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SELECTIVE DEPOLYMERISATION OF DERMATAN SULFATE: PRODUCTION OF RADIOLABELLED SUBSTRATES FOR α-L-IDURONIDASE, SULFOIDURONATE SULFATASE, AND β-D-GLUCURONIDASE

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ABSTRACT

Radiolabelled disaccharide substrates for α-L-iduronidase, β-D-glucuronidase, and sulfoiduronate sulfatase have been prepared from dermatan sulfate by application in sequence of *N*-deacetylation, deaminative cleavage, and reduction with $NaBT_4$. The yield of disaccharides was ~87% of the total oligosaccharide fraction. Five disaccharides were isolated and tentatively identified. The major disaccharide, *O*-(α-L-idopyranosyluronic acid)-(1→3)-2,5-anhydro-D-[1-^{3}H]talitol 4-sulfate (IdoA–anT4S), represented ~75% of the total disaccharide fraction. The other disaccharides were *O*-(α-L-idopyranosyluronic acid 2-sulfate)-(1→3)-2,5-anhydro-D-[1-^{3}H]talitol 4-sulfate (IdoA2S–anT4S), *O*-(β-D-glucopyranosyluronic acid)-(1→3)-2,5-anhydro-D-[1-^{3}H]talitol 4-sulfate (GlcA–anT4S), *O*-(β-D-glucopyranosyluronic acid)-(1→3)-2,5-anhydro-D-[1-^{3}H]talitol 6-sulfate (GlcA–anT6S), and *O*-(α-L-idopyranosyluronic acid)-(1→3)-2,5-anhydro-D-[1-^{3}H]talitol (IdoA–anT), which represented ~4.5, 11.2, 1.0, and 1.8%, respectively, of the total disaccharide fraction. When incubated with cultured skin-fibroblasts from normal controls, IdoA–anT4S was shown to be a sensitive substrate for α-L-iduronidase to produce 2,5-anhydro-D-talitol 4-sulfate (anT4S). Activity toward IdoA–anT4S was not observed with fibroblast homogenates from α-L-iduronidase-deficient patients (Mucopolysaccharidosis Type I). Similarly, normal-fibroblast homogenates degraded GlcA–anT6S to anT6S, and GlcA–anT4S to anT4S, at a rate 6 to 8 times greater than found for fibroblasts from β-D-glucuronidase-deficient patients (Mucopolysaccharidosis Type VII). IdoA–anT4S was hydrolysed by α-L-iduronidase at a rate 365 times greater than that for IdoA–anT. Sulfation of the anhydro-D-[1-^{3}H]talitol residues is an important structural determinant in the mechanism of action of α-L-iduronidase on disaccharide substrates. IdoA2S–anT4S was degraded to IdoA–anT4S and then to anT4S by normal-fibroblast homogenates, whereas fibroblasts from α-L-iduronidase-deficient and sulfoiduronate sulfatase-deficient (Mucopolysaccharidosis Type II) patients produced considerably decreased levels of anT4s and IdoA–anT4S (and anT4S), respectively.

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INTRODUCTION

Dermatan sulfate (DS) is degraded by the sequential action of at least four highly specific, lysosomal enzymes that act at the non-reducing end of this glycosaminoglycan chain. Three of these enzymes, namely, α-L-iduronidase, sulfoiduronate sulfatase, and β-D-glucuronidase, are also involved in the degradation of heparan sulfate (HS) and heparin[1-3]. Genetic disorders of glycosaminoglycan catabolism have been described where the activity of one of these lysosomal enzymes is grossly diminished, leading to the accumulation of partially degraded DS and HS in lysosomes and the expression of clinical defects[3]. Experience with the determination of the activities of mutant enzymes produced in genetic disorders has underlined the need to use substrates the structures of which closely match those of the natural substrate[4-8]. Radiolabelled disaccharides prepared from heparin *via* deamination with nitrous acid have been utilised as substrates for α-L-iduronidase, sulfoiduronate sulfatase, and β-D-glucuronidase[9-18]. The substrates derived from heparin all contain uronic acid residues α- or β-(1→4)-linked to 2,5-anhydro-D-mannitol or 2,5-anhydro-D-mannitol 6-sulfate, whereas the uronic acid residues in DS are α- or β-(1→3)-linked to 4- or 6-sulfated 2-acetamido-2-deoxy-D-galactose. We now report on radiolabelled disaccharides produced by degradation of *N*-deacetylated DS with nitrous acid and on their enzymic degradation by homogenates of cultured human-skin fibroblasts. A similar study with keratan sulfate has been reported[19].

RESULTS AND DISCUSSION

Isolation of radiolabelled disaccharide fragments from DS. — *N*-Deacetylation of hexosamine residues[20] with hydrazine has been applied to DS and thence, using nitrous acid followed by $NaBT_4$, various radiolabelled disaccharides have been produced. The effect of time, at 98° in hydrazine, on the *N*-deacetylation of DS was evaluated by fractionation on Sephadex G-25 of the products of degradation with nitrous acid. Two major uronic acid-positive fractions were observed (Fig. 1A). The more-retarded and the less-retarded peaks of uronic acid-positive material had the same elution position as the reference HS-derived disaccharide and tetrasaccharide standards, respectively. Treatment of DS with nitrous acid did not yield detectable amounts of di- or tetra-saccharides (Fig. 1A). The uronic acid-positive material in the disaccharide peak was reduced with $NaBT_4$, and the product was subjected to ion-exchange chromatography (Fig. 2). Similar profiles of radioactivity were obtained for the radiolabelled disaccharide fraction isolated from DS that had been *N*-deacetylated for 2 and 8 h. The major peak of radioactivity was eluted at the concentration of salt required to elute the monosulfated disaccharide IdoA–anM6S. The greatest proportion of disaccharide was produced from DS that had been treated with hydrazine for 8 h (Fig. 1A). The ratios of radiolabelled products that were eluted with unsulfated, monosulfated, and disulfated disac-

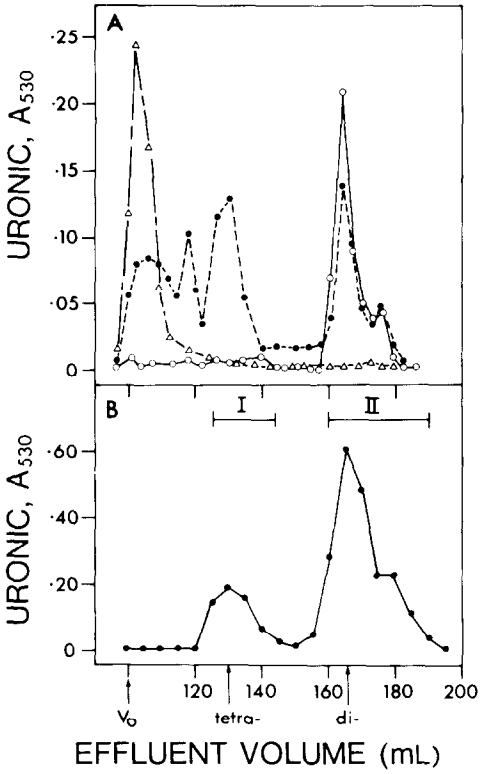


Fig. 1. Gel chromatography of products derived from DS and *N*-deacetylated DS by deamination with nitrous acid: A, DS *N*-deacetylated by treatment with hydrazine for 2 h (--●--), and 8 h (—○—), and DS (--△--) were deaminated (see Experimental), and the products were applied to a column (1 × 200 cm) of Sephadex G-25 and eluted with 0.2M NaCl in 0.05M sodium acetate buffer (pH 5.3) at 2–8 mL/h; B, DS *N*-deacetylated by treatment with hydrazine for 6 h. Arrows indicate peak elution of Blue Dextran (V_0), and tetrasaccharide and disaccharide standards.

charide-standards were not changed by *N*-deacetylation for 2 h or 8 h (Fig. 2A). Detailed structural studies were carried out on the products of *N*-deacetylation of DS with hydrazine for 6 h, followed by deamination and reduction with $NaBT_4$. After reduction with $NaBT_4$, electrophoresis of the disaccharide fraction (II, Fig. 1B) at pH 1.7 or pH 5.0 gave three radiolabelled fractions having mobilities similar to those reported[13,21] for unsulfated, monosulfated, and disulfated uronic acid disaccharides.

Ion-exchange chromatography of fraction II (from Fig. 1B) also gave three radioactive and uronic acid-positive sub-fractions (IIA–C; Fig. 2B), the relative ratios of which are recorded in Table I.

Characterisation of Fractions IIA–C. — Paper chromatography of IIA (solvent *1*) gave two radiolabelled fractions (IIA1 and IIA2), representing 1.2 and 3.3%, respectively, of the total radioactivity of fraction II. Fraction IIA1 was not studied further. Electrophoresis at pH 5.1 separated fraction IIA2 into two compo-

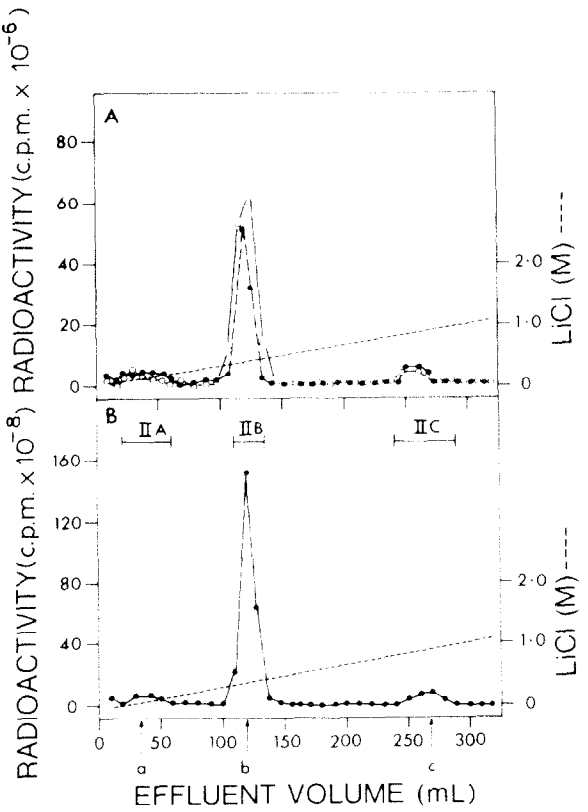


Fig. 2. Ion-exchange chromatography on AG 1-X4 of fraction II from Fig. 1B. Desalted II was reduced with $NaBT_4$, desalted, and applied to a column (2 × 10 cm) of AG 1-X4 washed with 20 mL of 0.05M Tris HCl buffer (pH 7.6) and eluted with a linear gradient generated from 0.05M Tris HCl buffer (pH 7.6) and 2M LiCl in 0.05M Tris HCl buffer (pH 7.6) by using an LKB 11300 Ultrograd gradient mixer: A, radiolabelled II isolated from DS *N*-deacetylated for 2 (—●—) and 8 h (—○—); B, radiolabelled II isolated from DS *N*-deacetylated for 6 h (—●—). Fractions were collected, assayed for radioactivity, combined where indicated (|——|), and desalted. Arrows indicate the peak position of standards: a, unsulfated disaccharide; b, monosulfated disaccharide; and c, disulfated disaccharide.

nents (IIA2a and IIA2b). Fraction IIA2a moved toward the anode with the same mobility as IdoA–anM and was degraded by β-D-glucuronidase-deficient and normal fibroblasts, but not by α-L-iduronidase-deficient fibroblasts, to a product that was not charged during electrophoresis at pH 5.1. Fraction IIA2b was not studied further. The relative ratios of fractions IIA2a and IIA2b are recorded in Table I. On the basis of these results and the products expected from *N*-deacetylated DS, IIA2a was concluded to be *O*-(α-L-idopyranosyluronic acid)-(1→3)-2,5-anhydro-D-[1-^{3}H]talitol (**1**, IdoA–anT) and represents ~1% of the total disaccharide isolated from DS.

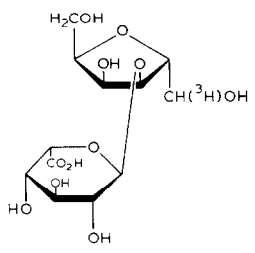
Fraction IIB gave (p.c., solvent *1*) three radiolabelled sub-fractions (IIB1, IIB2, and IIB3; Fig. 3), of which IIB2 and IIB3 migrated during electrophoresis at pH 1.7 and 5.1 as a single radiolabelled component having a mobility similar to that

TABLE I

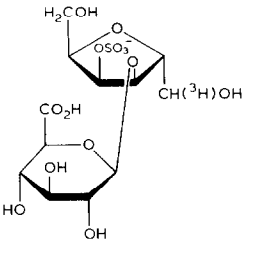
DATA FOR PRODUCTS OBTAINED FROM *N*-DEACETYLATED DS AFTER DEGRADATION WITH NITROUS ACID AND SUBSEQUENT REDUCTION WITH $NaBT_4$

Fraction	*Proposed structure*	*Yield (mCi)*	*Electrophoresis*		*P.c.*			
			pH 1.7 (Ra)[a]	*pH 5.1 (Rb)*[b]	*Solvent* 1		*Solvent* 2	
					(Rc)[a]	*(Rc)*[a]	*(Rd)*[a]	*(Rd)*[b]
I		4	mc[d]	mc	mc		mc	
II		22	mc	mc	mc		mc	
IIA		1	0.17	mc				
IIA1		0.26	[e]					
IIA2		0.74	0.26	mc				
IIA2a	IdoA–anT	0.17	0.22	0.55		0.94		1.05
IIA2b		0.40	0.26	0.71		0.97		1.08
	IdoA–anM		0.22	0.59		1.00		1.00
IIB		19.8	0.22		mc		mc	
IIB1		0.10	1.55	1.26	0.47		0.66	
IIB2		0.18			0.79		mc	
IIB3		12			0.99		mc	
IIB2a	GlcA–anT6S	0.09	0.96	1.01	0.79		0.68	
IIB2b	IdoA–anT4S	0.02	0.99	0.93			1.58	
IIB3a	GlcA–anT4S	1.1	0.93	1.03	0.97		1.14	
IIB3b	IdoA–anT4S	7	1.00	1.00	1.02		1.58	
IIC	IdoA2S–anT4S	0.98	1.54	1.23	0.44		0.69	
	GlcA–anT6S[c]		1.0	1.0			0.67	
	GlcA–anT4S[c]		1.0	1.0			1.14	

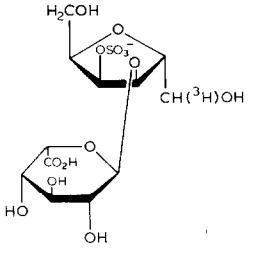
[a]Relative to standard monosulfated-disaccharide IdoA–anM6S. [b]Mobility of IIA components relative to IdoA–anM. [c]Derived from chondroitin sulfate. [d]Multiple components. [e]Not done.



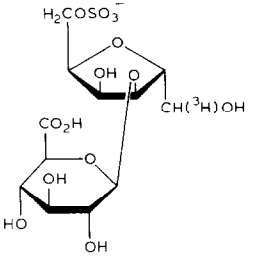
1 (IdoA–anT, IIA2a)



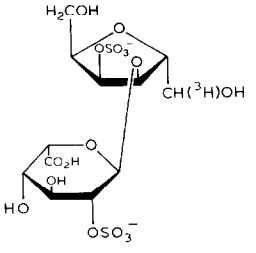
2 (GlcA–anT4S, IIB3a)



3 (IdoA–anT4S, IIB2b, IIB3b)



4 (GlcA–anT6S, IIB2a)



5 (IdoA2S–anT4S, IIc)

reported for monosulfated disaccharides derived from heparin[13]; IIB1 was not studied further. P.c. (solvent *2*) of IIB2 produced two components (IIB2a and IIB2b). Fraction IIB2b had mobilities (p.c., solvents *1* and *2*) similar to those of the major radiolabelled component in IIB3b, and was degraded by fibroblast homogenates from normal, β-D-glucuronidase-, and sulfoiduronide sulfatase-deficient patients, but not by α-L-iduronidase-deficient fibroblasts. Fraction IIB2a, representing 79% of the total radioactivity in IIB2 (Table I), migrated during electrophoresis at pH 1.7 as a single radiolabelled component having a mobility similar to that reported[13] for monosulfated disaccharides. IIB2a was degraded by α-L-iduronidase-deficient, sulfoiduronate sulfatase-deficient, and normal fibroblasts, but not by β-D-glucuronidase-deficient fibroblasts or purified α-L-iduronidase, to a product which, during electrophoresis at pH 1.7 or p.c. (solvent *3*), had mobilities similar to those of the radiolabelled component produced by incubation of GlcA–anT6S (derived from chondroitin sulfate, see Experimental) with normal fibroblasts (Table II). IIB2a migrated during p.c. (solvents *1* and *2*) with mobilities similar to those of GlcA–anT6S isolated from chondroitin sulfate (Table I). On the basis of these results and the products expected from *N*-deacetylated DS, IIB2a was assumed to be *O*-(β-D-glucopyranosyluronic acid)-(1→3)-2,5-anhydro-D-[1-^{3}H]-talitol 6-sulfate (**4**, GlcA–anT6S).

Fraction IIB3, representing 98% of the total radioactivity of IIB, gave (p.c., solvent *2*; Fig. 4) two sub-fractions (IIB3a and IIB3b), representing 14 and 86%, respectively, of the total IIB3 radioactivity (Table I). Incubation of IIB3a with α-L-iduronidase-deficient, sulfoiduronate sulfatase-deficient, or normal fibroblasts produced a radiolabelled component with an electrophoretic mobility at pH 1.7 similar to that of 2,5-anhydro-D-[1-^{3}H]mannitol 6-sulfate. Radiolabelled products were

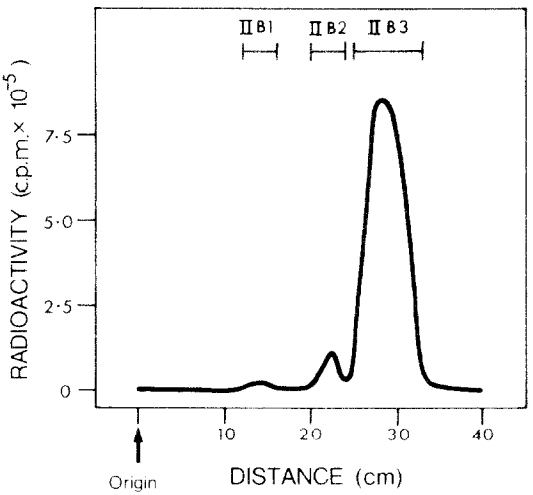


Fig. 3. Radiochromatographic scan after p.c. (42 h/25°, solvent *1*) of desalted fraction IIB from Fig. 2B. Fractions IIB, IIB2, and IIB3 were eluted with water.

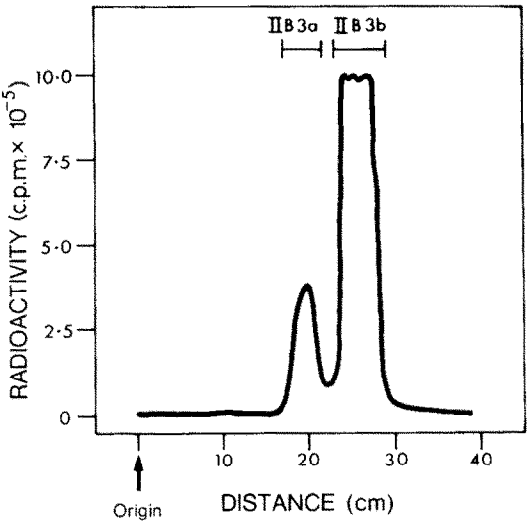


Fig. 4. Radiochromatographic scan after p.c. (64 h/25°, solvent *2*) of fraction IIB3 from Fig. 3. Fractions IIB3a, IIB3b were eluted with water.

not found following incubation of IIB3a with β-D-glucuronidase-deficient fibroblasts and purified α-L-iduronidase. The radiolabelled product from the incubation of normal fibroblasts and IIB3a had a mobility (p.c., solvent *3*) similar to that of the product obtained after incubation of GlcA–anT4S (isolated from chondroitin sulfate) with normal fibroblasts (Table II). Incubation of IIB3b with β-D-glucuronidase-deficient and sulfoiduronate sulfatase-deficient fibroblast lines produced a monosulfated monosaccharide that was not observed after incubation with the α-L-iduronidase-deficient fibroblasts. The radiolabelled components produced from incubation of IIB3a and IIB3b with normal fibroblasts had similar mobilities (p.c., solvent *3*; Table II), whereas the radiolabelled product obtained from incubation of IIB2a ran with a mobility lower than that observed for IIB3a and IIB3b (Table II). On the basis of these results, and the products expected from the *N*-deacetylated DS, IIB3a and IIB3b were concluded to be *O*-(β-D-glucopyranosyluronic acid)-(1→3)-2,5-anhydro-D-[1-^{3}H]talitol 4-sulfate (**2**, GlcA–anT4S) and *O*-(α-L-idopyranosyluronic acid)-(1→3)-2,5-anhydro-D-[1-^{3}H]talitol 4-sulfate (**3**, IdoA–anT4S), respectively. A digest of DS with chondroitinase ABC[22] contained ~4% of 6-sulfated disaccharide and 96% of 4-sulfated disaccharide. These values are consistent with the observed ratios of IIB2a, IIB3a, and IIB3b (Table I).

Unsulfated, radiolabelled disaccharide was not detected after incubation of any IIB fraction with normal fibroblasts. The possibility was therefore excluded that significant amounts of *O*-(α-L-idopyranosyluronic acid 2-sulfate)-(1→3)-2,5-anhydro-D-[1-^{3}H]talitol were present in IIB.

Only a single radiolabelled component was observed when IIC was subjected to p.c. (solvents *1*, *2*, or *4*). Only one radiolabelled component, with a mobility

TABLE II

P.C. (SOLVENT *3*, 4 DAYS AT 25°) OF DEGRADATION PRODUCT(S) FROM DISACCHARIDES AFTER INCUBATION WITH FIBROBLAST HOMOGENATES CULTURED FROM NORMAL INDIVIDUALS

Fraction	*Proposed structure of substrate*	*P.c. mobility of sulfated product (cm from origin)*	
		Run 1	*Run 2*
	IdoA2S–anM6S	15.2	16.8
	IdoA–anM6S	14.6	16.4
	GlcA–anM6S	16.0	[a]
IIC	IdoA2S–anT4S	17.0	20.4
IIB3b	IdoA–anT4S		18.3
IIB3a	GlcA–anT4S(DS)[b]	16.7; 17.8[c]	19.6
	GlcA–anT4S(CS)[b]	18.4	
IIB2a	GlcA–anT6S(DS)[b]	13.3	16.0
	GlcA–anT6S(CS)[b]	13.2	

[a]Not applicable or not done. [b]Derived from DS or chondroitin sulfate (CS). [c]Duplicate channel.

similar to that reported for a disulfated disaccharide, was observed when IIC was subjected to electrophoresis at pH 1.7 or 5.1. Incubation of IIC with β-D-glucuronidase-deficient fibroblasts produced two radiolabelled components, with mobilities [electrophoresis at pH 1.7 or p.c. (solvent *3*)] similar to those of the monosulfated disaccharide IIB3b and the radiolabelled product (Table II) resulting during the incubation of normal fibroblasts and IIB3b. Incubation of IIC with α-L-iduronidase-deficient fibroblasts produced just one radiolabelled component which had a mobility similar to that of IIB3b during electrophoresis at pH 1.7. Incubation of IIC with sulfoiduronate sulfatase-deficient fibroblasts failed to produce detectable levels of radiolabelled products.

These results suggest that the major radiolabelled component in IIC is *O*-(α-L-idopyranosyluronic acid 2-sulfate)-(1→3)-2,5-anhydro-D-[1-^{3}H]talitol 4-sulfate (**5**, IdoA2S–anT4S).

Assay of sulfate ester and uronic acid distribution in DS repeat-sequence. — Information about the structure of DS can be gained from the identification of the major radiolabelled components in fraction II as IdoA–anT4S (**3**), GlcA–anT4S (**2**), IdoA2S–anT4S (**5**), IdoA–anT (**1**), and GlcA–anT6S (**4**), representing (Table I) ~75, 11.2, 4.5, 1.8, and 1.0%, respectively, of the total radioactivity of II. The presence of small proportions of IdoA2S–anT and IdoA–anT6S in II isolated from this DS preparation cannot be excluded on the basis of the above negative data. The sequence *N*-deacetylation, nitrous acid deamination, and reduction with $NaBT_4$, to produce radiolabelled disaccharides, is useful for the study of the distribution of sulfate and uronic acid in DS from different sources.

Degradation of radiolabelled disaccharide fractions by homogenates of cultured human-skin fibroblasts. — The optimum conditions selected for incubation were similar to those reported to be optimal for the degradation of more-conven-

TABLE III

DEGRADATION OF IdoA-DISACCHARIDES WITH WHOLE-CELL HOMOGENATES PREPARED FROM CULTURED SKIN-FIBROBLASTS

Substrate	*α-L-Iduronidase activity (pmol/min/mg of protein)*	
	Normal fibroblasts	*MPS IH fibroblasts*
IdoA–anT	0.5	n.d.[a]
IdoA–anM	1.5	n.d.
IdoA–anT4S	292	n.d.
IdoA–anM6S	116	n.d.

[a]None detected.

TABLE IV

DEGRADATION OF IdoA2S-DISACCHARIDES WITH WHOLE-CELL HOMOGENATES PREPARED FROM CULTURED SKIN-FIBROBLASTS

Substrate	*Sulfoiduronate sulfatase activity (pmol/min/mg of protein)*	
	Normal fibroblasts	*MPS II fibroblasts*
IdoA2S–anM6S	45	1.1
IdoA2S–anT4S	42	1.7.

TABLE V

DEGRADATION OF GlcA-DISACCHARIDES WITH WHOLE-CELL HOMOGENATES PREPARED FROM CULTURED SKIN-FIBROBLASTS

Substrate	*β-D-Glucuronidase activity (pmol/min/mg of protein)*	
	Normal fibroblasts	*MPS VII fibroblasts*
GlcA–anT4S[a]	11.3	1.2
GlcA–anT6S[b]	10.8	1.6
GlcA–anM6S	11.3	0.6
GlcA–anT4S[c]	17.9	n.d.[d]
GlcA–anT6S[c]	11.3	1.5

[a]Fraction IIB3a. [b]Fraction IIB2a. [c]Isolated from chondroitin sulfate. [d]None detected.

tional disaccharide substrates derived from heparin[13–15,18], and will be reported in detail elsewhere.

(*a*) *α-L-Iduronidase.* IdoA–anT (**1**), IdoA–anT4S (**3**), and IdoA–anM6S (derived from heparin) were all degraded by normal fibroblasts (Table III). IdoA–anT was degraded at a rate ~145 and ~365 times less than those for IdoA–anM6S and IdoA–anT4S, respectively. No significant activity toward these three substrates was observed with MPS IH fibroblasts (Table III). These findings suggest that the degradation of these substrates requires the specific action of α-L-iduronidase and

that, as previously reported[15,23] for IdoA–anM6S, the presence of a sulfate ester group on the adjacent residue to the iduronide residue under attack is also an important determinant of enzyme activity.

(*b*) *Sulfoiduronate sulfatase.* IdoA2S–anT4S (**5**) isolated from DS and IdoA2S–anM6S from heparin were degraded to a similar extent by normal fibroblasts, but not by homogenates from MPS II fibroblasts (Table IV).

(*c*) *β*-D-*Glucuronidase.* Disaccharides Glc–anT4S and GlcA–anT6S (IIB3a and IIB2a, respectively) isolated from DS, the equivalent structures isolated from chondroitin sulfate, and GlcA–anM6S were degraded at similar rates by normal fibroblasts. Only ~10% of the normal enzyme activity towards each of these substrates was observed in incubations containing homogenates of MPS VII fibroblasts (Table V).

EXPERIMENTAL

High-voltage electrophoresis was performed on Whatman 3MM 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 Shandon Southern Model L-24 System cooled with water maintained at 10°. Descending p.c. was performed on Whatman 3MM paper with *1*, ethyl acetate–acetic acid–water (3:1:1); *2*, 1-butanol–acetic acid–7.4M ammonia (2:2:1); *3*, 1-butanol–acetic acid–7.4M ammonia (6:2:1); and *4*, isobutyric acid–2M ammonia (15:9). For preparative p.c., the paper was pre-washed with the irrigation solvent.

Reference (radiolabelled) unsulfated, monosulfated, and disulfated disaccharides were prepared from heparin and identified as *O*-(*α*-L-idopyranosyluronic acid)-(1→4)-2,5-anhydro-D-[1-^{3}H]mannitol (IdoA–anM), *O*-(*α*-L-idopyranosyluronic acid)-(1→4)-2,5-anhydro-D-[1-^{3}H]mannitol 6-sulfate (IdoA–anM6S), *O*-(*α*-L-idopyranosyluronic acid 2-sulfate)-(1→4)-2,5-anhydro-D-[1-^{3}H]mannitol 6-sulfate (IdoA2S–anM6S), and *O*-(*β*-D-glucopyranosyluronic acid)-(1→4)-2,5-anhydro-D-[1-^{3}H]mannitol 6-sulfate (GlcA–anM6S), respectively, as previously described[13].

Gel chromatography was conducted on columns of Sephadex G-10 or G-25 equilibrated with aqueous 10% ethanol or 0.2M NaCl in 0.05M sodium acetate buffer (pH 5.3). Fractions were desalted by elution from a column (1 × 100 cm) of Sephadex G-10 with aqueous 10% ethanol.

Uronic acid, protein, and radioactivity were assayed by methods previously described[21,23]. The relative amounts of 4- and 6-sulfate esters in DS and chondroitin sulfate A were determined by the method of Robinson and Dorfman[22].

Pig-skin chondroitin sulfate B (dermatan sulfate) and whole-cartilage chondroitin sulfate A (catalogue number C4134), shown[22] to contain ~30% of chondroitin 6-sulfate by chondroitinase ABC digestion, were obtained from Sigma. The anion-exchange resin used was Bio-Rad AG 1-X4 (100–200 mesh, chloride form). *α*-L-Iduronidase, purified from human liver, was a gift from Dr. Peter Clements of this Department. Anhydrous hydrazine was prepared as previously described[19].

Fibroblasts [α-L-iduronidase-deficient, Mucopolysaccharidosis Type IH (MPS IH); sulfoiduronate sulfatase-deficient, Mucopolysaccharidosis Type II (MPS II); and normal controls] were established from biopsies available to this hospital, and enzyme deficiencies were established by methods previously described[24]. β-D-Glucuronidase-deficient fibroblasts (GM-312-mucopolysaccharidosis Type VII) were obtained from the Human Genetic Cell Repository (Institute for Medical Research, Camden, N.J., U.S.A.). All lines were maintained and used for the preparation of cell homogenates 6–10 days after sub-culture as previously described[8,15].

N-*Deacetylation of DS.* — A mixture of DS (19 mg), anhydrous hydrazine (2 mL), and hydrazinium sulfate (150 mg) was kept in a capped glass-tube at 98°. After the times indicated in the text, the mixture was concentrated to 50 μL, water (2 mL) and glacial acetic acid were added to adjust the pH to <7, and the solution was dialysed against 0.15M NaCl for 4–5 h, followed by water for a further 4–5 h, and finally freeze-dried.

Deamination[25] *of* N-*deacetylated DS.* — A solution of *N*-deacetylated DS (8 mg) in water (500 μL), 33% acetic acid (750 μL), and 0.73M sodium nitrite (750 μL) was left at room temperature for 2 h. Ammonium sulfamate (50 mg) was then added to destroy the remaining nitrous acid, and the mixture was concentrated at <40° to dryness, adding 2 mL of 0.2M NaCl in 0.05M sodium acetate buffer (pH 5.2). The products were fractionated by chromatography on a column (200 × 1.6 cm) of Sephadex G-25, and uronic acid-positive fractions (Fig. 1A), which were eluted in the tetrasaccharide and disaccharide positions, were combined (I and II, respectively), desalted by elution from a column (100 × 1.6 cm) of Sephadex G-10 with aqueous 10% ethanol, and freeze-dried. Each residue was reduced with $NaBT_4$ (1.6 mg, 1.6 Ci/mmol) in 0.1M sodium borate buffer (1 mL, pH 8.1) for 4 h at 25°. $NaBH_4$ (5 mg) was then added and, after 4 h at 25°, each mixture was acidified with acetic acid [CAUTION, tritium gas] and desalted as described above. The reduced, radiolabelled disaccharides were further fractionated by ion-exchange chromatography on a column (5 × 1.6 cm) of Bio-Rad AG 1-X4 and p.c., as described in the text.

Preparation of GlcA–anT4S and GlcA–anT6S. — A mixture of chondroitin sulfate A (34 mg), anhydrous hydrazine (2.8 mL), and hydrazine sulfate (155 mg) was kept in a capped glass-tube at 98° for 4 h and then concentrated, dialysed, and degraded by deamination with nitrous acid. The uronic acid-positive disaccharide components, isolated on a column of Sephadex G-25, were reduced with $NaBT_4$ as described above for DS. The monosulfated disaccharide components were purified by ion-exchange chromatography and preparative p.c. (solvent *2*), to yield two radiolabelled components with relative mobilities (IdoA–anM6S, 1.0) 0.67 and 1.14 (major) (Table I). The major component was assumed to be *O*-(β-D-glucopyranosyluronic acid)-(1→3)-2,5-anhydro-D-[1-^{3}H]talitol 4-sulfate (GlcA–anT4S) and the other component was assumed to be *O*-(β-D-glucopyranosyluronic acid)-(1→3)-2,5-anhydro-D-[1-^{3}H]talitol 6-sulfate (GlcA–anT6S), both of which co-

migrated with standard IdoA–anM6S during electrophoresis at pH 1.7. Incubation of these preparations of GlcA–anT4S and GlcA–anT6S at pH 4.0 with normal fibroblast each produced a radiolabelled component that co-migrated with anM6S during electrophoresis at pH 1.7 but had different mobilities in p.c. with solvent *3* (Table II). These products were not detected when GlcA–anT6S and GlcA–anT4S were incubated with homogenates of β-D-glucuronidase-deficient fibroblasts. This finding corroborates the chemical data used to assign structures.

Preparation of skin fibroblasts for enzyme assay. — Skin fibroblasts, cultured in Falcon flasks (25 cm) to obtain 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 conditions previously described[8,15]. Fibroblasts were suspended in aqueous 0.1% Triton X-100 (0.5 mL), and disrupted by freezing (solid CO_2–ethanol) and thawing six times. Homogenates were dialysed for 17 h against 0.15M NaCl for assays measuring sulfoiduronate sulfatase.

Degradation of radiolabelled disaccharides by cultured skin-fibroblast homogenates. — Based on radioactivity measurement and specific radioactivity related to uronic acid, each of the disaccharide fractions isolated from DS, chondroitin sulfate, and heparin were diluted to 84 or 246μM with aqueous 10% ethanol.

For direct comparison of substrates, assays for α-L-iduronidase and β-D-glucuronidase were performed by incubating 6–7 μg of fibroblast-homogenate protein at 37° in 0.05M sodium acetate buffer (pH 4) for β-D-glucuronidase, or 0.05M sodium formate buffer (pH 3.6) for α-L-iduronidase. IdoA-disaccharides, assayed at a concentration of 84 pmol per incubation, were incubated for 15 min (IdoA–anT4S), 30 min (IdoA–anM6S), or 4 h (IdoA–anT and IdoA–anM). GlcA-disaccharides, assayed at a concentration of 246 pmol per incubation, were incubated for 2 h or 4 h (GlcA–anM).

Sulfoiduronate sulfatase activity was determined after a 16-h incubation (37°), using 70–100 pmol of substrate in 0.05M sodium acetate buffer (pH 4.0) with dialysed fibroblast-homogenate (12–14 μg). The total volume of each incubation mixture was 12 μL. After incubation with sulfated substrates, the whole reaction mixture was subjected to electrophoresis at pH 1.7 or p.c. (solvent *3*). Incubations containing unsulfated substrate were subjected to electrophoresis at pH 5.0.

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