Reaction of unsaturated uronic acid residues with mercuric salts

Cleavage of the hyaluronic acid disaccharide

2-acetamido-2-deoxy-3-*O*-(β -D-glucopyranosyluronic acid)-D-glucose

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Degradation of connective-tissue polysaccharides with bacterial or fungal eliminases and subsequent characterization of the reaction products are now part of standard methodology for the analysis of these compounds. However, the scope of preparative and analytical work based on the use of eliminases has been limited by the lack of procedures for specific removal of the unsaturated uronic acid residues generated in the eliminase reactions. In the present investigation, we have shown that these residues are cleaved by mercuric salts under mild conditions that are not likely to affect other structures in an oligo- or poly-saccharide molecule. Thus the disaccharide generated from hyaluronic acid by digestion with chondroitinase AC or ABC was cleaved into a keto acid and free *N*-acetylglucosamine within 10 min at room temperature upon exposure to 14 mM-mercuric acetate at pH 5. The reaction of the disaccharide with mercuric salts was used for ready determination of the distribution of radioactivity between the glucuronic acid and *N*-acetylglucosamine moieties in radioactive hyaluronic acid that had been synthesized by IMR-90 fibroblasts from ^3H -labelled monosaccharides. When the precursor was [^3H]galactose, over 95% of the incorporated radioactivity was found in the glucuronic acid moiety. In contrast, cells grown in the presence of [^3H]glucosamine synthesized a polysaccharide in which almost all of the label was located in the *N*-acetylglucosamine units. It is apparent from these experiments that the reaction of unsaturated uronic acid residues with mercuric salts provides a new tool with potential for many applications in the study of the structure and metabolism of connective-tissue polysaccharides.

INTRODUCTION

Bacterial and fungal eliminases are powerful tools in the study of connective-tissue polysaccharides and are used routinely in many laboratories to determine the composition of polysaccharide mixtures and to analyse the fine structure of individual polysaccharides (see, e.g. Linker *et al.*, 1956, 1960; Hoffman *et al.*, 1960; Linker & Hovingh, 1965, 1972; Yamagata *et al.*, 1968; Saito *et al.*, 1968; Suzuki *et al.*, 1968; Suzuki, 1972; Hascall *et al.*, 1972; Hiyama & Okada, 1976; Hiyama, 1976; Kennedy, 1979). Common to the reactions catalysed by these enzymes is the cleavage of hexosaminidic linkages to uronic acid residues by an elimination mechanism that results in the introduction of a double bond between C-4 and C-5 in the uronosyl groups. To extend the usefulness of eliminases as preparative and analytical tools, we have searched for methods by which the unsaturated uronic acid residues can be removed from the non-reducing terminal position of the reaction products without damage to adjacent structures. Using the disaccharide produced from hyaluronic acid by digestion with chondroitinase AC or ABC as the test compound, we have developed such a method, which is based on the known reaction of mercuric salts with the double bond of a vinyl ether structure (Norton, 1969; Schmidt *et al.*,

1969; Ferrier, 1980; and references cited therein). Details of this work are described here, together with the application of the new method to the determination of the distribution of radioactivity between the glucuronic acid and *N*-acetylglucosamine moieties in biosynthetically labelled hyaluronic acid. A preliminary report has appeared (Ludwigs *et al.*, 1985).

MATERIALS AND METHODS

Materials

Hyaluronic acid was prepared from umbilical cords essentially as described previously (Rodén *et al.*, 1972). Chondroitinase AC, chondroitinase ABC and *Streptomyces* hyaluronidase were purchased from Miles Laboratories, Elkhart, IN, U.S.A. Glucosamine and *N*-acetylglucosamine were from Pfanstiehl, Waukegan, IL, U.S.A., and glucuronic acid was from Sigma Chemical Co., St. Louis, MO, U.S.A. Twice-recrystallized papain was prepared from crude enzyme (type I; Sigma Chemical Co.) by the procedure of Kimmel & Smith (1954). Sephadex G-50 (fine grade), Sephadex G-10, PD-10 columns and DEAE-Sephacel were from Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A. AG 50W-X4 resin (200–400 mesh) and AG 1-X10 resin

Abbreviations used: MBTH, 3-methyl-2-benzothiazolinone hydrazone hydrochloride; $\Delta\text{Di-HA}$, 2-acetamido-2-deoxy-3-*O*-(β -D-glucopyranosyluronic acid)-D-glucose; $\Delta\text{Di-4S}$, 2-acetamido-2-deoxy-3-*O*-(β -D-glucopyranosyluronic acid)-4-*O*-sulpho-D-galactose.

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(200–400 mesh) were from Bio-Rad Laboratories, Richmond, CA, U.S.A.). MBTH was obtained from Aldrich Chemical Co., Milwaukee, WI, U.S.A. [^3H]Glucosamine (31.3 Ci/mmol) and [4,5- ^3H]galactose (44.1 Ci/mmol) were purchased from New England Nuclear, Boston, MA, U.S.A., and [^3H]galactose (10.4 Ci/mmol) was from Amersham Corp., Arlington Heights, IL, U.S.A. Other chemicals were of reagent grade.

Methods

Cell culture. The IMR-90 line of human-embryo lung fibroblasts (Nichols *et al.*, 1977) was obtained from the American Type Culture Collection (Hay *et al.*, 1982), Camden, NJ, U.S.A., and was maintained at 37 °C in a humidified environment of air/CO₂ (19:1) in Eagle's minimum essential medium with Earle's salts supplemented with 10% (v/v) fetal bovine serum (Grand Island Biological Co., Grand Island, NY, U.S.A.) (Elgavish *et al.*, 1985). The fibroblasts were grown to confluence in 70 cm² Corning culture flasks containing 15 ml of medium. Labelling was initiated 5–6 days after seeding, as the cultures were reaching confluency. The growth medium was removed, the cells were washed three times with serum-free medium (Eagle's minimum essential medium with Earle's salts), and 15 ml of serum-free medium was added, which contained [^3H]galactose (3 or 7 $\mu\text{Ci/ml}$), [4,5- ^3H]galactose (3 $\mu\text{Ci/ml}$) or [^3H]glucosamine (7 $\mu\text{Ci/ml}$). After incubation at 37 °C for 24 h, the medium was removed, and the cells were washed four times with 3 ml portions of 136.9 mM-NaCl/2.7 mM-KCl/4.3 mM-Na₂HPO₄/1.5 mM-KH₂PO₄, pH 7.5. Medium and cells were stored at –18 °C until processed further.

Analytical procedures. Uronic acid was measured by the carbazole method of Dische (1947), with D-glucuronic acid as standard, or by an automated modification described by Ford & Baker (1978); the latter was used exclusively for analysis of column effluents. *N*-Acetylhexosamine was measured by the Morgan–Elson procedure (Reissig *et al.*, 1955), with *N*-acetylglucosamine as standard. Hexosamine was measured by the MBTH method (Smith & Gilkerson, 1979) or by the Elson–Morgan procedure (Kraan & Muir, 1957), with glucosamine hydrochloride as standard. Keto acid analysis was carried out by the thiobarbituric acid assay as described by Weissbach & Hurwitz (1959), or by the *o*-phenylenediamine method as described by Lanning & Cohen (1951). Radioactivity was determined in a Packard model 2450 liquid-scintillation spectrometer, with 0.5 ml of aqueous sample and 4 ml of Scintiverse (Fisher Scientific, Springfield, NJ, U.S.A.).

Paper electrophoresis and chromatography. Electrophoresis was carried out in 0.12 M-pyridine/0.06 M-acetic acid, pH 5.3, at 3000 V for 30 min on 55 cm Whatman no. 3MM papers in a Gilson model D high-voltage Electrophorator or at 25 mA (constant current) for 60 min on 33 cm papers in a Camag horizontal-electrophoresis tank. Ascending paper chromatography was carried out on Whatman no. 3MM paper, which was developed overnight in isobutyric acid/0.5 M-NH₃ (5:3, v/v). After thorough drying (brief soaking in acetone was sometimes used as part of the drying process and improved the quality of the subsequent staining), the papers were stained with appropriate location reagents,

including AgNO₃ and alkali (Menzies & Seakins, 1969), the Morgan–Elson reagent (Salton, 1959) and the *o*-phenylenediamine reagent (Lanning & Cohen, 1951). For measurement of radioactivity, papers were cut into 1 cm strips, and each strip was extracted with 0.5 ml of water for 60 min and its radioactivity counted after addition of Scintiverse.

Gel chromatography. Separation of reaction products after treatment of $\Delta\text{Di-HA}$ with mercuric salts was carried out on a column (1.5 cm $\times$ 200 cm) of Sephadex G-10, which was eluted with 0.2 M-NH₄HCO₃ at a flow rate of 10 ml/h. Exact fraction sizes were determined by weighing. Fractions were analysed for uronic acid, *N*-acetylhexosamine, hexosamine and radioactivity as described above. Additional analyses by the thiobarbituric acid method or *o*-phenylenediamine method were carried out in some experiments. A column (1 cm $\times$ 50 cm) of Sephadex G-50 (fine grade), eluted with 0.2 M-NH₄HCO₃, was used for isolation of ^3H -labelled $\Delta\text{Di-HA}$.

H.p.l.c. The apparatus for h.p.l.c. (Constametric pump III and Spectrometer III) was purchased from Laboratory Data Control, Riviera Beach, FL, U.S.A., and the output was recorded on a 3390A Reporting Integrator (Hewlett–Packard, Avondale, PA, U.S.A.). Fractionation of reaction products after treatment of $\Delta\text{Di-HA}$ with mercuric salts was carried out on a Carbohydrate column (0.39 cm $\times$ 30 cm; Waters Associates), which was eluted with 10 mM-Na₂SO₄/50 mM-sodium acetate, pH 5.0, at a flow rate of 0.75 ml/min (see also Hjerpe *et al.*, 1979). The effluent was monitored at 232 nm. In some experiments the eluent contained no sodium sulphate. Usually the sample size was 10 μl .

Digestion of hyaluronic acid with chondroitinases. $\Delta\text{Di-HA}$ was obtained by incubation of hyaluronic acid (1 mg/ml) at 37 °C overnight with chondroitinase AC or ABC (0.01 unit/mg of polysaccharide) in 50 mM-Tris/50 mM-sodium acetate, pH 7.5. Reaction mixtures were checked for complete digestion by measurement of the absorbance at 232 nm before and after addition of excess enzyme to a sample of the digest. On electrophoresis and paper chromatography, the disaccharide preparation so obtained gave a single spot, as seen under short-wave u.v. light or by staining with the Morgan–Elson reagent or AgNO₃ and alkali. For most experiments the chondroitinase digests were used without further purification.

Isolation of ^3H -labelled hyaluronic acid. ^3H -labelled hyaluronic acid was isolated by the following procedure from the media of IMR-90 fibroblast cultures, grown in the presence of ^3H -labelled galactose or glucosamine. The medium (5–15 ml) was concentrated to dryness in a rotary evaporator, papain (1 mg) in 1 ml of 10 mM-EDTA/6 mM-cysteine hydrochloride/0.1 M-sodium acetate, pH 5.8, was added, and digestion was carried out overnight at 65 °C. After centrifugation at 5000 *g* for 10 min, the supernatant was applied to a column (1 cm $\times$ 50 cm) of Sephadex G-50, which was eluted with 0.15 M-NaCl or 0.2 M-NH₄HCO₃. Radioactive material eluted at the void volume was evaporated to a small volume and desalted on a PD-10 column, and was then evaporated to dryness and dissolved in 0.2 ml

Fig. 1. Chromatography on DEAE-Sephacel of ^3H -labelled glycosaminoglycans from the medium of IMR-90 fibroblasts grown in the presence of $[1\text{-}^3\text{H}]\text{galactose}$

●, Radioactivity; ○, NaCl concentration. The insert shows the profiles obtained on Sephadex G-50 chromatography of the major peak (eluted at approx. 0.35 M-NaCl) before (○) and after (●) digestion with *Streptomyces* hyaluronidase. For experimental details see the text.

of 50 mM-Tris/acetate, pH 7.0. (If NH_4HCO_3 had been used as eluent, desalting was accomplished by repeated evaporations.) The solution was then applied to a 2 ml column of DEAE-Sephacel, equilibrated in the same buffer, and the column was eluted over a 16 h period with a linear gradient of 0–1 M-NaCl in 50 mM-Tris/acetate, pH 7.0, at a flow rate of 10 ml/h. The gradient, which was generated by an Ultrograd 11300 gradient mixer (LKB Instruments, Bromma, Sweden), was checked by conductivity measurements on a Radiometer instrument (Copenhagen, Denmark), and the salt concentrations of individual fractions were determined by comparison with a standard curve of NaCl in buffer. Under the conditions chosen, a hyaluronic acid standard was eluted at an NaCl concentration of 0.3 M. As illustrated in Fig. 1, the papain digest from a culture grown in the presence of $[1\text{-}^3\text{H}]\text{galactose}$ yielded a radioactive peak in the same position, and this material was identified as hyaluronic acid by the following procedure. After concentration in a rotary evaporator and desalting on a PD-10 column, a sample (5000 c.p.m.) of the labelled product was mixed with 200 μg of hyaluronic acid and treated with 10 TRU (turbidity-reducing units) of *Streptomyces* hyaluronidase in 0.15 M-NaCl/50 mM-sodium acetate, pH 5.0, at 60 °C for 4 h. Whereas an untreated control sample emerged in the void volume from a column (1 cm $\times$ 50 cm) of Sephadex G-50, the *Streptomyces*-hyaluronidase digest yielded a broad peak of radioactivity in a retarded position. Radiolabelled $\Delta\text{Di-HA}$ was prepared from the ^3H -labelled hyaluronic acid by digestion with chondroi-

tinase AC or ABC, as described above, after the addition of various amounts of carrier hyaluronic acid.

The distribution of radioactivity between the uronic acid and *N*-acetylglucosamine moieties of $[1\text{-}^3\text{H}]\text{galactose}$ -labelled hyaluronic acid was determined as follows. A sample (approx. 30000 c.p.m.) of the labelled polysaccharide was mixed with *N*-acetylglucosamine (10 mg) and hydrolysed in 4 M-HCl for 16 h at 100 °C. The hydrolysate (0.32 ml) was diluted to 5 ml with water, and a 0.5 ml portion was applied to a column (bed volume 1 ml) of AG 50W resin (H^+ form), which was washed with four 2 ml portions of water and subsequently eluted with 2 ml of 4 M-HCl. The eluate and washes were analysed for radioactivity and glucosamine content (MBTH method) as described above; recoveries of applied radioactivity and glucosamine from the resin were quantitative.

Treatment of $\Delta\text{Di-HA}$ with mercuric chloride. Standard reaction mixtures contained equal volumes of $\Delta\text{Di-HA}$ (1 mg/ml) in 50 mM-Tris/50 mM-sodium acetate, pH 7.5, and 10 mM- HgCl_2 . The pH of this reaction mixture was 6.8. Incubations were carried out at 65 °C for 60–90 min, unless otherwise indicated.

Treatment of $\Delta\text{Di-HA}$ with mercuric acetate. Mercuric acetate (2.23 g) was dissolved in approx. 80 ml of water, and 0.5 ml of acetic acid was added to prevent the formation of a precipitate of HgO . The pH was then adjusted to 5.0 by addition of 2 M-sodium acetate, and

Fig. 2. Uronic acid analysis (carbazole method) after incubation of Δ Di-HA with 5 mM- HgCl_2 for 90 min at different temperatures

the final volume was made up to 100 ml, yielding a solution that was 70 mM in mercuric acetate. Standard reaction mixtures contained equal volumes of this reagent and a solution of Δ Di-HA (1 mg/ml). The pH of this mixture was 4.9–5.0.

Removal of Hg^{2+} ions from reaction mixtures containing mercuric acetate was accomplished by passing the samples over small columns (bed volume 1–2 ml) of AG 50W resin (200–400 mesh; H^+ or Na^+ form), which were eluted with water. In some experiments, further fractionation was carried out by passing the AG 50W eluates over columns (bed volume 1–2 ml) of AG 1-X10 resin (200–400 mesh; HCO_3^- form). In control experiments it was established that Δ Di-HA was retained quantitatively by the anion-exchange resin, as indicated by uronic acid analysis, whereas 90% of *N*-acetylglucosamine was recovered in the effluent. (The samples were applied as neutral or slightly basic solutions and were eluted with water.)

RESULTS

Reaction of Δ Di-HA with mercuric chloride

Evidence that the unsaturated uronic acid moiety of Δ Di-HA reacts with HgCl_2 was first obtained by the experiment shown in Fig. 2. When Δ Di-HA was incubated for 90 min at different temperatures in the presence of 5 mM- HgCl_2 , the uronic acid content of the reaction mixtures decreased, as shown by analysis with the carbazole method. At 65 °C and 100 °C close to 90% of the uronic acid was destroyed under the conditions chosen, whereas at 20 °C a decrease of only about one-third was observed.

The time course of the reaction at 65 °C, as monitored by the carbazole reaction, is shown in Fig. 3. After 10 min less than 50% of the uronic acid remained, and after 2 h no carbazole-positive material was present. In the interval between 10–15 min and 1 h, however, no significant change in the apparent uronic acid content occurred. The reason for this biphasic course of the reaction, observed in several experiments, is not clear. In control experiments no decrease in uronic acid content was observed in the absence of HgCl_2 , nor did HgCl_2

Fig. 3. Time course of reaction of Δ Di-HA with 5 mM- HgCl_2 at 65 °C, as determined by uronic acid analysis (carbazole method)

●, Control; ○, HgCl_2 -treated disaccharide; ■, HgCl_2 -treated hyaluronic acid; □, Δ Di-HA in 0.25 M-HCl without HgCl_2 . Reactions were stopped by addition of HCl to a final concentration of 0.25 M.

have any effect on intact hyaluronic acid. In yet another reaction mixture, which contained Δ Di-HA and 0.25 M-HCl but no HgCl_2 , the uronic acid content did not change significantly during the 2 h incubation at 65 °C.

Characterization of reaction products

The results of paper-electrophoretic and paper-chromatographic analyses of the reaction products are shown in Fig. 4. After treatment of Δ Di-HA with HgCl_2 or mercuric acetate, high-voltage paper electrophoresis at pH 5.3 showed the presence of two reaction products (Fig. 4a). One of these was Morgan–Elson-positive and was located near the origin in the same position as *N*-acetylglucosamine. The second product stained with the keto acid reagent *o*-phenylenediamine, and had migrated 11 cm towards the anode, i.e. slightly faster than glucuronic acid. Untreated disaccharide was located 8 cm from the origin in the direction of the anode on the electrophoretogram, as detected by staining with the Morgan–Elson reagent or by inspection under short-wave u.v. light. A sample that had been heated at 65 °C in buffer without HgCl_2 behaved similarly to the untreated disaccharide.

The formation of *N*-acetylglucosamine upon cleavage of Δ Di-HA by mercuric salts was also shown by paper chromatography followed by staining with the Morgan–Elson reagent (Fig. 4b). However, the *o*-phenylenediamine-reactive material observed after the electrophoretic separation was absent from the paper chromatogram. In contrast, a positive reaction with the keto acid reagent was seen on papers that were stained immediately after application of the sample or 15 min after the start of the chromatography. Although the lability of the putative keto acid indicated by these findings was initially surprising to us, we have subsequently learned that similar observations have been made by others, e.g. by Palleroni & Doudoroff (1956).

Further characterization of the reaction products was

Fig. 4. Paper electrophoresis (a) and paper chromatography (b) of reaction products after treatment of Δ Di-HA with mercuric acetate

Lane 1, glucuronic acid; lane 2, *N*-acetylglucosamine; lane 3, Δ Di-HA after mercuric acetate treatment; lane 4, untreated Δ Di-HA. A mixture of Δ Di-HA (0.5 ml; 3.3 mg) in 50 mM-Tris/50 mM-sodium acetate, pH 7.5, and 70 mM-mercuric acetate (0.5 ml), prepared as described in the Materials and methods section, was incubated at room temperature for 10 min. The sample was passed through a 1 ml column of AG 50W resin (Na^+ form), which was rinsed with 3 ml of water, and was then evaporated to dryness and taken up in 0.5 ml of water; 10 μ l portions of this solution were analysed by chromatography and electrophoresis. The control sample was incubated with 35 mM-sodium acetate instead of the mercuric salt but was otherwise treated in an identical manner. $\circ$, Morgan-Elson-positive spots; $\otimes$ [lanes 1 in (a) and 1 in (b)] indicates the position of glucuronic acid, as detected by silver staining; $\bullet$ [lane 3 in (a)] indicates the position of an *o*-phenylenediamine-positive reaction product. The spots in lanes 2–4 in (a) were also detectable by silver staining, although the *o*-phenylenediamine-positive spot stained faintly.

carried out by gel chromatography, and these experiments, which were also designed to determine the distribution of label in radioactive Δ Di-HA, are described below in a subsequent section.

Reaction of Δ Di-HA with mercuric acetate

To determine whether the cleavage of Δ Di-HA could be accelerated by the use of a mercuric salt that is more extensively dissociated than the chloride, we examined the reaction with mercuric acetate in a series of experiments illustrated in Figs. 5 and 6. In these experiments the reaction mixtures were freed of Hg^{2+} ions by passage through small columns of AG 50W resin (Na^+ form) and were then analysed by h.p.l.c. as described in the Materials and methods section. The effluent was monitored at 232 nm. As shown in Fig. 5, Δ Di-HA was cleaved rapidly by 35 mM-mercuric acetate at room temperature and was no longer detectable after 10 min. Choosing a 10 min incubation time, we then determined the effect of the mercuric acetate concentration on the reaction rate. As shown in Fig. 6, complete cleavage again occurred at a concentration of 35 mM, but a substantial proportion of unchanged disaccharide remained after exposure to 7 mM-mercuric acetate. Complete cleavage was also observed at a concentration of 14 mM (results not shown).

Analysis of cleavage products from biosynthetically labelled hyaluronic acid

The distribution of radioactivity between the glucuronic acid and *N*-acetylglucosamine moieties was determined for three preparations of hyaluronic acid, which had been isolated from IMR-90 fibroblast cultures grown in the presence of [$1\text{-}^3\text{H}$]glucosamine, [$1\text{-}^3\text{H}$]galactose and [$4,5\text{-}^3\text{H}$]galactose respectively. In all of these experiments the labelled polysaccharide was digested with chondroitinase AC, the digest was treated with HgCl_2 after the addition of sufficient amounts of

unlabelled Δ Di-HA to allow colorimetric analyses, and the reaction mixture was then analysed by chromatography on Sephadex G-10. Under the conditions chosen for the latter step, a standard mixture of Δ Di-HA and *N*-acetylglucosamine was almost completely resolved, with over 95% recovery of both components (Fig. 7a). Details of these experiments are described in the Materials and methods section and in the appropriate Figure legends.

The gel-chromatographic analysis of the '[$1\text{-}^3\text{H}$]glucosamine-labelled' reaction mixture is shown in Fig. 7(b). A single radioactive peak was observed, which coincided with a Morgan-Elson-positive peak in the same position as *N*-acetylglucosamine. No carbazole-positive material was found. These results are in accord with our previous conclusion that Δ Di-HA is completely cleaved by HgCl_2 under the conditions used and that *N*-acetylglucosamine is one of the reaction products. However, it was not possible, from this experiment alone, to draw any conclusion as to the distribution of radioactivity between the two moieties of the disaccharide, since the elution position of the reaction product generated from the unsaturated uronic acid by mercuric salts was not known. A full interpretation of the results became possible only after similar experiments with the '[^3H]galactose-labelled' material had been carried out, and these experiments are therefore described next.

HgCl_2 treatment of '[^3H]galactose-labelled' Δ Di-HA gave the results shown in Fig. 8. Unexpectedly, most of the radioactivity in the '[$1\text{-}^3\text{H}$]galactose-labelled' reaction mixture emerged in the same position as the untreated disaccharide, with a shoulder of the peak extending into the monosaccharide region (Fig. 8a). However, when glucuronic acid was chromatographed on the same column, it emerged ahead of and almost completely separated from glucosamine, which, in turn, was eluted slightly ahead of *N*-acetylglucosamine (results not shown). Since the added unlabelled Δ Di-HA had been

Fig. 5. Time course of the reaction of Δ Di-HA with 35 mM-mercuric acetate, as determined by h.p.l.c.

A solution of Δ Di-HA (20 mg/ml) in 50 mM-Tris/50 mM-sodium acetate, pH 7.5, was mixed with an equal volume of 70 mM-mercuric acetate, prepared as described in the Materials and methods section. After incubation at room temperature for the times indicated below, a 50 μ l sample was withdrawn and applied to a 1 ml column of AG 50W resin (Na^+ form), which was eluted with 1.95 ml of water; 10 μ l portions of the eluate were analysed by h.p.l.c. as described in the Materials and methods section except that the eluent did not contain Na_2SO_4 ; the effluent was monitored at 232 nm. Incubation times: (a) 0 min (untreated control); (b) 0.5 min; (c) 5 min; (d) 10 min.

completely degraded, as indicated by the position of the Morgan–Elson-positive peak and the absence of carbazole-positive material, it was therefore concluded that the major peak of radioactivity in Fig. 8(a) consisted of the product(s) of degradation of the unsaturated uronic acid. Consequently, the $[1\text{-}^3\text{H}]$ galactose used as a precursor of hyaluronic acid in this experiment had been incorporated largely into the glucuronic acid moiety. Another measure of the distribution of radioactivity was obtained as follows (for experimental details see the Materials and methods section). Disaccharide (approx. 2000 c.p.m.) from $[1\text{-}^3\text{H}]$ galactose-labelled hyaluronic acid was degraded with mercuric acetate, and the reaction mixture was then passed successively through small columns of AG 50W resin (Na^+ form) and AG 1 resin (HCO_3^- form), which were eluted with water. Approx. 95% of the applied radioactivity was adsorbed on the latter column, indicating that the label resided in a negatively charged molecule and not to any significant extent in *N*-acetylglucosamine, which was not retained by the resin.

Experiments with $[4,5\text{-}^3\text{H}]$ galactose-labelled

Δ Di-HA, similar to those shown in Fig. 8(a), gave the results presented in Fig. 8(b). Again, a peak of radioactivity was observed which was eluted ahead of *N*-acetylglucosamine in the same position as glucuronic acid and untreated disaccharide. In this case, however, there was no overlap into the *N*-acetylglucosamine peak. It should also be noted that a major peak of radioactivity was present at V_t in the profile shown in Fig. 8(b). This material presumably consisted of radioactive water formed during digestion of the polysaccharide with chondroitinase AC, which removes the hydrogen at C-5 of the glucuronic acid residues (note that the entire chondroitinase digest was used in this experiment rather than the isolated disaccharide).

We can now return to the interpretation of the analysis of the $[1\text{-}^3\text{H}]$ glucosamine-labelled disaccharide. Since it has been shown that the unsaturated uronic acid residue of Δ Di-HA gives rise to a product that is eluted before *N*-acetylglucosamine upon chromatography on Sephadex G-10, it is now evident that the radioactive peak in the experiment shown in Fig. 7(b) must have been *N*-acetylglucosamine and that no significant amount of radioactivity was incorporated into the uronic acid moiety of the polysaccharide from labelled glucosamine.

Fig. 6. Dependence of the reaction of Δ Di-HA with mercuric acetate on Hg^{2+} ion concentration

The reaction time was 10 min. The experimental conditions were similar to those described in the legend to Fig. 5, except that the mercuric acetate concentration was varied as indicated below and that the eluent for the h.p.l.c. analysis was 10 mM- Na_2SO_4 /50 mM-sodium acetate adjusted to pH 5.0 with acetic acid. Mercuric acetate concentrations: (a) 0 mM (untreated control); (b) 3.5 mM; (c) 7 mM; (d) 35 mM.

Fig. 7. Chromatography of degradation products from '[1-³H]glucosamine-labelled' ΔDi-HA on Sephadex G-10

(a) Profile for a calibration run with unlabelled ΔDi-HA (0.5 mg) (elution position 1) and *N*-acetylglucosamine (1.0 mg) (elution position 2). ³H₂O emerged in elution position 3. (b) Profile for a reaction mixture containing radioactive ΔDi-HA (5000 c.p.m.) and unlabelled disaccharide (0.5 mg) that had been treated with 5 mM-HgCl₂ at 65 °C for 90 min. For other experimental details see the text. ○, *N*-acetylhexosamine; □, radioactivity; ●, uronic acid.

This conclusion was verified by independent methodology in an experiment where glucosamine was isolated from an acid hydrolysate of the labelled polysaccharide by chromatography on AG 50W resin (see the Materials and methods section for details). This analysis showed that a maximum of 1% of the total radioactivity was located in the uronic acid moiety.

Analysis of reaction products by the thiobarbituric acid and *o*-phenylenediamine reactions

In addition to the results presented above, the experiment illustrated in Fig. 8(b) provided further information concerning the properties of the product formed in the reaction of the unsaturated uronic acid with mercuric salts. As seen from the Figure, the first radioactive peak coincided with a peak of thiobarbituric acid-positive material; this finding is in keeping with the conclusion that the product was a keto acid. A second, smaller, peak between the putative keto acid and *N*-acetylglucosamine was also observed; the nature of this material is not known.

Additional support for the notion that a keto acid is formed in the reaction under study was obtained when the progress of the reaction was monitored by the thiobarbituric acid and *o*-phenylenediamine reactions. As shown in Fig. 9, the absorbance in both analyses increased to 4 times the original value in the course of the reaction.

DISCUSSION

The unsaturated uronic acid residues formed during the cleavage of polyuronides by eliminases are useful components of the resultant fragments, since they have

Fig. 8. Chromatography of degradation products from (a) '[1-³H]galactose-labelled' (10000 c.p.m.) and (b) '[4,5-³H]galactose-labelled' (20000 c.p.m.) ΔDi-HA on Sephadex G-10

1, 2 and 3 indicate the elution positions of untreated disaccharide (and glucuronate), *N*-acetylglucosamine and ³H₂O respectively. For other experimental details see the text and legend to Fig. 7. ○, *N*-Acetylhexosamine; □, radioactivity; ●, uronic acid; ■, A₅₄₈ in the thiobarbituric acid reaction.

Fig. 9. Time course of the reaction of Δ Di-HA with 35 mM-mercuric acetate, as determined by analysis with the thiobarbituric acid and the *o*-phenylenediamine reactions

Experimental conditions were similar to those described in the legend to Fig. 5; 200 μ l portions of the eluates from the AG 50W resin column were analysed (approx. 50 μ g of Δ Di-HA). □, A_{548} (thiobarbituric acid reaction); ■, A_{330} (*o*-phenylenediamine reaction). As seen from the Figure, substantial cleavage of the disaccharide occurred in the 'zero-time' sample before Hg^{2+} ions could be removed by the ion-exchange step.

distinctive properties that aid in the analysis of these fragments. For example, their high absorbance at 232 nm allows easy monitoring of the progress of eliminase reactions and facilitates detection of the digestion products in chromatographic effluents. However, the very presence of the unsaturated uronic acids also limits the usefulness of the oligosaccharides produced by the eliminases, and it is therefore important to have access to methods by which these residues can be removed. Acid hydrolysis has been used for this purpose by Suzuki (1960), who observed that the uronidic linkage in a disaccharide with an unsaturated uronic acid is much more labile to such treatment than is the glucuronic linkage of a saturated glucuronic acid residue. However, even under the mild hydrolysis conditions used in these experiments (0.04 M-HCl; 100 °C; 60 min) some release of ester sulphate groups occurred, and, if larger oligosaccharides were to be subjected to the same treatment, it is likely that some cleavage of glycosidic linkages other than those of the unsaturated uronic acid residues would also take place. Thus acid hydrolysis is not suitable for specific removal of the latter. Among known agents that may be used for this purpose is a glycuronidase that is induced together with the eliminases in bacteria or fungi grown on mammalian connective-tissue polysaccharides (Linker *et al.*, 1960; Yamagata *et al.*, 1968; Saito *et al.*, 1968; Suzuki *et al.*, 1968; Dietrich, 1969; Dietrich *et al.*, 1973; Habuchi *et al.*, 1977; Kennedy, 1979). The glycuronidase cleaves Δ Di-HA and the isomeric disaccharide from chondroitin as well as the sulphated disaccharide from chondroitin 6-sulphate. However, it does not attack the disaccharide from chondroitin 4-sulphate (Suzuki *et al.*, 1968; Habuchi *et al.*, 1977), and its usefulness as an analytical and preparative tool is therefore somewhat restricted.

In view of the problems indicated above, we have searched for alternative means of removing the unsaturated uronic acid residues from fragments generated by eliminase action. It was hypothesized that the vinyl ether group, $-\text{CH}=\text{CH}-\text{O}-$, in the unsaturated uronic acid should be susceptible to reaction with mercuric salts (oxymercuration), by analogy with the behaviour of the same structure in plasmalogens and glycals (Norton, 1969; Schmidt *et al.*, 1969; Ferrier, 1980). Further, it was assumed that the mercury adduct formed in the initial reaction would be an unstable hemiketal and undergo spontaneous rearrangements resulting ultimately in cleavage of the glycosidic linkage and removal of the

Scheme 1. Proposed mechanism for the cleavage of Δ Di-HA by mercuric salts

In the structure of the disaccharide [formula (I)] R represents the *N*-acetylglucosamine moiety; X represents a univalent anion in the mercuric salt. Because of the mesomeric influence of the ring O atom, a nucleophilic centre will be located at C-4 of the unsaturated uronic acid residue. In the first, rate-limiting, step, addition of HgX^+ to the double bond will therefore occur at C-4, followed immediately by attack of a water molecule on C-5 to yield compound (II), with the release of a proton. The product (II) is a hemiketal and is therefore unstable; it is converted into the keto form (III) with concomitant cleavage of the pyranose ring. This compound is a hemiacetal and readily loses ROH (this step is the actual cleavage of the glycosidic linkage) to yield intermediate (IV). Hydrolysis of the Hg-C bond in the latter then yields the keto acid (V) or the free unsaturated uronic acid (VI). [Compounds (V) and (VI) are in equilibrium (Kiss, 1974), with the keto acid predominating.] Loss of the mercury may also be envisaged to occur at an earlier stage, concomitant with ring opening or glycosidic bond cleavage.

mercury by hydrolysis. The proposed reaction mechanism is outlined in Scheme 1. Although not proven by the experiments reported here, the indicated mechanism is plausible in view of the results of characterization of the reaction products formed from Δ Di-HA. That *N*-acetylglucosamine was one of the products was indicated by the finding that a Morgan–Elson-positive product was formed, which was uncharged and co-chromatographed with the authentic compound on paper chromatography and gel chromatography. The identity of the product formed from the unsaturated uronic acid was not established definitively, but the analyses were all in agreement with the notion that it was a keto acid. Thus it was found to be anionic at pH 5.3 and migrated with approximately the same mobility as glucuronic acid, it reacted with the keto acid reagents *o*-phenylenediamine and thiobarbituric acid, and it was of the same size as glucuronic acid, as evidenced by gel chromatography. Considering that it originated from Δ Di-HA, it was therefore assumed to be identical with the keto acid described by Linker *et al.* (1960) and Preiss & Ashwell (1963).

Although the main purpose of the present paper is to report on the reaction between unsaturated uronic acids and mercuric salts, it should be mentioned that this work was prompted by an on-going study of the role of galactose as a precursor of connective-tissue proteoglycans. Galactose is utilized via the following pathway, which leads to the formation of three of the nucleotide sugars required for proteoglycan synthesis: galactose $\rightarrow$ galactose 1-phosphate $\rightarrow$ UDP-galactose $\rightarrow$ UDP-glucose $\rightarrow$ UDP-glucuronic acid $\rightarrow$ UDP-xylose (Feingold, 1972; Schachter & Rodén, 1973; Schachter, 1978). From UDP-glucose, a connection exists with glycolysis and the pathway of hexosamine synthesis: UDP-glucose $\rightarrow$ glucose 1-phosphate $\rightarrow$ glucose 6-phosphate $\rightarrow$ fructose 6-phosphate $\rightarrow$ glucosamine 6-phosphate $\rightarrow$ UDP-*N*-acetylglucosamine $\rightarrow$ UDP-*N*-acetylgalactosamine (Feingold, 1972; Schachter & Rodén, 1973; Schachter, 1978). Radioactive galactose may therefore be incorporated into both the uronic acid and the *N*-acetylhexosamine components of hyaluronic acid and other connective-tissue polysaccharides. However, the radioactivity will not necessarily be evenly distributed between the two moieties of the repeating disaccharide units. Indeed, our analysis of the hyaluronic acid synthesized by IMR-90 fibroblasts showed that the added radioactive galactose had been converted largely into glucuronic acid, with less than 5% of the total radioactivity in the *N*-acetylglucosamine moiety. The reasons for this uneven distribution are complex and are not discussed in detail here. Suffice to say that the distribution will be influenced by such factors as the relative concentrations of glucose and galactose in the culture medium, the pool sizes of the various intermediates, the direction and magnitude of the fluxes through these pools, and the time for which the cells are exposed to the labelled precursor.

In summary, we have discovered that Δ Di-HA is cleaved by mercuric salts, presumably by initial formation of a mercury adduct (oxymercuration) and subsequent rearrangement of the product leading to cleavage of the glycosidic bond. In view of the general nature of the reaction, it should be possible to extend its application to the analogous disaccharides from the chondroitin sulphates and dermatan sulphate, and the

availability of a method for cleavage of Δ Di-4S will be particularly useful, since this disaccharide is not cleaved by the bacterial glucuronidase. Since the reaction with mercuric acetate is rapid and takes place under mild conditions, i.e. at room temperature and at a pH close to neutrality, it will probably be possible to extend the scope of the method even further and to remove, specifically, the unsaturated uronic acid residues from eliminase-treated proteoglycan preparations and from larger polysaccharide fragments such as oligosaccharides derived from heparin and heparan sulphate by digestion with heparinase and heparitinase.

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