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PERIODATE OXIDATION OF L-IDURONIC ACID RESIDUES IN DERMATAN SULPHATE*†

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ABSTRACT

The rate of periodate oxidation of dermatan sulphate and chondroitin sulphate was studied at various pH values, ionic strengths, and temperatures. The L-iduronic acid residues of dermatan sulphate were readily oxidized under a variety of conditions, whereas oxidation of D-glucuronic acid was essentially zero at pH 3 and 4°. Extensive depolymerization occurred at temperatures greater than 4°. Periodate oxidation, performed at relatively high ionic-strength, yielded hemiacetal-type cross-linkages between oxidized and unoxidized uronic acid residues in separate chains. After oxidation of a mixture of dermatan sulphate and $^{35}SO_4^{2-}$-labelled dermatan sulphate octasaccharide, a large proportion of the radioactivity was eluted with the polysaccharide on gel chromatography. Reductive cleavage of these cross-linkages allowed further consumption of oxidant. At the correct oxidation-limit for dermatan sulphate, >20% of the L-iduronic acid residues were resistant to periodate. Although some of these residues were undoubtedly sulphated at 2 or 3, the presence of a small proportion of L-iduronic acid residues in the *1C* conformation cannot be excluded.

INTRODUCTION

Polysaccharides which contain glycosyl units with HO-2 and HO-3 unsubstituted can be oxidized by periodate to yield oxypolysaccharides which, on reduction followed by mild, acid hydrolysis, undergo controlled (Smith) degradation[3]. Alternatively, periodate-oxidized, (1→4)-linked glycans may be degraded in alkaline solution[4], mainly *via* a β-elimination reaction at C-5.

Periodate oxidation of acidic polysaccharides in water proceeds very slowly because of repulsion between periodate ion and the polyanion[5,6]. This effect can be overcome by the addition of salt (0.2M sodium perchlorate). However, chondroitin sulphate is oxidized much more slowly than dermatan sulphate[6,7].

*Periodate oxidation of dermatan sulphate and chondroitin sulphate: Part I. For a preliminary report, see ref. 1.

†Whilst this work was in progress, a colorimetric method for the determination of dermatan sulphate, based on the selective periodate-oxidation of the L-iduronic acid, was reported[2].

We now report on the effect of pH, temperature, and ionic strength on the rate of periodate oxidation of chondroitin sulphate and dermatan sulphate.

EXPERIMENTAL

Materials. — Chondroitin 4-sulphate from beef nasal septum was the same preparation as described previously[8]. Dermatan sulphate was a 25% ethanol fraction of galactosaminoglycans solubilized from pig skin by papain digestion[9]. Radioactive ($^{35}SO_4^{2-}$) dermatan sulphate (glucuronic acid-rich fraction) obtained from rat skin was a gift from Dr. I. Sjöberg. Testicular hyaluronidase (20,000 i.u./mg) was purchased from AB Leo (Helsingborg, Sweden). Whatman Column Chromedia CF 11 was used for micro-column analysis. Sephadex gels and blue dextran 1000 were products of Pharmacia Fine Chemicals (Uppsala, Sweden). Instagel was purchased from Packard Instrument AB (Bandhagen, Sweden).

Periodate oxidation. — Solutions of polysaccharides (1–2 mg/ml) in 0.2M sodium perchlorate–20mM sodium periodate in 50mM buffers of sodium citrate (pH 3.0), sodium formate (pH 4.0), or sodium phosphate (pH 7.0) were incubated at 4° or 37° in the dark[5,6]. Samples were also incubated with 20mM sodium periodate–50mM sodium acetate buffer (pH 5.0). Aliquots were treated with a molar excess of D-mannitol and analyzed for uronic acid by the Dische carbazole method[10].

For preparative-scale oxidations, the reaction was terminated with D-mannitol, the mixture was directly applied to a Sephadex column, and oxydermatan sulphate was subsequently obtained.

Preparation of ^{35}S-labelled dermatan sulphate oligosaccharides. — Dermatan (^{35}S)-sulphate (~10 mg, 20,000 c.p.m.) in 1 ml of 0.15M sodium chloride–0.1M acetate buffer (pH 5.0) was digested with 0.1 mg of testicular hyaluronidase at 37° overnight. The digest was eluted from a column (1.1 × 200 cm) of Sephadex G-50 (superfine) with 0.2M pyridine acetate (pH 5.0) at 12 ml/h. Aliquots of the fractions were analyzed for uronic acid by an automated version[11] of the Bitter and Muir modification[12] of the carbazole method of Dische[10]. Fractions containing material corresponding to octasaccharide (elution volume, 125–150 ml) were combined and lyophilized (yield, 5,000 c.p.m.). The major octasaccharide component should have the structure[8]

GlcUA–GalNAc–(IdUA–GalNAc)$_2$–GlcUA–GalNAc.

SO_4 SO_4 SO_4

Periodate oxidation of a mixture of dermatan sulphate and radioactive octasaccharide. — A solution of dermatan sulphate (10 mg) and ^{35}S-labelled dermatan sulphate octasaccharide (3,000 c.p.m.) in 5 ml of 0.2M sodium perchlorate–20mM sodium periodate–50mM sodium phosphate (pH 7.0) was incubated at 4° for 24 h in the dark. In the control experiment, sodium perchlorate was omitted. After the addition of a molar excess of D-mannitol, the solutions were concentrated to 1 ml and subjected to gel chromatography on Sephadex G-50 as described above. Each fraction was analysed for uronic acid[11] and then lyophilized, the residue was dissolved in 4 ml

of water, and 5 ml of Instagel were added as scintillator. The radioactivity was measured in a TriCarb liquid scintillation spectrometer.

Alkaline degradation of oxydermatan sulphate. — A solution (5 mg/ml) of oxydermatan sulphate in water was adjusted to pH 12 with M NaOH. After 30 min at room temperature, the solution was neutralized with M acetic acid and concentrated. Oxygen was not excluded during treatment with alkali.

Reduction and mild, acid hydrolysis of oxydermatan sulphate. — A solution (10 mg/ml) of oxydermatan sulphate in water was treated with 2 mol. of potassium borohydride for 1 h at room temperature. Excess borohydride was destroyed with glacial acetic acid (final pH, 4–5). After desalting on Sephadex G-50, the product was dissolved in 25mM H_2SO_4 (2 mg/ml), and the solution was heated at 80° for 1 h and then neutralized with M NaOH.

Chromatographic methods. — Preparations of oxydermatan sulphate submitted to various experimental conditions were analyzed by the cetylpyridinium chloride–cellulose micro-column procedure[13]. The columns were eluted with neutral, aqueous magnesium chloride to yield information regarding molecular-size polydispersity[14]. Some samples were also eluted from a column (0.9 × 142 cm) of Sephadex G-200 with 0.2M pyridine acetate (pH 5.0) at 3.3 ml/h. The column effluents were analyzed for uronic acid[11].

RESULTS

Selective oxidation of L-*iduronic acid residues.* The rate of oxidation of dermatan sulphate and chondroitin sulphate was studied at various pH values and temperatures, and the results are shown in Fig. 1. At 4°, chondroitin sulphate was not oxidized (Fig. 1A), whereas at pH 7.0 and 37° there was considerable destruction of uronic acid (Fig. 1B, ●——●). The rate of oxidation of chondroitin sulphate was diminished as the pH decreased (⊙ – – ⊙) and was zero at pH 3.0 (●· · · · ●). In contrast, 50–60% of the uronic acid residues in dermatan sulphate were oxidised at 4° (Fig. 1C). Further oxidation (~70%) was achieved at neutral pH and 37° (Fig. 1D). Since D-glucuronic acid residues are oxidised at pH 7 but not at pH 3 (Fig. 1B), the difference in degree of oxidation of dermatan sulphate at these pH values (Fig. 1D) corresponds to the proportion of D-glucuronic acid residues in the polymer; a difference of ~15% was observed which is in fair agreement with the value of 10% determined by other methods[9].

When the periodate oxidation of chondroitin sulphate at 4° was monitored by the carbazole reaction, little or no destruction of uronic acid was observed (Fig. 1A). However, destruction of <5% uronic acid could not be detected by this method. Therefore, a more sensitive method was used in a few experiments. Since dialdehyde structures formed by periodate oxidation are labile in alkaline solution[4], a small number of oxidized uronic acid residues may be detected by gel chromatography after alkaline treatment. As shown in Fig. 2, chondroitin sulphate, which was oxidized with periodate at pH 5.0 (· · · · · ·), suffered cleavage of 1–2 D-glucuronic acid

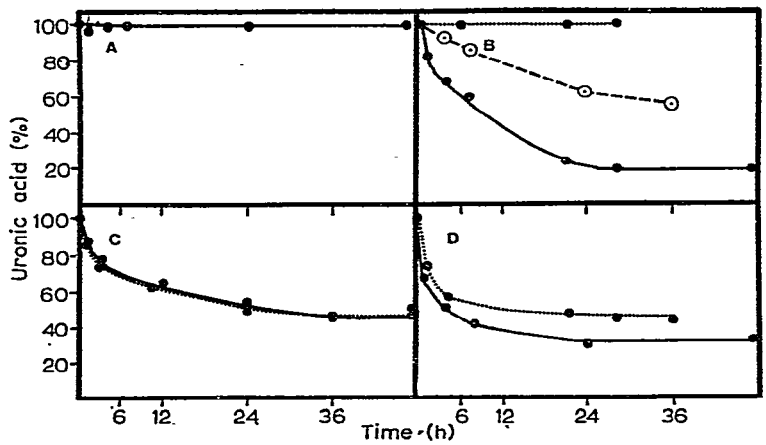


Fig. 1. Effect of pH and temperature on the rate of oxidation of chondroitin sulphate and dermatan sulphate: A and B, chondroitin sulphate at 4° and 37°; C and D, dermatan sulphate at 4° and 37°; ●····●, pH 3.0; ⊙— — —⊙, pH 4.0; ●————●, pH 7.0. The uronic acid content at zero time for dermatan sulphate was corrected for the low colour yield of L-iduronic acid. When chondroitin sulphate was oxidized exhaustively with periodate at 37° and pH 7.0 (B, ●————●), 15–20% of the original carbazole colour for uronic acid remained, presumably due to a non-specific reaction of oxychondroitin sulphate with carbazole–H_2SO_4; oxydermatan sulphate was assumed to behave in the same manner.

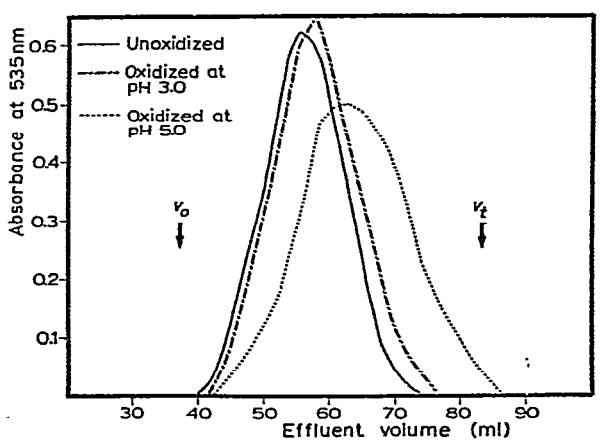


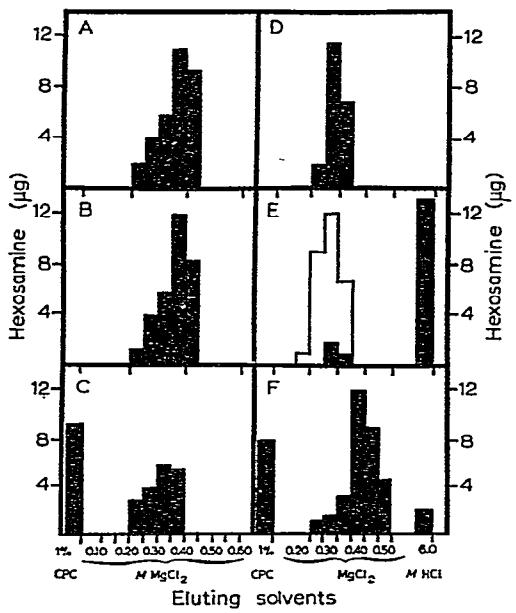
Fig. 2. Effect of periodate oxidation and alkaline elimination on chondroitin sulphate; oxidation at 4° with periodate in 50mM sodium citrate, pH 3.0 (–·–·–·–) or 50mM sodium acetate, pH 5.0 (·····). After treatment with alkali, the reaction mixture was neutralized and applied to the Sephadex column; v_o and v_t are the elution volumes of blue dextran and sodium chloride, respectively.

residues per chain (<5%). At pH 3.0, resistance to periodate was almost complete (–·–·–·). Thus, if a small number of L-iduronic acid residues are to be detected in a molecule where D-glucuronic acid preponderates, then oxidation must be performed at pH 3.0.

If oxidation of dermatan sulphate–chondroitin sulphate copolymers[8,15] with periodate at low temperature is combined with a suitable degradative method, then the procedure can be employed for the isolation of D-glucuronic acid-containing oligosaccharide segments from such copolymers.

Side reactions. During prolonged periodate oxidation of polysaccharides, side reactions are frequently encountered. Thus, depolymerizations catalyzed by free radicals[16] and unexpectedly low consumptions of periodate have been reported[17]. The latter phenomenon, which has been demonstrated for alginate[16,17], xylan[18], and amylose[19], is due to the formation of intrachain hemiacetal bonds between oxidized and unoxidized uronic acid (or hexose) residues[16–19].

The degree of polymerization occurring during periodate oxidation was therefore studied by product analysis on cetylpyridinium chloride–cellulose micro-columns



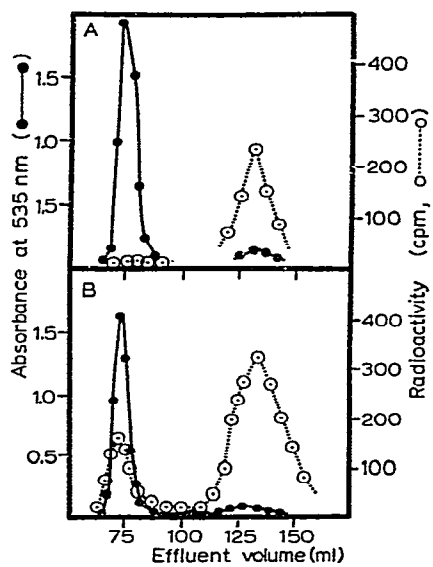


Fig. 3. Cetylpyridinium chloride–cellulose micro-column analyses of chondroitin sulphate and dermatan sulphate before and after periodate oxidations for 48 h in the presence of 0.2M perchlorate. A, chondroitin sulphate standard; B, chondroitin sulphate after oxidation at 4° and pH 3.0; C, chondroitin sulphate after oxidation at 37° and pH 3.0; D, dermatan sulphate standard; E, dermatan sulphate oxidized at 4° and pH 7.0 (unfilled bars, oxidized and reduced dermatan sulphate); F, dermatan sulphate oxidized at 37° and pH 7.0.

Fig. 4. Gel chromatography on Sephadex G-50: A, the products obtained on treating a mixture of dermatan sulphate and ^{35}S-labelled dermatan sulphate octasaccharide with periodate; B, the same mixture after oxidation with periodate in the presence of 0.2M sodium perchlorate.

(Fig. 3). No degradation was observed when chondroitin sulphate was oxidized by periodate at 4° (Fig. 3B), but considerable depolymerization took place at 37° (Fig. 3C). This depolymerization occurred without concomitant oxidation of D-glucuronic acid, since the reaction was performed at pH 3.0. Oxydermatan sulphate produced at 4° was eluted at much higher ionic-strength than the untreated material (Fig. 3D, E; filled bars), suggesting that polymerization had taken place. Subsequent reduction of oxydermatan sulphate produced a polymer with the same elution profile as the untreated material (Fig. 3E; unfilled bars). Although oxydermatan sulphate produced at 37° (Fig. 3F) was still partly retarded, the appearance of material soluble in 1% cetylpyridinium chloride indicated that substantial depolymerization had occurred. Thus, depolymerization could be minimized by running the reaction at 4°.

In order to investigate the possible formation of intermolecular hemiacetal linkages[16], a mixture of dermatan sulphate and a dermatan (^{35}S)-sulphate octasaccharide was oxidized. As shown in Fig. 4, approximately one-third of the radioactivity co-chromatographed with the polysaccharide after oxidation in the presence of 0.2M perchlorate, indicating cross-linking. It is conceivable that the perchlorate used to suppress polyanion–periodate repulsion[5–7] also suppressed polyanion–polyanion repulsion to some extent and thereby allowed the formation of intermolecular hemiacetal linkages between oxidized and unoxidized L-iduronic acid residues during the initial phase of the oxidation. Such linkages would protect some of the L-iduronic acid residues from further oxidation[16].

In order to compare the effect of low and high ionic-strength on the extent of oxidation, dermatan sulphate and chondroitin sulphate were oxidized with periodate in 50mM acetate buffer (pH 5.0) at 4°. It is clear from Fig. 5 that dermatan sulphate was extensively oxidized at relatively low ionic-strength, whereas condroitin sulphate was resistant. Both the rate and the extent of oxidation of dermatan sulphate were slightly increased (*cf.* Figs. 1C and 5). Almost 60% of the uronic acid residues in dermatan sulphate were destroyed.

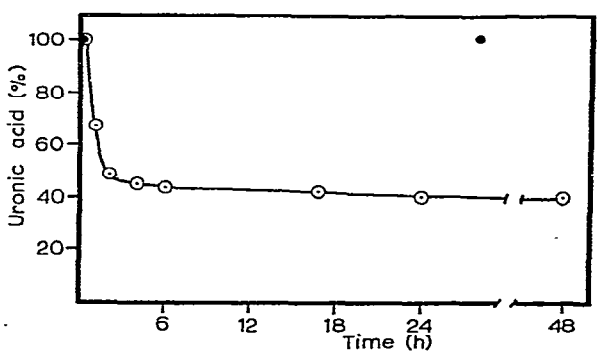


Fig. 5. Effect of low ionic-strength on the rate of oxidation of dermatan sulphate. Reactions were performed at 4° in 50mM acetateb uffer, pH 5.0 (○———○). A sample of chondroitin sulphate was oxidized in 50mM buffer as a control (●).

It has been demonstrated[16] that reductive cleavage of hemiacetal linkages allows further consumption of periodate. Dermatan sulphate was therefore oxidized with periodate in the presence of 0.2M sodium perchlorate until the initial oxidation-limit was attained (50–60%). After reduction with potassium borohydride, the material was reoxidized with periodate under the same conditions. Dialdehyde structures formed during the second oxidation were subsequently cleaved by treatment[20] with alkali, and the degree of fragmentation was then ascertained by chromatography on Sephadex G-50 (Fig. 6A). A marked degradation was observed, which suggests that the correct oxidation-limit was not reached during the first oxidation (Fig. 6A, —⊙—). Oxidized and reduced dermatan sulphate was used as a control to show that

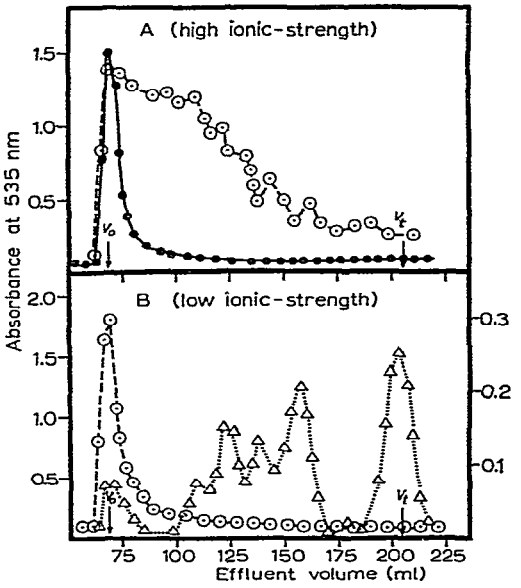


Fig. 6. Gel chromatography on Sephadex G-50 of dermatan sulphate: A, after exhaustive oxidation with periodate in the presence of 0.2M perchlorate, reduction, and treatment with alkali (●———●), and after oxidation, reduction, oxidation, and treatment with alkali (⊙— — — ⊙); B, after exhaustive oxidation in 50mM acetate buffer (pH 5.0), reduction, oxidation, and treatment with alkali (⊙— — — ⊙). This material was recovered and subjected to mild, acid hydrolysis (△····· △). The void volume (v_o) was determined by calibration with blue dextran 1000, and the total volume (v_t) was the elution volume of sodium chloride, as determined by the silver nitrate–nitric acid test.

the reduced material was resistant to alkali (Fig. 6A, —●—). In contrast, when dermatan sulphate was oxidized at low ionic-strength, complete oxidation of the susceptible uronic acid residues occurred, since oxidized–reduced–oxidized dermatan sulphate was not degraded by alkali (Fig. 6B, —⊙—). As expected, oxidized and reduced dermatan sulphate was susceptible to mild, acid hydrolysis[3] (Fig. 6B, · · · △ · · ·). At least four carbazole-positive oligosaccharide components were resolved on Sephadex G-50, although the material eluted in v_t did not give a typical carbazole

reaction. This indicates that periodate-resistant uronic acid residues are located in clusters of up to four disaccharide units.

DISCUSSION

Conformation of uronic acid residues. Sugars having the D-*gluco* configuration usually[21] adopt the *C1* conformation, and it has been generally assumed that this conformation is present in D-glucuronic acid residues in polysaccharides. On the other hand, sugars having the L- or D-*ido* configuration are often conformationally unstable[22] and, for β-L-idose, a skew-boat conformation has been proposed. P.m.r. data for dermatan sulphate seem to indicate a *1C* conformation for L-iduronic acid[23], whereas o.r.d. data have yielded conflicting results[24,25]. X-Ray difffraction studies on fibrous preparations of dermatan sulphate support the *C1* conformation for L-iduronic acid[26,27]; the axial periodicity observed makes *1C* conformations rather unlikely[26].

In the *C1* conformation, the carboxylate group of L-iduronic acid is axial and HO-2 and HO-3 are equatorial, whereas in the *1C* conformation, the reverse situation prevails. Since a diaxial glycol grouping is sterically unsuitable for periodate oxidation, the results reported herein are consistent with a *C1* conformation for L-iduronic acid. The L-iduronic acid residues in dermatan sulphate were readily oxidized under a variety of conditions, whereas the D-glucuronic acid residues of chondroitin sulphate could only be oxidized at elevated pH values and temperatures. Moreover, D-glucuronic acid was oxidized much more slowly than L-iduronic acid, as previously found by Scott[7] who proposed that formation of hydrogen bonds between the COOH group and HO-3 might be responsible for the observed difference in oxidation rates.

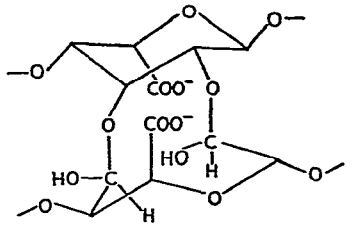
Oxidation of the C-2–C-3 glycol group in the D-glucuronic acid residues of a polysaccharide might be hindered if HO-2 and/or HO-3 were hydrogen bonded or involved in other types of linkage. Alternatively, if the D-glucuronic acid residues adopted a conformation in which HO-2 and HO-3 were axial, periodate oxidation would also be impeded. Although the boat and skew conformations of D-glucuronic acid should be energetically most unfavourable[28], hydrogen bonds within or between residues might stabilize an otherwise unfavourable conformation.

Chondroitin sulphate was completely resistant to periodate at pH 3.0. At this pH, the carboxyl group is extensively protonated[29] and the formation of hydrogen bonds should be promoted. Several boat conformations of D-glucuronic acid, but none for L-iduronic acid[7], allow hydrogen bonding between the carboxyl group and either HO-2 or HO-3. The finding that chrondroitin sulphate was oxidized with periodate solely at elevated temperatures is also consistent with the presence of hydrogen bonds. Hydrogen bonds between HO-2 or HO-3 of a D-glucuronic acid residue in the *C1* conformation and carbonyl groups or ring oxygens of the neighbouring 2-acetamido-2-deoxy-D-galactose residue should also be considered. It is possible that the peculiar resistance of glycosaminoglucuronans towards periodate is

due to extensive, intramolecular hydrogen-bonding involving COOH, NHAc, and HO groups and ring oxygens. It is of interest that D-glucuronic acid residues linked to hexose residues, as in pneumococcus type VIII polysaccharide, are sensitive to periodate[30] even at an acid pH.

Resistance to periodate has also been explained[2] by the formation of D-glucuronolactones. However, chondroitin sulphate was not oxidized at neutral pH and 4°, conditions under which lactones should be extremely unstable, Furthermore. lactone formation in D-glucuronic acid residues in a polymer would impose considerable strain on the pyranose ring.

Formation of intermolecular cross-links. On periodate oxidations of dermatan sulphate at relatively high ionic-strength, hemiacetal-type linkages are formed between oxidized and unoxidized L-iduronic acid residues located in separate chains (*e.g.*, as in **1**). When periodate oxidation was performed in solutions of relatively low ionic-strength, hemiacetal cross-linkages were not formed. When the oxidation limit was reached, ~30% (correcting for 15–20% background absorption of the oxypolysaccharide in the carbazole reaction) of the uronic acid residues in the dermatan sulphate preparation were periodate-resistant. Therefore, since the preparation used contained 10% of D-glucuronic acid, >20% of the L-iduronic acid residues were periodate-resistant. Some of these residues were undoubtedly sulphated[31], but the presence of a small proportion of unsubstituted L-iduronic acid residues in the *1C* conformation cannot be excluded.



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Periodate oxidation in combination with degradative methods. The major aim of the present study was to evaluate the use of periodate oxidation, in combination with either reduction and acid hydrolysis or alkali treatment, for degradative purposes. By selective oxidation of L-iduronic acid residues in dermatan sulphate–chondroitin sulphate copolymers[8,15] followed by suitable degradation, the D-glucuronic acid-containing oligosaccharide segments of the copolymer can be isolated as can oligosaccharide segments containing sulphated L-iduronic acid residues. This procedure may have value for structural studies on dermatan sulphate–chondroitin sulphate copolymers, in particular with regard to the length and distribution of the irregular oligosaccharide-segments. Moreover, a procedure for the isolation of D-glucuronic acid-containing segments will provide a tool for studying the action of the so-called "reversed epimerase". This enzyme, which is found in fibroblast secretions, converts[32]

L-iduronic acid residues in intact dermatan sulphate into D-glucuronic acid. By the procedure outlined above, it may be possible to estimate the size of the polysaccharide segments that bind to the enzyme, and this possibility is being investigated.

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