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# SELECTIVE DEPOLYMERISATION OF KERATAN SULFATE: PRODUCTION OF RADIOLABELLED SUBSTRATES FOR 6-*O*-SULFOGALACTOSE SULFATASE AND β-D-GALACTOSIDASE

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ABSTRACT

Keratan sulfate (KS) was *N*-deacetylated with anhydrous hydrazine and then degraded with nitrous acid, and the products were reduced with $NaBT_4$. Radiolabelled disaccharides constituted at least 76% of the total oligosaccharide fraction. Three major disaccharides were isolated and identified. Of the total disaccharide isolated from bovine intervertebral-disc and human costal-cartilage, 91 and 79%, respectively, was identified as a disulfated disaccharide, *O*-(β-D-galactopyranosyl 6-sulfate)-(1→4)-2,5-anhydro-D-[1-$^3$H]mannitol 6-sulfate (Gal6S–anM6S). The disaccharide fraction isolated from bovine-cornea KS contained only 14% of Gal6S–anM6S. The yield of monosulfated disaccharide, identified as *O*-β-D-galactopyranosyl-(1→4)-2,5-anhydro-D- [1-$^3$H]mannitol 6-sulfate, was 9, 17, and 84% of the total KS-disaccharide fraction isolated from intervertebral disc, costal cartilage, and cornea, respectively. For each of the KS type studied, the yield of unsulfated disaccharide was <4% of the total disaccharide-fraction. The tetrasaccharides were fractionated, on the basis of their sulfate content, into at least four species by paper electrophoresis, and some tentative structures are proposed. Disaccharide and tetrasaccharide species were evaluated as substrates for β-D-galactosidase and 6-*O*-sulfogalactose sulfatase.

## INTRODUCTION

Although the nature of lysosomal degradation of keratan sulfate (KS) is not yet well defined, it appears that at least four enzymes, β-D-galactosidase, 6-*O*-sulfogalactose sulfatase, 2-acetamido-2-deoxy-D-glucose 6-sulfate sulfatase, and β-*N*-acetylhexosaminidase act in concert at the non-reducing end of the glycosaminoglycan chain[1]. Each of these exo-enzymes may also be involved in the lysosomal degradation of other glycoconjugates. For example, β-D-galactosidase also hydrolyses $GM_1$-ganglioside[2], 6-*O*-sulfogalactose sulfatase acts on chondroitin 6-sulfate[3], 2-acetamido-2-deoxy-D-glucose 6-sulfate sulfatase acts on heparan sulfate[4], and β-*N*-acetylhexosaminidase acts on $GM_2$-ganglioside[5]. Genetic disorders have been

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described where the activity of β-D-galactosidase or 6-*O*-sulfogalactose sulfatase is grossly diminished, leading to the accumulation of partially degraded KS in lysosomes and the expression of clinical defects[6]. Experience with the determination of the activities of mutant enzymes produced in genetic disorders has underlined the need to use substrates whose structures closely match those of the natural substrate[7–11]. This is particularly important with these four exo-enzyme activities, since each also degrades another structurally different substrate. We have obtained radiolabelled disaccharides by degradation of *N*-deacetylated KS with nitrous acid and now report their enzymic degradation by homogenates of cultured human-skin fibroblasts. The method used to prepare these radiolabelled disaccharides from KS may also be used to characterise KS species from different tissue sources.

## RESULTS AND DISCUSSION

*Isolation of radiolabelled disaccharide fragments from KS.* — *N*-Deacetylation of hexosamine residues[12] with hydrazine has been applied to KS 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 KS was evaluated by fractionation on Sephadex G-25 of the products of degradation with nitrous acid. Two major hexose-positive fractions were observed (Fig. 1). The more-retarded (II) and the less-retarded (I) peaks of hexose-positive material had the same elution position as the reference disaccharide and tetrasaccharide, respectively. Treatment of KS with nitrous acid did not yield detectable amounts of di- or tetra-saccharides (Fig. 1). The greatest proportion of disaccharide was produced from KS that had been treated with hydrazine for 4 h, and detailed structural studies were carried out on these products after reduction with $NaBT_4$. The disaccharide fraction (II, Fig. 1), representing a recovery of 62% of the starting-hexose KS, was desalted by using Sephadex G-10 and then reduced with $NaBT_4$. Electrophoresis of the products at pH 1.7 gave radiolabelled fractions IIA–C (Fig. 2) having mobilities similar to those reported[13,14] for unsulfated, monosulfated, and disulfated uronic acid disaccharides, respectively. The relative ratios of fractions

TABLE I

DATA FOR PRODUCTS OBTAINED FROM KS BY *N*-DEACETYLATION, DEAMINATION WITH NITROUS ACID AND REDUCTION WITH $NaBT_4$

| | *Composition (%)* | | | |
|---|---|---|---|---|
| | *I* | *IIA* | *IIB* | *IIC* |
| KS Type | | | | |
| Human costal-cartilage | 20.0 | 3.2 | 13.6 | 63.2 |
| Bovine intervertebral-disc | 15.7 | 0.3 | 7.3 | 76.7 |
| Bovine cornea | 24.0 | 1.3 | 63.6 | 11.1 |

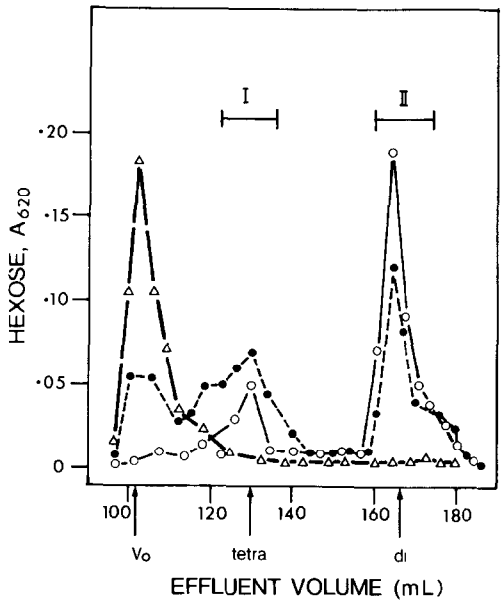



Fig. 1. Gel chromatography of products derived from intervertebral-disc KS and *N*-deacetylated KS by deamination with nitrous acids. KS *N*-deacetylated with hydrazine for 2 h (--●--) and 4 h (—○—) and KS (—△—) were deaminated (see Experimental), and the products were applied to a column (1 × 200 cm) of Sephadex G-25 and eluted with 0.2M NaCl in 0.05M sodium acetate buffer (pH 5.3) at 7–8 mL/h. Arrows indicate peak elution of Blue Dextran ($V_o$), tetrasaccharide, and disaccharide standards.

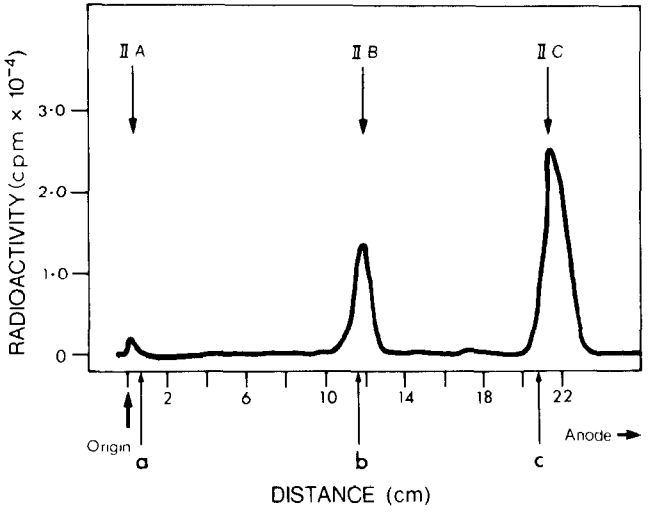



Fig. 2. Radiochromatographic scan after high-voltage electrophoresis at pH 1.7 of fraction II (Fig. 1). Arrows indicate the peak position of standard disaccharides: a, unsulfated disaccharide; b, monosulfated disaccharide; and c, disulfated disaccharide.

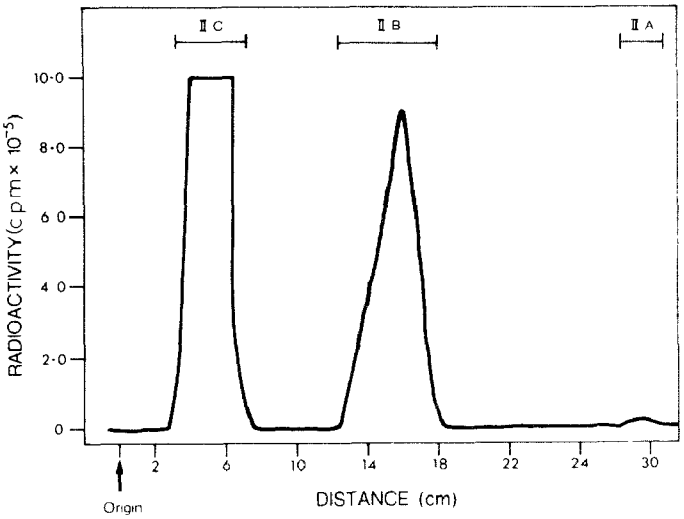
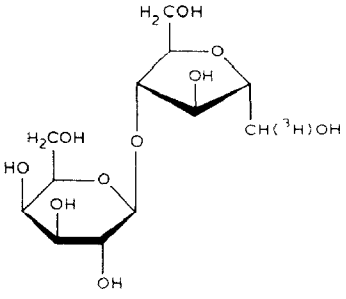
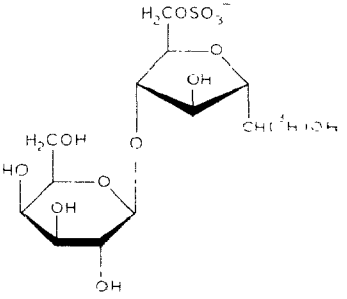


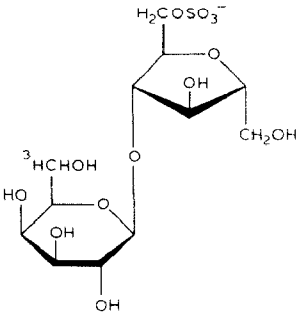

Fig. 3. Radiochromatographic scan after p.c (24 h, 25°, solvent *1*) of desalted fraction II from Fig. 1. Fractions IIA–IIC were eluted with water.



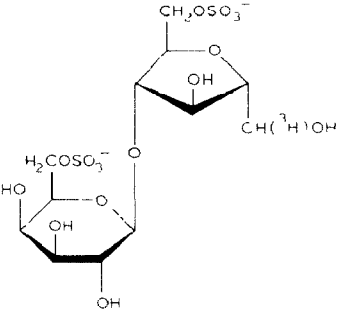
1 (Gal-anM, II A)



2 (Gal-anM6S, II B)



3 ([$^3$H]Gal-an M6S, II B)



4 (Gal 6S-anM6S, II c)

IIA–C for KS from human costal-cartilage, bovine intervertebral-disc, and bovine cornea are recorded in Table I. Paper chromatography of fraction II also gave fractions IIA–C (Fig. 3).

*Characterisation of Fractions IIA–C.* — These fractions were isolated by preparative p.c.; fraction IIA, which remained at the origin after electrophoresis at pH 1.7, was not studied further, but probably contained the unsulfated disaccharide, *O*-β-D-galactopyranosyl-(1→4)-2,5-anhydro-D-[1-$^3$H]mannitol (**1**, Gal–anM).

Fraction IIB migrated during electrophoresis at pH 1.7 as a single radiolabelled component having a mobility similar to that reported for monosulfated disaccharides derived from heparin[13,14]. The radiolabelled material in fraction IIB moved as a single component in p.c. using solvents (*2* and *3*) previously shown to separate monosulfated disaccharides on the basis of the position of the sulfate group[13,14]. Incubation of IIB with normal and 6-*O*-sulfogalactose sulfatase-deficient fibroblasts produced a radiolabelled fraction having an electrophoretic mobility at pH 1.7 similar to that of 2,5-anhydro-D-[1-$^3$H]mannitol 6-sulfate (Fig. 4). This product was not detected when IIB was incubated with β-D-galactosidase-deficient fibroblasts cultured from $GM_1$-gangliosidoses and mucopolysaccharidosis type-IVB (MPS-IVB) patients. Neutral, radiolabelled products were not detected in any of the fibroblast incubations with IIB. On the basis of these results and the products expected from *N*-deacetylated KS, IIB was assumed to contain mostly *O*-β-D-galactopyranosyl-(1→4)-2,5-anhydro-D-[1-$^3$H]mannitol 6-sulfate (**2**, Gal–an6S). The presence of a small proportion of *O*-(β-D-galactopyranosyl 6-sulfate)-(1→4)-2,5-anhydro-D-[1-$^3$H]mannitol in IIB cannot be excluded on the basis of the above negative data.

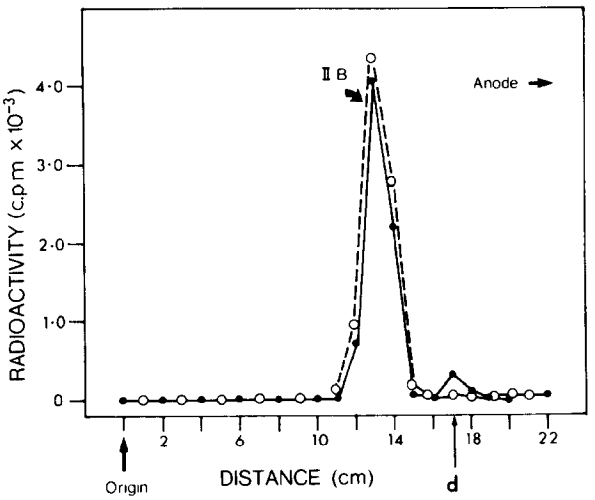



Fig. 4. Electrophoresis of incubation mixtures containing normal-fibroblast homogenate (—●—), MPS-IVB-fibroblast homogenate (--○--), and fraction IIB. Arrow d indicates the peak position of 2,5-anhydro-D-[1-$^3$H]mannitol 6-sulfate.

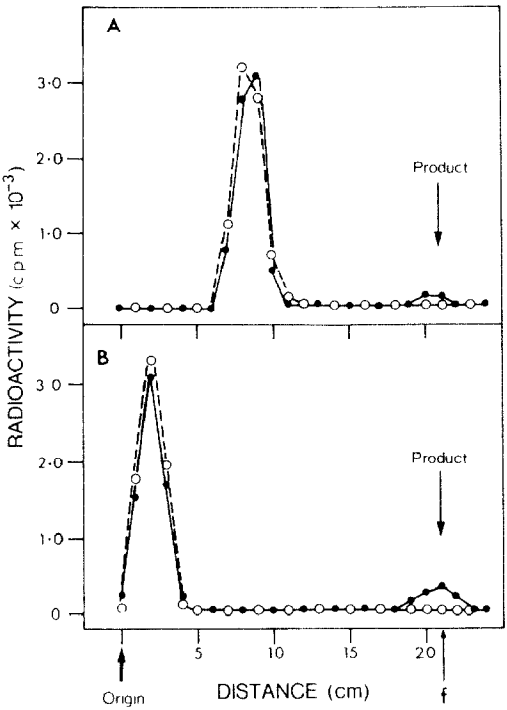



Fig. 5 P.c. (solvent *1*, 24 h) of incubation mixtures containing normal-fibroblast homogenate (—●—) or $GM_1$-gangliosidosis-fibroblast homogenate (--○--) A, [6-$^3$H]galactose-labelled IIB. B, [$^3$H]galactose-labelled IC Arrow f indicates the peak position of D-[U-$^{14}$C]galactose

Fraction II, produced using $NaBT_4$, was treated with D-galactose oxidase and then $NaBT_4$ to yield radiolabelled IIB which, on incubation with homogenates of normal fibroblasts, gave radiolabelled galactose (Fig. 5). Radiolabelled galactose was not produced on incubation of IIB with fibroblasts from $GM_1$-gangliosidosis and MPS-IVB patients. Thus IIB was assumed to contain mostly *O*-(β-D-[6-$^3$H]galactopyranosyl)-(1→4)-2,5-anhydro-D-mannitol 6-sulfate (**3**, [$^3$H]Gal–anM6S).

The radiolabelled material in fraction IIC moved as a single component during electrophoresis at pH 1.7 and had a mobility similar to that reported for a disulfated disaccharide derived from heparin [13,14]. Incubation of IIC with normal and β-D-galactosidase-deficient fibroblasts produced a radiolabelled fraction having an electrophoretic mobility similar to that of IIB (Fig. 6). This product was not detected when IIC was incubated with 6-*O*-sulfogalactose sulfatase-deficient fibroblasts. Radiolabel was not incorporated into IIC material when $NaBH_4$-reduced fraction II (Fig. 1) was treated with D-galactose oxidase and $NaBT_4$. On the basis of these results and from the expected deamination products of *N*-deacetylated KS, IIC was assumed to be *O*-(β-D-galactopyranosyl 6-sulfate)-(1→4)-2,5-anhydro-D-[1-$^3$H]mannitol 6-sulfate (**4**, Gal6S–anM6S).

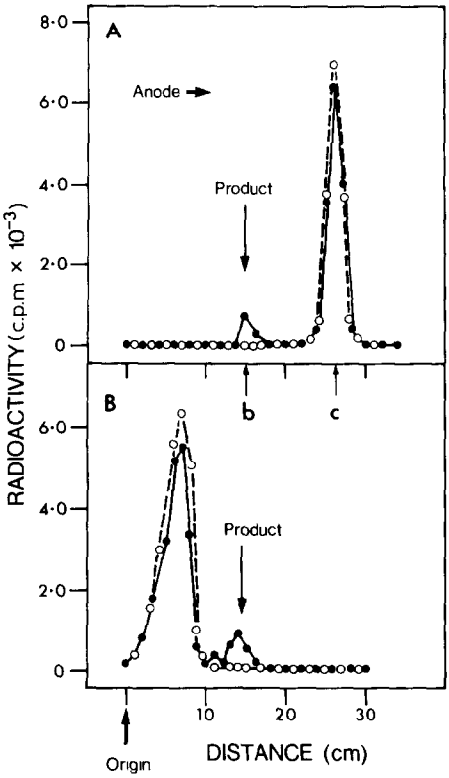



Fig. 6. Fractionation of incubation mixtures containing normal-fibroblast homogenate (—●—) or MPS-IVA-fibroblast homogenate (--○--): A, high-voltage electrophoresis of incubation mixtures containing IIC; B, p.c. (solvent *4*, 48 h) of incubation mixtures containing ID.

*Identification of tetrasaccharide fractions.* — It was assumed that tetrasaccharides were formed as a result of incomplete *N*-deacetylation and/or deamination, and these fractions contained many components. The identification of the major radiolabelled components in each fraction (IA–D) obtained by electrophoresis at pH 1.7 of fraction I (Fig. 7) is tentative. Based on their elution position from Sephadex G-25, their electrophoretic mobility relative to those of monosulfated and disulfated disaccharide-standards, and the known composition of KS, fractions IA–D were tentatively identified as the mono-, di-, tri- and tetrasulfated derivatives, respectively, of the tetrasaccharide: β-Gal-β-GlcNAc-β-Gal-2,5-anhydro-D-[1-$^3$H]mannitol. The tetrasulfated tetrasaccharide probably has the structure Gal6S–GlcNAc6S–Gal6S–anM6S. The positions of the sulfates on the partially sulfated tetrasaccharides are uncertain. Galactosyl end-groups in the $NaBH_4$-reduced fraction I were radiolabelled by oxidation with D-galactose oxidase and reduction with $NaBT_4$. Electrophoresis of this fraction at pH 1.7 produced the radioactivity profile shown in Fig. 7. Comparison of radioactivity profiles suggests that fractions IA, B, and C were radiolabelled by treatment with D-galac-

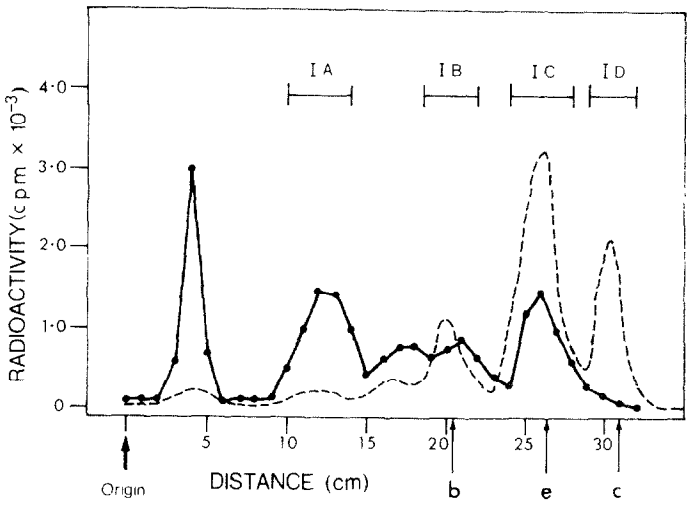



Fig. 7. High-voltage electrophoresis at pH 1.7 of fraction I (Fig. 1) reduced with $NaBT_4$ (----), and reduced with $NaBH_4$ and treated with D-galactose oxidase and $NaBT_4$ (—●—). Arrows indicate the peak position of standard oligosaccharides: b, monosulfated disaccharide; c, disulfated disaccharide; e, trisulfated tetrasaccharide.

tose oxidase and $NaBT_4$, whereas fraction ID was not. Thus, fractions IA, B, and C contain tetrasaccharides having unsulfated galactosyl groups at their non-reducing end. It is also probable that there are tetrasaccharide components in IA, B, and C that have galactosyl 6-sulfate end-groups which, as observed for fraction ID, will not be radiolabelled by treatment with D-galactose oxidase–$NaBT_4$.

*Degradation of oligosaccharide fractions by cultured, human-skin, fibroblast homogenates.* — The foregoing, radiolabelled oligosaccharides were specifically degraded by lysosomal enzymes, using fibroblast homogenates established from skin biopsies from patients having various mucopolysaccharide lysosomal-storage disorders. Although some optimisation was attempted, which will be reported elsewhere, the conditions selected for incubation were mostly those reported to be optimal for the degradation of more-conventional substrates.

*(a) 6-O-Sulfogalactose sulfatase.* Disaccharide Gal6S–anM6S and the presumed tetrasulfated-tetrasaccharide Gal6S–GlcNAc6S–Gal6S–anM6S (fractions IIC and ID, respectively) were partially *O*-desulfated at approximately the same rate (Table II) by normal-fibroblast homogenates, to produce a monosulfated disaccharide and a trisulfated tetrasaccharide, respectively (Fig. 6). The monosulfated product from Gal6S–anM6S had the same electrophoretic mobility at pH 1.7 as a major radiolabelled component in fraction IIB, tentatively identified as Gal–anM6S. Gal6S–anM6S (IIC) and the tetrasulfated tetrasaccharide (ID) were not *O*-desulfated when incubated with MPS-IVA (6-*O*-sulfogalactose sulfatase-deficient) fibroblasts (Table II). These findings suggest that *O*-desulfation of these substrates requires the specific action of 6-*O*-sulfogalactose sulfatase.

TABLE II

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

| *Substrate* | *6-O-Sulfogalactose sulfatase activity (pmol/min/mg of protein)* | | |
|---|---|---|---|
| | *Normal* | *MPS IVA* | *MPS IVB* |
| Gal6S–anM6S (IIC) | 2.1 | n.d.[a] | 2.8 |
| Gal6S–GlcNAc6S–Gal6S–anM6S (ID) | 3.0 | n.d. | —[a] |

[a]Key: n.d., none detected; —, not measured.

TABLE III

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

| *Substrate* | *β-D-Galactosidase activity (pmol/min/mg of protein)* | | | |
|---|---|---|---|---|
| | *Normal* | *MPS IVA* | *MPS IVB* | *$GM_1$-Gangliosidoses* |
| Gal–anM6S (IIB) | 0.8 | 1.2 | n.d.[b] | —[b] |
| [$^3$H]Gal–anM6S[a] | 0.6 | — | n.d | n.d |
| IA[a] | 24.0 | — | n.d | n.d |
| IB[a] | 21.8 | — | — | n.d |
| IC[a] | 26.7 | — | — | 0.6 |

[a]Radiolabel introduced by oxidation of galactose and reduction with $NaBT_4$ of $NaBH_4$-reduced, hexose-positive fractions I and II. [b]Key: n.d., none detected; —, not measured.

*(b) β-D-Galactosidase.* The disaccharide [$^3$H]Gal–anM6S and the [$^3$H]galactose-labelled tetrasaccharide-fractions IA, B, and C, when incubated with normal-fibroblast homogenates, gave [$^3$H]galactose (Table III, Fig. 5). Incubation of Gal–anM6S with normal-fibroblast homogenates produced a radiolabelled product tentatively identified as anM6S. β-D-Galactosidase activity assessed with the partially sulfated tetrasaccharide fractions (IA, B, and C) was ~30 times higher than the values obtained using [$^3$H]Gal–anM6S (Table III). These results demonstrate that, unlike the findings for the 6-*O*-sulfogalactose sulfatase, the extra group(s) on the tetrasaccharide, compared to the disaccharide, are important structural requirement(s) in the mechanism of action or binding of β-D-galactosidase toward β-linked D-galactosyl residues. Fractions IA, B, and C, with increasing content of sulfate groups per tetrasaccharide, were degraded at similar rates by fibroblast β-D-galactosidase, so that it is likely that GlcNAc and/or the extra galactose residue is responsible for the higher activity toward tetrasaccharides. Degradation was not observed when incubations were performed with $GM_1$-gangliosidoses and MPS-IVB fibroblast homogenates. These findings agree with the chemical data used to assign the structures to IIB and IA–C.

*Assay of sulfate ester distribution in KS repeat-sequence.* — The tentative identification of the major radiolabelled components in IIA–C as Gal–anM, Gal–anM6S, and Gal6S–anM6S, respectively, enabled a comparison of the repeat sequence in KS isolated from bovine intervertebral-disc and cornea and from human costal-cartilage. The results in Table I demonstrate that the major (>79%) disaccharide in intervertebral disc and costal cartilage is the disulfated disaccharide Gal6S–anM6S. In cornea, the level of Gal6S–anM6S is minor (15%), and the major component Gal–anM6S represents ~84% of the total disaccharide isolated; Gal6S–anM was not detected in any disaccharide fraction. Thus, the transfer of sulfate to galactose residues, during KS biosynthesis, is unlikely to occur unless proximal GlcNAc residues are sulfated. The sequence *N*-deacetylation, deamination with nitrous acid, and reduction with $NaBT_4$ to produce radiolabelled disaccharides is useful for the study of sulfate-ester distribution in KS.

## 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, 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*, 1-butanol–acetic acid–M ammonia (2:3:1); *2*, ethyl–acetate–acetic acid–water (3:1:1); *3*, isobutyric acid–2M ammonia (5:3); and *4*, ethyl acetate–acetic acid–water (4:3:3). For preparative p.c., the paper was prewashed with the irrigation solvent.

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.

Hexose, protein, and radioactivity were assayed by methods previously described[14].

Bovine intervertebral-disc KS was prepared as previously described[15]. Human costal-cartilage KS was a gift from Dr. M. B. Mathews (University of Chicago) and bovine-cornea KS was purchased from Seikagaku Kogyo Co. Ltd. (Tokyo, Japan). Standard tetrasaccharide and unsulfated, monosulfated, and disulfated disaccharides were isolated[13] from the products of nitrous acid deamination of heparin, and trisulfated tetrasaccharide[4] from bovine intervertebral-disc KS.

Anhydrous hydrazine was prepared by boiling hydrazine hydrate (90 g) over NaOH pellets (90 g) for 3 h under nitrogen. The mixture was distilled from fresh NaOH pellets (90 g) in a current of dry nitrogen (CAUTION: hydrazine is potentially explosive in presence of oxygen). The middle 60% was collected, and stored under nitrogen over molecular sieve 4A.

Fibroblasts [$\beta$-D-galactosidase-deficient, $GM_1$-ganglioside, and mucopolysaccharidosis Type IVB (MPS IVB)] were purchased from the Human Genetic

Cell Repository (Institute for Medical Research, Camden, N.J., U.S.A.). Fibroblasts [6-*O*-sulfogalactose sulfatase-deficient, mucopolysaccharidosis Type IVA (MPS IVA); β-D-galactosidase-deficient; $GM_1$-gangliosidosis; normal control] were established from biopsies made available to this hospital[16,17]. All cell lines were maintained and used for the preparation of cell homogenates 6–10 days after sub-culture as previously described[17].

N-*Deacetylation of KS.* — A mixture of KS (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 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*[18] *of* N-*deacetylated KS.* — A solution of *N*-deacetylated KS (10 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. The products were fractionated by chromatography on a column (200 × 1.6 cm) of Sephadex G-25, and hexose-positive fractions that 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 and desalted as described above. The reduced, radiolabelled disaccharides and tetrasaccharides were further fractionated by preparative high-voltage electrophoresis at pH 1.7 and preparative p.c. (solvent *1*), as described in the text.

*Treatment of hexose-positive fractions with* D-*galactose oxidase and* $NaBT_4$. — Desalted fractions I and II were reduced with 2M $NaBH_4$ in 0.05M sodium borate buffer (1 mL, pH 8.1) for 4 h at 25°, and then acidified with acetic acid and desalted as described above. The products were incubated with 15 Sigma units of D-galactose oxidase and 75 Sigma units of horse-radish peroxidase per mg of hexose-positive material, in 0.1M sodium phosphate buffer (1 mL, pH 6.0), for 48 h at 25°. The incubation mixture was reduced with 0.2M $NaBT_4$ (0.34 Ci/mmol) in 0.1M sodium borate buffer (1 mL, pH 8.1) at 25°. After 4 h, the reaction mixture was acidified with acetic acid and desalted as described above.

*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 0.1M NaCl (3 × 10 mL) under conditions previously described[16,17]. 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 disaccharides by cultured skin-fibroblast homogenates.* — Assays for β-D-galactosidase and 6-*O*-sulfogalactose sulfatase

were performed by incubating 15–30 $\mu$g of fibroblast-homogenate protein for 16 h at 37° in 0.05M sodium formate buffer (pH 4.0) containing 6mM $NaN_3$ and ~500 pmol of radiolabelled oligosaccharide substrate in a final volume of 20 $\mu$L. After incubation, reaction mixtures containing fraction IIC (Gal6S-anM6S) and IIB (Gal–anM6S) were subjected to electrophoresis at pH 1.7 in order to separate substrate from product. To separate substrate and product in reaction mixtures containing fraction ID and [$^3$H]Gal–anM6S, whole incubation mixtures were subjected to p.c. (solvent *4*, 48 h; and solvent *1*, 24 h; respectively).

ACKNOWLEDGMENTS

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