Periodate Oxidation for Sulfated Glycosaminoglycans, with Special Reference to the Position of Extra Sulfate Groups in Chondroitin Polysulfates, Chondroitin Sulfate D and Chondroitin Sulfate K

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(Received August 31, 1981)

Summary

The optimum conditions for periodate oxidation of sulfated disaccharides were investigated to determine the position of extra sulfate groups on the saturated disulfated disaccharides obtained from chondroitin polysulfates, chondroitin sulfates D and K. Under the conditions: 2 mM saturated disulfated disaccharide with 20 mM sodium periodate at 37° in the dark, the uronic acid residue in the disulfated disaccharide from chondroitin sulfate D was rapidly and completely destroyed, whereas that in the disulfated disaccharide from chondroitin sulfate K was quite stable, even after removal of the sulfate group at the 4 position of the *N*-acetylgalatosamine residue. Accordingly, it was concluded that the structure of the disaccharide disulfate from chondroitin sulfate D is 2-acetamido-2-deoxy-3-*O*-(2-*O*-sulfo-β-D-glucopyranosyluronic acid)-6-*O*-sulfo-D-galactose and that from chondroitin sulfate K; 2-acetamido-2-deoxy-3-*O*-(3-*O*-sulfo-β-D-glucopyranosyluronic acid)-4-*O*-sulfo-D-galactose.

Introduction

Three types of chondroitin polysulfate: chondroitin sulfates D, E and K (ChS-D, -E and -K) have previously been isolated from the cartilages of shark[1,2], squid[3] and king crab[4], respectively. These chondroitin polysulfates were found to differ both in the degree of sulfation and in the position of the sulfate groups. The structure of the unsaturated disulfated disaccharide, obtained from ChS-E after digestion with chondroitinase ABC, was proved to be 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-4, 6-di-*O*-sulfo-D-galactose[5]. However, the position of the extra sulfate groups in ChS-D and ChS-K have not long been determined, although these were assumed to be at the 2 or 3 position of the glucuronic acid residues[2,4,5].

In this paper, periodate oxidation of sulfated glycosaminoglycans, their tetra- and di-saccharides was investigated under various conditions to find the optimum

conditions for oxidation, with respect to both the rate and the extent of oxidation. Under optimum conditions for sulfated disaccharides, the position of sulfate groups in the saturated disaccharides, isolated from ChS-D and ChS-K after digestion with hyaluronidase followed by chondroitinase ABC, were determined.

Experimental

Materials. Sodium chondroitin sulfates A and C, and chondroitin polysulfates D and K were preparations from the cartilages of whale (*Balaenoptera physalus*, 1.5 M NaCl fraction)[6], shark (*Prionase glauca*, 1.5 M NaCl fraction)[6], shark (*Scolidon walbechmi*, 2.0 M NaCl fraction) and king crab (*Tachypleus tridentatus*, 3.0 M NaCl fraction)[4], respectively. Sodium chondroitin was isolated from squid skin, as described previously[7].

Bovine testicular hyaluronidase [EC 3.2.1.35] (280 NFunits/mg) was a commercial preparation from Sigma Chemical Co. Chondroitinase ABC [EC 4.2.2.4] and chondro-4-sulfatase [EC 3.1.6.9] and chondro-6-sulfatase [EC 3.1.6.10], from *Proteus vulgaris*, were purchased from Seikagaku Kogyo Co., Ltd.

2-Acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-4-*O*-sulfo-D-galactose (ΔDi-4S), 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-6-*O*-sulfo-D-galactose (ΔDi-6S) and 2-acetamido-2-deoxy-3-*O*-(2- or 3-*O*-sulfo-4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-6-*O*-sulfo-D-galactose (ΔDi-diS_D) were obtained from the digestion products of chondroitin sulfates A, C and D with chondroitinase ABC, respectively[5].

Tetrasaccharides "I", *O*-(β-D-glucopyranosyluronic acid)-(1→3)-*O*-(2-acetamido-2-deoxy-4-*O*-sulfo-β-D-galactopyranosyl)-(1→4)-*O*-(β-D-glucopyranosyluronic acid)-(1→3)-2-acetamido-2-deoxy-4-*O*-sulfo-D-galactose and "II", *O*-(β-D-glucopyranosyluronic acid)-(1→3)-*O*-(2-acetamido-2-deoxy-6-*O*-sulfo-β-D-galactopyranosyl)-(1→4)-*O*-(β-D-glucopyranosyluronic acid)-(1→3)-2-acetamido-2-deoxy-6-*O*-sulfo-D-galactose were obtained from the digestion products of chondroitin sulfates A and C with testicular hyaluronidase[8].

2-Acetamido-2-deoxy-3-*O*-(β-D-glucopyranosyluronic acid)-4-*O*-sulfo-D-galactose (Di-4S), 2-acetamido-2-deoxy-3-*O*-(β-D-glucopyranosyluronic acid)-6-*O*-sulfo-D-galactose (Di-6S), 2-acetamido-2-deoxy-3-*O*-(2- or 3-*O*-sulfo-β-D-glucopyranosyluronic acid)-6-*O*-sulfo-D-galactose (Di-diS_D) and 2-acetamido-2-deoxy-3-*O*-(2- or 3-*O*-sulfo-β-D-glucopyranosyluronic acid)-4-*O*-sulfo-D-galactose (Di-diS_K) were isolated from chondroitin sulfates A, C, D and K by digestion with testicular hyaluronidase followed by treatment with chondroitinase ABC[8].

Monosulfated disaccharides, Di-monoS_D and Di-monoS_K, were prepared from the digestion products of Di-diS_D with chondro-6-sulfatase and of Di-diS_K with chondro-4-sulfatase[4].

Methods. Periodate oxidation of samples was carried out at 37° in the dark. Polysaccharides (1–2 mg) or tetrasaccharides (2 mM) were incubated in 20 mM sodium periodate, or in 20 mM sodium periodate-50 mM phosphate buffer (pH 7.0) in the presence of 0.2 M sodium perchlorate[9]. In the case of sulfated disac-

charides, 0.2 mM or 2 mM samples were incubated with 2 mM or 20 mM sodium periodate, respectively. At time intervals, aliquots were analysed for any uronic acid remaining, periodate consumption and formaldehyde formation. After addition of 1% sodium sulfite or a molar excess of D-mannitol (where sodium perchlorate was used), uronic acid and formaldehyde were determined by the carbazole reaction[10] and by the chromotropic acid reaction[11], respectively. Periodate consumption was measured by the spectrophotometric method of Aspinall and Ferrier[12] at 223 nm.

Paper chromatography of disaccharide disulfates and monosulfates was carried out on Toyo No. 51A paper (60 cm long) using n-butyric acid-0.5 M ammonia (5 : 3, by vol.)[5].

Paper electrophoresis of the disaccharides was performed on Toyo No. 51A paper (60 cm long) in 0.1 M acetic acid-pyridine buffer (pH 5.0) at 40 volt/cm for 1 h or in borate buffer (boric acid 7.45 g, NaOH 4.0 g in 1 *l* of water, pH 10.0)[13] at 40 volt/cm for 3 h.

Results and Discussion

Effect of ionic strength on periodate oxidation of glycosaminoglycans

When non-sulfated glycosaminoglycan, chondroitin, was oxidized in water, about 60% of the uronic acid residues were destroyed after 20 h, the effect of sodium perchlorate addition was small, as shown in Fig. 1. On the other hand, chondroitin sulfates A and C were hardly oxidized in water, while in the presence of 0.2 M perchlorate at pH 7.0, considerable destruction of the uronic acid residues was observed as described previously by Fransson[9].

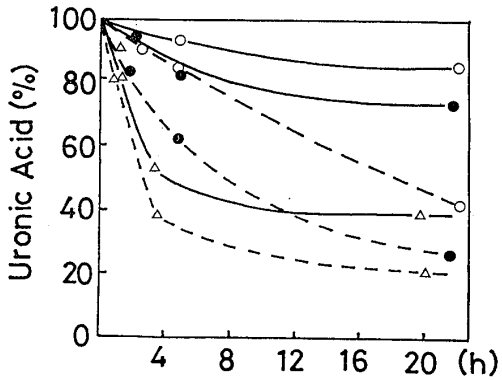


Fig. 1. Periodate oxidation of chondroitin (△), chondroitin sulfate A (●) and C (○). Each polysaccharide (1-2 mg) was oxidized with 20 mM periodate at 37° in the dark in water (——) or in 50 mM phosphate buffer (pH 7.0) in the presence of 0.2 M perchlorate (- - - -).

Effect of ionic strength on periodate oxidation of sulfated tetrasaccharides

To study the effect of ionic strength on the oxidation of oligosaccharides, 2 mM tetrasaccharides "I" and "II" obtained from ChS-A and -C were oxidized with 20 mM periodate in water or in 0.2 M perchlorate-50 mM phosphate buffer

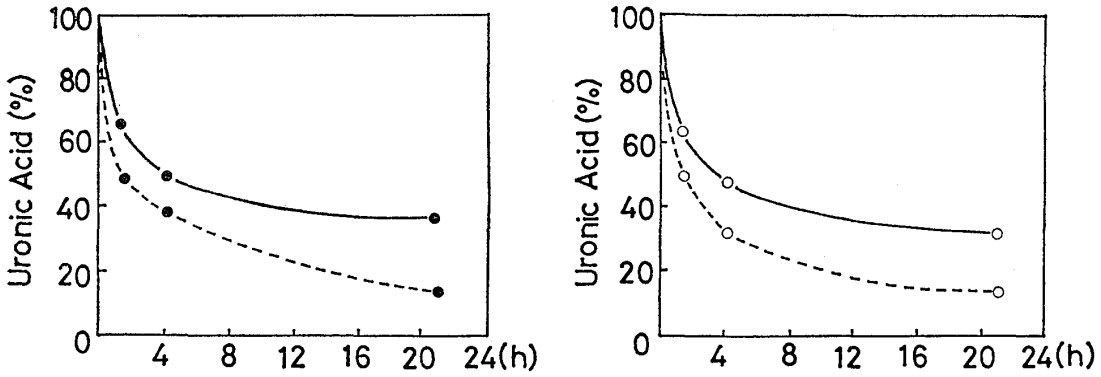


Fig. 2. Periodate oxidation of sulfated tetrasaccharide "I" (●) and "II" (○). Reactions were performed under the same conditions as in Fig. 1.

(pH 7.0). It is clear from Fig. 2 that the sulfated tetrasaccharides were extensively and rapidly oxidized, even in water and the effect of high ionic strength was less than that on oxidation of sulfated glycosaminoglycans (ChS-A and -C).

From these results, the effect of ionic strength on the rate and the extent of oxidation of smaller sulfated oligosaccharides, such as sulfated disaccharides, was assumed to be smaller.

Effect of concentrations of sample and periodate on oxidation of sulfated disaccharides

The effect of concentrations of both sample and periodate on the oxidation of sulfated disaccharides was investigated. The rate of oxidation of all disaccharide monosulfates examined at a higher concentration was faster than at a lower concentration, in spite of the fact that the molar ratio of periodate to disaccharide was the same (10 : 1), as shown in Fig. 3. The effect of concentra-

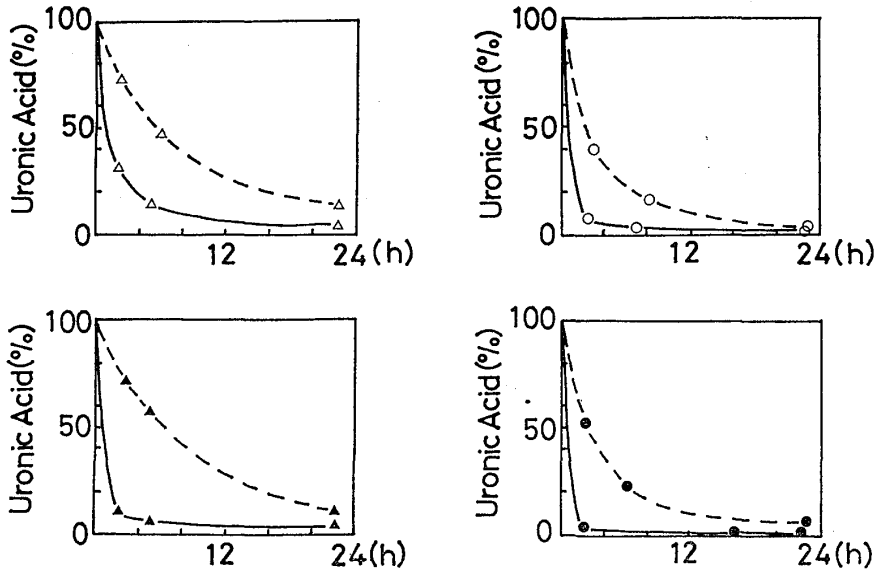


Fig. 3. Periodate oxidation of sulfated disaccharides. 0.2 mM sample was incubated with 2 mM periodate (- - - -) or 2 mM sample with 20 mM periodate (——) at 37° in the dark. Δ4Di-4S (△), Di-4S (▲), Δ4Di-6S (○) and Di-6S (●).

tions, for Di-4S and ⊿Di-4S oxidation, was larger than for Di-6S and ⊿Di-6S. This may be dependent upon the axial sulfate group at the 4 position of the *N*-acetylgalactosamine residue. From these results, it is clear that the conditions, 2 mM sample with 20 mM periodate at 37° in the dark, are suitable for the oxidation of the uronic acid moiety in sulfated disaccharides.

Paper chromatography and paper electrophoresis of Di-diS$_D$, Di-diS$_K$, Di-monoS$_D$ and Di-monoS$_K$

On paper chromatography, using n-butyric acid-ammonia, Di-monoS$_D$ showed the same behavior as Di-monoS$_K$, although Di-diS$_D$ and Di-diS$_K$ were separated well, as shown in Fig. 4. In this solvent system, axial and equatorial isomers such as ⊿Di-4S and ⊿Di-6S separate well, however, it seems difficult to separate "equatorial from equatorial" such as Di-monoS$_D$ and Di-monoS$_K$, in which the sulfate groups are both equatorial at the 2 or 3 positions of the glucuronic acid residues. The disaccharide monsulfates were therefore subjected to high voltage paper electrophoresis under two conditions. After electrophoresis, even in borate buffer (pH 10.0) for 3 h, however, both disaccharides migrated to the same position.

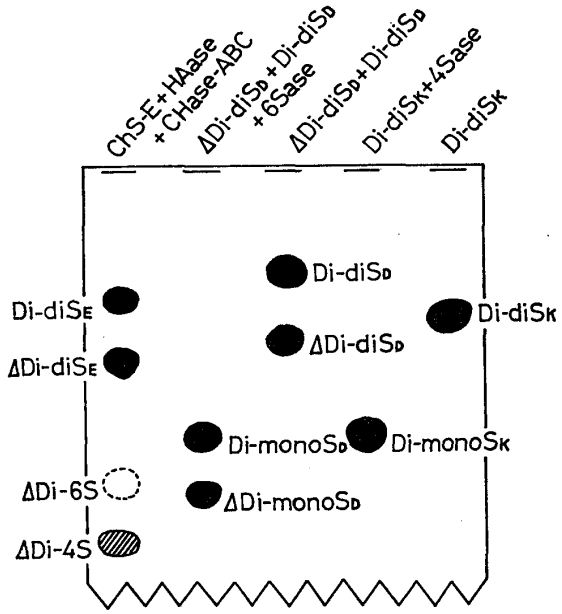


Fig. 4. Paper chromatogram of sulfated disaccharides from chondroitin sulfate D and K.

Periodate oxidation of sulfated disaccharides from ChS-D and ChS-K

Periodate oxidation of ⊿Di-diS$_D$ and ⊿Di-monoS$_D$ was previously examined by Suzuki[2] reporting that ⊿Di-diS$_D$ rapidly consumed one mole of periodate and ⊿Di-monoS$_D$ 3 moles of periodate. Based on this finding, he tentatively concluded that a novel sulfate group is located at the 2 or 3 position of the glucuronic acid moiety, without determination of the uronic acid content during oxidation.

In the present study, ⊿Di-diS$_D$ rapidly consumed over one moles of periodate

and after 20 h oxidation, 3 moles of periodate were taken up without a decrease of the uronic acid content. It therefore appears, that the use of unsaturated disaccharides to determine that 2 or 3 position of the extra sulfate group on the uronic acid residues by periodate oxidation, may not be particularly effective.

When 2 mM of saturated disaccharide disulfates, Di-diS_D and Di-diS_K, were incubated with 20 mM periodate at 37° in the dark, the uronic acid residue in Di-diS_D was rapidly and completely oxidized, whereas that in Di-diS_K was quite stable (Fig. 5). Di-diS_D consumed about 7 moles of periodate, although the theoretical value would be 2 moles. This may be the result of over oxidation[14,15] of both the uronic acid and the galactosamine moieties. After oxidation for 24 h, Di-diS_K consumed nearly one mole of periodate and released 0.4 mole of formaldehyde. After 48 h, the liberation of formaldehyde increased to 0.73 mole. To remove the effect of the sulfate group at the 4 position of *N*-acetylgalactosamine residue in Di-diS_K on oxidation, Di-monoS_K was oxidized under the same conditions. The uronic acid residue in Di-monoS_K was also resistant to oxidation and rapid liberation of about one mole of formaldehyde was found, as shown in Fig. 5.

From these results, it is concluded that the sulfate group, in the glucuronic acid residue of Di-diS_D, is located at the 2 position, while that of Di-diS_K is at

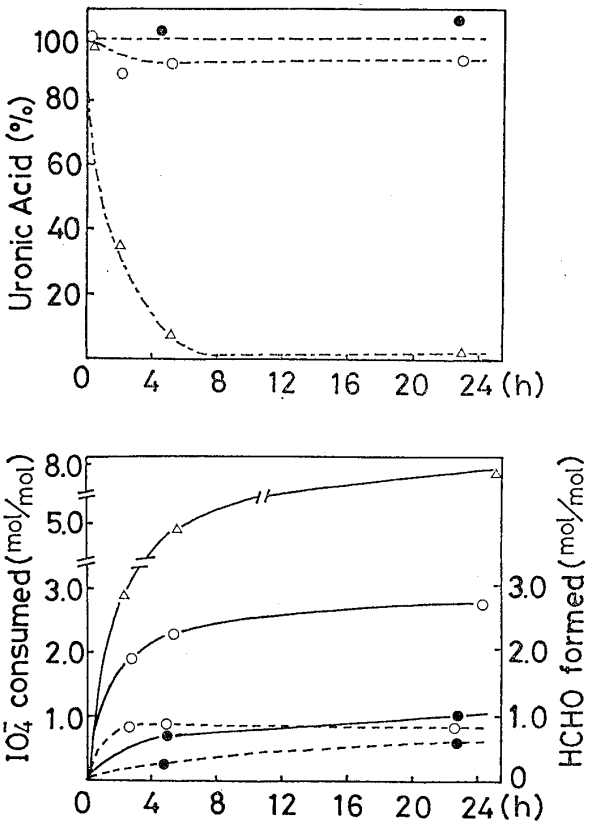
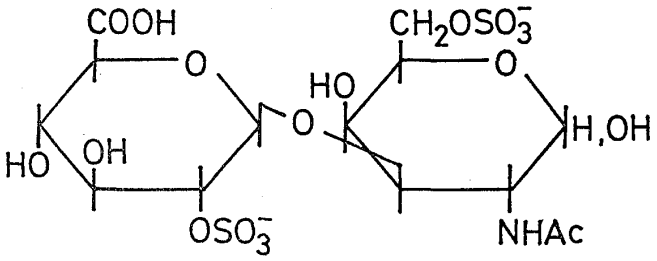
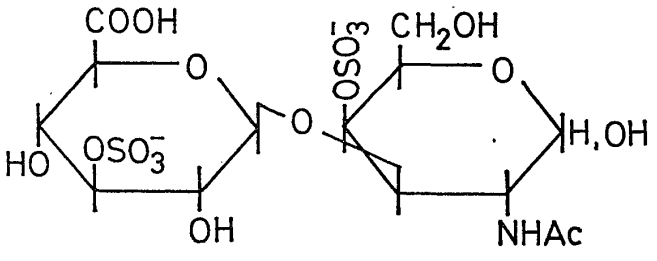


Fig. 5. Periodate oxidation of Di-diS_D (△), Di-diS_K (●) and Di-monoS_K (○). The sample (2 mM) was incubated with 20 mM periodate at 37° in the dark; the uronic acid remaining: (— — — —), the periodate consumed: (————) and the formaldehyde formed (- - - - -).

the 3 position. Accordingly, the structure of Di-diS_D is concluded to be 2-acetamido-2-deoxy-3-*O*-(2-*O*-sulfo-β-D-glucopyranosyluronic acid)-6-*O*-sulfo-D-galactose and that of Di-diS_K to be 2-acetamido-2-deoxy-3-*O*-(3-*O*-sulfo-β-D-glucopyranosyluronic acid)-4-*O*-sulfo-D-galactose, as shown in Fig. 6.



Di-diS_D



Di-diS_K

Fig. 6. Structure of Di-diS_D and Di-diS_K.

Acknowledgments

The authors are indebted to Emeritus Prof. Kimiko Anno for her encouragement.

This work was supported in part by research grants from the Ministry of Education, Science and Culture of Japan.

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