Biochem. J. (1979) **179**, 7–13
Printed in Great Britain

Sequential Degradation of a Chondroitin Sulphate Trisaccharide by Lysosomal Enzymes from Embryonic-Chick Epiphysial Cartilage

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(*Received* 4 *August* 1978)

The disulphated trisaccharide D-*N*-acetylgalactosamine sulphate–β-D-glucuronic acid–β-D-*N*-acetylgalactosamine sulphate prepared from ^{35}S- or ^{14}C-labelled chondroitin sulphate was incubated with a preparation of lysosomal enzymes from embryonic-chick epiphysial cartilage. Degradation was demonstrated by analysis of the reaction products. By use of the appropriate intermediate products as substrates, in conjunction with specific enzyme inhibitors, it was shown that the degradation proceeded sequentially from the non-reducing end. It was initiated by sulphatase (preferentially hydrolysing sulphate ester groups at the 6-position), followed by β-*N*-acetylgalactosaminidase and β-glucuronidase, converting the substrate into monosaccharides and inorganic sulphate. The latter enzyme preferentially attacked disaccharides carrying their sulphate ester group at C-4 of the hexosamine residue. Generation of chondroitin sulphate oligosaccharides may occur by the action of an endoglycosidase, previously demonstrated in embryonic-chick cartilage. Endo- and exo-enzymes may thus form a functional unit in lysosomal degradation of chondroitin sulphate.

Hydrolytic enzymes operating on ground-substance constituents of cartilage may be involved in degenerative processes such as osteoarthritis (Ali & Evans, 1973; Schwartz *et al.*, 1974). Sulphatases and glycosidases capable of degrading chondroitin sulphate have been demonstrated in cartilage (Morrison, 1970; Wasteson *et al.*, 1972; Amadò *et al.*, 1974; Wasteson *et al.*, 1975), but their role in a co-ordinated attack on the polymer has not been elucidated. We have previously initiated studies on chondroitin sulphate-degrading enzymes in cartilage, using embryonic-chick epiphysis as a model tissue, from which lysosomal enzymes and defined natural substrates of chondroitin sulphate were prepared. The present paper describes the sequential degradation of a disulphated trisaccharide from chondroitin sulphate to monosaccharides and inorganic sulphate. A preliminary report has been published (Ingmar & Wasteson, 1976).

Materials and Methods

Chemicals

Chondroitinase ABC (EC 4.2.2.4), the unsaturated disaccharides ΔDi-4S, ΔDi-6S and ΔDi-0S (Yamagata *et al.*, 1968) and chondroitin sulphate A and C (Super Special Grade) were obtained from Seikagaku Kogyo Co., Tokyo, Japan. GalNAc-4-SO_4 and GalNAc-6-SO_4 were prepared by acid hydrolysis of ΔDi-4-S and ΔDi-6-S respectively (Suzuki *et al.*, 1968), and purified by paper electrophoresis at pH 1.7; the disaccharides used for these preparations were obtained by chondroitinase ABC digestion of chondroitin sulphate A and C respectively. Testicular hyaluronidase (Hyalas; EC 3.2.1.35) was a product of AB Leo, Helsingborg, Sweden. β-Glucuronidase (type B-10) (EC 3.2.1.31) and D-saccharo-1,4-lactone was purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Sephadex gels (G-25, G-15 and G-10) were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden, and DEAE-cellulose (Whatman DE-52) was from Whatman Biochemicals, Maidstone, Kent, U.K. Carrier-free inorganic [^{35}S]sulphate and [^{14}C]glucose (225 mCi/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

Substrates

^{35}S- or ^{14}C-labelled chondroitin sulphate was prepared from epiphysial cartilage of tibias and femurs from 13-day chick embryos after labelling *in vitro* with [^{35}S]sulphate or [^{14}C]glucose (Amadò *et al.*, 1974). Tetrasaccharide was prepared by incubation of the corresponding polysaccharide with testicular hyaluronidase, followed by chromatography on Sephadex G-25 and desalting (Amadò *et al.*, 1974). Trisaccharide was prepared by incubation of tetrasaccharide with β-glucuronidase. The diges-

Abbreviations used: ΔDi-4S, 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-4-*O*-sulpho-D-galactose; ΔDi-6S, 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-6-*O*-sulpho-D-galactose; ΔDi-0S, 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-D-galactose; GalNAc-4-SO_4 and GalNAc-6-SO_4, *N*-acetylgalactosamine 4- and 6-sulphate; GlcUA, glucuronic acid.

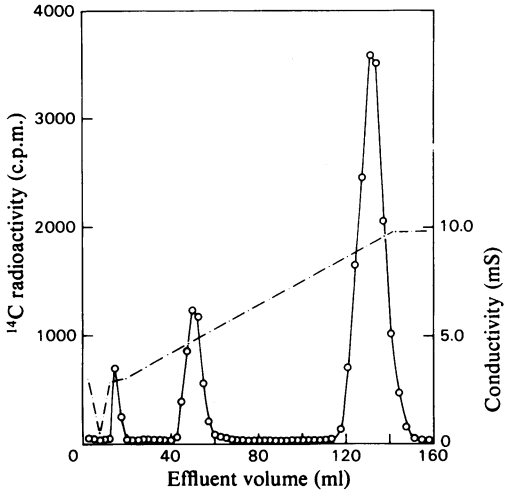

Fig. 1. *Ion-exchange chromatography on DEAE-cellulose of trisaccharides from ^{14}C-labelled chondroitin sulphate*
Trisaccharides were prepared from ^{14}C-labelled chondroitin sulphate by hyaluronidase digestions, followed by treatment of tetrasaccharides with β-glucuronidase. The trisaccharide fraction was applied to a column (1 cm × 5 cm) of DEAE-cellulose, equilibrated with 0.03 M-NaCl. Elution was with a linear gradient of NaCl, from 0.03 M to 0.1 M (conductivity, –·–·–). Effluent fractions were analysed by liquid-scintillation counting (○). The most retained fraction (118–147 ml) was identified as disulphated trisaccharide. The middle (42–60 ml) and first (14–20 ml) peaks consisted of mono- and non-sulphated trisaccharide respectively.

tion was performed for 16 h at 37°C in 0.05 M-sodium acetate buffer, pH 5.0, with 100 units of enzyme/μg of uronic acid. The resulting trisaccharide was isolated by chromatography on Sephadex G-25 and desalted. Disulphated trisaccharide was isolated by ion-exchange chromatography on DEAE-cellulose, which separates the different trisaccharide species according to degree of sulphation (Fig. 1). The specific radioactivities of ^{35}S- and ^{14}C-labelled disulphated trisaccharides were 7000 and 44000 c.p.m./μg of uronic acid respectively. The identity of ^{35}S- or ^{14}C-labelled disulphated trisaccharide was ascertained by digestion with chondroitinase ABC and separation of the degradation products on DEAE-cellulose (results not shown). Only two components were detected. The peak eluted first from the DEAE-cellulose column was identified as GalNAc-SO_4 by gel chromatography on Sephadex G-15 and paper chromatography in solvent C. The second peak behaved like the unsaturated monosulphated disaccharide ΔDi-4S or ΔDi-6S on Sephadex G-15 chromatography and paper chromatography in solvent C. ^{35}S-labelled trisaccharide had a ratio of mono- to di-saccharide (as ^{35}S) of 51:49, in good agreement with the expected value (50:50). The analogous ratio of ^{14}C-labelled mono- to di-saccharide (as ^{14}C) was 37:63, slightly deviating from the ratio 33:67 expected for the products from ^{14}C-labelled trisaccharide with a perfectly homogeneous distribution of label between the glucuronic acid and the *N*-acetylgalactosamine residues.

Lysosomal enzyme preparation

A lysosomal enzyme preparation (approx. 0.5 mg of protein/ml) was obtained from epiphysial cartilage of 13-day chick embryos as described (Amadò *et al.*, 1974).

Analytical methods

Uronic acid was determined as described by Bitter & Muir (1962), and protein as described by Lowry *et al.* (1951), with human serum albumin as standard.

Radioactivity was measured either with Packard liquid-scintillation spectrometers models 2002 and 2450 with Insta-Gel (Packard Instruments) as the scintillation medium, or in a Packard model 7201 radiochromatogram scanner.

Gel chromatography was performed at 4°C on columns of Sephadex G-25 (1 cm × 190 cm) or G-15 (1 cm × 156 cm), with 1 M-NaCl as eluent. Desalting of trisaccharides and disaccharides was carried out on columns of Sephadex G-15 (1 cm × 50 cm or 2 cm × 50 cm), equilibrated with 10% (v/v) ethanol. The monosaccharides GlcUA and GalNAc-SO_4 were desalted on a column (1 cm × 145 cm) of Sephadex G-15, equilibrated with 0.2 M-NH_4HCO_3. Preparations of *N*-acetylgalactosamine were desalted by paper electrophoresis at pH 5.3 and eluted with water. Samples were concentrated by freeze-drying.

Ion-exchange chromatography was carried out on DEAE-cellulose at 4°C. The columns (1 cm × 5 cm or 2 cm × 5 cm) were equilibrated with 0.03 M-NaCl and eluted with linear gradients of NaCl from 0.03 M to 0.06 M or 0.1 M. The gradients were monitored by conductivity measurements. Final elution was made with 0.3 M-NaCl until no absorption at 280 nm was detectable in column eluates.

High-voltage paper electrophoresis was performed on desalted fractions as described by Amadò *et al.* (1974).

Descending paper chromatography was carried out on desalted fractions on Whatman 3MM paper at 20°C in the following systems: solvent A, ethyl acetate/acetic acid/water (3:1:1, by vol.) for 16 h; solvent B, butan-1-ol/ethanol/water (10:3:5, by vol.) for 40 h; solvent C, butan-1-ol/acetic acid/1 M-NH_3 (2:3:1, by vol.) for 18 h; solvent D, isobutyric acid/2 M-NH_3 (5:3, v/v) for 24–44 h. Reducing sugars were detected by staining with a silver dip reagent (Smith, 1960). Unsaturated sugars were also detected by their u.v. absorption at 234 nm.

Digestion with chondroitinase ABC was performed as described by Yamagata *et al.* (1968), but with some modifications: NaF and ΔDi-0S were added to inhibit contaminating sulphatase (Suzuki, 1960) and glucuronidase respectively. The incubation mixtures contained, in final volumes of 720 μl: ^{35}S- or ^{14}C-labelled trisaccharide, 5–6 μg as glucuronic acid; Tris/HCl, pH 8.0, 4 μmol; NaF, 25 μmol; sodium acetate, 18 μmol; bovine serum albumin, 50 μg; ΔDi-0S, 40 μg, and 0.5 unit of chondroitinase ABC. The mixtures were incubated at 37°C for 6h.

Incubation with lysosomal enzymes

Oligosaccharide substrates were incubated with the lysosomal enzyme preparation at 37°C for 20–24h. To 5.0ml of enzyme preparation were added 2.0ml of 0.1M-sodium acetate buffer, pH 4.8, and 1.0ml of substrate solution, containing 3–8 μg of uronic acid. Sodium formate buffer (final concentration 0.05M, pH 4.4) was substituted for acetate buffer in some experiments. Occasionally NaF (final concentration 0.023M) or saccharolactone (final concentration 0.001M) was added to inhibit sulphatase (Suzuki, 1960) and β-glucuronidase activities (Conchie *et al.*, 1967) respectively. Incubations were terminated by heat-inactivation at 100°C for 4min.

Results

Degradation of disulphated trisaccharide

DEAE-cellulose chromatography of ^{35}S- or ^{14}C-labelled disulphated trisaccharide after incubation with lysosomal enzymes yielded the product patterns shown in Figs. 2 and 3; see also Table 1. Four new peaks of radioactivity (fractions I–IV) appeared, in addition to unchanged substrate (fraction V). (The numbering of peaks is consistent throughout this paper.)

Fraction I was a product of ^{14}C-labelled substrate only. It was not retained on the DEAE-cellulose column and did not migrate on paper electrophoresis at pH 5.3. It migrated like a monosaccharide on a calibrated column of Sephadex G-15 (Fig. 4). Paper chromatography in solvent A or B identified it as [^{14}C]GalNAc.

Fraction II behaved like a monosaccharide on Sephadex G-15 (Fig. 4). Paper chromatography in solvent C of ^{35}S-labelled fraction II showed two components with the mobilities of GalNAc-4-SO_4 (82%) and GalNAc-6-SO_4 (18%). ^{14}C-labelled fraction II showed one non-migrating (IIA) and one migrating (IIB) component on paper electrophoresis at pH 1.7; the ratio of ^{14}C in fraction IIA to that in

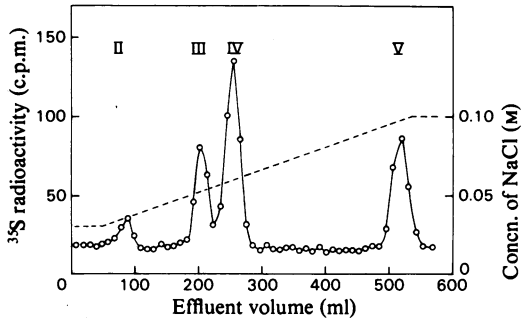

Fig. 2. *Ion-exchange chromatography on DEAE-cellulose of ^{35}S-labelled disulphated trisaccharide after incubation with lysosomal enzymes*

^{35}S-labelled disulphated trisaccharide was incubated with lysosomal enzymes in acetate buffer as described in the text. The reaction mixture was applied to a column (2cm × 5cm) of DEAE-cellulose, equilibrated with 0.03M-NaCl. Elution was with a linear gradient of NaCl, from 0.03M to 0.1M (----). Effluent fractions were analysed by liquid-scintillation counting (○). Fraction II, GalNAc-SO_4; fraction III, monosulphated trisaccharide, GalNAc-GlcUA-GalNAc-SO_4; fraction IV, monosulphated disaccharide, GlcUA-GalNAc-SO_4, plus inorganic [^{35}S]-sulphate; fraction V, unchanged disulphated trisaccharide, GalNAc-SO_4-GlcUA-GalNAc-SO_4. The proportions of ^{35}S in fractions II:III:IV:V were 7:21:44:28.

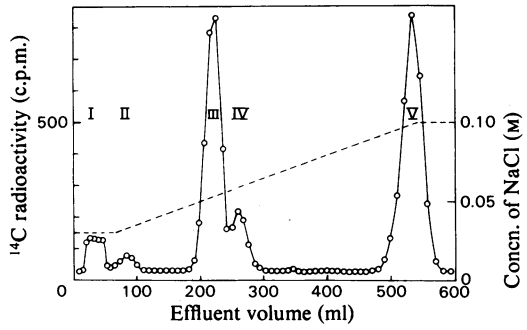

Fig. 3. *Ion-exchange chromatography on DEAE-cellulose of ^{14}C-labelled disulphated trisaccharide after incubation with lysosomal enzymes*

^{14}C-labelled disulphated trisaccharide was incubated with lysosomal enzymes as described in the text. The reaction mixture was applied to a column (2cm × 5cm) of DEAE-cellulose, equilibrated with 0.03M-NaCl. Elution was with a linear gradient of NaCl, from 0.03 to 0.1M (----). Effluent fractions were analysed by liquid-scintillation counting (○). Fraction I, GalNAc; fraction II, GlcUA plus GalNAc-SO_4; fraction III, monosulphated trisaccharide, GalNAc-GlcUA-GalNAc-SO_4; fraction IV, monosulphated disaccharide, GlcUA-GalNAc-SO_4; fraction V, unchanged disulphated trisaccharide, GalNAc-SO_4-GlcUA-GalNAc-SO_4. The proportions of ^{14}C in fractions I:II:III:IV:V were 5:2:38:8:47.

Table 1. *Analysis of products obtained after degradation of ^{35}S- or ^{14}C-labelled disulphated trisaccharide with lysosomal enzymes*

Fractions I–V were those shown in Fig. 2 (^{35}S) and Fig. 3 (^{14}C) respectively. In addition the analysis of the original disulphated trisaccharide substrates is given. —, Not applicable.

Fraction	Composition	Ratio of 4-sulphate:6-sulphate at Non-reducing terminus ^{35}S	Non-reducing terminus ^{14}C	Reducing terminus ^{35}S	Reducing terminus ^{14}C
I	GalNAc	—	—	—	—
IIA	GlcUA	—	—	—	—
IIB	GalNAc-SO_4	—	—	82:18	91:9
III	GalNAc-GlcUA-GalNAc-SO_4	—	—	35:65	30:70
IVA	GlcUA-GalNAc-SO_4	—	—	52:48	59:41
IVB	SO_4^{2-}	—	—	—	—
V	GalNAc-SO_4-GlcUA-GalNAc-SO_4	95:5	100:0	50:50	77:23
Original substrate	GalNAc-SO_4-GlcUA-GalNAc-SO_4	33:67	55:45	42:58	51:49

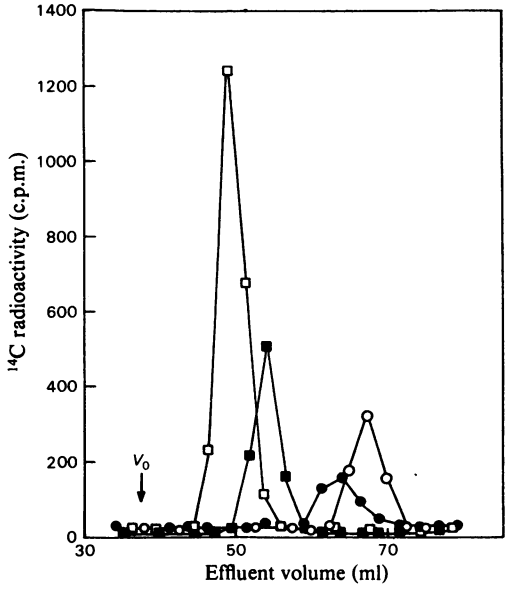

Fig. 4. *Gel chromatography on Sephadex G-15 of products obtained from ^{14}C-labelled disulphated trisaccharide after incubation with lysosomal enzymes*

^{14}C-labelled fraction I (○), II (●), III (□) and IV (■) (see legend to Fig. 3) were chromatographed on a column (1 cm × 156 cm) of Sephadex G-15 in 1 M-NaCl. Effluent fractions were analysed by liquid-scintillation counting. The following standards were used to calibrate the column: trisaccharide (peak 49 ml); monosulphated disaccharide (peak 54 ml); monosulphated ΔDi-4S (peak 57 ml); GlcUA (peak 64 ml). V_0, void volume.

fraction IIB was 2:3. Fraction IIA was identified as [^{14}C]GlcUA by paper electrophoresis at pH 5.3 and by paper chromatography in solvent A. Fraction IIB was resolved by paper chromatography in solvent C into two components, with the mobilities of GalNAc-4-SO_4 (91%) and GalNAc-6-SO_4 (9%).

Fraction III had the size of a trisaccharide on Sephadex G-15 chromatography (Fig. 4). Digestion with chondroitinase ABC quantitatively converted ^{35}S-labelled fraction III into unsaturated disaccharides, as demonstrated by DEAE-cellulose chromatography followed by paper chromatography in solvent C (results not shown). The ratio of [^{35}S]ΔDi-4S to [^{35}S]ΔDi-6S was 7:13. Chondroitinase ABC treatment of ^{14}C-labelled fraction III yielded a labelled component behaving like GalNAc on DEAE-cellulose chromatography, paper electrophoresis at pH 5.3 and paper chromatography in solvent A, in addition to the ^{14}C-labelled unsaturated disaccharides ΔDi-4S (30%) and ΔDi-6S (70%). In conclusion, these findings identify fraction III as monosulphated trisaccharide, carrying sulphate exclusively at the reducing terminus.

^{35}S-labelled fraction IV was resolved by Sephadex G-25 chromatography into two components, fractions IVA and IVB, migrating like a disaccharide (17%) and inorganic sulphate (83%) respectively (results not shown). Interestingly, chromatography on Sephadex G-15 failed to separate these two elements; in this system inorganic sulphate is eluted anomalously early, similarly to a monosulphated disaccharide. Fraction IVA gave rise to two peaks of ^{35}S radioactivity on paper chromatography in solvent D, migrating like the saturated monosulphated disaccharides GlcUA-GalNAc-4-SO_4 (52%) and GlcUA-GalNAc-6-SO_4 (48%). Fraction IVB was identified as inorganic [^{35}S]sulphate by paper electrophoresis at pH 5.3. ^{14}C-labelled fraction IV consisted of disaccharides only; paper chromatography in solvent D showed two components migrating like the saturated monosulphated disaccharides

GlcUA-GalNAc-4-SO_4 (59%) and GlcUA-GalNAc-6-SO_4 (41%).

Fraction V behaved like non-converted trisaccharide substrate on DEAE-cellulose chromatography. Its constituent components were identified by chondroitinase ABC treatment, followed by DEAE-cellulose chromatography and paper chromatography in solvent C; the procedure was as described for the original trisaccharides (see the Materials and Methods section). As shown in Table 1, fraction V differed markedly from the latter in that sulphates at the non-reducing terminus were exclusively at position 4, rather than equally distributed between the 4- and 6-positions. Fraction V remained intact (about (94%) after repeated incubation with fresh enzyme.

In summary, the analysis of the degradation products obtained led to the conclusion that sulphatase, *N*-acetylgalactosaminidase and glucuronidase were present in chick epiphysial cartilage. Sulphate ester groups at the 6-position were preferentially attacked; sulphates at the 4-position essentially resisted degradation under the experimental conditions used. Incubation of ^{14}C-labelled disulphated trisaccharide with lysosomal enzymes in the presence of NaF (a sulphatase inhibitor) failed to produce significant amounts of degradation products (results not shown). However, the ^{14}C-labelled monosulphated trisaccharide (fraction III, Fig. 3) was readily degraded in the presence of NaF (see below). It was concluded that the action of sulphatase had to precede that of other hydrolases involved.

Degradation of monosulphated trisaccharide

Incubation of ^{14}C-labelled monosulphated trisaccharide (i.e. fraction III, Fig. 3) with lysosomal enzymes gave rise to the products shown in Fig. 5(*a*): fractions I (GalNAc), IIA and IIB (GlcUA and GalNAc-SO_4 respectively) and fraction IV (saturated monosulphated disaccharide, GlcUA-GalNAc-SO_4). The degradation was less efficient in acetate than in formate, probably owing to inhibition of *N*-acetylhexosaminidase by acetate (cf. Fig. 3) (Frohwein & Gatt, 1967). In the presence of saccharolactone (a β-glucuronidase inhibitor), however, no fraction II was formed; fractions I and IV were the only reaction products (Fig. 5*b*). These findings indicate that degradation of monosulphated trisaccharide was initiated by *N*-acetylgalactosaminidase, converting its substrate into free GalNAc and monosulphated disaccharide. Addition of NaF had no influence on this step (results not shown). The appearance of the monosaccharides GlcUA and GalNAc-SO_4 in the absence of saccharolactone would suggest that the disaccharide may be ultimately degraded by a β-glucuronidase.

Degradation of monosulphated disaccharide

Incubation of ^{14}C-labelled monosulphated disaccharide [fraction IV (Fig. 5*b*) reisolated by paper electrophoresis at pH 1.7; ratio of 4-sulphate:6 sulphate isomers 37:63] led to the formation of fraction II (25%), as revealed by DEAE-cellulose chromatography (Fig. 6). Paper electrophoresis at pH 1.7

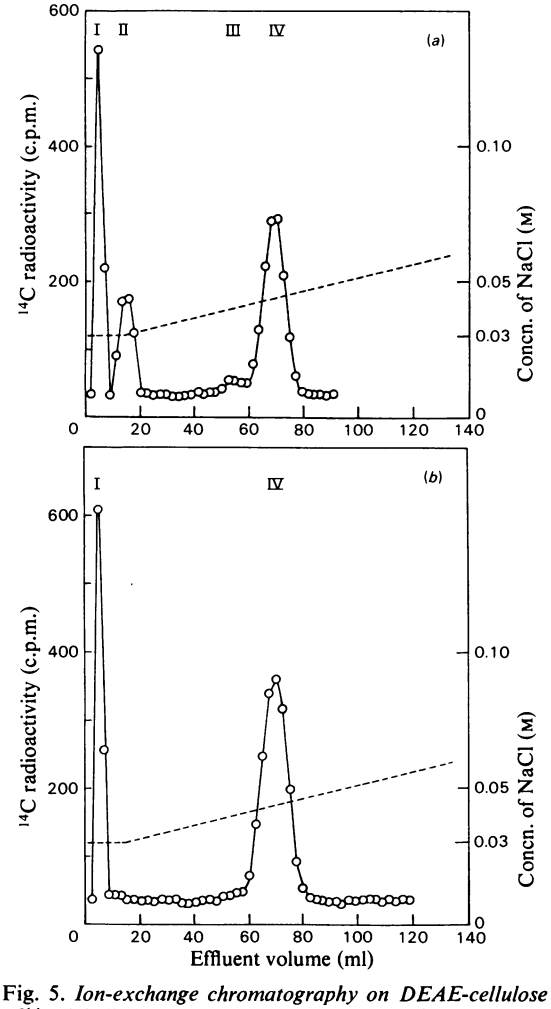

Fig. 5. *Ion-exchange chromatography on DEAE-cellulose of ^{14}C-labelled monosulphated trisaccharide after incubation with lysosomal enzymes*

^{14}C-labelled monosulphated trisaccharide (fraction III; see the legend to Fig. 3) was incubated with lysosomal enzymes in the absence (*a*) or presence (*b*) of 1 mM-saccharolactone, in formate buffer, as described in the text. The reaction mixture was applied to a column (1 cm × 5 cm) of DEAE-cellulose, equilibrated with 0.03 M-NaCl. Elution was made with a linear gradient of NaCl, from 0.03 M to 0.06 M (----). Effluent fractions were analysed by liquid-scintillation counting (○). Fraction numbers are explained in the legend to Fig. 3.

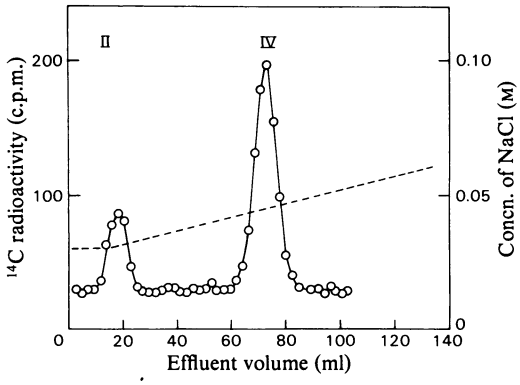

Fig. 6. *Ion-exchange chromatography on DEAE-cellulose of ^{14}C-labelled monosulphated disaccharide after incubation with lysosomal enzymes*

^{14}C-labelled monosulphated disaccharide (fraction IV; see the legend to Fig. 5*b*) was incubated with lysosomal enzymes (in formate buffer) as described in the text and chromatographed as described in the legend to Fig. 5. Fraction numbers are explained in the legend to Fig. 3.

followed by paper chromatography in solvents A and C showed fraction II to consist of [^{14}C]GlcUA and [^{14}C]GalNAc-SO_4 in the proportions 39:61. The ratio of the 4-sulphate to 6-sulphate isomers of GalNAc-SO_4 was 81:19, whereas that of the unchanged disaccharide was 23:77. These findings support the notion that a β-glucuronidase may convert the disaccharide GlcUA-GalNAc-SO_4 into monosaccharides, and further that the glucuronidic bond to 4-sulphated GalNAc residues was more susceptible to hydrolysis than that to 6-sulphated ones. Since no [^{14}C]GalNAc was detectable, GalNAc-SO_4 was not desulphated under the experimental conditions.

Discussion

The present results demonstrate that the chondroitin sulphate trisaccharide GalNAc-SO_4-GlcUA-GalNAc-SO_4 was sequentially degraded to monosaccharides and inorganic sulphate by a lysosomal enzyme preparation from chick epiphysial cartilage, confirming the hypothesis suggested previously that oligosaccharides of glycosaminoglycans may be degraded by the concerted action of exoenzymes (Muir, 1973; Buddecke & Kresse, 1974). The degradation was initiated by sulphatase, apparently a 6-sulphatase. This enzyme activity is probably identical with that deficient in Morquio disease (Singh *et al.*, 1976; Di Ferrante *et al.*, 1978). In contrast, no significant 4-sulphatase activity was detected in the present study, although the conditions were similar to those previously used to demonstrate this enzyme (O'Brien *et al.*, 1974). The possibility remains that 4-sulphatase is active only with polymeric substrates (cf. Held & Buddecke, 1967; Matalon *et al.*, 1974).

The second degradative step was catalysed by *N*-acetylgalactosaminidase, converting monosulphated trisaccharide into GalNAc and the monosulphated disaccharide, GlcUA-GalNAc-SO_4. In the absence of glucuronidase inhibitor the degradation of disaccharide could proceed further, glucuronidase liberating GlcUA and GalNAc-SO_4 as monosaccharides. The latter reaction was demonstrated more directly by incubating isolated disaccharide with lysosomal enzymes. Again the monosaccharides GlcUA and GalNAc-SO_4 were formed. The analogous disaccharide from hyaluronic acid has been reported to be resistant to treatment with β-glucuronidase from testicular or liver tissue (Linker *et al.*, 1955). The finding that disaccharides carrying the sulphate ester group at position 4 (rather than position 6) were preferentially attacked favours the view that structural features at the *N*-acetylhexosamine residue may influence the susceptibility of the disaccharide to β-glucuronidase.

We have previously demonstrated the presence in chick epiphysial cartilage of a chondroitin sulphate-degrading endopolysaccharidase (Amadò *et al.*, 1974; Wasteson *et al.*, 1975). The smallest fragments generated by this enzyme included tetrasaccharides. Therefore it is possible that suitable substrates for the enzymes studied in the present investigation may be formed endogenously, within the cartilage lysosomes. This form of co-operativity may be an important feature in glycosaminoglycan degradation: the exoenzymes are probably not efficient in the degradation of large polysaccharide fragments (Weissman *et al.*, 1975; Amadò *et al.*, 1974; Hayashi, 1978). In the degradation of chondroitin 4-sulphate by lysosomal enzymes from liver tissue, decasaccharide was reported to be the largest substrate for β-glucuronidase (Hayashi, 1978). The intralysosomal balance between degradation by endo- and exoenzymes is not known, but may be influenced by local factors such as pH or ionic strength.

The functional role of chondroitin sulphate-degrading enzymes in cartilage is not known. They may participate in remodelling of cartilage during growth and development, which probably involves an active turnover of ground substance. Morrison (1970) demonstrated release of sulphate from chick-embryo bone rudiments in organ culture; the desulphation was ascribed to a degradative process of intact living cells. Intracellular desulphation of chondroitin sulphate was similarly inferred from studies with rat costal cartilage (Wasteson *et al.*, 1972). Enzymes similar to those described in the present paper may further contribute to the elevated

catabolism of chondroitin sulphate in degenerative joint processes (Gold *et al.*, 1976).

We are indebted to Dr. R. Amadò for helpful discussions. The skilful technical assistance of Mrs. F. Carlsson, Miss Y. Öhgren and Mr. P. Thebo is gratefully acknowledged. This work was supported by the Swedish Medical Research Council (13X-4486; 13X-2309), Konung Gustaf V:s 80-årsfond, Svenska Sällskapet för Medicinsk Forskning and the University of Uppsala.

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