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SELECTIVE DEPOLYMERISATION OF HEPARIN TO PRODUCE RADIO-LABELLED SUBSTRATES FOR SULFAMIDASE, 2-ACETAMIDO-2-DEOXY-α-D-GLUCOSIDASE, ACETYL-CoA:2-AMINO-2-DEOXY-α-D-GLUCOSIDE *N*-ACETYLTRANSFERASE, AND 2-ACETAMIDO-2-DEOXY-D-GLUCOSE 6-SULFATE SULFATASE

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ABSTRACT

Heparin was carboxyl-reduced with $NaBT_4$, and degraded under conditions of acid hydrolysis that selectively cleaved the 2-*O*-sulfo-L-idopyranosidic linkages. The resulting, radiolabelled-disaccharides and -tetrasaccharides were isolated by gel chromatography, and then fractionated by ion-exchange chromatography, paper chromatography, and paper electrophoresis. Of the nine disaccharides isolated and identified, eight were probably derived from the major repeating-disaccharide unit in heparin (2-deoxy-2-sulfoamino-D-glucosyl 6-sulfate → L-idosyluronic acid 2-sulfate). Sodium borotritide reduction and/or HNO_2 deamination of these eight disaccharide fractions indicated four to contain L-idopyranose residues and the other four to contain 1,6-anhydro-L-idopyranose residues as terminal units. The latter, terminal unit probably represents a minor component formed during the acid hydrolysis. On the basis of *N*-acetylation, *N*-sulfation, and HNO_2-deamination studies, and the known positions and configurations of the glycosidic and sulfate linkages in heparin, four disaccharides were identified as *O*-(2-amino-2-deoxy-α-D-glucopyranosyl)-(1→4)-L-[6-^{3}H]idopyranose, *O*-(2-amino-2-deoxy-α-D-glucopyranosyl 6-sulfate)-(1→4)-L-[6-^{3}H]idopyranose, *O*-(2-amino-2-deoxy-α-D-glucopyranosyl)-(1→4)-L-[6-^{3}H]idopyranose 2-sulfate, and *O*-(2-amino-2-deoxy-α-D-glucopyranosyl 6-sulfate]-(1→4)-L-[6-^{3}H]idopyranose 2-sulfate. A similar set of four disaccharides contained 1,6-anhydro-L-[6-^{3}H]idopyranose residues in place of the L-[6-^{3}H]idopyranose residues. The other disaccharide was tentatively identified as *O*-(2-acetamido-2-deoxy-α-D-glucopyranosyl)-(1→4)-L-[6-^{3}H]idopyranose, the isolation of which suggests the presence of an IdA(OSO_3^-)–GlcNAc–IdA(OSO_3^-) sequence in the heparin preparation, which accounts for at least 1% of its total sequence. The tetrasaccharides were fractionated, on the basis of their sulfate content, into at least five species by ion-exchange chromatography or by paper electrophoresis. These were fractionated further into species with and without carboxyl groups, and with L-idopyranose or 1,6-anhydro-L-idopyranose residues as terminal units. Tentative

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structures for some of these tetrasaccharides are proposed. Disaccharide and tetrasaccharide species were evaluated before and after *N*-acetylation or *N*-sulfation, as substrates for sulfamidase, acetyl-CoA: 2-amino-2-deoxy-α-D-glucoside *N*-acetyltransferase, 2-acetamido-2-deoxy-α-D-glucosidase, or 2-acetamido-2-deoxy-D-glucose 6-sulfate sulfatase in human-skin fibroblasts.

INTRODUCTION

Heparin and heparan sulfate are degraded by the sequential action of at least seven, highly specific, lysosomal enzymes that act at the non-reducing end of the glycosaminoglycan chains. Of these enzymes, α-L-iduronidase, 2-*O*-sulfo-L-idosiduronic acid 2-sulfatase, and β-D-glucuronidase are also involved in the degradation of dermatan sulfate[1-3]. The remaining four lysosomal enzymes, sulfamidase (2-deoxy-2-sulfoamino-α-D-glucoside 2-sulfamidase), 2-acetamido-2-deoxy-α-D-glucosidase, acetyl-CoA:2-amino-2-deoxy-α-D-glucoside *N*-acetyltransferase, and 2-acetamido-2-deoxy-D-glucose 6-sulfate sulfatase are only involved in the degradation of heparin and heparan sulfate[1,2,4,5,6].

Genetic disorders of glycosaminoglycan catabolism have been described where the activity of one of these lysosomal enzymes is grossly reduced, leading to the accumulation of partially degraded glycosaminoglycans in lysosomes and the expression of clinical defects[1,2,6]. 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 the structures of the presumed natural substrate[7-11]. Radiolabelled disaccharides prepared from heparin *via* deamination with nitrous acid may be utilised as substrates for α-L-iduronidase, β-D-glucuronidase, and 2-*O*-sulfo-L-idosiduronic acid 2-sulfatase[11-19]. Selective depolymerisation by degradation with nitrous acid produces oligosaccharides having glycosyluronic acid non-reducing end-groups. The other lysosomal enzymes involved in the degradation of heparin and heparan sulfate specifically require 2-amino-2-deoxy-α-D-glucosyl non-reducing end-groups. We have taken advantage of the extreme acid-lability of the 2-*O*-sulfo-L-idopyranosidic bond in carboxyl-reduced heparin (CR-heparin)[20] to produce radiolabelled oligosaccharides (mostly disaccharides) having 2-amino-2-deoxy-α-D-glucosyl groups at their non-reducing end, which are useful substrates for 2-acetamido-2-deoxy-α-D-glucosidase, acetyl CoA:2-amino-2-deoxy-α-D-glucoside *N*-acetyltransferase, 2-acetamido-2-deoxy-D-glucose 6-sulfate sulfatase, and sulfamidase. We now report on these oligosaccharides and their enzymic degradation or modification by homogenates of cultured human-skin fibroblasts.

RESULTS AND DISCUSSION

Isolation of radiolabelled disaccharide and tetrasaccharide fragments from the

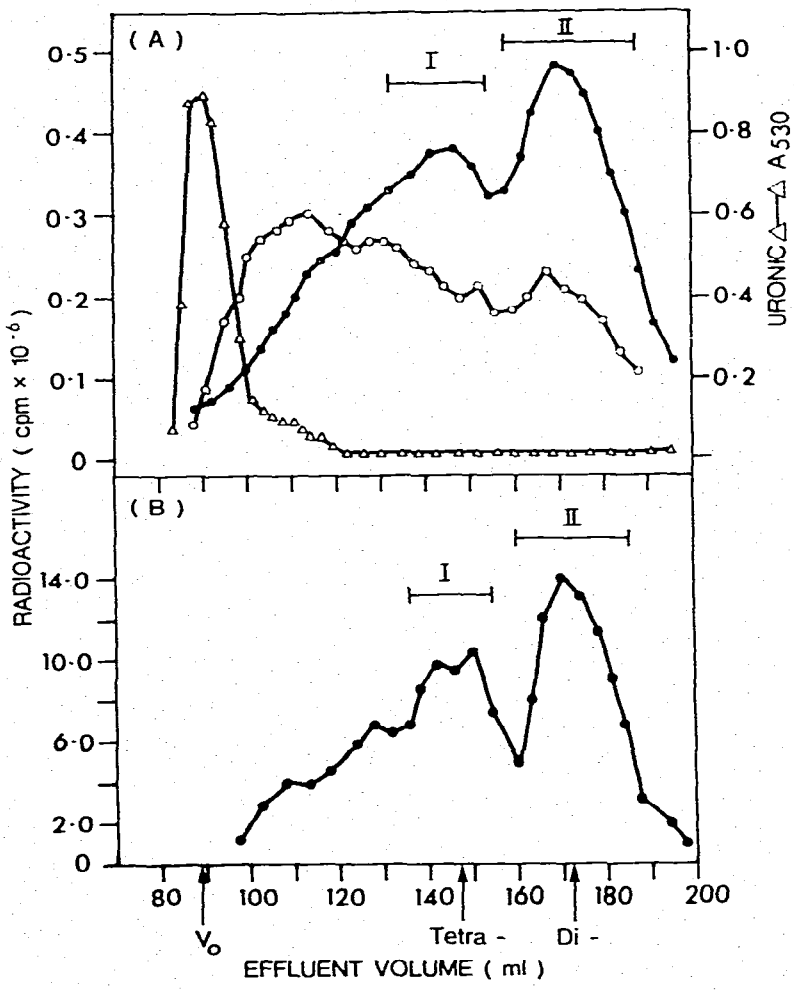


Fig. 1. Gel chromatography of products derived from the acid hydrolysis of CR-heparin and heparin. (A) CR-Heparin (1.7 mg), prepared using the second procedure (see Experimental), was heated at 100° in 0.1M HCl (1.0 ml) for 30 min (○—○) or in 0.2M HCl for 1 h (●—●). Heparin (5 mg) was heated at 100° in 0.2M HCl (2.5 ml) for 1 h (△—△). The acid hydrolysate was neutralised with 2M NaOH, 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 7–8 ml/h. (B) CR-heparin was acid-hydrolysed, applied to and eluted from Sephadex G-25 as described for the procedure of choice (see Experimental). Arrows indicate peak elution of Blue Dextran (V_0), tetrasaccharide, and disaccharide standards. Fractions were combined as indicated (|——|).

acid hydrolysate of CR-heparin. — The acid lability[21,22] of 2-*O*-sulfo-L-idopyranosidic bonds has been used as a basis for the selective cleavage of CR-heparin and thence, using $NaBT_4$, to produce a variety of radiolabelled disaccharides and tetrasaccharides.

The effects of acid concentration and time of hydrolysis on the degradation of CR-heparin were evaluated by fractionation of the hydrolysates by gel filtration on Sephadex G-25; two major, radiolabelled fractions were isolated (Fig. 1A). The more-

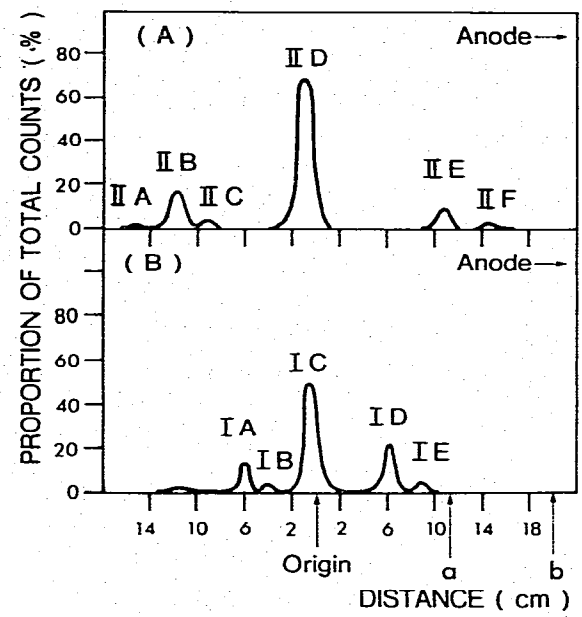


Fig. 2. H.v.e. at pH 1.7: (A), fraction II (Fig. 1B); (B), fraction I (Fig. 1B). Arrows indicate the peak position of standard disaccharides: a, *O*-(α-L-idopyranosyluronic acid)-(1→4)-(2,5-anhydro-D-[1-^{3}H]mannitol 6-sulfate)[16]; b, *O*-(α-L-idopyranosyluronic acid 2-sulfate)-(1→4)-(2,5-anhydro-D-[1-^{3}H]mannitol 6-sulfate)[16].

retarded (fraction II) and less-retarded (fraction I) peaks of radiolabelled material had the same elution position as the reference disaccharide and tetrasaccharide standards, respectively. Heparin, under similar conditions of acid hydrolysis, did not yield significant amounts of tetrasaccharides or disaccharides (Fig. 1A). The extreme acid-lability of 2-*O*-sulfo-L-idopyranosidic bonds[20,21] compared to the stability of 2-amino-2-deoxy-α-D-glucosidic bonds[22] dictates the selection of 2-amino-2-deoxy-D-glucoside oligosaccharides and limits the production of monosaccharides (idopyranose and its derivatives) and trisaccharides.

Since a relatively greater proportion of disaccharide was produced in the 0.2M HCl hydrolysate, these conditions were chosen to produce the oligosaccharide fractions for detailed structural studies (Fig. 1B). The disaccharide fraction (II, Fig. 1B) derived from CR-heparin by procedure *I* was subjected to h.v.e. at pH 1.7 to give six radioactive sub-fractions (IIA–F, Fig. 2), whereas the "tetrasaccharide" fraction (I, Fig. 1B) gave at least five (fractions IA–E, Fig. 2B).

Clearly, the primary requirement of this method for the production of disaccharides is a repeating sequence of 2-*O*-sulfo-L-idopyranosyl-containing fragments. Thus, the yield of disaccharide should be related to the length of this repeat-sequence within a particular preparation of heparin. For the production of maximum yields of disaccharides from heparin, it is therefore important to carboxyl-reduce all of the 2-*O*-sulfo-L-iduronic acid residues. The absence of detectable uronic acid and the

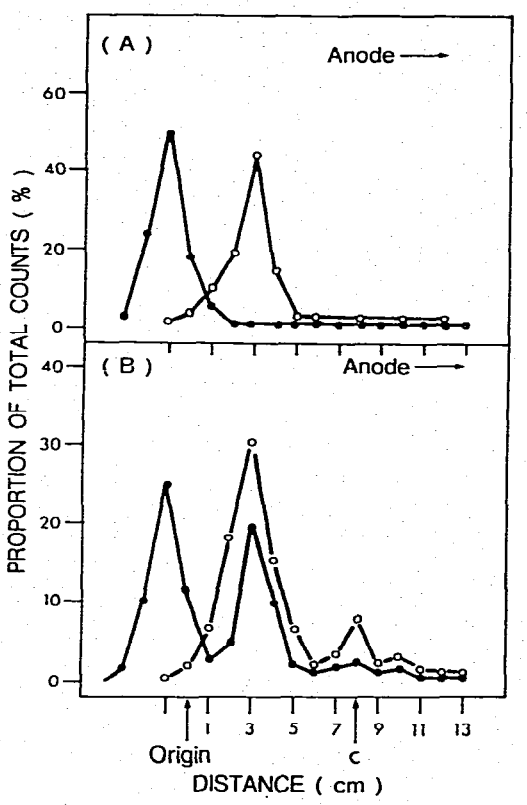


Fig. 3. H.v.e. at pH 5.1 of fraction IC (●—●) and fraction ID (○—○) recovered from positions IC and ID, respectively (Fig. 2B). Fractions (IC and ID) were isolated from CR-heparin, using (A) the first method (see Experimental) and (B) the second method of carboxyl-reduction and acid hydrolysis under the same conditions as used in (A). Arrows indicate the origin and peak position of the standard disaccharide c, *O*-(α-L-idopyranosyluronic acid)-(1→4)-(2,5-anhydro-D-[1-^{3}H]mannitol)[16].

similar electrophoretic mobilities at pH 1.7 and 5.1 of the various tetrasaccharides isolated from CR-heparin suggested that carboxyl-reduction of all 2-*O*-sulfo-L-iduronic acid residues had been achieved (Fig. 3A). On the other hand, the tetrasaccharides derived from heparin, using two other two methods of carboxyl-reduction, contained species that *do* require an extra negative charge at pH 5.1 (Fig. 3B). These results suggest that reduction of all 2-*O*-sulfo-L-iduronic acid carboxyl-groups in heparin is achieved by using procedure *1*, but not procedure *2* or *3*.

The disaccharide fraction (II, Fig. 1B) was desalted on a column of Sephadex G-10 and fractionated by ion-exchange chromatography on CM-Sephadex C-25 into three radiolabelled sub-fractions (Fig. 4). These fractions were subsequently shown by h.v.e. to contain fractions IID–F in the void peak, and IIB,C and IIB in the two bound-peaks. The amount of IIB in fractions IIB,C was diminished by a further fractionation on CM-Sephadex C-25. Fraction IID–F was subjected to ion-exchange chromatography on Dowex 1, to give two major peaks of radioactive material

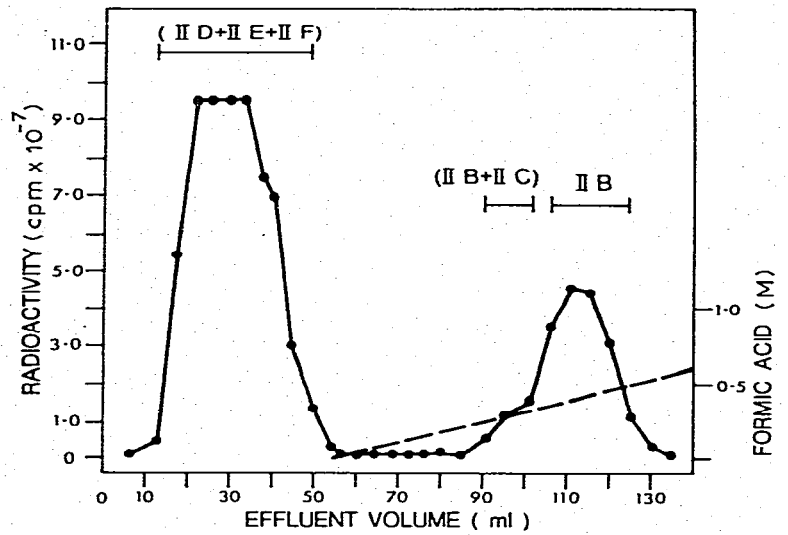


Fig. 4. Ion-exchange chromatography on CM-Sephadex C-25 of fraction II from Fig. 1B. Desalted II was applied to a column (2 × 10 cm) of CM-Sephadex C-25 (H^+ form), washed with water (25 ml), and eluted at 26 ml/h at 25° with a linear gradient generated from water and 1.5M formic acid by using an LKB 11300 Ultrograd gradient mixer. Fractions of ~5 ml were collected, assayed for radioactivity (●—●), and combined as indicated (|——|).

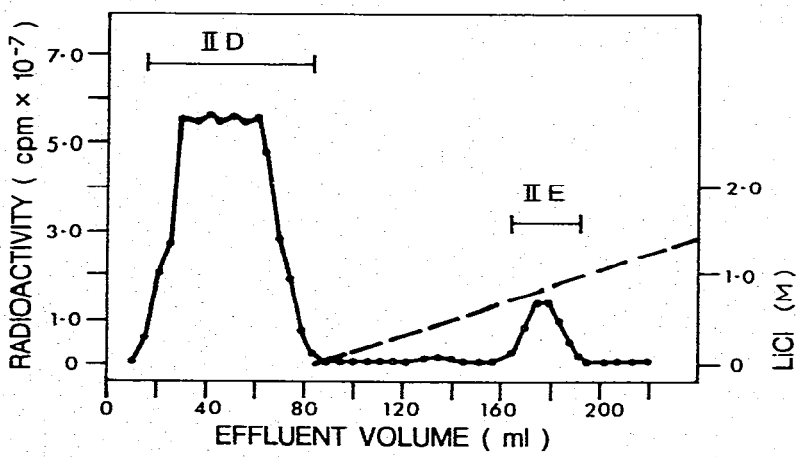


Fig. 5. Ion-exchange chromatography of fraction IID–F from Fig. 4. Fraction IID–F was rotary-evaporated, diluted to 50 ml with water, and applied to a column (2 × 10 cm) of Dowex 1 (Cl^- form), washed with water (40 ml), and eluted at 26 ml/h at 25° with a linear gradient generated from water and 2.0M LiCl by using an LKB 11300 Ultrograd gradient mixer. Fractions of ~5 ml were collected, assayed for radioactivity (●——●), and combined as indicated (|——|).

(Fig. 5). Based on h.v.e., the void peak contained IID and the bound peak IIE. Identification of other (minor) peaks bound to Dowex 1 was not attempted.

The recovery of radiolabelled material from both ion-exchange chromatographic steps was >85%. Fractions IIB (Fig. 4), IID, and IIE (Fig. 5) were each further

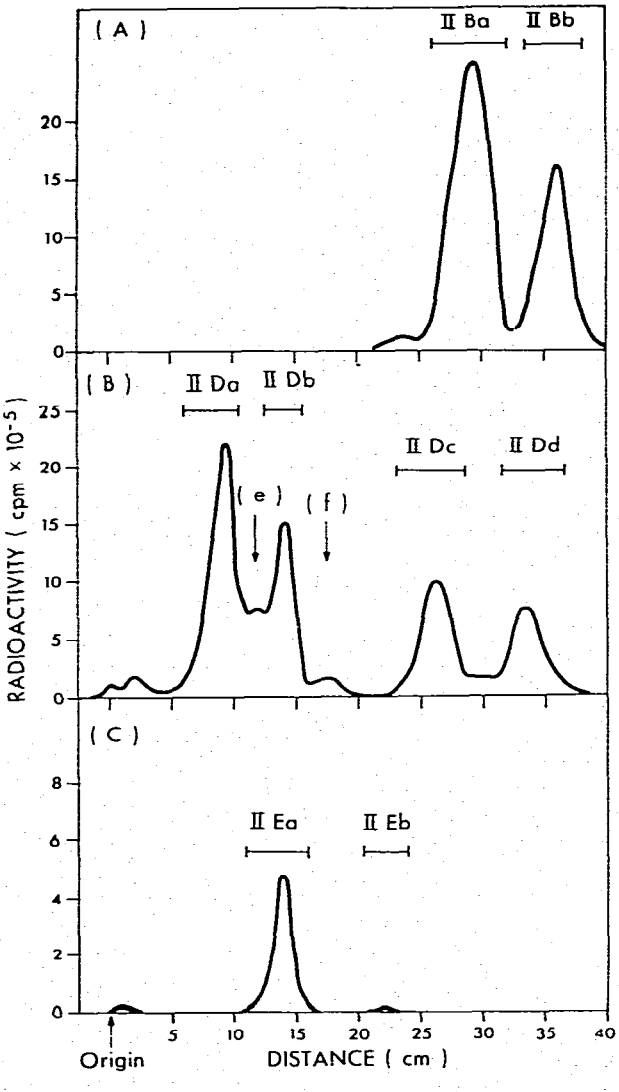


Fig. 6. Preparative p.c. (solvent *1*) of desalted fractions IIB, IID, and IIE from Figs. 4 and 5. Radiochromatography scans of (A) IIB run for 22 h; (B) IID run for 28 h; and (C) IIE run for 84 h at 25°. Radiolabelled material from zones indicated (|——|) were eluted with water.

fractionated by preparative p.c. into two (IIBa and IIBb), four (IIDa, IIDb, IIDc, and IIDa), and two (IIEa and IIEb) radiolabelled components, respectively (Fig. 6). The relative amounts of these components are shown in Table I.

Fraction I (Fig. 1B) was desalted on a column of Sephadex G-10 and separated into two fractions by ion-exchange chromatography on CM-Sephadex C-25 (Fig. 7). The void peak and bound peak of radioactive material were shown by h.v.e. to contain fractions IC–E and IA,B, respectively. Preparative h.v.e. was then used to

TABLE I

DATA FOR PRODUCTS OBTAINED FROM CR-HEPARIN BY PROCEDURE *1*

Product	*Yield (mCi)*	*H.v.e. (pH 1.7)* (R_a[a])	*P.c. (solvent 1)* ($R_{GlcN\ 6\text{-}SO_4^-}$[d])
CR-Heparin	3.0	—[b]	—
I	1.30	—	—
II	1.10	—	—
IAa	0.15	−0.55	0.50
IAb	0.02	−0.55	1.05
IB	0.09	−0.30	0.10
IC	0.65	0	—
ID	0.30	+0.55	—
IE	0.10	+0.80	—
IIA	0.02	−1.25	—
IIBa	0.13	−1.00	1.75
IIBb	0.06	−1.00	2.35
IIC	0.04	−0.75	—
IIDa	0.30	0	0.65
IIDb	0.23	0	0.90
IIDc	0.12	0	1.80
IIDd	0.10	0	2.45
IIEa	0.07	+0.95	0.20
IIEb	trace[c]	+0.95	0.35

[a]Relative to standard monosulfated disaccharide a (Fig. 2). [b]Not studied. [c]~2% of IIEa. [d]Values for reference compounds: 2-amino-2-deoxy-D-glucose, 2.80; D-galactose, 2.0; 2-acetamido-2-deoxy-D-glucose 6-sulfate, 1.40; 2,5-anhydromannitol 6-sulfate, 1.40; 2-deoxy-2-sulfoamino-D-glucose, 1.00; D-glucose 6-sulfate, 0.65; and 2-deoxy-2-sulfoamino-D-glucose 6-sulfate, 0.20.

isolate each individual component, the relative amounts of which are shown in Table I.

Identification of disaccharide fractions. — (*a*) *Fractions IIBa and IIBb.* Both fractions migrated with a net positive charge in h.v.e. After *N*-acetylation, both remained at the origin; after *N*-sulfation, both species had an h.v.e. mobility similar to that of a monosulfated disaccharide standard (Fig. 8B,C).

Nitrous acid deamination (procedure *1*) of IIBa and IIBb produced major, radiolabelled components having mobilities in p.c. similar to those reported for L-idofuranose and 2,5-anhydro-L-idopyranose, respectively[20] (Fig. 8D). Reduction of IIBa with $NaBT_4$ gave a product having increased specific radioactivity and which, on degradation with nitrous acid (procedure *1*), yielded iditol (identified by p.c.). The specific radioactivity of IIBb was not increased following treatment with $NaBT_4$. On the basis of these results and according to current concepts of heparin structure, the radiolabelled components in IIBa and IIBb may be identified tentatively as *O*-(2-amino-2-deoxy-α-D-glucopyranosyl)-(1→4)-L-[6-^{3}H]idopyranose (GlcN–Id) and *O*-(2-amino-2-deoxy-α-D-glucopyranosyl)-(1→4)-1,6-anhydro-L-[6-^{3}H]idopyranose (GlcN–anId), respectively (Fig. 10).

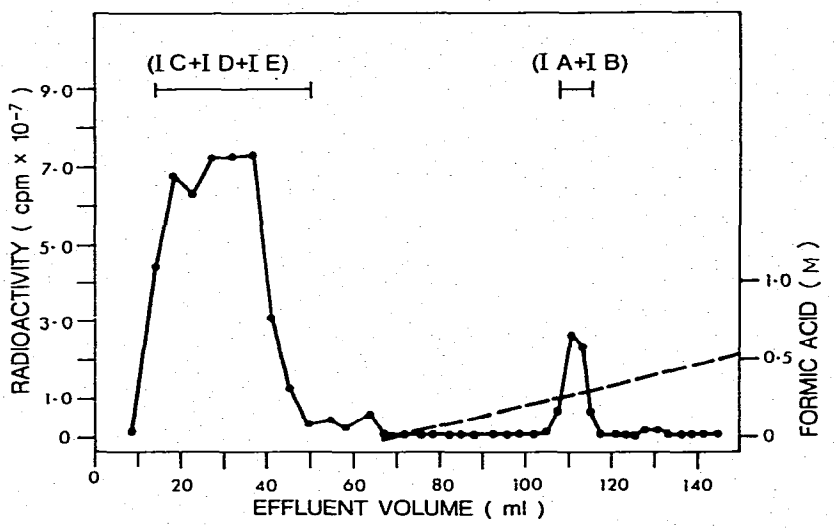
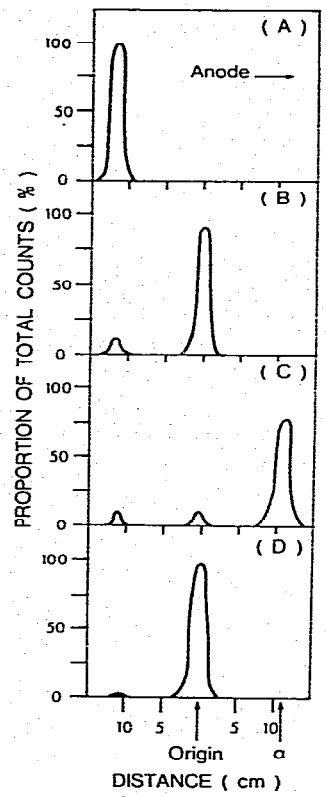


Fig. 7. Ion-exchange chromatography of fraction I from Fig. 1B. Desalted I was applied to a column (2 × 10 cm) of CM-Sephadex C-25 (H^+ form), washed with water (30 ml), and eluted as described in the legend to Fig. 4. Fractions of ~3 ml were collected, assayed for radioactivity (●——●), and combined as indicated (|——|).

(*b*) *Fractions IIDa and IIDb*. Both fractions had a net charge of zero. However, after *N*-acetylation, both IIDa and IIDb migrated in h.v.e. as monosulfated disaccharides or, after *N*-sulfation, as disulfated disaccharides (Figs. 9B,C; IIDb not shown). Nitrous acid degradation (procedure *1*) of IIDa and IIDb and h.v.e. of the products at pH 1.7 suggested the presence of at least three radiolabelled species in both IIDa and IIDb (Fig. 9D; IIDb data not shown). Nitrous acid degradation of IIDa produced a major radiolabelled component (89% of total radioactivity) that was not charged (h.v.e.) and which, in p.c. (solvent *3*), produced two components (sub-fractions iii and iv, Fig. 11A) making up 14% and 75%, respectively, of the total IIDa radioactivity and migrating with the same mobilities as L-idopyranose and L-idofuranose, respectively. The two minor radiolabelled components produced by the nitrous acid degradation of IIDa migrated in h.v.e. as a monosulfated monosaccharide and monosulfated disaccharide representing ~2.4% and ~8.6%, respectively, of the total IIDa radioactivity (Fig. 9D). The production of a sulfated disaccharide having a net negative charge of one at pH 1.7, from nitrous acid degradation of IIDa, most probably results from deamination, followed by internal *ring contraction* to *2-deoxy-2-C-formyl-D-pentofuranosyl* residues without cleavage of glycosidic bonds[20,23–25]. When IIDa was heated with 0.4M HCl for 1.5 h at 100°, a major radiolabelled component (75% of the total radioactivity) was produced, which migrated in h.v.e. with the same mobility as IIBa. On the basis of these results, IIDa contains two radiolabelled components, tentatively identified as *O*-(2-amino-2-deoxy-α-D-glucopyranosyl 6-sulfate)-(1→4)-L-[6-^{3}H]idopyranose [GlcN(OS)-Id], and *O*-(2-amino-2-deoxy-α-D-glucopyranosyl)-(1→4)-L-[6-^{3}H]idopyranose 2-sulfate



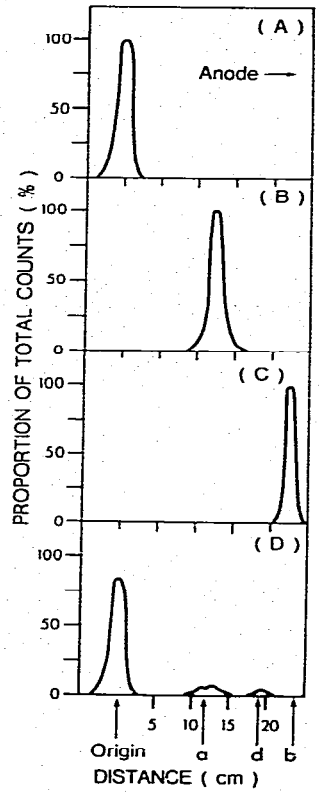


Fig. 8. Identification by h.v.e. at pH 1.7 of products formed from the chemical modification of IIBa. Paper strips were cut into 1-cm segments and counted to locate and quantitate radiolabelled reaction-products. Panels show (A) unmodified IIBa; (B) *N*-acetylated IIBa; (C) *N*-sulfated IIBa; and (D) IIBa treated with nitrous acid (procedure *I*). Arrows indicate the origin and peak position of the mono-sulphated disaccharide a (see legend to Fig. 2).

Fig. 9. Identification by h.v.e. at pH 1.7 of products formed from the chemical modification of IIDa. Samples were analysed as described in the legend to Fig. 8. Panels show (A) unmodified IIDa; (B) *N*-acetylated IIDa; (C) *N*-sulfated IIDa; and (D) IIDa treated with nitrous acid (procedure *I*). Arrows indicate: origin; peak positions of standards a and b (see legend to Fig. 2); and 2,5-anhydro-mannitol 6-sulfate[16] (d).

[GlcN–Id(OS)] (Fig. 10), making up ~98% and ~2%, respectively, of the total radioactivity in this fraction.

Degradation of fraction IIDb with nitrous acid produced a major (81% of total radioactivity) radiolabelled component (sub-fraction v, Fig. 11B) that was not charged at pH 1.7 and migrated in p.c. (solvent *3*) with the mobility reported for 1,6-anhydro-L-idopyranose[20]. The other radiolabelled component (sub-fraction i plus ii, Fig. 11B) migrated in h.v.e. as a monosulfated disaccharide and a monosulfated monosaccharide, which represent ~3% and ~16%, respectively, of the total radio-activity present in IIDb. Only traces (<5%) of L-idopyranose and L-idofuranose were detected (Fig. 11B). These results suggest that IIDb contained *O*-(2-amino-2-

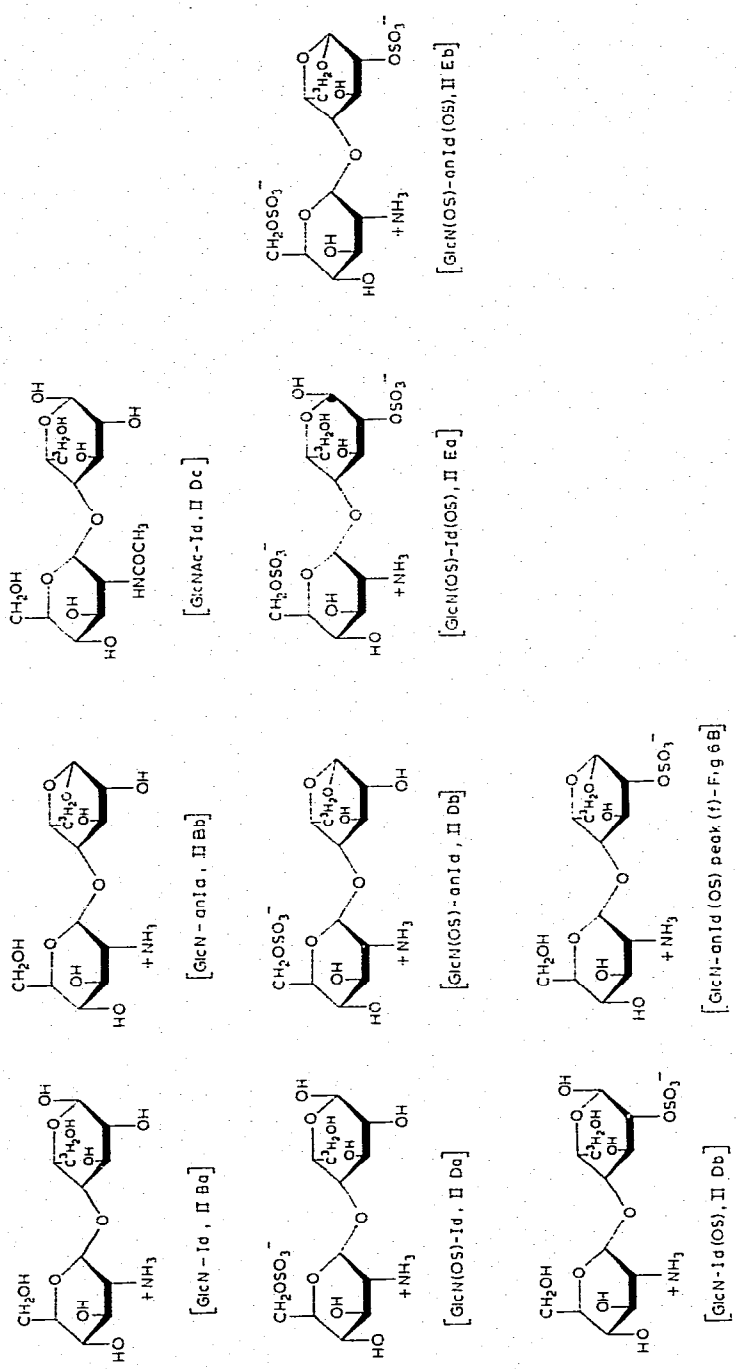


Fig. 10. Proposed structures of disaccharides isolated from acid-hydrolysis products of CR-heparin. The notations in square brackets are those used throughout the text for structure reference and fraction identification, respectively.

deoxy-α-D-glucopyranosyl 6-sulfate)-(1→4)-1,6-anhydro-L-[6-^{3}H]idopyranose [GlcN(OS)–anId] and *O*-(2-amino-2-deoxy-α-D-glucopyranosyl)-(1→4)-L-[6-^{3}H]-idopyranose-2-sulfate [GlcN–Id(OS)] (Fig. 10) representing ~84% and ~16%, respectively, of the total radioactivity present in this fraction. Minor peak (e) (Fig. 6A) appeared to contain a mixture of GlcN(OS)–Id, GlcN(OS)–anId, and GlcN–Id(OS). The concentration of GlcN–Id(OS), identified from the products of nitrous acid degradation, represents ~28% of the total radioactivity present in peak (e) and ~2% of that of IID. Preparative p.c. (solvent *1*) of the material of peak (e) produced a fraction that contained >90% of GlcN–Id(OS). The presence of minor component GlcN–Id(OS) in IIDa, IIDb, and peak (e) probably resulted from its intermediate mobility and therefore overlap with the two major species GlcN(OS)–Id and GlcN(OS)–anId identified in IIDa and IIDb, respectively. The overall concentration of GlcN–Id(OS) in IID is ~10%. Minor peak (f) (Fig. 6B) is tentatively identified as *O*-(2-amino-2-deoxy-α-D-glucopyranosyl)-(1→4)-1,6-anhydro-L-[6-^{3}H]idopyranose 2-sulfate [GlcN–anId(OS), Fig. 10], which represents ~1% of the total radioactivity in IID.

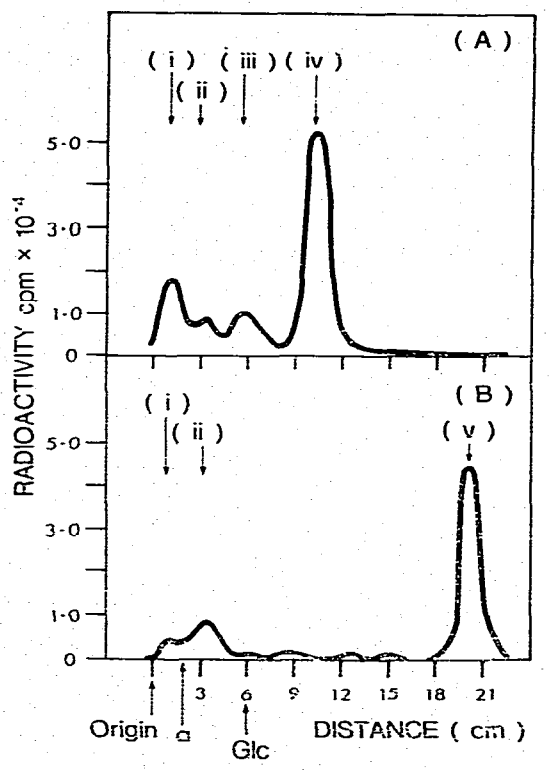


Fig. 11. Identification by p.c. in system *3* of products formed after nitrous acid treatment (procedure *1*) of (A) IIDa and (B) IIDb. Tracings of radiochromatographic scans are shown. Peaks indicated (i)–(v) were eluted with water, and the recovered, radiolabelled material was subjected to h.v.e. at pH 1.7. Arrows indicate the origin, and peak positions of standard d (Fig. 9) and D-[^{14}C]glucose (Glc).

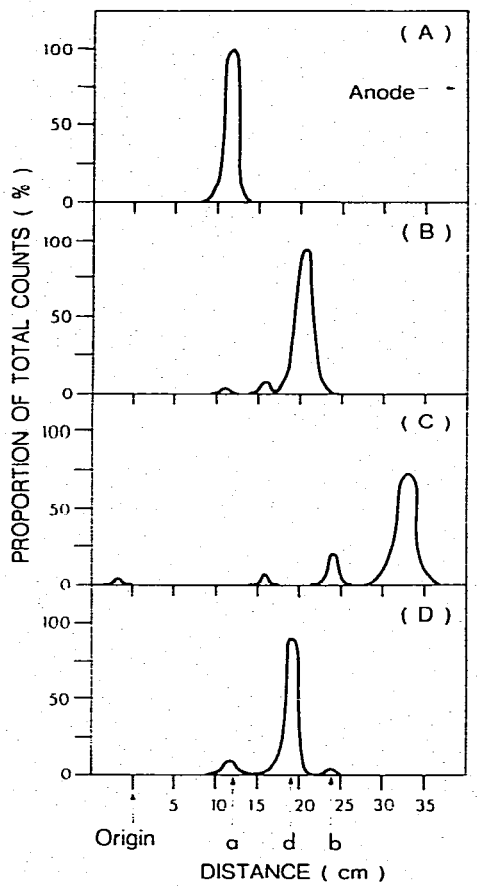


Fig. 12. Identification by h.v.e. at pH 1.7 of products formed from the chemical modification of IIE. Samples were analysed as described in the legend to Fig. 8. Panels show (A) unmodified IIE; (B) *N*-acetylated IIE; (C) *N*-sulfated IIE; and (D) IIE treated with nitrous acid (procedure *1*). Arrows indicate the origin, and peak positions of standards a, b, and d (see legends of Figs. 2 and 9).

(*c*) *Fractions IIDc and IIDd*. After h.v.e., both fractions remained at the origin before and after treatment under conditions for *N*-acetylation, *N*-sulfation, or nitrous acid deamination (procedures *1* and *2*). Fraction IIDc gave two major components in p.c. (solvent *2*). The slower moving component (30% of the radioactivity in IIDc) had the same mobility as *N*-acetylated IIBa in p.c. (solvents *1–3*). The faster moving component had the same mobility as idopyranose in p.c. (solvents *2* and *3*) and represented ~70% of the radioactivity in IIDc. Fraction IIDd migrated with a mobility slightly different from that of *N*-acetylated IIBb in p.c. (solvent *3*). On the basis of these results, it is tentatively proposed that the radiolabelled disaccharide component in fraction IIDc is *O*-(2-acetamido-2-deoxy-α-D-glucopyranosyl)-(1→4)-L-[6-^{3}H]idopyranose (GlcNAc–Id, Fig. 10); ~5% of the total radioactivity in IID is GlcNAc–Id. The structure of the component(s) in IIDd, representing ~13% of the total radioactivity in IID, is unknown.

Although GlcNAc–Id was tentatively identified in IID, GlcNAc(OS)–Id was not detected in IIE. This finding, if confirmed by other methods, indicates that, although this heparin preparation contains GlcNAc regions, it is devoid of *O*-sulfated GlcNAc residues. The occurrence of GlcNAc linked (1→4) to 2-*O*-sulfo-L-idopyranosyl residues is an unexpected finding in view of the reported structural requirements of glucuronosyl C-5-epimerase, which displays very low activity toward glycosyluronic acid residues sandwiched between GlcNAc residues[26]. To produce the proposed disaccharide structures GlcNAc–Id in IIDc, the heparin preparation used must contain the sequence IdA(OSO_3^-)–GlcNAc–IdA(OSO_3^-). This sequence represents ~1.3% of the total heparin structure (Table I) and would concentrate in the tetrasaccharide and higher oligosaccharide fractions that result from nitrous acid degradation of this heparin preparation previously reported[16] to account for ~20% of the total structure.

Nitrous acid (procedure *2*) quantitatively deaminates 2-deoxy-2-sulfoamino-D-glucosyl residues and then, in most cases, cleaves the glycosidic linkage to produce 2,5-anhydro-D-mannose residues, or, occasionally, yields 2-deoxy-2-*C*-formyl-D-pentofuranosyl residues without cleavage of glycosidic linkages[23,25]. Thus, estimations of GlcNAc content, by reference to the level of tetrasaccharide and higher oligosaccharides produced by nitrous acid degradation, are probably over-estimates. Lindberg *et al.*[25] and Shively[27] reported that the glycosidic bond of 2-deoxy-2-*C*-hydroxymethyl-D-pentofuranosyl residues is labile in dilute acid. Of the aldehyde-reduced tetrasaccharides produced from nitrous acid degradation of this heparin preparation[16], ~50% were cleaved to disaccharides by heating in 0.1M HCl at 100° for 30 min (Muller and Hopwood, unpublished observations). These results suggest that this heparin may contain up to 10% of GlcNAc residues. Cifonelli and King[28,29], using a combined periodate–nitrous acid degradation system, presented evidence for the presence of a GlcNAc–IdA(OSO_3^-)–GlcNAc(OSO_3^-) sequence in heparin and, particularly, in heparan sulfate. They reported[28] that GlcNAc residues constituted ~14% of the hexosamine residues of a hog-mucosal heparin preparation and that some GlcNAc may be associated with sulfated uronic acid residues. Thus, the presence of 1.3% of GlcNAc–Id and the suggestion that sulfate-free GlcNAc residues are linked to O-4 of 2-*O*-sulfo-L-iduronic acid residues are consistent with these findings.

Nitrous acid deamination has been used to study sequence repetition of 2-deoxy-2-sulfoamino-D-glucosyl residues in heparan sulphate and heparin[16,19,20,23,27,29]. Likewise, selective cleavage of 2-*O*-sulfo-L-idopyranosidic bonds could be used to study and identify the sequence of 2-*O*-sulfo-L-iduronic acid disaccharide-units in glycosaminoglycans that contain these residues. It should also be possible to use this method for the sequence analysis of GlcNAc–IdA(OSO_3^-) regions in heparin-like polysaccharides, which has proved difficult in the past because of the lack of specific methods of degradation directed at this residue. The development of new methods for the elucidation of repeat-sequences in glycosaminoglycans (heparin, heparan sulfate, and dermatan sulfate) that contain 2-*O*-sulfo-L-iduronic acid is important, as such procedures would facilitate studies of the effect of glycosaminoglycan struc-

ture on biological function[30]. For example, the binding of heparin to antithrombin, a prerequisite to blood anticoagulation, requires one or more specific sequences of the many possible in heparin[31–33].

(*d*) *Fractions IIEa and IIEb.* Both fractions migrated with a net negative charge consistent with a monosulfated disaccharide. Insufficient IIEb was available for further study. After *N*-acetylation or *N*-sulfation, IIEa migrated with a net negative charge consistent with a disulfated and trisulfated disaccharide, respectively (Fig. 12B,C). Nitrous acid deamination (procedure *1*) produced a single, major, radioactive component which migrated in h.v.e. as a monosulfated monosaccharide and in p.c. (system *1*) had the same mobility as 2-*O*-sulfo-L-idopyranose.

On the basis of these results and those described for the other disaccharide fractions, the major radiolabelled components in fraction IIEa and, with less certainty, IIEb may be tentatively identified as *O*-(2-amino-2-deoxy-α-D-glucopyranosyl 6-sulfate)-(1→4)-L-[6-^{3}H]idopyranose 2-sulfate [GlcN(OS)–Id(OS)] and *O*-(2-amino-2-deoxy-α-D-glucopyranosyl 6-sulfate)-(1→4)-1,6-anhydro-L-[6-^{3}H]idopyranose 2-sulfate [GlcN(OS)–anId(OS)], respectively (Fig. 10); ~98% of the total radioactivity was due to GlcN(OS)–Id(OS).

The yield of 2-sulfated disaccharides [GlcN–Id(OS), GlcN–anId(OS), GlcN(OS)–Id(OS), and GlcN(OS)–anId(OS)] produced from the acid cleavage of 2-*O*-sulfo-L-idopyranosidic bonds in CR-heparin was ~10% of the total disaccharide fraction (II). Shively and Conrad[20] reported that there may be two rates of ester-sulfate hydrolysis for 2-*O*-sulfo-L-idopyranosides, involving (*a*) a concerted cleavage of glycosidic and sulfate-ester bonds and (*b*) glycosidic hydrolysis to yield 2-*O*-sulfo-L-idopyranose which undergoes a lower rate of ester-sulfate hydrolysis. Acid hydrolysis of *O*-(α-L-idopyranosyluronic acid 2-sulfate)-(1→4)-(2,5-anhydro-D-[1-^{3}H]-mannitol 6-sulfate)[16], under similar conditions used to produce fraction II (Fig. 1B), left a residue of at least 6% of 2-sulfated disaccharide. This result suggests that acid cleavage of 2-*O*-sulfo-L-idopyranosidic bonds, under these conditions, occurred without the concomitant loss of 2-sulfate esters, to produce 2-sulfated disaccharides that were subsequently desulfated by acid hydrolysis.

Identification of tetrasaccharide fractions. — Preliminary studies suggested the presence of many components in these fractions. We have, therefore, only attempted the tentative identification of the major radiolabelled component(s) in each fraction (IA–E).

(*a*) *Fraction IA.* Based on its electrophoretic migration toward the cathode and the chromatographic migration of the major radiolabelled component (IAa) at ~50% of the rate for IIBa, and its elution position from Sephadex G-25, IA was tentatively identified as a tetrasaccharide having a single, positive net charge. The radiolabelled species in IA do not contain unreduced uronic acid residues, since they have the same electrophoretic migration at pH 1.7 and 5.1. P.c. (solvent *1*) showed IA to contain two components, IAa and IAb (Table I). Sub-fraction IAa accounted for >80% of the total radioactivity and, after *N*-acetylation, migrated electrophoretically toward the anode at a rate expected for a monosulfated tetrasaccharide.

After nitrous acid degradation (procedure *1*), IAa produced approximately equal amounts of a neutral and a negatively charged component. Thus, it is proposed that the major radiolabelled component in IAa is probably a monosulfated GlcN–Id(or Glc)–GlcN–Id tetrasaccharide.

Following *N*-acetylation, IAb remained at the origin after h.v.e. at pH 1.7, which suggested that, unlike IAa, it is not sulfated and probably has the structure mono-*N*-acetylated GlcN–Id(or Glc)–GlcN–Id. It must be emphasised that these proposals for IAa and IAb are very tentative.

(*b*) *Fraction IB.* Its chromatographic and electrophoretic migration-rates before and after *N*-acetylation suggest that it may be a disulfated hexasaccharide.

(*c*) *Fractions IC–E.* Identification of IC, ID, and IE produced from CR-heparin (procedure *1*) were not studied further than by h.v.e. at pH 5.1, to indicate the absence of carboxyl groups. IC and ID, isolated from CR-heparin (procedure *2*), had a net zero and negative charge, respectively, in h.v.e., and each gave at least two, major, radiolabelled species when subjected to h.v.e. at pH 5.1 (Fig. 3B). Approximately 50% of IC remained at the origin and 40% moved toward the anode at a rate expected for a tetrasaccharide having a single negative charge (Fig. 3B). Approximately 75% of ID remained with a mobility expected for a tetrasaccharide having a net negative charge of one, and 20% moved as a tetrasaccharide having a net negative charge of two. These results suggest that IC and ID both contain tetrasaccharide species having carboxyl groups.

Degradation (modification) of oligosaccharide fractions by cultured human-skin fibroblast homogenates. — Demonstration that some of the foregoing, radiolabelled oligosaccharides are specifically modified (or degraded) by lysosomal enzymes was achieved by using fibroblast homogenates established from skin biopsies from patients having various mucopolysaccharide, lysosomal storage disorders (mucopolysaccharidoses). It must be stressed that, although some optimisation has been attempted, the conditions selected for incubation of the various oligosaccharide fractions were those reported to be optimal for the degradation of more conventional substrates.

(*a*) *Sulfamidase.* Disaccharides GlcN–Id, GlcN–anId, GlcN(OS)–Id, GlcN–Id(OS), and GlcN(OS)–Id(OS) (Fig. 10) and tetrasaccharide IA were *N*-sulfated and evaluated as substrates for sulfamidase. GlcNS(OS)–Id, GlcNS(OS)–Id(OS), *N*-sulphated IA oligosaccharides, and $GlcNSO_3^-$ were not degraded at a detectable level by normal fibroblast homogenates incubated between pH 3.0 and 6.0 for up to 24 h. Disaccharides GlcNS–Id and GlcNS–Id(OS), and the *N*-sulfated tetrasaccharide, prepared from fraction IA following acid hydrolysis to remove *O*-sulfate groups, were *N*-desulfated by normal fibroblast homogenates. The tetrasaccharide and GlcNS–Id were degraded at a rate 400 times less than that observed with *N*-[^{35}S]sulfated heparin (Table II). In contrast, the sulfamidase activity measured with GlcNS–Id(OS) was 100 times greater than observed for GlcNS–Id. We have recently shown that *N*-sulfated disaccharides with adjacent-residue carboxyl groups [*e.g.*, *O*-(2-deoxy-2-sulfoamino-α-D-glucopyranosyl)-(1→3)-2,5-anhydro-L-[6-^{3}H]idonic acid and *O*-(2-deoxy-2-sulfoamino-α-D-glucopyranosyl)-(1→3)-L-gulonic acid] are

TABLE II

DEGRADATION OF *N*-SULFATED OLIGOSACCHARIDES WITH WHOLE-CELL HOMOGENATES PREPARED FROM CULTURED HUMAN-SKIN FIBROBLASTS

Substrate	*Sulfamidase activity (pmol/min/mg)*[a]	
	Normal fibroblasts	*Sanfilippo A fibroblasts*
N-[^{35}S]sulfated heparin	4.00	n.d.
GlcNS–Id[b]	0.01	n.d.
GlcNS(OS)–Id[c]	n.d.	—
GlcNS(OS)–Id(OS)[d]	n.d.	n.d.
GlcNS–Id(OS)[e]	1.00	n.d.
N-sulfated IA	n.d.	—
N-sulfated IA (acid-hydrolysed)[f]	0.01	—
GlcNS	n.d.	n.d.

[a]Key: n.d., none detected; —, not measured. [b–e]Prepared by *N*-sulfation of the radiolabelled species present in fractions IIBa, IIDa, IIEa, and peak (e), respectively. [f]Hydrolysed with 0.4M HCl at 100° for 1.5 h before *N*-sulfation.

TABLE III

DEGRADATION OF *N*-ACETYLATED OLIGOSACCHARIDES WITH WHOLE-CELL HOMOGENATES PREPARED FROM CULTURED HUMAN-SKIN FIBROBLASTS

Substrate	*2-Acetamido-2-deoxy-α-D-glucosidase activity (pmol/min/mg)*	
	Normal fibroblasts	*Sanfilippo B fibroblasts*
GlcNAc–Id (IIDc)	2.1	0.18
GlcNAc–Id (*N*-acetylated IIBa)	1.7, 1.8	0.06, 0.05
GlcNAc–anId (*N*-acetylated IIBb)	0.3, 0.6	n.d.
IIDd	n.d.[a]	n.d.
GlcN–Id (IIBa)	n.d.	—[a]
GlcNAc–Id (OS) (*N*-acetylated peak e)	0.6	0.04

[a]Key: n.d., not detected; —, not measured.

degraded by sulfamidase at 400 times the rate observed[34] for GlcNS–Id. These differences suggest that the presence of a 2-sulfate group and a C-5 carboxyl group on the sugar residue adjacent to the GlcNS moiety under enzymic attack are important structural determinants for the action of sulfamidase. The absence of measurable sulfamidase activity on GlcNS(OS)–Id and GlcNS(OS)–Id(OS) suggests that the 6-sulfate residue prevents the action of sulfamidase on these disaccharides.

N-Desulfation of GlcNS–Id and GlcNS–Id(OS) could not be detected when they were incubated with Sanfilippo A (sulfamidase-deficient) fibroblasts (Table II).

TABLE IV

N-ACETYL TRANSFER FROM ACETYL-CoA TO OLIGOSACCHARIDES BY WHOLE-CELL HOMOGENATES PREPARED FROM CULTURED HUMAN-SKIN FIBROBLASTS

Substrate	*Acetyl-CoA: 2-amino-2-deoxy-α-D-glucoside* N-*acetyl transferase activity (pmol/min/mg)* *Normal fibroblasts*
GlcN–Id[a]	5.8
GlcN–anId[b]	5.6
GlcN(OS)–Id[c]	n.d.[e]
GlcN(OS)–Id(OS)[d]	n.d.
IA	n.d.

[a–d]Radiolabelled species present in fractions IIBa, IIBb, IIDa, and IIEa, respectively. [e]n.d., not detected.

This finding suggests that degradation of these substrates requires the specific action of sulfamidase.

(*b*) *2-Acetamido-2-deoxy-α-D-glucosidase.* *N*-Acetylated IIBa and the disaccharide component in IIDc were degraded to L-[6-^{3}H]idopyranose, whereas *N*-acetylated IIBb gave 1,6-anhydro-L-[6-^{3}H]idopyranose after incubation with normal fibroblast homogenates. Fraction IIDd was not degraded when incubated with fibroblast homogenate. As yet it has not been possible to identify the structure(s) in IIDd. Degradation was not observed when incubations were performed with Sanfilippo B (2-acetamido-2-deoxy-α-D-glucosidase-deficient) fibroblast homogenates (Table III). These findings corroborate the chemical data used to assign the structures shown in Fig. 10. 2-Amino-2-deoxy-α-D-glucosidase activity, which would cleave the glycosidic bond in GlcN–Id, could not be detected in fibroblast homogenates. This finding is consistent with observations[6] that the enzyme acetyl-CoA:2-amino-2-deoxy-α-D-glucoside *N*-acetyltransferase in the presence of acetyl-CoA acetylates 2-amino-2-deoxy-α-D-glucosyl residues to produce substrates that are then degraded by 2-acetamido-2-deoxy-α-D-glucosidase.

(*c*) *Acetyl-CoA: 2-amino-2-deoxy-α-D-glucoside* N-*acetyltransferase.* The GlcN–Id component in IIBa and GlcN–anId in IIBb were *N*-acetylated by incubation with normal fibroblast homogenates. It was not possible to detect *N*-acetylated, radiolabelled products after incubation of IIDa [GlcN(OS)–Id] or IIEa [GlcN(OS)–Id(OS)] with normal fibroblast homogenates (Table IV). These results suggest that, under the conditions of incubation, the 2-amino group will not be acetylated if there is a sulfate residue at position 6 of the GlcN residue. Tetrasaccharide IAa was not *N*-acetylated under the enzyme conditions evaluated, suggesting that the GlcN residue at the non-reducing end of IAa may be 6-sulfated.

(*d*) *2-Acetamido-2-deoxy-D-glucose 6-sulfate sulfatase.* Only the disaccharide fractions were evaluated as substrates for this enzyme. *N*-Acetylated or *N*-sulfated IIDa and IIE were all degraded by normal fibroblast homogenates at a rate similar

TABLE V

DESULFATION OF OLIGOSACCHARIDES WITH DIALYSED WHOLE-CELL HOMOGENATES PREPARED FROM CULTURED HUMAN-SKIN FIBROBLASTS

Substrate	*2-Acetamido-2-deoxy-D-glucose 6-sulfate sulfatase activity (pmol/min/mg) Normal fibroblasts*
GlcNS(OS)–Id (*N*-sulfated IIDa)	0.5
GlcNS(OS)–Id(OS) (*N*-sulfated IIE)	1.5
GlcNAc(OS)–Id(OS) (*N*-acetylated IIE)	1.2
GlcN(OS)–Id(OS) (IIE)	None detected
GlcNAc(OS)	0.6

to that observed for 2-acetamido-2-deoxy-D-glucose 6-sulfate (Table V). Under the incubation conditions used, the radiolabelled components in IIDa and IIE were not desulfated unless the amino group on each was acetylated or sulfated.

Degradation of IIE by concentrated, urinary proteins. — Incubation of *N*-acetylated or *N*-sulfated IIE with urinary protein gave a series of products that accounted for 45% of the total radioactivity (Fig. 13). H.v.e. of incubations containing *N*-sulfated IIE, with a net negative charge of three, showed initial degradation to a radiolabelled disaccharide having a net negative charge of two (product A1, Fig. 13A) and then to a component (A2) which remained near the origin. It is proposed that A1 is GlcNS–Id(OS) and a result of the action of 2-acetamido-2-deoxy-D-glucose 6-sulfate sulfatase on *N*-sulfated IIE [GlcNS(OS)–Id(OS)], whereas A2 is probably GlcN–Id(OS) and results from the action of sulfamidase on A1. Further degradation of A2 by the action of 2-amino-2-deoxy-α-D-glucosidase or 2-amino-2-deoxy-α-D-glucoside *N*-acetyltransferase and then 2-acetamido-2-deoxy-α-D-glucosidase in the presence or absence of acetyl-CoA was not detected. *N*-Acetylated IIE [GlcNAc(OS)–Id(OS)], with a net negative charge of two, was degraded to disaccharide having a net negative charge of one (product B1) and then to a monosulfated monosaccharide (B2, Fig. 13B). It is proposed that B1 [GlcNAc–Id(OS)] results from the action of 2-acetamido-2-deoxy-D-glucose 6-sulfate sulfatase on GlcNAc(OS)–Id(OS) and that B2 (2-*O*-sulfo-L-idopyranose) is derived by the action of 2-acetamido-2-deoxy-α-D-glucosidase on B1. Thus, detection of 2-acetamido-2-deoxy-D-glucose 6-sulfate sulfatase in conjunction with sulfamidase or 2-acetamido-2-deoxy-α-D-glucosidase activities is possible with *N*-sulfated IIE and *N*-acetylated IIE, respectively. Interestingly, *no* detectable breakdown was observed when unmodified IIE was incubated with urinary protein under conditions where 47% degradation of *N*-acetylated or *N*-sulfated IIE was observed. This finding strengthens the observations made with fibroblasts that 2-acetamido-2-deoxy-D-glucose 6-sulfate sulfatase will not act on substrates having a free 2-amino group, even under conditions where 47% of the 6-sulfate is removed from *N*-acetylated or *N*-sulfated IIE. Thus, 2-acetamido-2-deoxy-D-glucose 6-sulfate sulfatase may also be termed a 2-deoxy-

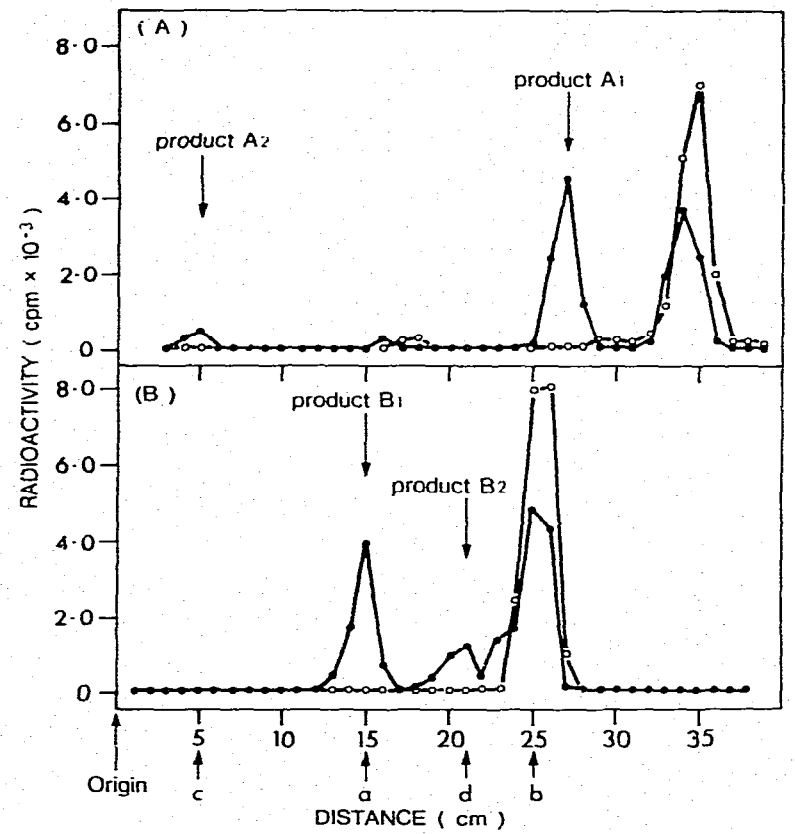


Fig. 13. Separation by h.v.e. at pH 1.7 of radiolabelled products (●——●) formed after incubation of (A) *N*-sulfated IIE (○——○) and (B) *N*-acetylated IIE (○——○) with urinary protein. Arrows indicate the origin, and peak positions of products and standards a–d (see legends of Figs. 2, 3, and 10).

2-sulfoamino-D-glucose 6-sulfate sulfatase but *not* a 2-amino-2-deoxy-D-glucose 6-sulfate sulfatase.

EXPERIMENTAL

Materials. — Heparin (Grade II, from pig-intestinal mucosa, Sigma Chemical Co.) was purified[35] by repeated precipitation with cetylpyridinium chloride from 1.2M NaCl. *N*-[^{35}S]Sulfated heparin (150 mCi/mg), $NaBT_4$ (10 Ci/mmol), D-[U-^{14}C]glucose (336 mCi/mmol), 2-acetamido-2-deoxy-D-[1-^{3}H]glucose (5 Ci/mmol), and 2-amino-2-deoxy-D-[1-^{14}C]glucose hydrochloride (55 mCi/mmol) were obtained from The Radiochemical Centre (Amersham, Bucks, U.K.). Blue Dextran, Sephadex G-10 and G-25 (superfine), and CM-Sephadex C-25 were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden), and Dowex 1-X4 (100–200 mesh) and Dowex 50W-X4 (100–200 mesh) resins from Bio-Rad Laboratories (Richmond, CA, U.S.A.).

1-Ethyl-3-(3-dimethylaminopropyl)carbodi-imide hydrochloride (EDC) and acetyl-CoA were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Monosulfated and disulfated disaccharide and tetrasaccharide reference-compounds were prepared by nitrous acid degradation of heparin as previously described[16].

Fibroblasts [sulfamidase-deficient, GM-312-Mucopolysaccharidosis Type IIIA (MPS IIIA)], Sanfilippo A; 2-acetamido-2-deoxy-α-D-glucosidase-deficient, GM-156-MPS IIIB, Sanfilippo B] were obtained from the Human Genetic Cell Repository (Institute for Medical Research, Camden, NJ, U.S.A.). Similar enzyme-deficient lines and normal fibroblasts were established from skin biopsies available to this hospital. All lines were maintained in culture as described by Carey and Pollard[36], and used for the preparation of cell homogenate 6–10 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 Shandon Southern Model L-24 System. Unless otherwise stated in the text, h.v.e. was conducted at pH 1.7, a pH at which all carboxyl groups were assumed to be uncharged. Paper chromatography (p.c.) was performed on Whatman 3MM paper with descending development in the following solvents: *1*, 1-butanol–acetic acid–M aqueous ammonia (2:3:1); *2*, 1-butanol–ethanol–water (4:1:1); and *3*, on Whatman No. 1 paper with ethyl acetate–acetic acid–formic acid–water (18:3:1:4). Hexitols were separated[37] on Whatman 3MM paper sprayed with 0.5mM $Na_2B_4O_7$ and developed in ethyl acetate–pyridine–5mM H_3BO_3 (3:2:1) (solvent *4*). Preparative experiments were conducted with paper strips, pre-washed with the solvent to be used. Reference radiolabelled 6-*O*-sulfo and 2-deoxy-2-sulfoamino monosaccharides were prepared by methods described below for chemical modification of oligosaccharide fractions.

Gel chromatography was conducted by using glass columns filled with Sephadex G-10 or G-25 equilibrated with 10% aqueous ethanol or 0.2M NaCl in 0.05M sodium acetate buffer (pH 5.3), respectively, as described in the text or legends to Figures. Fractions were desalted by passage through a column (1 × 100 cm) of Sephadex G-10 eluted with 10% aqueous ethanol.

Uronic acid was determined by the carbazole method of Bitter and Muir[38], using D-glucurono-6,3-lactone as the standard; total hexose by the anthrone method[39] with D-glucose as the standard; and protein by the Folin method of Lowry *et al.*[40], using crystalline bovine serum albumin (Sigma) as the standard.

Radioactivity was measured with either a Searle Analytic Model 6868 ISOCAP/300 Ambient Temperature Liquid Scintillation Counter, or a Packard Radiochromatogram Scanner model 7201. For scintillation counting, aqueous samples (1.0 ml) were mixed with 2 ml of PCS solubiliser (Amersham/Searle, Arlington Heights, IL, U.S.A.) in 5-ml polypropylene vials (Medical Plastics Pty. Ltd., Edwardstown, S.A., Australia), inserted in a Pico-Holder (Packard, Chicago, IL, U.S.A.). Under these conditions, tritium was determined at ~30% efficiency.

Borotritide reduction of EDC-activated heparin. — Heparin was carboxyl-reduced by a modification of the procedure described by Taylor and Conrad[41] and

Shively and Conrad[23]. Under the conditions of Taylor and Conrad, the reduced product retained all of the *N*- and *O*-sulfate groups[20]. Three methods were used for the carboxyl-reduction of heparin. In two, radiolabel was introduced by reduction of EDC-activated heparin with $NaBT_4$, whereas, in the third method, carboxyl groups in EDC-activated heparin were first reduced with $NaBH_4$, the product was hydrolysed to oligosaccharides with acid, and then the radiolabel was introduced by aldehyde reduction with $NaBT_4$.

Unless otherwise referred to in the text, the following procedure (*1*) was the method of choice for the preparation of radiolabelled oligosaccharides from heparin. Heparin (50 mg) in water (5.0 ml) was maintained at pH 4.75 with 0.1M HCl by a Radiometer Copenhagen Titrator II whilst EDC (300 mg) was added in one portion. The reaction at 25° consumed ~1.0 ml of 0.1M HCl during 0.5 h; the end-point was indicated by no further consumption of acid. The reaction mixture was dialysed against three changes of water (1 litre) at 4° over a 3-h period and rotary-evaporated at 30°, and the total volume was adjusted to 0.5 ml with water. $NaBT_4$ (38 mg; 250 Ci/mol) was added and the mixture was then heated at 50° for 2 h, cooled to room temperature, and acidified with acetic acid in a fume hood to release tritium gas. The reaction mixture was dialysed successively against two changes of water (2 litres), four changes of 0.2M NaCl (2 litres), and two changes of water (2 litres), applied to a column (1 × 100 cm) of Sephadex G-10, and eluted with 10% aqueous ethanol. The uronic acid- and hexose-containing fractions and radiolabelled material, all of which was eluted in the void volume, were combined and freeze-dried, and the residue was hydrolysed with 0.2M HCl (20 ml) at 100° for 1 h. The hydrolysate was neutralised with 2M NaOH, 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 7–8 ml/h.

Where indicated in the text, a second method was used to produce CR-heparin in which fewer carboxyl groups were reduced with $NaBT_4$. Heparin (10 mg) in 1.7 ml of water was maintained at pH 4.75 with 0.1M HCl, as described above, whilst EDC (30 mg) was added in one portion. The mixture was maintained at 25° for 0.5 h, dialysed as described above, and rotary-evaporated to a total volume of 0.5 ml. $NaBT_4$ (7.6 mg; 500 Ci/mmol) was added to a 0.1-ml aliquot, which was then heated at 50° for 2 h, cooled to room temperature, acidified, dialysed, and subjected to gel chromatography on Sephadex G-10 as described above.

In the third method, a solution of heparin (20 mg) in water (1.7 ml) was stirred with EDC (15 mg) and maintained at pH 4.75, as described above, for 45 min at 25°. $NaBH_4$ (130 mg) was added, and the reaction mixture was heated at 50° for 2 h, cooled to room temperature, acidified with acetic acid, dialysed, desalted on Sephadex G-10, acid-hydrolysed, and fractionated on Sephadex G-25 as in the first method described above. Material eluted in the tetrasaccharide region from Sephadex G-25 was desalted on a column of Sephadex G-10, freeze-dried, and incubated with $NaBT_4$ (11.4 mg; 220 Ci/mol) in 0.3M sodium borate buffer (0.3 ml, pH 7.8). After 1 h at 25°, the reaction mixture was acidified with acetic acid in a fume hood, rotary-

evaporated to dryness in the presence of methanol, dissolved in water (1 ml), and desalted on Sephadex G-10 as described above.

Chemical modification of radiolabelled oligosaccharide fractions isolated from the acid hydrolysate of CR-heparin. — (*a*) *Sulfation of amino groups.* Two procedures were used; both were adaptations of the method of Lloyd *et al.*[42]. The following method, unless otherwise stated in the text, was the method used for *N*-sulfation. A mixture consisting of ~10 nmol of radiolabelled oligosaccharide (or 2-amino-2-deoxy-D-[1-^{14}C]glucose hydrochloride), 3.5 μmol of $NaHCO_3$, and 2.0 μmol of sulfur trioxide–trimethylamine complex in a total volume of 12 μl was heated at 55° for 3 h and then subjected to h.v.e. at pH 1.7. In the other method, used as a preparative procedure, a mixture consisting of ~0.5 μmol of radiolabelled oligosaccharide (or 2-amino-2-deoxy-D-[1-^{14}C]glucose hydrochloride), 93 μmol of $NaHCO_3$, and 50 μmol of sulfur trioxide–trimethylamine complex in a total volume of 0.35 ml was heated for 3 h at 55°, cooled to room temperature, mixed with 50 μl of M $BaCl_2$, and centrifuged at 2000*g* for 10 min, and the supernatant solution was applied to a column containing Dowex 50 (H^+) resin (5 g) and washed with water (~20 ml). The eluate was neutralised with 0.2M NaOH, freeze-dried, and, where necessary, desalted on Sephadex G-10. Disaccharide preparations were purified by preparative p.c. (solvent *1*) or, like all tetrasaccharide preparations, subjected to ion-exchange chromatography on Dowex 1 resin, as previously described[16], except that the linear gradient was generated from 0.05M sodium acetate buffer (pH 5.1) and 2.0M LiCl in 0.05M sodium acetate buffer (pH 5.1). Combined fractions were desalted on Sephadex G-10.

(*b*) *N-Acetylation.* For small-scale reactions, a mixture of ~1 nmol of radiolabelled oligosaccharide (or 2-amino-2-deoxy-D-[1-^{14}C]glucose hydrochloride), 25 μmol of $NaHCO_3$, 10 nmol of acetic anhydride, and 150 nmol of methanol in a total volume of 50 μl was maintained at 4° for 1 h and then subjected to h.v.e. at pH 1.7. For preparative purposes, a mixture of ~50 nmol of radiolabelled oligosaccharide, 0.75 mmol of $NaHCO_3$, 300 nmol of acetic anhydride, and 3.5 μmol of methanol in a total volume of 0.75 ml was maintained at 4° for 1 h, acidified with formic acid, and fractionated by preparative p.c. (solvent *1*).

(*c*) *Sulfation of primary hydroxyl groups.* This reaction was carried out under anhydrous conditions and is an adaption of the method of Suzuki and Strominger[43]. To a mixture of 100 nmol of radiolabelled monosaccharide and 300 μl of pyridine at 0° was added, during 10 min, a solution of chlorosulfuric acid (75 μmol) in chloroform (30 μl). After 1 h at 4°, the mixture was left at 25° for 2 h, mixed with water (2 ml), neutralised with M KOH, and rotary-evaporated to dryness. A solution of the residue in water was mixed with M $BaCl_2$ (150 μl) and centrifuged at 2000*g* for 10 min. The supernatant solution was applied to a column of Dowex 50 (H^+) resin (5 g) and washed with water (~20 ml). The eluate was neutralised with M KOH, rotary-evaporated to ~0.1 ml, and purified by preparative p.c. (solvent *1*).

Nitrous degradation of radiolabelled oligosaccharides. — Two methods were used to discriminate species containing glucosamine residues having unsubstituted amino groups[44] (procedure *1*) from those containing sulfamate groups[23] (procedure

2). In the first procedure, <5 nmol of radiolabelled oligosaccharide was mixed with 1 μmol of H_2SO_4 and 27.5 μmol of $NaNO_2$ in a total volume of 7 μl and incubated at 25° for 15 min. In the second procedure, HNO_2 was prepared by mixing 0.5 mmol of H_2SO_4 with 0.5 mmol of $Ba(NO_2)_2$ in a total volume of 2 ml, as described by Shively and Conrad[23]. The deamination reaction was performed by mixing ~5 nmol (or less) of radiolabelled oligosaccharide in water (5 μl) with the HNO_2 solution (20 μl) and incubating at 25° for 15 min. The extent of deamination and the nature of the products were assessed by h.v.e. or p.c. of the whole reaction mixture.

Preparation of skin fibroblasts for enzyme assay. — Skin fibroblasts, cultured in 25-cm^2 Falcon Flasks (Bio Quest, Cockeysville, MD, U.S.A.) 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 the conditions described by Carey and Pollard[36]. Fibroblasts were suspended in 0.1% aqueous Triton X-100 (0.5 ml) and disrupted by freezing (solid CO_2–ethanol) and thawing six times.

Degradation of radiolabelled oligosaccharides by cultured skin-fibroblast homogenates. — Sulfamidase activity[45] was estimated by incubation of ~400 pmol of sulfamated, radiolabelled oligosaccharide with fibroblast homogenate (9 μl; containing ~40 μg of protein) in 16 μl of 0.13M sodium acetate buffer (pH 5.0; containing 6mM NaN_3 and 0.15M KCl) for 15 h at 45°. After incubation, the whole reaction mixture was subjected to h.v.e. at pH 1.7.

2-Acetamido-2-deoxy-α-D-glucosidase activity[45,46] was estimated by incubation of ~420 pmol of *N*-acetylated, radiolabelled oligosaccharide with fibroblast homogenate (4 μl, containing ~34 μg protein) in 12 μl of 0.05M sodium acetate buffer (pH 4.5; containing 6mM NaN_3) for 24 h at 37°. After incubation, the whole reaction mixture was subjected to p.c. (solvent *2*).

The assay for acetyl-CoA: 2-amino-2-deoxy-α-D-glucoside *N*-acetyltransferase activity[6] was performed by incubating ~40 pmol of radiolabelled oligosaccharide with fibroblast homogenate (3 μl, containing ~50 μg of protein) in 17 μl of 0.06M sodium acetate buffer (pH 5.5; containing 7.6mM NaN_3 and 4mM acetyl-CoA) for 15 h at 37°. After incubation, the whole reaction mixture was subjected to h.v.e. at pH 1.7.

For the estimation of 2-acetamido-2-deoxy-D-glucose 6-sulfate sulfatase activity[47], fibroblast homogenate was centrifuged at 600*g*, and the supernatant solution was dialysed for 16 h at 4° against 30mM sodium acetate buffer (pH 5.5); 10 μl of this preparation, containing ~90 μg of protein, was incubated with 400–500 pmol of radiolabelled oligosaccharide in 10 μl of 0.1M sodium acetate buffer (pH 6.0; containing 10mM NaN_3) for 15 h at 37°. After incubation, the whole reaction mixture was subjected to h.v.e. at pH 1.7.

Degradation of radiolabelled oligosaccharides by urinary proteins. — Urine from normal subjects was concentrated 50-fold and dialysed against water at 10° in an Amicon Hollow Fiber Dialyser/Concentrator fitted with a Diaflo Hollow Fiber Type H1P10. Radiolabelled oligosaccharide (~400 pmol) and urinary protein (150 μg) in 10 μl of concentrate and 10 μl of 0.1M sodium acetate buffer (pH 6.0;

containing 10mM NaN_3) was incubated at 45° for 15 h. The whole incubation mixture was then subjected to h.v.e. at pH 1.7.

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REFERENCES

1 A. Dorfman and R. Matalon, *Proc. Natl. Acad. Sci. U.S.A.*, 73 (1976) 630–637.
2 C. P. Legum, S. Schorr, and E. R. Berman, *Adv. Pediatr.*, 22 (1976) 305–347.
3 V. A. McKusick, E. F. Neufeld, and T. E. Kelly, in J. B. Stanbury, J. B. Wyngaarden, and D. S. Fredrickson (Eds.), *The Metabolic Basis of Inherited Disease*, 4th edn., McGraw-Hill, New York, 1978, pp. 1282–1307.
4 H. Kresse, E. Paschke, K. von Figura, W. Gilberg, and W. Fuchs, *Proc. Natl. Acad. Sci. U.S.A.*, 77 (1980) 6822–6826.
5 R. Basner, H. Kresse, and K. von Figura, *J. Biol. Chem.*, 254 (1979) 1151–1158.
6 U. Klein, H. Kresse, and K. von Figura, *Proc. Natl. Acad. Sci. U.S.A.*, 75 (1978) 5185–5189.
7 J. F. Tallman, R. O. Brady, R. Navon, and B. Padeh, *Nature (London)*, 252 (1974) 254–255.
8 M. Owada, T. Sakiyama, and T. Kitagawa, *Pediatr. Res.*, 11 (1977) 641–646.
9 J. S. O'Brien, A. G. W. Norden, A. L. Miller, R. G. Frost, and T. E. Kelly, *Clin. Genet.*, 11 (1977) 171–183.
10 Y. Ben-Yoseph and H. L. Nadler, *J. Clin. Pathol.*, 31 (1978) 1091–1093.
11 J. J. Hopwood and V. Muller, *Clin. Sci.*, 57 (1979) 265–271.
12 T. W. Lim, I. G. Leder, G. Bach, and E. F. Neufeld, *Carbohydr. Res.*, 37 (1974) 103–109.
13 P. Dinatale, I. G. Leder, and E. F. Neufeld, *Clin. Chim. Acta*, 77 (1977) 211–218.
14 J. J. Hopwood and V. Muller, *Uppsala J. Med. Sci.*, 82 (1977) 134 (Abstr.).
15 J. N. Thompson, *Clin. Chim. Acta*, 89 (1978) 435–443.
16 J. J. Hopwood, *Carbohydr. Res.*, 69 (1979) 203–216.
17 J. J. Hopwood, V. Muller, and A. C. Pollard, *Clin. Sci.*, 56 (1979) 591–599.
18 J. J. Hopwood, V. Muller, A. Smithson, and N. Baggett, *Clin. Chim. Acta*, 92 (1979) 257–265.
19 I. Jacobsson, M. Höök, I. Pettersson, U. Lindahl, O. Larm, E. Wirén, and K. von Figura, *Biochem. J.*, 179 (1979) 77–87.
20 J. E. Shively and H. E. Conrad, *Biochemistry*, 15 (1976) 3943–3950.
21 P. F. Lloyd and P. F. Forrester, *Carbohydr. Res.*, 19 (1971) 430–431.
22 R. C. G. Moggridge and A. Neuberger, *J. Chem. Soc.*, (1938) 745–750.
23 J. E. Shively and H. E. Conrad, *Biochemistry*, 15 (1976) 3932–3942.
24 C. Erbing, B. Lindberg, and S. Svensson, *Acta Chem. Scand.*, 27 (1973) 3699–3704.
25 B. Lindberg, J. Lönngren, and S. Svensson, *Adv. Carbohydr. Chem. Biochem.*, 31 (1975) 185–240.
26 I. Jacobsson and U. Lindahl, *J. Biol. Chem.*, 255 (1980) 5094–5100.
27 J. E. Shively, Ph.D. Dissertation, University of Illinois, 1975.
28 J. A. Cifonelli and J. King, *Connect. Tissue Res.*, 3 (1975) 97–104.
29 J. A. Cifonelli and J. King, *Biochemistry*, 16 (1977) 2137–2141.
30 U. Lindahl and M. Höök, *Annu. Rev. Biochem.*, 47 (1978) 385–417.
31 J. J. Hopwood, M. Höök, A. Linker, and U. Lindahl, *FEBS Lett.*, 69 (1976) 51–54.
32 R. D. Rosenberg, G. Armand, and L. Lam, *Proc. Natl. Acad. Sci. U.S.A.*, 75 (1978) 3065–3069.
33 J. Riesenfeld, M. Höök, and U. Lindahl, *Proc. Int. Symp. Glycoconjugates, 5th*, 1979, pp. 68–69 (Abstr.).
34 J. J. Hopwood and H. Elliott, *Proc. Aust. Biochem. Soc.*, 13 (1980) 1.
35 U. Lindahl, J. A. Cifonelli, B. Lindahl, and L. Rodén, *J. Biol. Chem.*, 240 (1965) 2817–2820.
36 W. F. Carey and A. C. Pollard, *Aust. J. Exp. Biol. Med. Sci.*, 55 (1977) 245–252.
37 H. E. Conrad, E. Varboncoeur, and M. E. James, *Anal. Biochem.*, 51 (1973) 486–500.
38 T. Bitter and H. M. Muir, *Anal. Biochem.*, 4 (1962) 330–334.

39 W. E. TREVELYAN AND J. R. HARRISON, *Biochem. J.*, 50 (1952) 298–303.
40 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR, AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265–275.
41 R. L. TAYLOR AND H. E. CONRAD, *Biochemistry*, 11 (1972) 1383–1388.
42 A. G. LLOYD, G. EMBERY, AND L. J. FOWLER, *Biochem. Pharmacol.*, 20 (1971) 637–648.
43 S. SUZUKI AND J. L. STROMINGER, *J. Biol. Chem.*, 235 (1960) 267–273.
44 U. LINDAHL, G. BÄCKSTRÖM, L. JANSSON, AND A. HALLÉN, *J. Biol. Chem.*, 248 (1973) 7234–7241.
45 P. WHITEMAN AND E. YOUNG, *Clin. Chim. Acta*, 76 (1977) 139–147.
46 K. VON FIGURA, M. LÖGERING, G. MERSMANN, AND H. KRESSE, *J. Pediatr.*, 83 (1973) 607–611.
47 A. L. HORWITZ AND A. DORFMAN, *Biochem. Biophys. Res. Commun.*, 80 (1978) 819–825.