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Identification of oversulphated galactosaminoglycans in intestinal-mucosal mast cells of rats infected with the nematode worm *Nippostrongylus brasiliensis*

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The oversulphated galactosaminoglycans synthesized by rat mucosal mast cells were isolated from the small intestine of animals infected with the nematode *Nippostrongylus brasiliensis*, which causes proliferation of these cells. The ^{35}S-labelled polysaccharides were degraded by digestion with chondroitinase ABC, and the structures of the disaccharide products were determined by cleavage with mercuric acetate followed by electrophoretic characterization of the resultant sulphated monosaccharides. It was concluded that about half of the disulphated disaccharide units in the polysaccharide consisted of chondroitin sulphate E-type structures [GlcA-GalNAc(4,6-di-OSO_3)], in which both sulphate groups were located on the *N*-acetylgalactosamine unit. The remainder consisted of isomeric structures with one sulphate group on the *N*-acetylgalactosamine residue and one on the hexuronic acid unit and presumably represented the dermatan sulphate-type sequence [IdoA(2-OSO_3)-GalNAc(4-OSO_3)].

INTRODUCTION

The secretory granules of mast cells generally contain sulphated glycosaminoglycans, hence their strongly basophilic staining properties. Recent studies have shown that various types of mast cells differ with regard to the structure of the stored polysaccharide. Thus heparin, by tradition considered the hallmark of a mast cell, is essentially restricted to connective-tissue-type mast cells (Yurt *et al.*, 1977; Robinson *et al.*, 1978), whereas the polysaccharides in other related cells, such as the cultured interleukin 3-dependent bone-marrow-derived mouse mast cell (Razin *et al.*, 1982) and the rat basophil-leukaemia cell (Seldin *et al.*, 1985), appear to be largely galactosaminoglycans. Also, the so-called 'mucosal mast cell' of the rat, which undergoes thymus-dependent proliferation in the small intestine in response to helminthic infections, appears to contain mainly galactosaminoglycans (Enerbäck *et al.*, 1985; Stevens *et al.*, 1986). Isolation of such a polysaccharide, labelled with inorganic [^{35}S]sulphate *in vivo* after infection of rats with the nematode helminth *Nippostrongylus brasiliensis*, yielded 'oversulphated' species which contained appreciable amounts of disulphated disaccharide units. However, the identification of these units, recovered as Δ4,5-unsaturated disaccharides after digestion of the polysaccharide with the bacterial eliminase chondroitinase ABC, gave conflicting results. Whereas Enerbäck *et al.* (1985) concluded that the disulphated disaccharide residues had the predominant structure:

-GlcA-GalNAc(4,6-di-OSO_3)-

with both sulphate groups on the *N*-acetylgalactosamine moiety (sometimes referred to as a 'chondroitin sulphate E structure'), Stevens *et al.* (1986) claimed that the sulphate groups were largely distributed between both sugar moieties in the disulphated di-B sequence:

-IdoA(2-OSO_3)-GalNAc(4-OSO_3)-

In the present study this problem was reinvestigated by using a novel method to characterize saccharide sequences with non-reducing-terminal hexuronic acid units (Ludwigs *et al.*, 1987). The method is based on selective elimination of such units by treatment with mercuric ions under conditions that leave the remaining saccharide unmodified.

EXPERIMENTAL

Materials

^{35}S-labelled polysaccharides from rat mucosal mast cells were isolated essentially as described in detail previously (Enerbäck *et al.*, 1985). Briefly, rats were infected with the nematode *Nippostrongylus brasiliensis*, and polysaccharides were isolated from the small intestine

Abbreviations used: HexA, unspecified hexuronic acid; GlcA, D-glucuronic acid; IdoA, L-iduronic acid; GalNAc, 2-deoxy-2-acetamido-D-galactose (*N*-acetyl-D-galactosamine); GlcNAc, 2-deoxy-2-acetamido-D-glucose (*N*-acetyl-D-glucosamine); $aMan_R$, 2,5-anhydro-D-mannitol formed by reduction of 2,5-anhydromannose residues; ΔDi-4S, 2-acetamido-2-deoxy-3-*O*-(β-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-diS_B, 2-acetamido-2-deoxy-3-*O*-(2-*O*-sulpho-β-D-gluco-4-enepyranosyluronic acid)-4-*O*-sulpho-D-galactose; ΔDi-diS_E, 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-4,6-di-*O*-sulpho-D-galactose; chondroitinase ABC, chondroitin ABC lyase (EC 4.2.2.4); chondroitinase AC, chondroitin AC lyase (EC 4.2.2.5); chondro-6-sulphatase, 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-6-sulpho-D-galactose 6-sulphohydrolase; heparinase, heparin lyase (EC 4.2.2.7); in abbreviated saccharide sequences the positions of sulphate groups are indicated in parentheses.

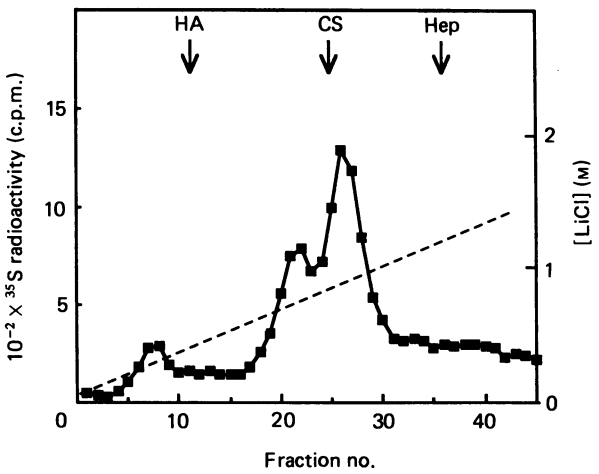

Fig. 1. Ion-exchange chromatography of ^{35}S-labelled polysaccharide from rat mucosal mast cells

A sample of small intestine was prepared from a rat, injected with inorganic [^{35}S]sulphate after infection with *N. brasiliensis* (see Enerbäck *et al.*, 1985). After proteolytic digestion of the tissue, followed by alkali treatment, a sample (30×10^3 c.p.m. of ^{35}S) of the digest was mixed with glycosaminoglycan standards (see below) and applied to a column (1 cm × 4 cm) of DEAE-cellulose, equilibrated with 0.05 M-NaCl/0.05 M-Tris/HCl, pH 8.0. The column was washed extensively with 0.05 M-LiCl in 0.05 M-acetate buffer, pH 4.0, and was then eluted with a linear gradient (----) extending from 0.05 M to 1.5 M-LiCl in the same acetate buffer. Fractions (approx. 3 ml) were collected and analysed for ^{35}S radioactivity and uronic acid (carbazole reaction). In preparative separations (performed under identical conditions, except that 0.3 mg of carrier chondroitin sulphate was substituted for the glycosaminoglycan standards), labelled material corresponding to fractions 18–31 was pooled, dialysed against water and retained for further analysis. The arrows on the top of the Figure indicate the peak elution positions of hyaluronan (HA), chondroitin sulphate (CS) and heparin (Hep) standards.

after intraperitoneal injection of $Na_2{}^{35}SO_4$. The tissue was digested with Pronase, and the digest was treated with $NaOH/NaBH_4$ and was then centrifuged at 20000 *g* for 20 min. After dialysis against water, the supernatant was adjusted to pH 8.0 by the addition of Tris and was then applied to a column of DEAE-cellulose (DE-52, Whatman). The column was washed with 0.05 M-NaCl/0.05 M-Tris buffer, pH 8.0, then with 0.05 M-LiCl/0.05 M-acetate buffer, pH 4.0, and was finally eluted with a gradient of LiCl from 0.05 to 1.5 M, in acetate buffer, pH 4.0. As shown in Fig. 1, a major portion of the labelled material was eluted somewhat later than was a chondroitin sulphate standard, but ahead of heparin. Despite the heterogeneous appearance of the elution profile, which suggested the presence of several incompletely resolved components, subdivision of the eluate was not necessary for the purpose of this investigation. Labelled material was pooled as described in the legend to Fig. 1 and dialysed against water. In some preparations, the labelled polysaccharides were recovered from the ion-exchange column by batchwise elution with 1.5 M-LiCl in 0.05 M-acetate buffer, pH 4.0, after exhaustive washing with 0.2 M-NaCl in the same acetate buffer.

The unsaturated disulphated ^3H-labelled disaccharide ΔDi-diS_E was prepared from chondroitin sulphate E, kindly given by Dr. N. Seno, Department of Chemistry, Ochanomizu University, Tokyo, Japan. The polysaccharide was *N*-[^3H]acetyl-labelled by the procedure of Höök *et al.* (1982), which involved *N*-deacetylation by hydrazinolysis (100 °C for 2 h) followed by reaction with [^3H]acetic anhydride. Hexuronic acid residues are known to be partially converted into hydrazides during hydrazinolysis (Shaklee & Conrad, 1984), and carboxy groups were therefore regenerated by dialysis of the polysaccharide against 0.25 M-HIO_3, and then against several changes of water. The product (sp. radioactivity 300×10^3 c.p.m. of $^3H/\mu g$ of hexuronic acid) was digested with chondroitinase ABC, and the resulting disaccharides were isolated by gel chromatography on a column (1 cm × 170 cm) of Sephadex G-15, eluted with 0.2 M-NH_4HCO_3. After freeze-drying, the disulphated disaccharide (ΔDi-diS_E) was separated from non-sulphated and monosulphated disaccharides by preparative high-voltage paper electrophoresis at pH 1.7 (see below) and was finally eluted from the paper with water.

^3H-labelled unsaturated monosulphated disaccharides were prepared in a similar manner from chondroitin sulphate isolated from bovine nasal cartilage and kindly given by Dr. Å. Wasteson, Department of Cell Biology, University of Linköping, Linköping, Sweden. The corresponding 4- or 6-sulphated *N*-[^3H]acetylgalactosamine monosaccharides, obtained by treatment of the unsaturated disaccharides with mercuric acetate, were isolated by preparative paper electrophoresis (pH 1.7) and were finally separated by paper chromatography as described below (see also Fig. 4 below). The two monosaccharide sulphates were identified in a separate experiment by modifying this protocol such that a sample of the intact disaccharides was digested with chondro-6-sulphatase before cleavage with mercuric acetate. The resulting paper chromatogram lacked the slower-migrating component, which was therefore assumed to represent *N*-acetylgalactosamine 6-sulphate. *N*-[^3H]Acetylgalactosamine 4,6-disulphate was isolated in a similar fashion, after cleavage of labelled ΔDi-diS_E.

A ^{35}S-labelled trisulphated unsaturated disaccharide with the structure:

$$\Delta HexA(2\text{-}OSO_3)\text{-}GlcNSO_3(6\text{-}OSO_3)$$

was obtained by heparinase digestion (Linhardt *et al.*, 1986) of [^{35}S]heparin synthesized by mouse mastocytoma cells in culture. Confluent cultures were incubated with inorganic [^{35}S]sulphate for 1 h, and labelled polysaccharide was isolated as described (Jacobsson *et al.*, 1985). Chondroitinase ABC-resistant material (1.7×10^6 c.p.m.) was digested at 30 °C for 15 h with heparinase (3.5 μg of protein) in 1 ml of 2.5 mM-calcium acetate/0.25 M-sodium acetate, pH 7.0. Trisulphated disaccharide was isolated from the incubation mixture by gel chromatography and high-voltage paper electrophoresis as described above; it was the major disaccharide product and migrated faster than a disulphated disaccharide standard on paper electrophoresis at pH 1.7 (see the Results section).

HexA-[^3H]aMan_R disaccharides with *O*-sulphate groups in various positions were obtained as described (Pejler *et al.*, 1987), after deaminative cleavage of heparin with HNO_2. 2,5-Anhydro[1-^3H]mannitol(6-OSO_3) monosaccharide was prepared by digestion of GlcA-

[^{3}H]aMan$_R$(6-OSO$_3$) with bovine liver β-D-glucuronidase (Jacobsson *et al.*, 1979).

[^{3}H]Acetic anhydride (500 mCi/mmol) and inorganic [^{35}S]sulphate (carrier-free) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Chondroitinase ABC (chondroitin ABC lyase; EC 4.2.2.4) and chondro-6-sulphatase (EC 3.1.6.10) were purchased from Seikagaku Fine Chemicals, Tokyo, Japan, and Sigma Chemical Co., St. Louis, MO, U.S.A., respectively. Bacterial heparinase (heparin lyase; EC 4.2.2.7) was a gift from Dr. R. J. Linhardt, College of Pharmacy, University of Iowa City, Iowa City, IA, U.S.A.; Sephadex G-15 was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden; DEAE-cellulose (DE-52) was from Whatman Biochemicals, Maidstone, Kent, U.K.; Dowex 50W-X8 (200–400 mesh) was from Serva Feinbiochemica, Heidelberg, Germany. Bovine serum albumin and Pronase were from Sigma Chemical Co. Mercuric acetate (analytical grade) was a product of E. Merck, Darmstadt, Germany.

Methods

Hexuronic acid was determined by the carbazole method of Bitter & Muir (1962).

Glycosidic linkages of non-reducing-terminal unsaturated hexuronic acid units were cleaved by treatment with 10 mM-mercuric acetate at room temperature for 30 min (Ludwigs *et al.*, 1987), as described in the legend to Fig. 3 (below). The reaction products were either directly spotted on paper for high-voltage electrophoresis, or passed through small columns of Dowex 50W-X8 (Na^+ form) for removal of mercuric ions before further analysis.

Digestions of polysaccharides with chondroitinase ABC and of unsaturated disaccharides with chondro-6-sulphatase (Yamagata *et al.*, 1968; Saito *et al.*, 1968) were performed as described in the legends to Figs. 2 and 7 (below) respectively.

Paper chromatography was performed on Whatman no. 3MM paper, developed with ethyl acetate/acetic acid/water (3:1:1, by vol.). After development for the appropriate periods of time, the strips were dried and cut into 1 cm segments, which were transferred to vials for liquid-scintillation counting of radioactivity. The paper segments were extracted with 1 ml of water for 1 h before the addition of 5 ml of scintillation cocktail {0.2 g of POPOP [1,4-bis-(5-phenyloxazol-2-yl)benzene] and 3.3 g of PPO (2,5-diphenyloxazole) per litre of toluene/Triton X-100 (2:1, v/v)}. High-voltage electrophoresis was conducted on Whatman no. 3MM paper in 0.83 M-pyridine/0.5 M-acetic acid (pH 5.3; 80 V/cm) or in 1.6 M-formic acid (pH 1.7; 40 V/cm). After drying, papers were cut in 1 cm segments, which were analysed for radioactivity as described above.

RESULTS

Enzymic degradation of ^{35}S-labelled polysaccharides and isolation of disaccharides

The ^{35}S-labelled polysaccharides isolated (see the Experimental section) from rat small intestine ($\sim 500 \times 10^3$ c.p.m./g of tissue from infected rats as compared with $\sim 100 \times 10^3$ c.p.m./g from uninfected control animals) were digested with chondroitinase ABC, and the resulting unsaturated disaccharides were separated from resistant material by gel chromatography as described in the legend to Fig. 2. The degraded galactosaminoglycans corresponded to 50–60% of the total labelled macromolecules, in agreement with previous findings (Enerbäck *et al.*, 1985). This previous study had revealed a close correlation between the content of labelled polysaccharide, the histamine content and the number of mast cells in the intestinal mucosa. Moreover, autoradiography revealed a selective labelling of the mucosal mast cells. It was therefore concluded that the polysaccharide originated from these cells (Enerbäck *et al.*, 1985). The proportion of labelled heparin/heparan sulphate ($\sim$ 13% of the total labelled macromolecules) was less than half of that in the control material, in accord with the notion that the rat mucosal mast cell does not produce heparin.

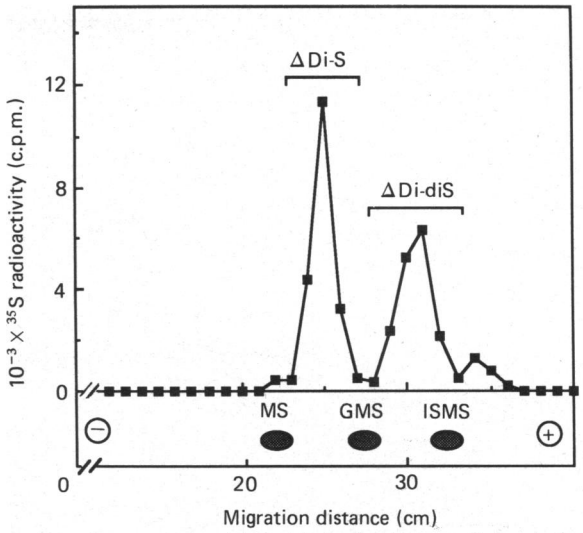

Fig. 2. Paper electrophoresis of disaccharides generated by digestion of ^{35}S-labelled galactosaminoglycan with chondroitinase ABC

A sample ($\sim 5 \times 10^6$ c.p.m.) of labelled polysaccharide, isolated as described in the legend to Fig. 1, was digested with 0.2 unit of chondroitinase ABC in 1 ml of 0.05 M-Tris/HCl/0.03 M-sodium acetate, pH 8.0, containing 100 μg of bovine serum albumin/ml. After incubation for 15 h at 37 °C, the digest was passed through a column (1 cm × 170 cm) of Sephadex G-15, equilibrated with 0.2 M-NH_4HCO_3, to separate disaccharides from undigested labelled polysaccharide. The disaccharides were freeze-dried and were then fractionated further by high-voltage paper electrophoresis at pH 5.3, as described in the Experimental section. Guide strips were analysed for ^{35}S as indicated. The zones corresponding to the peaks designated 'ΔDi-S' and 'ΔDi-diS' were cut out, and the labelled components were eluted from the strips. The standards shown below the tracing are: MS, [^{3}H]aMan$_R$(6-OSO$_3$); GMS, GlcA-[^{3}H]aMan$_R$(6-OSO$_3$); ISMS, IdoA(2-OSO$_3$)-[^{3}H]aMan$_R$(6-OSO$_3$) (see the Experimental section).

Fractionation of the unsaturated disaccharides by high-voltage paper electrophoresis at pH 5.3 gave the results shown in Fig. 2. Two major components were observed, and comparison with reference compounds derived from heparin (see the legend to Fig. 2 for details) indicated that the slower-migrating component (ΔDi-S) consisted of monosulphated disaccharide(s), whereas the faster-migrating component (ΔDi-diS) contained disulphated disaccharide(s). ΔDi-diS accounted for approx. 40% of the total radioactivity recovered as disaccharide

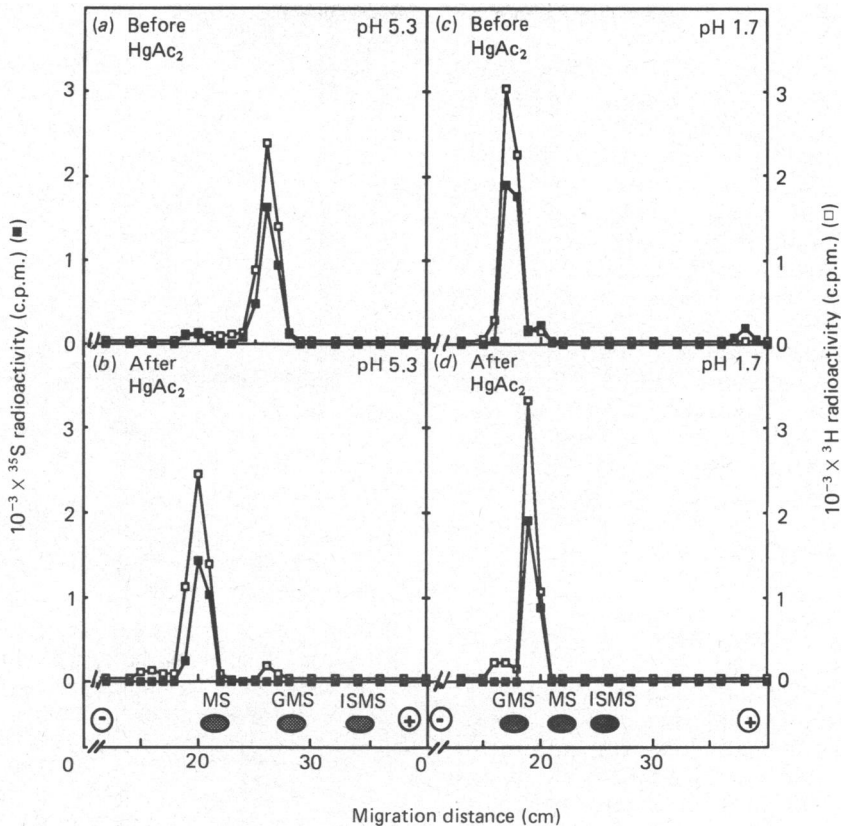

Fig. 3. Paper electrophoresis of unsaturated mono[^{35}S]sulphated disaccharides (*Δ*Di-S) before and after treatment with mercuric acetate ($HgAc_2$)

Monosulphated, unsaturated disaccharide [*Δ*Di-S in Fig. 2; 4000 c.p.m. of ^{35}S (■)] derived from mucosal mast-cell galactosaminoglycan, and the corresponding *N*-[^{3}H]acetyl-labelled disaccharide [6000 c.p.m. of ^{3}H (□)] prepared from cartilage chondroitin sulphate (see the Experimental section), were subjected to paper electrophoresis at pH 5.3 or at pH 1.7, either before or after treatment with mercuric acetate, as indicated. Samples (10 μl) were mixed with 10 μl of 20 mM-mercuric acetate/130 mM-sodium acetate, and the reaction was allowed to proceed at room temperature for 30 min. Before electrophoresis, mercuric ions were removed by passage of the reaction mixtures through small columns of Dowex 50 (Na^+ form ; 200–400 mesh). The standards below the tracings are the same as in Fig. 2.

after digestion with chondroitinase ABC. Sufficient amounts of the labelled disaccharide(s) were isolated by preparative electrophoresis, and further characterization was carried out as follows.

Characterization of monosulphated disaccharide(s) (*Δ*Di-S)

The unsaturated uronic acid component of the monosulphated disaccharide(s) was released by treatment with mercuric acetate, as described in detail in the legend to Fig. 3, and the reaction products as well as the untreated *Δ*Di-S material were analysed by high-voltage paper electrophoresis (Fig. 3). After mercuric acetate treatment, a single product was observed on electrophoresis at pH 5.3 (*b*) that migrated more slowly than the untreated disaccharide fraction (*a*). Compared with the reference standards, the reaction product had a slightly lower mobility than did anhydromannitol 6-sulphate. At pH 1.7, the untreated disaccharide fraction yielded a single peak, which was located in the same position as GlcA-aMan(6-OSO_3) (*c*). The single component observed after mercuric acetate treatment was located just ahead of the untreated control, between the monosulphated reference disaccharide and anhydromannitol 6-sulphate (*d*). Fig. 3 also shows the corresponding electrophoretic patterns for a [^{3}H]acetyl-labelled monosulphated unsaturated dissaccharide fraction (mixture of 4- and 6-sulphated species) derived from chondroitin sulphate (see the Experimental section). The ^{3}H-labelled standard invariably co-migrated with the ^{35}S-labelled samples before, as well as after, treatment with mercuric acetate.

These results indicate that the ^{35}S-labelled component released on mercuric acetate treatment of the monosulphated disaccharide fraction was *N*-acetylgalactosamine 4- or 6-sulphate. The parent disaccharide fraction, derived from the mucosal mast cells, would thus consist of one or both of the two species *Δ*Di-4S and *Δ*Di-6S. In particular, the migration characteristics of the products of mercuric acetate treatment clearly ruled out the presence of a sulphated uronic acid residue. On electrophoresis at pH 5.3, a sulphated uronic acid monosaccharide would have a higher charge density than a monosulphated disaccharide and therefore would migrate faster than the untreated disaccharide. No such fast-migrating product was observed, even when the separation was performed immediately after the mercuric acetate treatment (see below).

Further characterization of the putative *N*-acetyl-

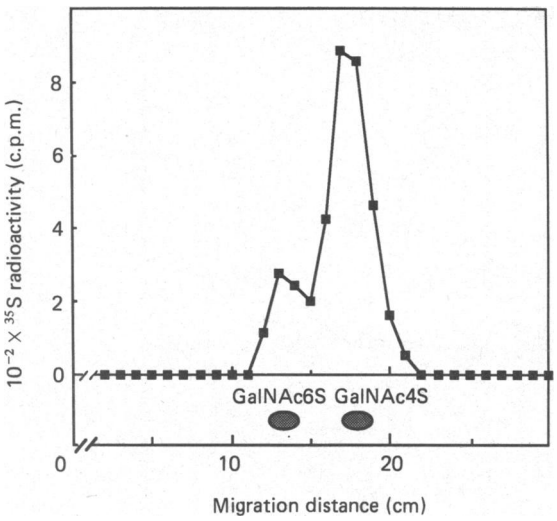

Fig. 4. Paper chromatograph of *N*-acetylgalactosamine [^{35}S]-sulphate formed by mercuric acetate treatment of unsaturated monosulphated disaccharides (*Δ*Di-S)

The ^{35}S-labelled product obtained by degradation of *Δ*Di-S with mercuric acetate was isolated by preparative paper electrophoresis at pH 1.7, as shown in Fig. 3(*d*), and was then subjected to paper chromatography (see the Experimental section). Below the tracings are indicated the migration positions of standard *N*-[^{3}H]acetylgalactosamine 6-sulphate (GalNAc6S) and *N*-[^{3}H]acetylgalactosamine 4-sulphate (GalNAc4S).

galactosamine [^{35}S]sulphate formed on mercuric acetate treatment of *Δ*Di-S was carried out by paper chromatography (Fig. 4). Most of the radioactivity coincided with the position of standard *N*-acetylgalactosamine 4-sulphate, but a minor component was also seen in the position of *N*-acetylgalactosamine 6-sulphate. The *Δ*Di-S fraction thus consisted mainly of the 4-sulphated disaccharide, *Δ*Di-4S, with smaller amounts of the 6-sulphated isomer, *Δ*Di-6S. These results are in agreement with previous analyses based on ion-exchange chromatography (Enerbäck *et al.*, 1985), and therefore indicate that the reaction with mercuric salts, previously studied only with a non-sulphated model disaccharide derived from hyaluronic acid (Ludwigs *et al.*, 1987), can be applied also to the structural analysis of sulphated unsaturated disaccharides.

Characterization of disulphated disaccharide(s) (*Δ*Di-diS)

Fraction *Δ*Di-diS was further separated by paper electrophoresis at pH 1.7. This purification step was required to remove a faster-moving component that migrated as a distinct peak ahead of the disulphated disaccharide(s) at this pH, but co-migrated with the latter at pH 5.3 (results not shown). This component represented approx. 10% of the total radioactivity in *Δ*Di-diS and was found in all preparations. It has not yet been characterized further.

The purified preparation of disulphated disaccharide(s) was treated with mercuric acetate as described in the Experimental section, and the reaction mixture was analysed by high-voltage paper electrophoresis. As seen in Figs. 5(*a*) and 5(*b*), the products separated into two fractions at pH 1.7: one which migrated more slowly than the untreated disaccharide(s) (peak I-pH 1.7), and one which migrated faster (peak II-pH 1.7). The mobility of the latter component was also greater than that of the standard disulphated disaccharide from heparin, which, in turn, migrated somewhat faster than untreated *Δ*Di-diS. At pH 5.3 (Figs. 5*c* and 5*d*, untreated *Δ*Di-diS migrated to approximately the same position as the disulphated standard (partially overlapping with, and partially just behind, the latter; see also Fig. 2). The mercuric acetate-treated sample again showed the presence of two peaks, which both migrated more slowly than did the untreated material. With reference to other compounds, the most retarded fraction, peak I-pH 5.3, migrated somewhat more slowly than the slowest standard (anhydromannitol 6-sulphate), whereas the faster-moving peak, II-pH 5.3, had a slightly lower mobility than had the untreated parent disaccharide, intermediate between those of the mono- and di-sulphated heparin disaccharide standards.

Keeping in mind those structural features of the oversulphated galactosaminoglycans which are already known, we are presumably dealing with one or both of the two disulphated disaccharides *Δ*HexA-GalNAc(4,6-di-OSO_3) and *Δ*HexA(2-OSO_3)-GalNAc(4-OSO_3), which originate from glucuronic acid-containing disaccharide units in chondroitin sulphate E and iduronic acid-containing units in dermatan sulphate respectively. Cleavage of the former disaccharide by mercuric acetate would be expected to yield a single ^{35}S-labelled product, namely *N*-acetylgalactosamine 4,6-disulphate, which has a higher charge density than the parent disaccharide at pH 1.7 and should therefore migrate faster. Peak II-pH 1.7 in Fig. 5(*b*) satisfies this criterion. At pH 5.3, the charge density of *N*-acetylgalactosamine 4,6-disulphate should be approximately the same as at pH 1.7, whereas the dissociation of the uronic acid carboxy group will increase the charge density of the parent disaccharide. The two compounds should thus have similar mobilities at pH 5.3; as seen in Figs. 5(*c*) and 5(*d*), peak II-pH 5.3 migrated only slightly behind the untreated disaccharide fraction. That the faster-migrating components did indeed correspond to *N*-acetylgalactosamine disulphate was ascertained by electrophoresis of a mercuric acetate-treated disulphated disaccharide, which had been isolated after chondroitinase ABC digestion of an authentic sample of [^{3}H]acetyl-labelled chondroitin sulphate E. The single labelled compound formed co-migrated with ^{35}S-labelled peak II-pH 5.3 (Fig. 5*d*) as well as with peak II-pH 1.7 (Fig. 5*b*).

It may be predicted that *Δ*HexA(2-OSO_3)-GalNAc(4-OSO_3), that is, the second disulphated disaccharide likely to be a component of fraction *Δ*Di-diS, will yield two ^{35}S-labelled products on treatment with mercuric acetate. Like the non-sulphated unsaturated uronic acid in the hyaluronic acid disaccharide *Δ*HexA-GlcNAc, the sulphated *Δ*4,5-hexuronosyl residue of the disulphated disaccharide may be expected to undergo immediate rearrangement into an oxo ('keto') acid after release as a monosaccharide (Ludwigs *et al.*, 1987). One of the potential degradation products, therefore, would be a sulphated oxo acid, formed along with the monosulphated monosaccharide *N*-acetylgalactosamine 4-sulphate. With regard to the electrophoretic properties, we may postulate that, at pH 1.7, these two fragments will migrate to the same position, since at this pH they are both monosulphated monosaccharides with a charge of -1. (The dissociation of the uronosyl carboxy group is

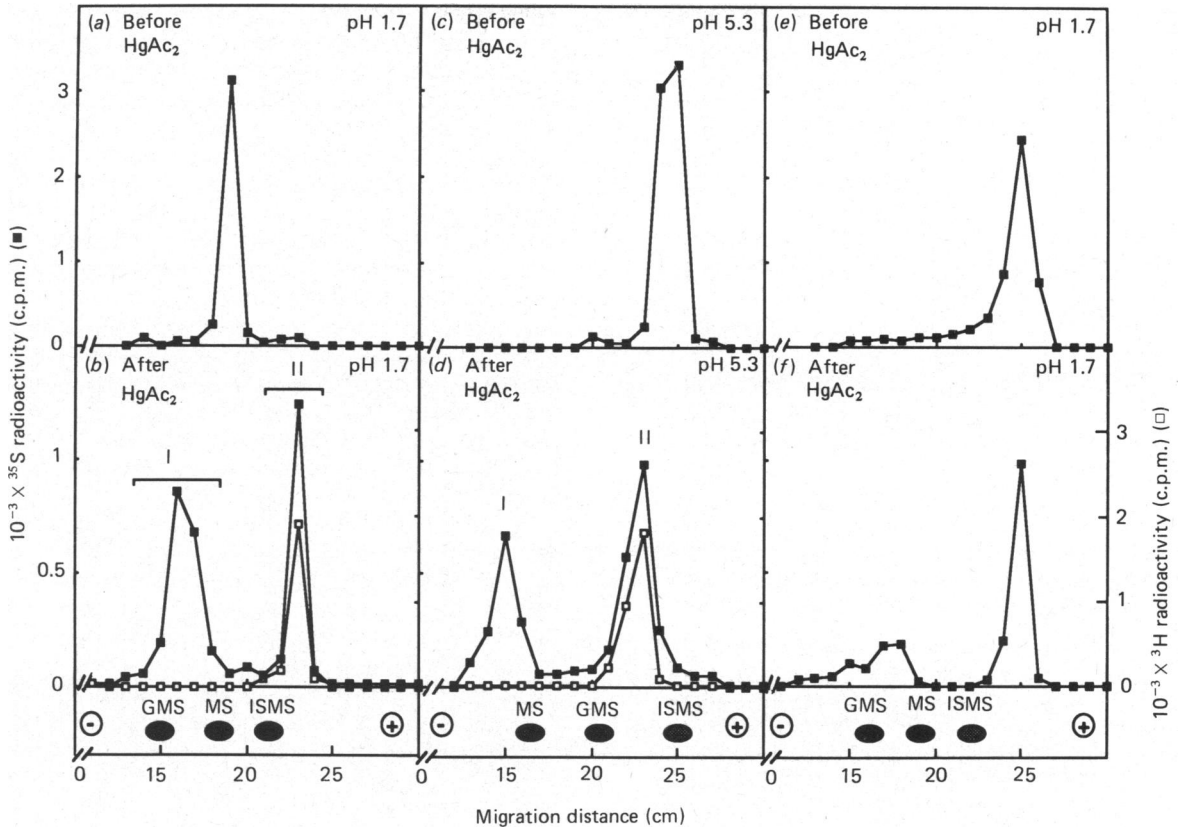

Fig. 5. Paper electrophoresis of unsaturated di[^{35}S]sulphated disaccharides (*Δ*Di-diS), before and after treatment with mercuric acetate ($HgAc_2$)

Disulphated unsaturated disaccharide [*Δ*Di-diS in Fig. 2; 5000 c.p.m. of ^{35}S (■)], derived from mucosal mast-cell polysaccharide (*a–d*) and trisulphated unsaturated disaccharide [5000 c.p.m. of ^{35}S (■)] derived from mastocytoma heparin (*e*, *f*) were subjected to paper electrophoresis at pH 1.7 or at pH 5.3, either before or after treatment with mercuric acetate, as indicated. Superimposed in (*b*) and (*d*) are peaks corresponding to 4000 c.p.m. of *N*-[^{3}H]acetylgalactosamine 4,6-disulphate standard (□). Additional standards, indicated below the tracings, are as described in the legend to Fig. 2. ^{35}S-labelled components corresponding to peak I-pH 1.7 and peak II-pH 1.7 (indicated by braces in *b*) were isolated by preparative paper electrophoresis and re-run at pH 5.3 (see Fig. 6).

suppressed at the acidic pH.) Moreover, they should be located close to the monosulphated monosaccharide standard derived from heparin. The mobility of peak I-pH 1.7 (Fig. 5*b*) was in agreement with the prediction. At pH 5.3 a different pattern is to be expected; whereas the charge density of *N*-acetylgalactosamine 4-sulphate is not substantially altered by the change in pH, the carboxy group of the oxo acid becomes ionized, and the sulphated oxo acid would acquire a charge density similar to that of *N*-acetylgalactosamine 4,6-disulphate. Since the position of the latter component corresponds to that of peak II-pH 5.3, the sulphated oxo acid would be expected to coincide with, or migrate close to, this peak. The validity of these predictions, relating to the sulphated oxo acid, was verified in experiments with the tri[^{35}S]-sulphated disaccharide *Δ*HexA(2-OSO_3)-$GlcNSO_3$(6-OSO_3) isolated after digestion of biosynthetically labelled heparin with bacterial heparinase (see the Experimental section). After treatment of this disaccharide with mercuric acetate, paper electrophoresis at pH 1.7 yielded two ^{35}S-labelled components (Fig. 5*f*).

The major product, presumably $GlcNSO_3$(6-OSO_3), corresponded to about two-thirds of the total radioactivity, and had migrated similar to the untreated disaccharide (Fig. 5*e*), distinctly faster than the disulphated IdoA(2-OSO_3)-aMan_R(6-OSO_3) standard. A slower-migrating fraction, comprising about one-third of the total ^{35}S (as expected for the sulphated oxo acid derivative), appeared at approximately the same position (Fig. 5*f*) as peak I-pH 1.7 (cf. Fig. 5*b*); the somewhat smeared appearance of this fraction is explained below. When the degradation products of the heparin disaccharide were instead analysed at pH 5.3, all the radioactivity was accumulated in a broad peak located between the mono- and di-sulphated disaccharide standards (not shown); no slower-migrating ^{35}S-labelled component was observed.

On the basis of these considerations, and assuming that the initial disulphated disaccharide fraction, *Δ*Di-diS, contained the *Δ*HexA(2-OSO_3)-GalNAc(4-OSO_3) species in addition to the *Δ*HexA-GalNAc(4,6-di-OSO_3) component, the following composition may be anticipated for the various fractions formed by mercuric acetate treatment: peak I-pH 1.7, *N*-acetylgalactosamine 4-sulphate and sulphated oxo acid; peak II-pH 1.7, *N*-acetylgalactosamine 4,6-disulphate; peak I-pH 5.3, *N*-acetylgalactosamine 4-sulphate; peak II-pH 5.3, sulphated oxo acid and *N*-acetylgalactosamine 4,6-disulphate.

To substantiate these assumptions, peak I-pH 1.7 was

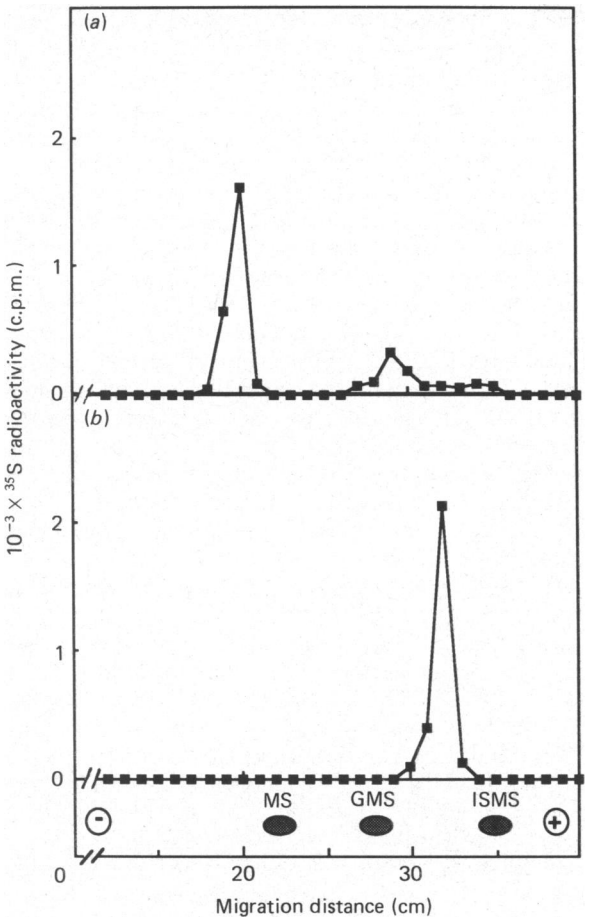

Fig. 6. Paper electrophoresis at pH 5.3 of ^{35}S-labelled products isolated at pH 1.7 after mercuric acetate treatment of ΔDi-diS

The di[^{35}S]sulphated unsaturated disaccharide fraction, ΔDi-diS, obtained from mucosal mast-cell polysaccharide, was treated with mercuric acetate, and the products were fractionated by paper electrophoresis at pH 1.7 as shown in Fig. 5(*b*). Peak I-pH 1.7 and peak II-pH 1.7 were isolated and subjected to repeated electrophoresis, at pH 5.3; the results are shown in (*a*) for peak I-pH 1.7 and in (*b*) for peak II-pH 1.7. The standards below the tracings are as described in Fig. 2.

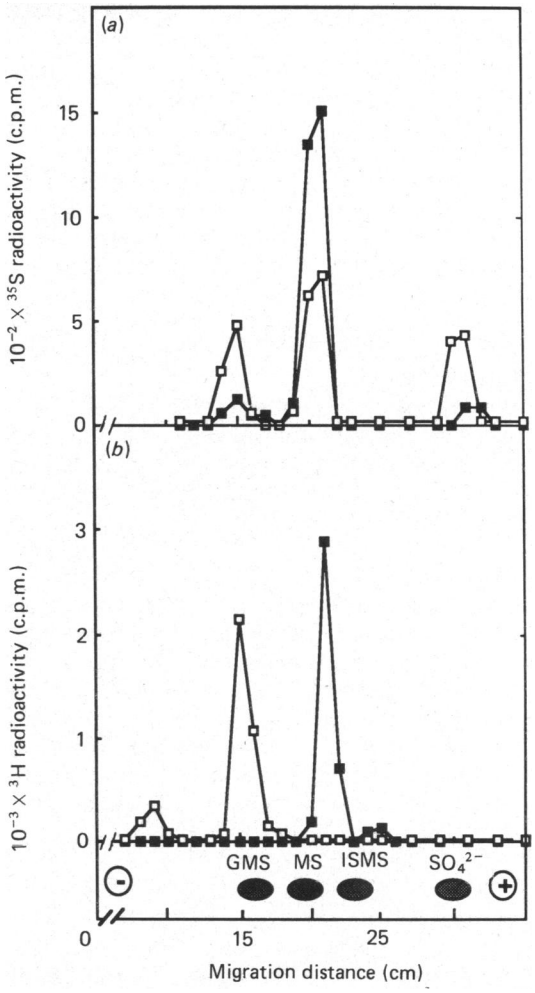

Fig. 7. Digestion of ΔDi-diS with chondro-6-sulphatase

Samples of Δi-diS derived from mucosal mast-cell polysaccharide (*a*; 5000 c.p.m. of ^{35}S) and of *N*-[^{3}H]acetyl-labelled ΔDi-diS_E standard (*b*: 6000 c.p.m. of ^{3}H), were incubated for 60 min at 37 °C with 0.1 unit of chondro-6-sulphatase in 50 μl of 0.05 M-Tris/HCl/0.03 M-sodium acetate, pH 8.0, containing 100 μg of bovine serum albumin/ml. Each disaccharide sample was analysed by high-voltage paper electrophoresis at pH 1.7, either before (■) or after (□) enzyme digestion. The standards shown below the tracings are as described in the legend to Fig. 2, and, in addition, inorganic [^{35}S]sulphate (SO_4^{2-}).

isolated by preparative paper electrophoresis at pH 1.7 and was then subjected to electrophoresis at pH 5.3. A major component was seen (Fig. 6*a*), presumably *N*-acetylgalactosamine 4-sulphate, which migrated slightly behind the monosulphated monosaccharide standard from heparin (similar to peak I-pH 5.3; see Fig. 5*d*). A second, less abundant, component was also observed, which migrated faster and was located between the monosulphated and the disulphated heparin disaccharide standards (essentially similar to peak II-pH 5.3; see Fig. 5*d*). This material, along with an additional component of somewhat greater mobility, constituted approx. 30% of the total radioactivity of the sample. Although these findings confirm, in a qualitative sense, the presence of the two postulated monosulphated monosaccharide moieties in peak I-pH 1.7, their evaluation is complicated by the slurred profile of the faster-migrating material and the deviation from the theoretical distribution of radioactivity between the two monosaccharide sulphates (expected ratio 1:1). However, previous studies (Ludwigs *et al.*, 1987, and references cited therein) showed that the oxo acid (hence presumably also the sulphated oxo acid) is unstable and is converted into products which have not been well characterized. Thus it was not possible to detect the oxo acid after paper chromatography overnight, whereas paper electrophoresis immediately after the completed reaction with mercuric acetate allowed the revelation of an *o*-phenylenediamine-staining component, before extensive degradation had occurred. This lability of the oxo acid could account for the observed diffuse electrophoretic pattern as well as for the low yield of the faster-migrating material, smearing degradation products being difficult to distinguish from background radioactivity. Similar analysis of peak II-pH 1.7 yielded a single peak only, with the expected

mobility of *N*-acetylgalactosamine 4,6-disulphate at pH 5.3 (Fig. 6*b*).

Peak I-pH 1.7 and peak II-pH 1.7 were further characterized by paper chromatography (results not shown). Peak II-pH 1.7 co-chromatographed with authentic [^{3}H]acetyl-labelled *N*-acetylgalactosamine 4,6-disulphate, prepared by mercuric acetate treatment of ΔDi-diS$_E$ (see the Experimental section), and migrated slightly faster than the undegraded disaccharide. Peak I-pH 1.7 was separated into three components. The major ^{35}S-labelled component had the same R_F value as *N*-[^{3}H]acetylgalactosamine 4-sulphate, whereas approx. 15% of the ^{35}S remained at the origin and 20% showed intermediate mobility.

These results are consistent with the notion that the disulphated disaccharides obtained by chondroitinase ABC digestion are a mixture of ΔDi-diS$_B$ and ΔDi-diS$_E$. Owing to the lability of the sulphated oxo acid, they do not, however, provide a reliable measure of the proportions of these components. Therefore an additional experiment was undertaken in which the entire disulphated disaccharide fraction (ΔDi-diS) was digested with chondro-6-sulphatase. Analysis of the products by paper electrophoresis (Fig. 7*a*) showed that approx. 45% of the ^{35}S-labelled material was resistant to digestion (and thus representative of ΔDi-diS$_B$ disaccharide), whereas the remainder was converted into ΔDi-4S and inorganic sulphate. Under identical conditions, a standard of *N*-[^{3}H]acetyl-labelled ΔDi-diS$_E$ was quantitatively converted into [^{3}H]ΔDi-4S (Fig. 7*b*).

DISCUSSION

In contrast with the unsaturated monosulphated disaccharides obtained by eliminase digestion of galactosaminoglycans, which are readily separated by various chromatographic procedures, the disulphated species are difficult to characterize. Selective removal of sulphate groups from the *N*-acetylgalactosamine units by digestion of such disaccharides with specific chondrosulphatases has been successfully employed (Suzuki *et al.*, 1968), but is critically dependent on the purity of the enzymes. Sulphate groups on the uronic acid moieties have been demonstrated (Suzuki, 1960) and in some cases located (Seno & Murakami, 1982) by using periodate oxidation of unsaturated or saturated disaccharides. Stevens *et al.* (1986) based their identification of ΔDi-diS$_B$ on h.p.l.c., although the data presented (see Fig. 4*a* in the cited paper) showed only partial separation of this disaccharide from ΔDi-diS$_E$. Nevertheless, these authors claimed that ΔDi-diS$_B$ accounted for $> 90\%$ of the disulphated disaccharide units obtained from the mast-cell polysaccharide, and suggested that the discrepancy in relation to our previous results (Enerbäck *et al.*, 1985) might be ascribed largely to differences in the analytical techniques employed.

In the present study the problem has been approached by a novel technique involving cleavage of the glycosidic bond between the unsaturated hexuronic acid unit and the *N*-acetylgalactosamine moiety by mercuric ions. The method was developed by using the unsaturated disaccharide from hyaluronan as a model compound (Ludwigs *et al.*, 1987), but it is obviously applicable also to eliminase digestion products of sulphated glycosaminoglycans. Our results show, in contrast with the proposal of Stevens *et al.* (1986), that ΔDi-diS$_E$ is not a minor component but accounts for about half of the total disulphated disaccharide generated by chondroitinase ABC digestion of the mucosal mast-cell polysaccharide. This estimate is based primarily on the ratio between peak I-pH 1.7 (which represents the sum of the *N*-acetylgalactosamine monosulphate and the sulphated oxo acid from ΔDi-diS$_B$) and peak II-pH 1.7 (*N*-acetylgalactosamine 4,6-disulphate from ΔDi-diS$_E$) (Fig. 5*b*), and allows for some underestimation of peak I-pH 1.7 (attributable to partial loss of the labile oxo acid). Moreover, it is in good agreement with the value calculated from the composition of the products obtained on digestion of the total disulphated disaccharide with chondro-6-sulphatase (Fig. 7). It is unclear at present whether the discrepancy between our conclusions and those of Stevens *et al.* (1986) should indeed be ascribed to the different analytical techniques employed, or to variations in the biosynthetic capacity of the mucosal mast cells under study.

Finally, a world of caution regarding the identity of the disulphated disaccharide referred to as 'ΔDi-diS$_B$' in this report and in that of Stevens *et al.* (1986). It has become increasingly apparent that galactosaminoglycans occur in a large variety of structures, and it is particularly notable that both D-glucuronic and L-iduronic acid units may be sulphated at C-2 and/or C-3 (Seldin *et al.*, 1984). In our opinion the evidence available is not sufficient to establish conclusively the identity of the sulphated hexuronic acid unit in the galactosaminoglycan of mucosal mast cells, nor the position of the sulphate group. Owing to loss of the asymmetric centre at C-5 on introduction of the double bond, cleavage by chondroitinase ABC of a galactosaminidic linkage to D-glucuronic acid or L-iduronic acid will generate the same Δ-4,5-unsaturated uronic acid residue. In previous experiments, digestion of the mast-cell polysaccharide with chondroitinase ABC yielded larger amounts of disulphated disaccharides than did digestion with chondroitinase AC, as expected for an iduronic acid-containing sequence (Enerbäck *et al.*, 1985). On the other hand, galactosaminoglycan preparations may resist chondroitinase AC digestion in spite of a high glucuronic acid content (Yanagashita *et al.*, 1979), presumably owing to inhibitory effects of sulphate groups. Furthermore, attempts at defining the location of sulphate groups in unsaturated disulphated disaccharides, using sulphatase digestion, have been inconsistent. The alleged ΔDi-diS$_B$ described by Stevens *et al.* (1986) thus was claimed to resist digestion by chondro-4-sulphatase, in apparent disagreement with the original finding of Suzuki *et al.* (1968). Although this discrepancy may well have a trivial explanation, the occurrence in the mast-cell galactosaminoglycan of as yet unrecognized sequences cannot be excluded.

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