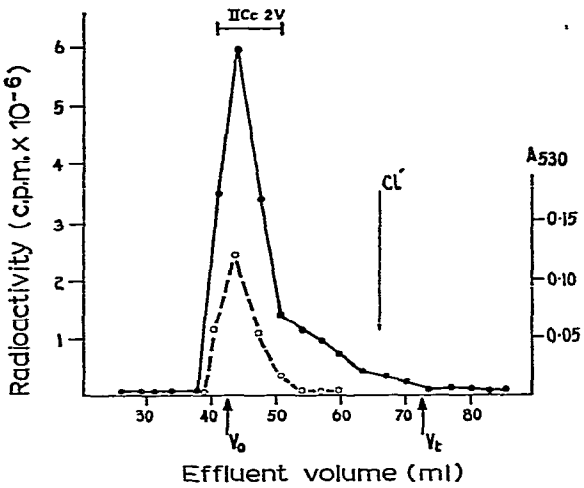

Fig. 7. *Gel chromatography of Fraction IICc2.* Fractions containing IICc2 were combined, concentrated to ~5 ml, and applied to a column (1 × 100 cm) of Sephadex G-10, which was eluted with 10% (v/v) aqueous ethanol. Fractions (~3 ml) were analysed for radioactivity (●—●), and uronic acid-containing material (O---O) as shown by the carbazole reaction (A_{530}), and combined as indicated (I—I). Chloride ion was detected by precipitation with Ag^{+}.

TABLE III

ANALYSIS OF PRODUCTS OBTAINED FROM THE ACID HYDROLYSIS OF FRACTION IIC (TABLE I)

Fraction	*Uronic acid (μg)*	*Sulfate/uronic acid (molar ratio)*	*Yield (% of total recovered d.p.m.)*		
IIC	1000	1.93	—		
IICa	n.d.[a]	—[b]	7		
IICb	248	n.d.	25		
IICc	578	—	62		
IICc1	22[c]	—		20[c]	
IICc2	539[c]	0.88		80[c]	
IICc2V	539	—			
IICc2Va	427	—			85
IICc2Vb	75	—			15
IICd	59	1.75	6		

[a]n.d., None detected. [b]—, Not measured. [c]Cross contamination of 2,5-anhydro-D-mannitol-*1-t* 6-sulfate (Ms) and IdA–Ms in Fractions IICc1 and IICc2. All of the uronic acid-positive and 20% of the labelled material in Fraction IICc1 was IdA-Ms; <0.5% of the labelled material in Fraction IICc2 was Ms.

combined as fraction IICc1, and the remaining material as fraction IICc2. Most of the radiolabelled material in IICc1 was shown to be 2,5-anhydro-D-mannitol-*1-t* 6-sulfate by p.c. and h.v.e., its conversion into 2,5-anhydro-D-mannitol-*1-t* in 2M trifluoroacetic acid at 100°, and its instability to treatment with periodate. More than 95% of the radioactive material in fraction IICc2 was identified as a monosulfated

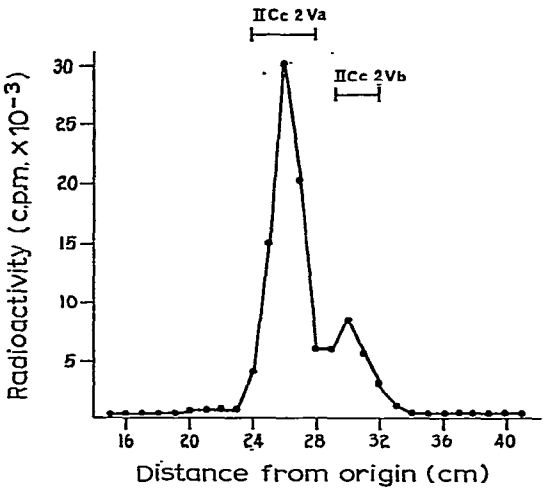

Fig. 8. *Paper chromatography (Solvent 3) of Fraction IICc2V (Fig. 7).* For preparative chromatography, the paper was pre-washed with Solvent *3*, each 1-cm segment was eluted with water, and the eluates were analysed for radioactivity (● —●) and combined as indicated (I—I).

TABLE IV

DEGRADATION OF DISACCHARIDE SUBSTRATES WITH WHOLE-CELL HOMOGENATES PREPARED FROM HUMAN, PERIPHERAL BLOOD LEUCOCYTES AND CULTURED HUMAN-SKIN FIBROBLASTS

Cell type	*Enzyme activity (pmol/min/mg of protein)*					
	Substrate					
	IdA–Ms	*IdA–M*	*GlcA–Ms*	*Fraction IICc2Vb*	*IdAs–Ms*	*IdAs–M*
Leucocytes						
Normal	64	2.3	—	—	12	—
MPS-IH (Hurler)	4	n.d.[b]	—	—	15	—
MPS-II (Hunter)	—[a]	—	—	—	n.d.	—
Fibroblasts						
Normal	340	9	143	19	27	0.64
MPS-IH (Hurler)	4	n.d.	180	0.6	23	0.35
MPS-IS (Scheie)	5	—	—	—	—	—
MPS-II (Hunter)	282	—	—	25	n.d.	n.d.
MPS-VII	176	7	2	—	—	—

[a]—, Not studied. [b]n.d., None detected.

IdA–M (p.c. and h.v.e.). Chromatography of the radiolabelled material in IICc2 on Sephadex G-10 separated monosulfated disaccharide from sulfated anhydromannitol and chloride ion (Fig. 7). Fraction IICc2V in Fig. 7 was shown by h.v.e. at pH 1.7 to contain 99.5% of monosulfated disaccharide and 0.5% of 2,5-anhydro-D-mannitol-*1-t* 6-sulfate.

The radioactive material in fraction IICc2V (Fig. 7) was fractionated into two tritiated components (IICc2Va, and IICc2Vb) by paper chromatography in Solvent *3* (Fig. 8). Fractions IICc2Va and IICc2Vb co-chromatographed on paper (Solvent *3*) with IdA–Ms (fraction IIBc1) and IdAs–M (fraction IIBc2), respectively. Fraction IICc2Va, ~85% of the total radioactivity, was assumed to be IdA–Ms from its degradation with fibroblast homogenates from normal subjects and with fibroblast homogenates deficient in 2-sulfo-L-iduronate 2-sulfatase (Table IV), to produce 2,5-anhydro-D-mannitol-*1-t* 6-sulfate, which, as expected, was degraded by periodate.

The labelled material in IICc2Vb was degraded by cell homogenates from normal fibroblasts and, unlike fraction IIBc2, by (2-sulfo-L-iduronate 2-sulfatase)-deficient fibroblasts (Table IV), to produce labelled material that (*a*) was degraded by periodate, and (*b*) migrated as a single component at the same rate as 2,5-anhydro-D-mannitol-*1-t* 6-sulfate when subjected to h.v.e. at pH 1.7 or paper chromatography in Solvents *3* or *4*. The rate of degradation of IICc2Vb by cell homogenates from normal fibroblasts was 18 times less than the rate observed for IdA–Ms (IICc2Va, Table IV). Incubation of the trititiated material in IICc2Vb with cell homogenates prepared from α-L-iduronidase-deficient fibroblasts gave 2,5-anhydro-D-mannitol-*1-t* 6-sulfate at a rate 30-times less than that found for control cell-preparations (Table IV). These results suggest that the radiolabelled material in IICc2Vb is not IdAs–M or IdA–M-3-sulfate. A detailed investigation of IICc2Vb will be needed to reveal the structure of the radiolabelled material and its relation to the original heparin structure.

Radiolabelled material in IICd was assumed to be IdAs–Ms on the basis of its mobility in h.v.e. at pH 1.7 and its resistance to degradation by fibroblast homogenates deficient in 2-sulfo-L-iduronate 2-sulfatase.

Preparative methods of choice. — IdA–Ms is best prepared by acid hydrolysis of fraction IIC (Fig. 6); the overall yield of fraction IICc2Va from heparin is 24% (Table III), compared to ~1.1% for IIBc1 (Table I). Acid hydrolysis of IIC is also the best method for the preparation of IdA–M (fraction IICb, Table III); the yield of IICb from heparin is 14% by acid hydrolysis of IIC, compared to <0.2% for IIAb (Table I).

Degradation of disaccharide fractions by leucocytes and cultured human-skin fibroblast homogenates. — Cleavage rates of the radiolabelled disaccharides measured with IdA–M (fraction IICb, Table III), IdA–Ms (IICc2Va, Table III), and IICc2Vb in the incubation mixture described (Experimental) as optimum for α-L-iduronidase activity are recorded in Table IV. Whole-cell homogenates from α-L-iduronidase-deficient cell lines (MPS Types IH and IS) were shown, using IdA–Ms as substrate, to have <1.5% of the activity found for normal fibroblasts. These results corroborate the chemical evidence used above to assign structures to the fractions shown in Table II. α-L-Iduronidase activity measured with IdA–Ms (fraction IICc2Va) was more than 25-times greater than the value obtained with IdA–M (IICb). This result (Table IV) is consistent with the finding that the presence of a sulfate residue at C-6 of the anhydromannitol residue considerably increases V_{max} and decreases K_m for

the enzyme under conditions where the α-L-iduronidase activity is linear for time and protein concentration (J. J. Hopwood and V. Muller, unpublished observations). Under the conditions described above, the released 2,5-anhydro-D-mannitol-*1-t* 6-sulfate is not further desulfated.

The 2-sulfo-L-iduronate 2-sulfatase activity of cultured skin fibroblasts was studied by using IdAs–Ms (fraction IIC) and IdAs–M (IIBc2) as substrates. IdAs–Ms is first degraded to a product (IdA–Ms) that is further degraded by α-L-iduronidase, to produce 2,5-anhydro-D-mannitol-*1-t* 6-sulfate; IdAs–M is degraded to IdA–M and then by α-L-iduronidase to 2,5-anhydro-D-mannitol-*t*. Analogously to α-L-iduronidase, 2-sulfo-L-iduronate 2-sulfatase activity measured by using IdAs–Ms was more than 45-times greater than the value obtained with IdAs–M (Table IV). Thus, it seems that C-6 sulfation of the penultimate sugar residue is an important structural determinant in the mechanism of action of both α-L-iduronidase and 2-sulfo-L-iduronate 2-sulfatase on disaccharide substrates.

Fractions IIC (IdAs–Ms) and IIBc2 (IdAs–M) were degraded by MPS-IH fibroblast homogenates to IdA–Ms and IdA–M, respectively, but were not degraded by (2-sulfo-L-iduronate 2-sulfatase)-deficient MPS-II fibroblasts. The finding that monosulfated-disaccharide fractions IICc2Va and IICc2Vb were degraded by MPS-II fibroblast homogenates to produce sulfated anhydromannitol-*t* (Table IV) confirms that the sulfate residue in these components is linked to the anhydromannitol residue and not C-2 or C-3 of the iduronic acid residue. These findings corroborate the chemical data used to assign the structures shown in Table II.

In addition, it was shown that GlcA–Ms was rapidly cleaved by both normal and iduronidase-deficient cells, but this rate was at least 50-fold less than that with β-D-glucuronidase-deficient (MPS-VII) cells (Table IV).

After the conclusion of the present study, a report appeared giving a preparative method for IdA–M and showing its use as a substrate for the estimation of α-L-iduronidase activity[17]. With this substrate, the authors were unable to detect residual α-L-iduronidase activity in fibroblast preparations isolated from a Hurler patient[17], but further, extensive studies in this department have recently confirmed that IdA–Ms is a 25-times more-sensitive substrate than IdA–M for the assay of α-L-iduronidase activity; with IdA–Ms, it is possible to detect and measure the kinetic constants of residual α-L-iduronidase activity in fibroblast homogenates isolated from both Hurler and Scheie patients (Table IV; see also J. J. Hopwood and V. Muller, refs. 37 and 38). This capacity to detect residual α-L-iduronidase activity in Hurler fibroblasts and peripheral blood leucocytes has proved to be extremely helpful in a recent, prenatal, diagnostic assessment for Hurler's Syndrome (J. J. Hopwood, V. Muller, A. C. Pollard, and J. Rogers, presented at the inaugural meeting of the Human Genetics Society of Australasia, Monash, Australia, August, 1977, [ref. 39]).

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