

## A Further Illustration of Nearest-neighbour Auto-inhibitory Effects in the Oxidation of Alginate by Periodate Ion

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Samples of sodium alginate from *Laminaria digitata* were treated separately with dilute, aqueous sodium metaperiodate until 5 %, 10 %, 20 %, and 30 %, respectively, of the hexuronic-acid residues had been oxidised. The products were then reduced with sodium borohydride, and oxidised again with an excess of periodate under conditions that suppress overoxidation. In each case, the additional consumption of periodate was  $0.42 \pm 0.02$  mol per 198 g.

It was shown theoretically that this result is to be expected, on the assumption that both aldehyde groups of oxidised hexuronic-acid residues spontaneously form highly stable, intramolecular hemiacetals with the secondary hydroxyl groups on unoxidised residues adjacent to them in the chains.

When sodium alginate is oxidised in dilute, aqueous sodium metaperiodate under conditions that suppress depolymerisation and overoxidation, reaction ceases when only 44 % of the hexuronic-acid residues have been oxidised.<sup>1</sup> This oxidation-limit corresponds closely to that calculated on the assumption that only one residue in a given chain is oxidised at a time, and that oxidised residues, once formed, spontaneously protect the two adjacent, unoxidised residues in the chain from subsequent oxidation.<sup>1,2</sup>

The protective mechanism consists in the spontaneous formation of six-membered hemiacetal rings between the aldehyde groups of the oxidised residue and the closest hydroxyl groups on the two adjacent, unoxidised residues, thus removing their vicinal-diol functions.<sup>1</sup> When the hemiacetal rings are cleaved by treatment of the limit-oxidised alginate with sodium borohydride, the vicinal-diol groups in the remaining, unoxidised hexuronic-acid residues are again exposed.<sup>1</sup>

The molecule then consumes further periodate until a second oxidation-limit, corresponding to the oxidation of a total of about 88 % of the original hexuronic-acid residues, is reached. A second reduction with borohydride then permits oxidation of the remaining 12 % of intact hexuronic-acid residues.<sup>1</sup>

Although further evidence in support of these deductions is perhaps not needed, it was of interest to enquire how much additional periodate a sample of alginate would consume, if reduction with borohydride was carried out before the first oxidation-limit of 44 % had been reached. The results, which are now reported, are pleasingly simple, and provide a further illustration of the operation of the nearest-neighbour, auto-inhibitory mechanism.

### THEORY

After oxidation to its first limit, the remaining, unoxidised hexuronic-acid residues in alginate exist in the chains either as "singlets", with oxidised residues in both neighbouring positions, or as "doublets", in which each member has only one oxidised residue in a neighbouring position.<sup>1</sup> The ratio of singlets to doublets is 2.35:1. Reduction with borohydride and further treatment with periodate result in complete oxidation of all the singlets, but of only one member in each doublet. This implies that the second oxidation-limit should be exactly twice the first, and the experimental result agrees well with this.<sup>1</sup>

When oxidation has been stopped before the first limit has been reached, the remaining, unoxidised residues must exist in the chains not only as singlets and doublets, but also as triplets, quadruplets, *etc.* All these groups must be isolated from one another by an oxidised residue, so that, after reduction of the oxidised residues, they must undergo further oxidation independently of one another. The total population of chains can therefore be regarded, for purposes of calculation, as consisting of a fraction,  $x_1$ , of chains containing singlets only, a fraction,  $x_2$ , containing doublets only, a fraction,  $x_3$ , containing triplets only, and so on.

The hypothetical group of chains containing singlets only will, upon reduction and re-oxidation, consume 1 mol of periodate for every unoxidised residue, corresponding to 0.5 mol, based upon the weight of the starting-material. Those containing doublets only will consume only 1 mol of periodate for every doublet, corresponding to 0.33 mol, based upon the starting material. The behaviour of those containing larger groups can be readily calculated from published data,<sup>2</sup> as follows.

For a group of  $N$  contiguous, oxidisable units, the oxidation-limit,  $D_N$ , expressed as a fraction of these units, is known.<sup>2</sup> For every such group, there is one unit that was oxidised in the first treatment with periodate, and then reduced. Therefore, the fraction of the total number of units in the chains that are oxidised in the second treatment with periodate is  $ND_N/(N+1)$ . The results for various values of  $N$  are shown in Table 1.

Formally, it is now necessary to evaluate  $x_1, x_2, x_3, \text{etc.}$ , but it is seen from Table 1 that, for values of  $N$  greater than 2, the results for each group are approximately the same. It is clearly necessary to evaluate  $x_1$  and  $x_2$ . This can be done by considering the two groups together, as follows.

At very low degrees of oxidation, singlets and doublets must be generated with equal frequency, because, on the assumption of random attack, the probability of the nearest neighbour of a singly-protected unit being attacked

*Table 1.* Calculation of the periodate consumed by hypothetical alginate molecules containing groups of  $N$  contiguous, oxidisable hexuronic-acid residues, each being separated by a single residue which has previously been oxidised by periodate and reduced by borohydride.  $D_N$  is the oxidation-limit of each group, and  $ND_N/(N+1)$  expresses it as a fraction of all the units in the chain.

$N$	$D_N$	$ND_N/(N+1)$
1	1.000	0.500
2	0.500	0.333
3	0.556	0.417
4	0.500	0.400
5	0.493	0.411
6	0.481	0.412
7	0.475	0.416
8	0.469	0.417
10	0.462	0.420
15	0.452	0.424
20	0.447	0.426
40	0.440	0.429

must be identical with the probability of its next-nearest neighbour being attacked. Initially, then,  $x_1 = x_2$ , and the average, limiting degree of oxidation of the two groups will be  $(0.5x_1 + 0.333x_2)/(x_1 + x_2) = 0.416$ .

As the degree of oxidation increases, the probability that an oxidisable unit will lie next to a singly-protected unit will increase until, at the first oxidation-limit,  $x_1/x_2 = 2.35$ , giving a value for the limit of 0.44 as previously shown.<sup>1,2</sup> It is therefore safe to conclude that, regardless of the degree of oxidation at which reduction with borohydride is carried out, the additional units oxidised in the second treatment with periodate will always be  $42 \pm 2\%$  of the total number of units in the original sample of alginate.

## EXPERIMENTAL

*Materials.* Sample of 5%, 10%, 20%, and 30% periodate-oxidised alginates were isolated as their sodium salts as described by Smidsrød and Painter.<sup>3</sup> Their intrinsic viscosities in 0.01 M sodium chloride at 20° were 8.10, 5.75, 4.15, and 3.95 dl/g, respectively. They were reduced by adding the freeze-dried solids directly to freshly-prepared, 20% w/v aqueous sodium borohydride at room temperature, and stirring until solution was complete (ca. 1 h). The solutions were then brought to pH 6 with acetic acid, dialysed exhaustively against water, centrifuged, and freeze-dried. The intrinsic viscosities of the products in 0.01 M sodium chloride at 20° were 5.90, 3.85, 3.30, and 2.03 dl/g, respectively.

*Periodate oxidation.* A solution of each sample (200 mg) in a mixture of 0.2 M sodium acetate-acetic acid buffer (pH 5.0, 40 ml) and 1-propanol (5 ml) was cooled to 0° in an ice-bath, and reaction was initiated by addition of 0.25 M sodium metaperiodate (5 ml), previously cooled to 0°. At intervals, portions (5 ml) of reaction mixture were added to a mixture of ice-cold, 0.5 M sodium phosphate buffer (pH 7.0, 25 ml) and aqueous potassium iodide (30% w/v, 4 ml), and the liberated iodine was rapidly titrated with 0.01 M sodium thiosulphate.

*Measurement of the rate of reduction of limit-oxidised alginate with sodium borohydride.* Portions (5 ml) of aqueous (4% w/v) limit-oxidised alginate<sup>1</sup> at 20° were mixed rapidly

with aqueous sodium borohydride (5 ml, variable concentration) at the same temperature. After the required interval of time, a few drops of 1-butanol were added to break foam, and then an amount of glacial acetic acid, calculated to bring the pH to about 6, was added quickly while shaking. After the effervescence had subsided, the volume of the mixture was made up to 25 ml with water, and the optical rotation was measured.

From the result, and the known specific rotations of the unreduced and fully reduced materials (+102° and -36°, respectively), the extent of reaction was calculated. With the large excess of borohydride, the reactions were approximately pseudo-first order, and the times of half-change were calculated.

## RESULTS

*Suppression of depolymerisation and overoxidation.* To avoid complications arising from end-group effects, it was essential to keep the molecular weights of the samples as high as possible during the initial oxidation with periodate<sup>3</sup> and the subsequent reduction with borohydride.

It was known from earlier work<sup>1</sup> that depolymerisation occurs during the initial oxidation with periodate, apparently by a free-radical mechanism which is mediated by impurities. By including 1-propanol in the reaction mixture to serve as a radical-scavenger, and by working with very pure alginate, it was possible to suppress depolymerisation and consequent end-group effects to such an extent that the first oxidation limit could be measured reproducibly with an accuracy of  $\pm 1$  %.

Propanol was therefore used in the initial preparation<sup>3</sup> of the partially-oxidised alginates for the present work, and it was also included in the second oxidation with periodate. However, poor reproducibility (about  $\pm 5$  %) was still obtained in the second oxidation, until it was recognised that considerable depolymerisation was also occurring during reduction with borohydride.

It was logical to assume that this kind of depolymerisation was occurring by the well-known  $\beta$ -alkoxycarbonyl elimination<sup>4</sup> under the alkaline conditions of the reduction. Although this elimination is general-base catalysed,<sup>5</sup> it seemed likely that hydroxyl ions would be the major catalytic species under the conditions of the reduction. Sodium borohydride is a buffer, and the pH of a freshly-prepared solution varies very little with concentration (see Table 2). On the other hand, the rate of reduction of an aldehyde or hemiacetal should

Table 2. Effect of the concentration of sodium borohydride upon the pH of an aqueous solution, and upon the rate of reduction of limit-oxidised alginate, expressed as the time of half-change ( $t_{1/2}$ ).

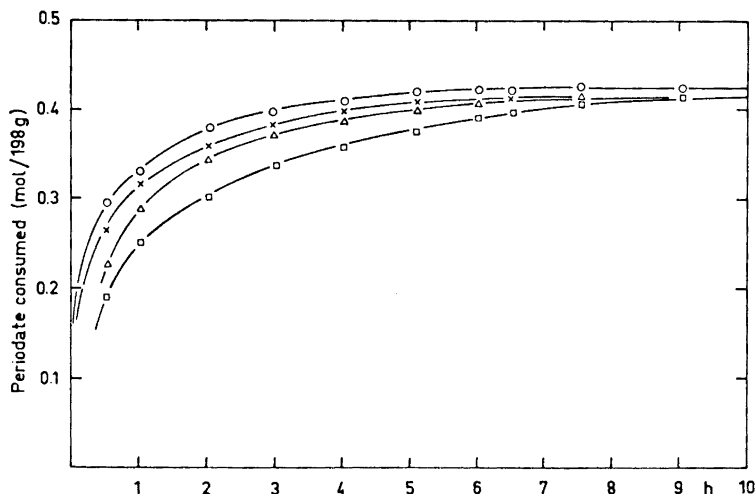
NaBH <sub>4</sub> (% w/v)	pH of an aqueous solution at the following times (in h) after preparation:					$t_{1/2}$ (min)
	0	1	2	3	4	
2	9.6	10.1	10.2	10.4	10.4	3.8
4	9.5	10.2	10.3	10.3	10.5	2.3
10	9.4	10.3	10.4	10.6	10.6	1.1
20	9.3	10.4	10.4	10.6	10.6	0.4

be directly proportional to the concentration of borohydride ions. It should therefore be possible to increase the rate of reduction relative to the rate of alkaline depolymerisation by increasing the concentration of sodium borohydride in the reaction mixture.

Pertinent experimental data are given in Table 2. The hydroxyl-ion activity apparently decreases slightly with increasing concentration of borohydride in freshly-prepared solutions, but increases as the solutions age, and the borohydride ions undergo hydrolysis to give borate ions. As expected, the rate of reduction of limit-oxidised alginate is approximately proportional to the concentration of borohydride. A freshly-prepared, concentrated solution of sodium borohydride was therefore indicated as the best means of minimising alkaline depolymerisation during reduction.

Confirmation of these ideas was provided by measurements of intrinsic viscosity. Thus, when the 5 % oxidised sample of alginate was reduced with 2 % w/v aqueous sodium borohydride as described in the earlier work,<sup>1</sup> its intrinsic viscosity in 0.01 M sodium chloride at 20° dropped from 8.10 to 1.35 dl/g, but when it was reduced with 20 % borohydride as described in the present paper, the intrinsic viscosity fell only to 5.90 dl/g. Similarly, 2 % borohydride decreased the intrinsic viscosity of 10 % oxidised alginate from 5.75 to 0.59 dl/g, while 20 % borohydride lowered it only to 3.85 dl/g.

*Measurement of the additional periodate consumed by the borohydride-reduced samples.* The second oxidation of each of the four samples was carried out at 0° to suppress overoxidation in the accepted manner. Good reproducibility was obtained in parallel experiments, and, as shown in Fig. 1, the reaction



*Fig. 1.* Oxidation at pH 5.0 and 0° in 25 mM sodium metaperiodate of 5 % (O), 10 % (x), 20 % (Δ), and 30 % (□) periodate-oxidised alginates, after they had been reduced with sodium borohydride. The consumption of periodate is expressed as mol per 198 g of material; that is, for every hexuronate residue present in the original sample of un-oxidised alginate.

in each case became very slow after the consumption of about 0.425 mol of periodate per 198 g of material. The different initial rates of oxidation are, of course, due to the different amounts of oxidisable hexuronic-acid residues initially present, and in accordance with this, the ratio of the initial slopes is about 95:90:80:70 for the 5 %, 10 %, 20 %, and 30 % oxidised samples, respectively.

#### DISCUSSION

The status of periodate oxidation as a tool in structural polysaccharide chemistry rests upon the assumption that it measures the total proportion of vicinal diol functions in the substrate. The recognition that the formation of inter-residue hemiacetals sometimes prevents it from doing this, led the authors, in an earlier paper,<sup>1</sup> to propose that reduction of the product obtained after the initial treatment with periodate, followed by a second oxidation step, should always be carried out to determine whether or not the true, Malapradian oxidation-limit has been reached. It was also suggested that this procedure should be repeated, until no further periodate is consumed.<sup>1</sup>

In principle, this proposal is sound, but if depolymerisation occurs during the oxidation steps, and again in the reduction steps, new end-groups will constantly be exposed, and the material will continue to consume periodate indefinitely.

Apart from its theoretical interest, the present project provided an opportunity of determining how accurately a second oxidation-limit can be measured. It is concluded that the use of 1-propanol to minimise free-radical depolymerisation in the oxidation steps, and concentrated aqueous sodium borohydride for the reduction steps, allows accurate results to be obtained. These conditions are now used routinely in this laboratory.

The authors are indebted to Kjersti Andresen for skilful technical assistance, to Dr. Olav Smidsrød for the measurements of intrinsic viscosity, and to Prof. N. A. Sørensen for his continued interest.

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Received February 14, 1973.