Periodate Oxidation and the Shapes of Glycosaminoglycuronans in Solution

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1. It is proposed that periodate oxidation of glycol groups in the repeating units of polysaccharide molecules can be used to probe differences in polymer shapes in solution. 2. Measurement of second-order rate constants (k_2) of periodate–glycol reactions may be compared between polymers and relevant monomers, to assess perturbations due to polymer configuration. 3. Factors effecting the measurement and interpretation of k_2 are discussed. Over-oxidation, free-radical side reactions, end-group effects, Donnan equilibria and polymer (or molecular-weight) effects are relevant, but their importance is either small or can be minimized in practice. 4. A small group of glycosaminoglycuronans (chondroitin 4- and 6-sulphates and hyaluronate) are oxidized 50–100 times more slowly than three other glycosaminoglycuronans of similar composition, relevant monomers or three homopolyuronides. 5. A stable configuration in solution is postulated for the periodate-resistant polymers, involving carboxylate, acetamido and hydroxy groups in hydrogen-bonded sequences on alternate sides of the molecule. The more easily oxidizable polyuronides are unable to form this configuration. 6. The effect of temperature on the postulated configuration is investigated through the Arrhenius plot of k_2 , measured on hyaluronate, chondroitin 6-sulphate and methyl 4-*O*-methyl- α -D-glucopyranoside. Probable transitions at high (around 90°C) temperatures were observed for both polymers, with an additional transition at about 37°C in the case of hyaluronate. 7. L-Iduronic acid can take up different conformations depending on the polymer environment.

The low reactivity towards periodate of the C-2–C-3 glycol group in the uronic acid moiety of the glycosaminoglycuronans has been mysterious and controversial. Erroneous structures were allocated to chondroitin sulphate as a consequence (Meyer *et al.*, 1948). At room temperature, hyaluronate required 10 or more days for complete oxidation (Montgomery & Nag, 1963). The slowness of the reaction was due, in part, to a Donnan exclusion of periodate anions from the domains of polyanionic substrates (Scott & Harbinson, 1968). Although this effect could be largely eliminated by adding a large excess of NaClO₄, the reactivity of D-glucuronic acid in chondroitin 4-sulphate and 6-sulphate etc. was still low (Scott, 1968). However, the C-5 epimer L-iduronic acid in dermatan sulphate, was oxidized considerably faster (Scott, 1968), suggesting that the conformation was different, perhaps because a hydrogen bond could be formed between the carboxy groups and the C-3 hydroxy group of D-glucuronate, which was not possible in the L-uronic acid (Scott, 1968). Alternatively, the different reactivities of iduronic acid and glucuronic acids may be due to the polymer environments, which in some way are determined by the equatorial or axial position of the carboxy group at C-5 of the

uronic acids. A decision between the two possibilities has been sought by measuring the second-order reaction rates of a number of glycosaminoglycuronans with periodate, and comparing them with that of the 'monomer', (methyl 4-*O*-methyl- α -D-glucuronid)ate. A further comparison of the glucuronide with the corresponding glucoside permits an assessment of the role of the carboxy group in determining the conformation of the 'monomer'. The evidence favours the presence of a preferred configuration in certain glycosaminoglycuronans, involving the co-operation of three adjacent sugars, that hinders periodate oxidation of D-glucuronic acid in these polymers.

A preliminary report of some of this work has appeared (Scott & Tigwell, 1975).

Materials and Methods

Materials

Methyl (methyl 4-*O*-methyl- α -D-glucopyranosid)-uronate (Kovacs, 1973) was a gift from Dr. P. Kovacs. The sample (a syrup, 64.3 mg) dissolved easily in water to a nominal concentration of 1.62% (w/v) (69 mm), pH 4.5–5.0. Uronic acid determination or saponification by 0.047 M-NaOH at room tempera-

ture (20°C) each yielded a uronate concentration of 64mM (1.52%, w/v). This value was used subsequently. $A_{1\text{cm}, 222.5}$ was 5.35.

Electrophoresis on filter paper in 0.05M-sodium formate/acetate buffers at pH 3.0, 4.5 and 6.0 showed only one band of zero mobility at each pH, with alkaline AgNO_3 as location reagent.

Sodium (methyl 4-*O*-methyl- α -D-glucopyranosid)-uronate was prepared as a 64mM stock solution (see above) and, with an equal volume of 1.0M- Na_2CO_3 , was left at room temperature for 2 or 4h. Portions were then electrophoresed in 0.05M-sodium formate/acetate buffers at pH 3.0, 4.5 and 6.0 and showed only one band in each case, migrating slightly slower than galacturonate. The recovery, by uronic acid determination on the hydrolysate, was 110%.

Methyl 4-*O*-methyl- α -D-glucopyranoside (m.p. 95–96°C) was a gift from Professor B. Lindberg, Stockholm.

Hyaluronate, chondroitin 4-sulphate and dermatan sulphate were prepared by the method of Scott (1960) from human mesothelioma fluid, lamb nasal septa and pig-skin respectively.

Chondroitin 6-sulphate and chondroitin sulphate D were from Seikagaku, Tokyo.

Heparan sulphate was a gift from Dr. L. Rodén; heparan sulphate II was a gift from P.-H. Iverius. The uronic acid was composed of glucuronic acid (71%) and iduronic acid (29%) (Höök *et al.*, 1974).

Teichuronic acid (Wright & Heckels, 1975) was a gift from Dr. Heckels.

Desulphated *N*-acetylated heparin was prepared by Dr. L. Rodén, and was a gift from Dr. L.-A. Frånsson. The uronic acid was composed of glucuronic acid (25%) and iduronic acid (75%) (L. A. Frånsson, unpublished work).

Chondroitin methyl ester was prepared by continuously mixing solid chondroitin sulphate D for 1 week in acetyl chloride/methanol (1:49; v/v) according to the method of Kantor & Schubert (1957). It had an intrinsic viscosity in 0.4M- NaClO_4 of 23.5ml/g that was not appreciably different from that in water (19.4ml/g). Practically no cetylpyridinium-precipitable material was present in the product.

Chondroitin was prepared by saponification of chondroitin methyl ester in 0.1M- NaOH . The reactant solutions were degassed, by using a water pump. The reaction tubes were filled to the stoppers to exclude air. After 5h in the dark at room temperature, the pH was adjusted to 7.0 with 1M-acetic acid. The product was precipitated as the cetylpyridinium salt, from which it was recovered as the sodium salt, washed with ethanol and ether and dried (Scott, 1960). A yield of 8.5mg of chondroitin was recovered from 17.0mg of chondroitin methyl ester.

Hyaluronic acid methyl ester was prepared in the same way as chondroitin methyl ester (see above). The product was insoluble in water.

'Reduced' hyaluronate was prepared from hyalurononic acid methyl ester by sodium borohydride reduction (Wolfrom & Juliano, 1960). The products, with their properties, are listed in Table 1. No reactivity was shown in the HNO_2 /indole test for 2-aminohexose. Solutions were opalescent.

Methods

Uronic acid was determined by the Dische carbazole method in the presence of borate (Bitter & Muir, 1962). In addition, polymer-bound uronate was estimated by cetylpyridinium titration (Scott, 1960).

Hexosamine was determined after overnight hydrolysis in 3M-HCl at 102°C in stoppered tubes by the method of Kraan & Muir (1957). Hydrolysates were de-acidified by extraction with 33% (v/v) 1M-*NN*-didecylmethylamine in chloroform (Scott & Newton, 1975).

Hexose was determined by the Dische cysteine/ H_2SO_4 reaction (Dische, 1955).

2-Aminohexose (polymer-bound) was determined by the Dische-Borenfreund HNO_2 -indole reaction (Lindahl, 1973).

Periodate consumption was measured by the spectrophotometric method of Aspinall & Ferrier (1957) at 222.5nm with a Perkin-Elmer-Hitachi 124 u.v.-visible recording spectrophotometer. Glass-stoppered reaction tubes were kept in the dark in thermostatted water baths. Solutions were allowed to reach the reaction temperature before being mixed. For experiments at high temperatures (>70°C), or with rapidly reacting substrates, the two reactants were placed in the side arm and base respectively of a Warburg flask that was immersed in the water bath. Mixing was by rapid inversion. Measurements were usually made on solutions containing 0.2M- NaClO_4 (Scott & Harbinson, 1968).

Viscosity. The time taken for the solution to run out of a 0.1ml Warburg pipette from the 0 to 0.1ml calibrations with the tip immersed 2mm below the level of the solution was measured and compared with that of the control solution. Polyanions were assayed in the presence of 0.4M- NaClO_4 .

Electrophoresis. This was carried out on no. 54 Whatman paper in a Shandon horizontal tank at about 10V/cm with a maximum current of 0.5mA/1cm width of paper. Detection was by the AgNO_3 /NaOH stain (Trevelyan *et al.*, 1950). Thiourea was the zero-mobility marker.

Second-order rate constants (k_2). These were calculated from either

$$k_2 = \frac{1}{t} \frac{x}{a(a-x)}$$

when the reactants were present in equivalent amounts, or

$$k_2 = \frac{1}{t(a-b)} \frac{b(a-x)}{a(b-x)}$$

when one or other was in appreciable excess, where a and b are initial concentrations of the reactants, x is the amount of periodate consumed and t is the time in seconds.

Results and Discussion

The use of periodate-glycol reaction second-order rate constants to probe molecular configurations of polysaccharides has not, as far as we know, been proposed previously, and it is therefore necessary to consider the assumptions and limitations behind the measurements of k_2 .

Oxidation is assumed to be bimolecular and second-order. Second-order kinetics are observed for a considerable part (more than 60%) of the reaction (Fig. 1 and Table 2).

Overoxidation

Overoxidation leads to the accumulation of by-products that react further with concomitant increases in the consumption of oxidant. The increasing k_2 in the later stages of the oxidation of monomer (Fig. 1), which was also observed in the case of hyaluronate etc., may be due to this effect. The values of k_2 in Table 3 are on the basis of measurements made before periodate consumption had reached 50% of the theoretical value.

End groups

End groups may have more than one oxidizable glycol group and be more than usually accessible to the reagent. They are therefore oxidized more rapidly than glycol groups in the polymer chain. The glycosaminoglycuronans are of high molecular weight (with the exception of teichuronic acid, and possibly acetylated desulphated heparin) and the effect may be neglected. There is no observable change from a higher to a lower rate of oxidation for hyaluronate etc. in the early stages (Table 2). Polyguluronate and polymannuronate, and possibly teichuronic acid, show signs of this rapid phase. This atypical phase must be taken into account when extracting a typical k_2 for the polymer as a whole.

Hemi-acetal formation

Hemi-acetal formation in the homopolysaccharides (polyguluronate, polymannuronate and polygalacturonate) progressively limits the rate of oxidation as the reaction proceeds (Painter & Larsen, 1970). In these cases, rates obtained at higher than about 30–40% of the theoretical consumption of oxidant are ignored. In the glycosaminoglycuronans (Fig. 2), the glycol groups are separated from each other by a sugar that is not a substrate for periodate oxidation. The formation of hemi-acetals, if it occurs, therefore does not influence the consumption of periodate.

Table 1. Composition, physical properties and second-order rate constants of periodate oxidation of hyaluronate, reduced hyaluronate and sodium (methyl 4-O-methyl- α -D-glucopyranosid)uronate

	Anhydro-uronic acid (% weight)			Anhydro hexose (% weight)			Reduced viscosity (mg/ml)	Periodate consumption (% of theoretical maximum in 7h)	k_2 (litre·mol ⁻¹ ·s ⁻¹)
	Loss on oxidation			Loss on oxidation					
	Original	Total	% of original	Original	Total	% of original			
Anhydro-hexosamine (% weight)									
Hyaluronate	32	38.4		2.5			1300		8.8×10^{-4}
Reduced hyaluronate I	33	10.8		30			21		Approx. 3.5×10^{-3}
Reduced hyaluronate II	36	15.6		30	8	26	25	43	Approx. 6.0×10^{-3}
Sodium (methyl 4-O-methyl- α -D-glucopyranosid)uronate			22					32	1.78×10^{-2}

Table 2. *Second-order rate constants (k_2) at 25°C in 0.2M- NaClO_4 of the oxidation of glycol-containing polymers by periodate as a function of time*

Polymer	Time (s)	$100 \times \{[\text{Periodate consumed (mM)}] / [\text{*Glycol in soln. (mM)}]\}$	k_2 (litre $\cdot$ mol $^{-1} \cdot$ s $^{-1}$)
10mm-Polygalacturonate with [periodate] = 10mm	300	8.5	3.1×10^{-2}
	600	15.0	2.9×10^{-2}
	1200	20.0	2.1×10^{-2}
	1800	27.7	2.1×10^{-2}
	2700	30.9	1.7×10^{-2}
	3600	36.8	1.6×10^{-2}
	5400	46.2	1.6×10^{-2}
	9000	53.4	1.3×10^{-2}
	14400	60.3	1.1×10^{-2}
	19800	68.5	1.1×10^{-2}
1.44mm-Polymannuronate with [periodate] = 2mm	600	11.9	11.4×10^{-2}
	1200	16.6	8.1×10^{-2}
	1800	20.1	6.8×10^{-2}
	2400	21.4	5.3×10^{-2}
	3000	24.3	5.0×10^{-2}
	3600	27.1	5.0×10^{-2}
	4560	29.1	4.3×10^{-2}
	9000	39.6	3.3×10^{-2}
	17100	47.2	2.3×10^{-2}
6.5mm-Hyaluronate with [periodate] = 8mm	7620	5.5	9.6×10^{-4}
	11100	6.8	8.1×10^{-4}
	16140	9.8	8.4×10^{-4}
	63960	33.2	9.3×10^{-4}
	77700	38.1	9.3×10^{-4}
	96900	43.0	9.1×10^{-4}
	161940	59.6	10.0×10^{-4}
4.7mm-Chondroitin 4-sulphate with [periodate] = 8mm	12420	9.3	9.6×10^{-4}
	59640	23.6	6.0×10^{-4}
	74160	25.4	5.3×10^{-4}
	85740	30.4	5.8×10^{-4}
	147360	38.9	4.8×10^{-4}
	176100	40.6	4.2×10^{-4}
	407640	73.4	5.6×10^{-4}
4.0mm-Dermatan sulphate with [periodate] = 8mm	1830	11.0	8.2×10^{-3}
	3660	22.0	9.0×10^{-3}
	5460	34.0	10.5×10^{-3}
	7500	41.0	9.9×10^{-3}
	12540	52.0	8.6×10^{-3}
	59520	77.0	4.1×10^{-3}
	147240	82.0	2.0×10^{-3}
	407640	94.0	1.3×10^{-3}
7.8mm-Heparan sulphate with [periodate] = 9mm	1830	17.3	9.0×10^{-3}
	3480	24.9	8.5×10^{-3}
	7140	35.7	7.5×10^{-3}
	10770	41.0	6.3×10^{-3}
	14400	47.3	6.1×10^{-3}
	18000	51.9	5.9×10^{-3}
	71010	73.7	3.6×10^{-3}
	106470	80.7	3.4×10^{-3}

Table 2—continued

Polymer	Time (s)	$100 \times \{[\text{Periodate consumed (mm)}] / [\text{*Glycol in soln. (mm)}]\}$	k_2 (litre $\cdot$ mol $^{-1} \cdot$ s $^{-1}$)
1.4mm-Polyguluronate with [periodate] = 2mm	390	15.2	2.3×10^{-1}
	630	21.5	2.1×10^{-1}
	990	27.7	1.8×10^{-1}
	1230	31.9	1.8×10^{-1}
	1710	36.8	1.6×10^{-1}
	2190	40.2	1.4×10^{-1}
	2670	44.4	1.4×10^{-1}
	3150	46.5	1.2×10^{-1}
	16290	70.8	0.57×10^{-1}
5mm-Chondroitin with [periodate] = 8mm	3720	8.0	2.9×10^{-3}
	5520	8.8	2.1×10^{-3}
	7140	13.6	2.7×10^{-3}
	10680	20.8	2.9×10^{-3}
	14520	24.0	2.6×10^{-3}
	19860	31.2	2.6×10^{-3}
	25440	36.0	2.5×10^{-3}
4mm-Teichuronic acid with [periodate] = 4mm	1830	21.0	3.6×10^{-2}
	3720	36.5	3.9×10^{-2}
	5460	47.0	4.0×10^{-2}
	7200	58.0	4.8×10^{-2}
	9000	66.0	5.5×10^{-2}
	10860	68.0	4.8×10^{-2}
	12780	77.0	6.5×10^{-2}
	16320	84.0	8.0×10^{-2}
7.7mm-Chondroitin 6-sulphate with [periodate] = 8mm	16020	4.0	2.8×10^{-4}
	63840	13.0	2.8×10^{-4}
	77520	14.0	2.5×10^{-4}
	96720	17.7	2.6×10^{-4}
	161700	27.0	2.7×10^{-4}
	190740	30.1	2.7×10^{-4}
	276540	40.0	2.8×10^{-4}
10.6mm-Heparan (from heparin) with [periodate] = 8mm	570	9.7	4.0×10^{-1}
	1800	17.4	1.4×10^{-1}
	6900	35.8	4.2×10^{-2}
	14760	37.5	2.0×10^{-2}
	24300	43.9	1.0×10^{-2}
	30600	45.4	1.1×10^{-2}
	78300	58.6	5.8×10^{-3}
	107100	58.1	4.2×10^{-3}
	162000	64.4	3.3×10^{-3}
10mm-Reduced hyaluronate with [periodate] = 8mm	1800	14.0	8.9×10^{-3}
	3720	20.5	6.8×10^{-3}
	5520	27.5	6.6×10^{-3}
	7560	29.0	5.2×10^{-3}
	11220	37.0	5.0×10^{-3}
	14460	47.0	5.6×10^{-3}
	18330	51.0	5.2×10^{-3}
	21690	59.0	5.8×10^{-3}
	26220	64.0	5.8×10^{-3}

* Assuming no extra 'end-group glycol'.

Donnan effects

It is necessary to carry out periodate oxidations in the presence of a great excess of inert electrolyte to

eliminate the Donnan effect (Scott & Harbinson, 1968), and it is then possible to compare non-ionized with ionized substrates. The presence of

Fig. 1. Plot to show second-order kinetics of periodate oxidation of methyl (methyl 4-*O*-methyl- α -D-glucopyranosid)uronate (Δ) and methyl 4-*O*-methyl- α -D-glucopyranoside ($\bullet$)

Each sugar was present at a concentration of 0.8 mM (*b*) in 1.0 mM-sodium periodate (*a*) solution at 25°C.

extra electrolyte or buffer salts made no significant difference to the rate of oxidation of the non-ionized monomers (Table 4).

The polymer effect

A polymer molecule in solution represents a high concentration of monomers within a confined domain. In the limit of very dilute solutions, there are infrequent volumes of highly concentrated substrate, surrounded by large volumes of substrate-free reactant. In the limit of concentrated polymer solutions, the solution is homogeneous with respect to the distribution of 'monomer'. The experiments described in the present paper were carried out in conditions between the two extremes. Measurements performed at three concentrations of hyaluronate, from 4.3 to 8.7 mM, gave the same value of k_2 within the experimental error of each determination. The rate of reaction, as measured in the present paper, is not subject to the polymer effect.

Free-radical effects and hydroxyl-radical scavengers

Hydroxyl ($\text{OH}^\bullet$) radicals, which have been demonstrated and measured in periodate solutions (Scott & Page-Thomas, 1976), depolymerize polysaccharides and produce periodate-oxidizable groups (Scott & Tigwell, 1973). The addition of *n*-propanol, a

hydroxyl free-radical scavenger, minimizes these effects (Scott *et al.*, 1976). The addition of 10% (v/v) *n*-propanol to either methyl 4-*O*-methylglucoside or chondroitin sulphate D or of 5% (v/v) *n*-propanol to polygalacturonate solutions during periodate oxidation in the standard conditions had no effect on k_2 . It is concluded that side reactions caused by hydroxyl free radicals generated by periodate in the standard conditions (i.e. in the dark; Scott & Page-Thomas, 1976) may be neglected in the measurement of k_2 .

Effect of temperature

Sodium hyaluronate (0.8%, w/v) in 0.2M- NaClO_4 was heated in a water bath to 100°C for 5 min, and cooled to 25°C. The k_2 value that was measured on this sample at 25°C was identical with that of an unheated portion of the same solution.

The k_2 value was measured over a range of temperatures, from 4°C to 90°C on chondroitin 6-sulphate, hyaluronate and methyl 4-*O*-methyl- α -D-glucopyranoside. The results are shown in Fig. 3 as an Arrhenius plot ($\ln k_2$ against the reciprocal of the temperature in K). Marked increases in the slopes in the region of 89–91°C were observed for both polymers. In the case of hyaluronate, but not of chondroitin 6-sulphate or the glucoside, a change in the slope occurred at about 37°C.

Effect of pH

The k_2 values of the sodium salts of chondroitin 6-sulphate, hyaluronate and (methyl 4-*O*-methylglucuronid)ate show measurable, but small (10–30%), decreases with decreasing pH in the region of pH 2.4–6.0. So also does the k_2 value of methyl 4-*O*-methylglucoside (Table 4). These measurements were made after adding the unbuffered substrate to the buffer plus periodate. Similar results were obtained if the polysaccharide was preincubated with buffer and NaClO_4 for 30 min before adding the periodate.

Fransson (1974) and Di Ferrante *et al.* (1971) commented on an apparently decreased rate of oxidation of chondroitin 4-sulphate in acid compared with neutral solutions.

Methylation of carboxy groups

At pH 6 and in 0.2M- NaClO_4 the k_2 value of the methyl ester of (methyl 4-*O*-methylglucuronid)ate is noticeably (about 80%) higher than that of the sodium salt (Table 4). There is also an increase, but smaller, in the k_2 value of the methyl ester of chondroitin compared with that for the sodium salt (Table 3).

Polymer k_2 values

The k_2 values of the unmodified glycosaminoglycuronans fall into two separate groups that differ

Fig. 2. Formulae of repeating units of chondroitin 4-sulphate (A), dermatan sulphate (B), chondroitin 6-sulphate (C), hyaluronate (HA), methyl(methyl 4-O-methyl- α -D-glucopyranosid)uronate (I) and methyl 4-O-methyl- α -D-glucopyranoside (II)
R = CH₃, H or Na⁺ (as described in the text).

by at least an order of magnitude. Chondroitin 6-sulphate is oxidized approximately 100 times less rapidly than teichuronic acid.

The k_2 value for chondroitin sulphate D is calculated on the assumption that part of the glucuronic acid is esterified by sulphate that is present in excess of 1 mol/mol of repeating unit (the ratio of sulphate/carboxy anion is 1.29). If some of the excess sulphate were on the hexosamine, the true value of k_2 would be lower than that recorded in Table 3.

The k_2 values of the first phases of oxidation of the heparan sulphates and dermatan sulphate are calculated on the assumption that all the uronic acid in the molecule is equally involved in the reaction. Since it is probable that an important proportion of the uronic acid is oxidized at much lower rates, if at all, the k_2 values given in Tables 2 and 3 are conservative. The pronounced downward drift of the k_2 value at high degrees of oxidation in these cases contrasts with the behaviour of chondroitin 6-sulphates and hyaluronate, in which the rate tends to rise, presumably because of overoxidation, as consumption of periodate approaches the theoretical value.

Both preparations of reduced hyaluronate (listed

in Table 1) show increased values of k_2 compared with the parent hyaluronate. They show a downward drift of the k_2 value as oxidation proceeds. Both hexose and uronic acid are oxidized, as indicated colorimetrically (Table 1). The values are not interpretable quantitatively since uronic acid contributes a little to the cysteine reaction for hexose, and the carbazole reaction apparently underestimates the destruction of uronic acid by periodate (cf. the model monomer; Table 1).

Polymer shapes

In principle, chemical reactions could be used to investigate shapes of polymer molecules in solution, if the reagent were sensitive to (i) the conformation of the monomers or if (ii) the configuration of the polymer systematically affected the reaction. By comparing intrinsic reaction rates in the polymer with those of relevant monomers, the influence of the polymer configuration could be detected. For instance, periodate would be expected to react at similar speeds with all glycols of a similar torsional angle to each other, if no other steric effects were present. In each glycosaminoglycuronan the repeating

Fig. 3. Arrhenius plot [$\ln(k_2 \times 10^5)$ against $10^3/T$] of reaction of periodate with the glycol-containing compounds methyl 4-*O*-methyl- α -D-glucopyranoside (▲), chondroitin 6-sulphate (■) and hyaluronate (●).

The vertical lines show the spread of values of $\ln k_2$ at each temperature point. The plotted point is the arithmetical average. The plots are drawn by inspection. All solutions contained 0.2M- NaClO_4 . The glucoside concentrations were all 0.4mM in 0.4mM- NaIO_4 . Hyaluronate concentrations were either 6.53mM or 8.7mM in either 8.0mM- or 10mM- NaIO_4 , except for the readings at 364 and 358K, which were 1.9mM in polymer and 2mM in NaIO_4 . Chondroitin 6-sulphate concentrations were 7.72mM in 8.0mM-periodate.

unit contains one glycol group in which, assuming C1 ring forms, the hydroxy groups would be expected to be at 60° and in much the same position with regard to the rest of the uronic acid ring. Unexpectedly, dermatan sulphate was more rapidly oxidized than the isomeric chondroitin 4- and 6-sulphates (Scott, 1968). This was confirmed by Frånsson (1974) and by Di Ferrante *et al.* (1971).

No relevant substituted monomeric hexuronic acids were available, until the synthesis (Kovacs, 1973) of (methyl 4-*O*-methylglucopyranosid)uronate. A comparison with the analogous glucoside showed little difference in the speeds of oxidation (Table 3). It is therefore unlikely that unusual conformations are present in glucuronic acid residues *per se*. The

Table 3. Second-order rate constants (k_2) at 25°C in 0.2M- NaClO_4 of the oxidation of glycol-containing compounds by periodate

Glycol-containing compound	k_2 (litre $\cdot$ mol $^{-1}$ $\cdot$ s $^{-1}$)
Sodium (methyl 4- <i>O</i> -methyl- α -D-glucopyranosid)uronate	1.78×10^{-2}
Methyl 4- <i>O</i> -methyl- α -D-glucopyranoside	3.29×10^{-2}
Polygalacturonate	2×10^{-2}
Polymannuronate	5×10^{-2}
Polyguluronate	1.8×10^{-1}
Dermatan sulphate	9×10^{-3} *
Heparan sulphate	8×10^{-3}
Heparan sulphate (HS II)	7×10^{-3}
Desulphated <i>N</i> -acetylated heparin	10^{-1} – 10^{-2} *
Chondroitin 4-sulphate	5.2×10^{-4}
Chondroitin 6-sulphate	2.7×10^{-4}
Chondroitin sulphate D	7.9×10^{-4}
Hyaluronate	8.8×10^{-4}
Teichuronic acid	3×10^{-2} – 4×10^{-2}
Chondroitin	2.6×10^{-3}
Reduced hyaluronate	5×10^{-3}
Chondroitin methyl ester	2.9×10^{-3}

* First phase.

basic assumption that polysaccharides in which the glycol groups are present in similar situations react at similar rates is supported by the comparison of the monomers with polymannuronic acid, polyguluronic acid and polygalacturonic acids (Table 3). L-Guluronic acid is normally in the 1C form (Atkins *et al.*, 1973), but the angle between glycol COH groups is 60° as in the C1 form. Although polyguluronate is oxidized more rapidly than polymannuronate, in agreement with previous results, the difference is only about 3-fold. The difference between dermatan sulphate and either chondroitin 4- or 6-sulphate is of the order of 50–100-fold (Table 3).

At acid pH, in which lactones might form, or the carboxy group would be undissociated, k_2 values are similar to those at higher pH, in which carboxy groups would be dissociated, and lactones would not be expected to be present (Table 4). Differences between the uronic acids cannot be attributed to differential formation of lactones.

The strong resistance to oxidation demonstrated in a restricted group of compounds suggests that there is a stable configuration present throughout these molecules that is not present in the monomers nor in polygalacturonate etc. If we reject the suggestion that the uronic acids in the resistant chondroitin sulphate and hyaluronate polymers have abnormal conformations (and available evidence is to the contrary; e.g., Atkins & Laurent, 1973; Winter *et al.*, 1975), it follows that the polymer configuration is

Table 4. *Effect of pH and added electrolyte on the second-order rate constants (k_2) of monomeric and polymeric glycols*

Glycol	Temperature (°C)	Added Na ⁺ (M)	pH	k_2 (litre·mol ⁻¹ ·s ⁻¹)
Hyaluronate	30	0.2	Unbuffered HIO ₄ (pH 2.4)	1.0×10^{-3}
			4.2	1.2×10^{-3}
Chondroitin 6-sulphate	25	0.2	3.0	2.3×10^{-4}
			6.0	3.0×10^{-4}
Methyl (methyl 4-O-methyl- α -D-glucopyranosid)uronate	25	0.0	Unbuffered NaIO ₄ (pH 5.5–5.8)	1.0×10^{-2}
		0.2	6.0	1.1×10^{-2}
Sodium (methyl 4-O-methyl- α -D-glucopyranosid)uronate	25	0.2	3.0	1.0×10^{-2}
			6.0	1.8×10^{-2}
Methyl 4-O-methyl- α -D-glucopyranoside	25	0.0	Unbuffered HIO ₄	2.9×10^{-2}
			Unbuffered NaIO ₄	3.3×10^{-2}
		0.2	3.0	3.4×10^{-2}
			6.0	4.0×10^{-2}

responsible. One can then tentatively identify the groups that are involved in stabilizing the assumed structure, arguing from the premise that there is one configuration in common, involving immediately neighbouring sugars. Other alternatives, that there are different sorts of configuration in the resistant polymers or that other than neighbouring sugars (e.g. on other polymers) are involved, are not amenable to analysis at the moment.

The sulphate group is not a necessary participant in the assumed interaction, since hyaluronate is a resistant polymer. Positions C-4 and C-6 of the hexosamine can be ruled out, since both chondroitin 4- and 6-sulphate are resistant to oxidation. Furthermore, the hydroxy group at position C-4 can be axial or equatorial, as in chondroitin 6-sulphate or hyaluronate respectively, and the resistant configuration is still present. The carboxylic acid group is strongly implicated, since in dermatan sulphate the oxidation rate is similar to that of the monomers, in contrast with that of isomeric chondroitin 4-sulphate, which differs only in the orientation of the carboxy group. Thus only carboxy (directly) and acetamido (by elimination) groups can be involved.

Although the analysis limits the features that may participate, there still remain too many possibilities to allow a detailed conclusion, without help from other sources. The polymer configurations derived from X-ray studies of fibres (Atkins *et al.*, 1972) are relevant. They indicate that the carboxy and acetamido groups are very close together on the same side of the polymer chain. These are the groups that our analysis implies are important in stabilizing the resistant structure in solution. Courtauld space-filling models of the X-ray fibre structures (Fig. 4) show that the carboxy and acetamido groups are sufficiently close to permit easy hydrogen-bond formation between them. When this occurs, the hydroxy group on position C-2 on the neighbouring uronic acid

can join in a second hydrogen bond, resulting in a three-group hydrogen-bonded sequence:

The glycol group of the uronic acid is then considerably hindered, probably much more so than if the hydroxy group was not involved in a hydrogen bond. In chondroitin 4- and 6-sulphates there is an exactly similar possibility, as there is in chondroitin sulphate D. On the other hand, a hydrogen bond between the carboxy and neighbouring acetamido groups of dermatan sulphate could only form if the chain is very considerably twisted, in which case the glycol groups are not hindered. Arnott and co-workers (Winter *et al.*, 1975) independently suggested that their X-ray fibre data were compatible with a hydrogen bond between acetamido and carboxy groups in hyaluronic acid.

The oxidation of the glycol group of chondroitin 6-sulphate is less rapid than that of chondroitin 4-sulphate, and the rate increases considerably on desulphation (Table 3). This suggests that the sulphate groups (that are on the neighbouring sugar) are in a relatively fixed position with regard to the glycol group. In the proposed structure, the sulphate group on galactosamine residues of chondroitin 6-sulphate is located close to the glycol group of the neighbouring uronic acid, but is less close in the case of chondroitin 4-sulphate (Fig. 4).

The sugar residues on the proposed structure are approximately co-planar because the glycosidic bonds are equatorial. Co-planarity of neighbouring units is important to the proposed extended sequence of hydrogen bonds down alternate sides of the molecule. If one of the β -glycosidic

Fig. 4. Model of a pentasaccharide of chondroitin constructed from Courtauld space-filling atomic units '4' and '6' indicate the position at which the sulphate ester group is located in chondroitin 4- or 6-sulphate respectively. 'H-bond' indicates the proposed hydrogen bonds between hydroxy, acetamido and carboxy groups.

bonds is α , the co-planarity is disturbed, and analogous hydrogen bonds are not formed without gross twisting of the molecule, with a considerable change in the accessibility of the glycol group to periodate. Heparan sulphate and teichuronic acid are particularly relevant, since in both at least one of the glycosidic bonds is α . Both contain glucuronic acid and both are rapidly oxidized. This is further confirmation, but from polymer-bound glucuronic acid, that the rate of oxidation of glucuronic acid is not of itself slow. The much slower rate of oxidation of chondroitin as compared with teichuronic acid is consistent with the presence of the proposed structure in chondroitin, but not in teichuronic acid, since in teichuronic acid and chondroitin all other details, apart from the α -link, are similar.

The major uronic acid of heparan sulphate is glucuronic acid (at least 70%). Thus there is no doubt from the extent of the rapid oxidation that glucuronic acid is being oxidized at a rate that approaches that of the monomer (Table 3). Fransson (1978) confirmed that glucuronic acid is rapidly oxidized in heparan sulphate. The glycosidic bonds are 1 $\rightarrow$ 4,1 $\rightarrow$ 4 compared with 1 $\rightarrow$ 4,1 $\rightarrow$ 3 in the compounds previously considered, which would be sufficient to disrupt the extended hydrogen-bonded sequence.

Since the proposed structure involves the carboxy

and acetamido groups, modification of either ought to affect the hydrogen-bonded sequence. Thus the reduction of some carboxy groups of hyaluronic acid to a primary alcohol was attended by an increased rate of periodate oxidation (Table 1). Conversion was incomplete, and the results are only semi-quantitative.

Conversely, modifications of the carboxy group that do not seriously hinder its capacity to form hydrogen-bonds ought not to affect the reaction. Neither methylation nor change in the ionization of the carboxy group appreciably alters the k_2 value (Table 4).

Thermal stability

The proposed structure contains hydrogen bonds whose existence is facilitated by the formation of another hydrogen bond. This element of co-operativity suggests that there should be a temperature effect on the stability of the structure.

An Arrhenius plot of the second-order constants (Fig. 3) shows no change in the slope in the case of monomeric glucoside, or in the case of chondroitin 6-sulphate at $<90^\circ\text{C}$, but in the case of hyaluronate there is a significant change at about 37°C . This result suggests that one configuration is preferred at temperatures below physiological, whereas another configuration exists at higher temperatures. It may be

relevant that hyaluronate is able to assume different configurations in the fibre (Atkins *et al.*, 1972; Sheehan *et al.*, 1974). As a corollary, chondroitin 6-sulphate exists in only one configuration in aqueous solution at temperatures below 90°C in our experimental conditions.

The speed of oxidation of hyaluronate increases disproportionately with temperature at about 90°C. The rate of increase in the case of chondroitin 6-sulphate is much less marked, whereas that of the monomer is normal, which eliminates the possibility that the mechanism of periodate oxidation changes in this region of temperature. Over a short temperature interval the glycol group of hyaluronate becomes much more accessible. The configuration at lower temperatures is very stable, since it breaks only at temperatures close to boiling point and it re-forms on cooling. As a corollary, the configuration of chondroitin 6-sulphate is even more stable. Measurements at high temperatures are not precise and further work is needed. There is no other indication from any technique that sharp changes in configuration occur with temperature in this group of glycosaminoglycuronans, although there is a steady change with temperature, which is reversible, in the circular dichroism of hyaluronate solutions (J. E. Scott & P. J. Bayley, unpublished work).

Since all three compounds give rise to plots with similar slopes the reactions have similar activation energies, which is consistent with the assumption that the mechanisms of oxidation of monomer and polymers are identical. The intercepts on the $1/T$ axis differ, implying lower frequency factors for the polymers, compared with monomer, which could be due to steric hindrance of the kind discussed above.

The ratio k_2 (monomer)/ k_2 (polymer) $\approx$ 30–100 at a given temperature, which corresponds to a difference in the free energy of activation between monomer and polymer of 8.4–12.6 kJ/mol of glycol. This is approximately the energy of a hydrogen bond, which would support the suggestion that a hydrogen bond to a hydroxy group in the polymer glycol group must be broken before oxidation can proceed.

Probably both steric effects and transition-state energy operate against the oxidation of resistant glycosaminoglycuronans.

Molecular rigidity and specific interactions

The proposed array of hydrogen bonds down each side of these molecules should endow them with greater rigidity. Hyaluronate behaves as an unusually stiff polymer in solution (Laurent, 1957). Close-range interactions between stiff molecules and other more-or-less rigid structures such as fibres, globular proteins and membranes require precise complementarity of reacting sites, i.e. they are specific.

Polymer-chain stiffness may contribute to the striking specificity of hyaluronate interactions with,

e.g., whole cells (Pessac & Defendi, 1972; Westermarck & Wasteson, 1973; Wiebkin & Muir, 1973) and proteoglycans (Hardingham & Muir, 1972), as well as hindering or preventing similar interactions by other almost identical polymers.

Iduronic acid conformations

Desulphated acetylated heparin is identical with natural heparan sulphate in the arrangement of sugars in the polymer backbone, but it contains a considerably greater molar ratio of iduronic acid/glucuronic acid (3:1). By analogy with heparan, it is not surprising that the initial oxidation phase is rapid, but it is surprising that the larger part of the uronic acid resists periodate oxidation. This implies that the iduronic acid behaves differently in heparan sulphate from that in dermatan sulphate, which is rapidly oxidizable. L. A. Fransson (unpublished work) has shown that iduronic acid in desulphated acetylated heparin is less easily subject to oxidation than the glucuronic acid contained therein. This resistance depends on the conformation, which n.m.r. studies have shown (G. Ghatti & B. Casu, unpublished work) to be $1C_4$, in which the glycol hydroxy groups are at 180°C and therefore not subject to oxidation by periodate. The periodate kinetics indicate that the conformation of iduronic acid in dermatan sulphate is different from that in heparin or heparan. Presumably iduronic acid can take up different conformations depending on the polymer environment.

These results are relevant to the claim (Di Ferrante *et al.*, 1971) that it was possible, on the basis of its higher rate of oxidation compared with other glycosaminoglycuronans, to assay dermatan sulphate in urine of suspected mucopolysaccharidoses. Heparan sulphate was said to react as chondroitin sulphate A etc. The situation is not so simple, and the method must be regarded as unsafe.

Non-bonding interactions

The data point to a regular structure that is stable, featuring the glycol group in a situation that is present throughout the polymer chain. Steric hindrance and non-bonding interactions between neighbouring sugars may be sufficient to stabilize glycosaminoglycans into preferred configurations (Cleland, 1971; Potenzzone & Hopfinger, 1975). However, the calculated minimum-energy configurations are compatible with structures derived from X-ray-fibre results and in these cases the interatomic distances (Winter *et al.*, 1975) and molecular models (Fig. 4) suggest that hydrogen bonds are easily formed. The resulting structure accounts satisfactorily for a wide range of biochemical and physical observations.

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