

## Formation of Hemiacetals between Neighbouring Hexuronic Acid Residues during the Periodate Oxidation of Alginate

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The anomalously low consumption of periodate by alginate (0.45–0.50 mol per anhydrohexuronic acid unit) was shown to be due to the formation of 6-membered hemiacetal rings between the aldehyde groups of oxidised hexuronic-acid residues and the closest hydroxyl groups of unoxidised residues, immediately preceding and following them in the chains.

Under ordinary analytical conditions, the spontaneous formation of these hemiacetals is fast compared to the rate of oxidation, and in sufficiently dilute periodate, the observed oxidation-limit can be explained quantitatively on the assumption of initially random oxidation, followed by instantaneous protection of the two nearest neighbours of the oxidised residues from subsequent oxidation.

Reductive cleavage of the hemiacetal rings at intermediate stages in the oxidation allows the consumption of oxidant to proceed up to 1.0 mol per anhydrohexuronic-acid unit, as expected for a 1,4-linked polyuronide.

Whereas methylation and other data<sup>1-3</sup> strongly indicate that alginate is an entirely 1,4-linked polyuronide, this material does not, under conditions that avoid overoxidation, consume more than 0.50–0.55 mol of periodate per anhydrohexuronic-acid unit.<sup>1</sup> Under conditions that allow overoxidation to occur, alginate can be induced to consume 1 mol or more of periodate, but then the molecule still contains intact D-mannuronic- and L-guluronic-acid residues, so that the additional consumption of oxidant can be attributed to the overoxidation reaction alone.<sup>1</sup>

This conflict between the results obtained by two classical methods of structural analysis, methylation and periodate oxidation, has aroused much discussion.<sup>1,4-8</sup> It prompted a careful re-examination of the methylation data, obtained with nine different samples of alginate from different algal species and different types of algal tissue, but in every case the results only confirmed the original formulation<sup>2</sup> of alginate as a uniformly 1,4-linked polyuronide.<sup>4</sup> It was pointed out<sup>1,4</sup> that the methylation data do not exclude the possible presence of 1,5-linked hexuronic-acid residues, and that, in their furanoid forms, both D-mannuronic and L-guluronic acids could readily form 3,6-

lactones which would be resistant to periodate. No evidence for the presence of such lactones was, however, obtained.<sup>1,4</sup> This is in agreement with experience in this laboratory, where no significant *O*-substitution of any kind has ever been detected in ordinary preparations of sodium alginate of marine algal origin. In one sample, it was estimated that less than 0.01 % of the carboxyl groups were esterified.<sup>9</sup>

Other ideas that have been put forward for discussion are that the partial resistance of alginate to periodate is due to some unusual effect of conformation in the macromolecule as a whole,<sup>4</sup> or in some of the monomeric units.<sup>7</sup> Two authors<sup>5,7</sup> have looked to the binary copolymeric nature of alginate for an explanation, and have proposed diversely that the L-guluronic-acid residues<sup>5</sup> or the D-mannuronic-acid residues<sup>7</sup> are, for some physical reason, unsusceptible to periodate. It has also been pointed out that, in solutions of low ionic strength, the Donnan effect should depress the rate of oxidation of anionic polysaccharides in general, but this was not held to account for the well-defined oxidation-limit shown by alginate, because this limit was also observed in solutions of high ionic strength.<sup>6</sup>

In this laboratory, it was desired to make extensive use of periodate oxidation for the study of other hexuronic-acid containing polysaccharides and glycoproteins from both benthic<sup>10</sup> and planktonic algae, and it was necessary to ascertain to what extent it would be safe to do so. A study of the literature gave some reason to believe that alginate was not unique in its anomalous behaviour with periodate. There is evidence<sup>5,11</sup> that pectates react incompletely with periodate<sup>5</sup> and lead tetra-acetate,<sup>11</sup> and that the consumption of oxidant increases with increasing reduction of the carboxyl groups at C(6) to hydroxymethyl groups.<sup>11</sup> Wolfrom and Juliano<sup>12</sup> noted that the oxidation of 3-*O*- $\beta$ -D-glucuronamidopyranosyl-2-acetamido-2-deoxy-D-galactitol was anomalous, and Toro-Feliciano<sup>13</sup> had suggested that this was due to the formation of a stable hemiacetal ring between the two moieties of a partially oxidised intermediate. Moreover, while the present work was still in progress, Sloneker *et al.* reported a clear anomaly in the periodate oxidation of a bacterial polysaccharide containing residues of D-mannuronic acid, and suggested the formation of hemiacetals between adjacent sugar residues in the chains as an explanation.<sup>14</sup>

The present work establishes that the anomaly in the case of alginate is caused by intramolecular, inter-residue hemiacetal formation similar to that previously envisaged by Toro-Feliciano<sup>13</sup> and Sloneker *et al.*,<sup>14</sup> proposes a kinetic mechanism that accounts quantitatively for the anomalous oxidation-limit that is observed under typical analytical conditions, and shows how the problem can be surmounted, permitting the valid application of periodate oxidation to hexuronic acid-containing polysaccharides. A preliminary communication describing some of the results has been published.\*

\* Attention is drawn to two errors in the preliminary communication.<sup>15</sup> The stereochemistry in the Figure is erroneous, and the theoretical oxidation-limit for long chains is 0.435 mol, not 0.48 mol as stated. The errors are corrected in Fig. 8 and Table 1 of this paper.

## THEORY

The experimental results showed that the aldehyde groups of oxidised hexuronic-acid residues form hemiacetals with the hydroxyl groups of unoxidised residues in the same chain. Since the data were consistent only with hemiacetal formation between nearest neighbours, there are two possibilities, namely, that an oxidised residue can protect both its nearest neighbours from subsequent oxidation, or that it can protect only one of them. The present calculations were carried out to test these two possibilities for their capacity to account quantitatively for the observed oxidation-limit.

The simplest possible assumptions in each case are that oxidation initially proceeds in a random manner, that only one monomeric unit in a given chain is oxidised at a time, and that protection of either one or both neighbouring units ensues immediately, before the next oxidative attack on the chain occurs. This kind of mechanism has parallels in other processes, such as the  $\alpha$ -amylolysis of amylose,<sup>16</sup> and indeed the theoretical oxidation-limit for two-sided protection in a very long chain (0.435 mol) is identical with the limiting degree of scission in the first stage of  $\alpha$ -amylolysis, namely, that giving rise to maltose and maltotriose.<sup>16,17</sup>

For the present work, it was convenient to adopt a synthetic method of calculation, consisting in a direct simulation of the assumed process in the digital computer. An analytical solution has also been arrived at and will be published elsewhere.

A polymer chain of  $N$  monomer units was represented by a unidimensional array of  $N$  integers. A procedure for generating pseudo-random numbers of a rectangular density-distribution<sup>18</sup> was adapted to supply random integers in the interval from unity to  $N$  inclusive. If the first random number to be supplied was for example, 15, then the number 2 was inserted in the fifteenth position in the array. For protection of both nearest neighbours, the number 1 was then inserted in both the fourteenth and sixteenth positions, while for protection of only one neighbour, a 1 was placed in the fourteenth position only.

If the next random number to be supplied corresponded to a position in the array occupied by a 1 or a 2, then it was rejected. If the next random number was acceptable, then a 2 was placed in the appropriate position of the array, together with a 1 in the appropriate neighbouring position or positions, where this or these were not already occupied by a 1 or a 2. This process was continued until the array was filled with 1's and 2's, whereupon the number of 2's was counted, divided by  $N$ , and the result was stored. The array was then emptied, and the process repeated. The resultant degree of oxidation was then averaged with that obtained for the first chain, and the variance about the mean was also calculated. Further chains were then generated and "oxidised" in the same way until the probable error in the mean degree of oxidation was of the order of  $10^{-3}$ . The results for both one- and two-sided protection are shown in Table 1.

Table 1. Mean degree of oxidation (DEGO) as a function of the degree of polymerisation ( $N$ ), for protection of either one or both nearest neighbours. ( $\sigma$ ) is the standard deviation in the mean, and the number of chains scanned in each case is approximately 10000/ $N$ .

$N$	One neighbour protected		Both neighbours protected	
	DEGO	( $\sigma$ ) $\times 10^3$	DEGO	( $\sigma$ ) $\times 10^3$
3	0.721	2.74	0.552	3.52
4	0.701	2.72	0.500	0.00
5	0.689	3.26	0.492	2.58
6	0.676	2.93	0.481	1.69
10	0.660	2.79	0.464	2.15
15	0.645	2.73	0.452	2.04
18	0.649	2.54	0.447	2.41
50	0.636	2.82	0.442	2.08
500	0.633	2.54	0.434	1.04
1000	0.631	2.94 <sup>a</sup>	0.432	1.72 <sup>a</sup>
5000	0.634	1.42 <sup>a</sup>	0.429	1.60 <sup>a</sup>

<sup>a</sup> These figures are not reliable because an insufficient number of chains was sampled.

## EXPERIMENTAL

*Materials and general methods.* Sodium alginate was prepared in the laboratory<sup>19</sup> from *Laminaria digitata*, this alga being chosen to secure minimum contamination with phenolic material and other polysaccharides. After purification by high-speed centrifugation of an aqueous solution, followed by graded precipitation with ethanol, a perfectly white product showing negligible absorbance in the ultraviolet was obtained. It had an intrinsic viscosity of 12.4 dl/g in 0.1 M sodium chloride at 20°, and contained residues of mannuronic and guluronic acids in the ratio<sup>20</sup> of 1.6:1.

Fragments (A, B, and C) of alginate, all having a number-average degree of polymerisation of  $20 \pm 1$ , and containing 65 %, 92 %, and 13 % of mannuronic acid, respectively, were prepared as described by Haug *et al.*<sup>21</sup> Methyl alginate was prepared by treatment of alginic acid with diazomethane,<sup>22</sup> and alginate was acetylated by the method of Schweiger.<sup>23</sup>

Hexuronic acid was determined by a modification<sup>24</sup> of the Dische carbazole method, and total carbohydrate by the phenol method.<sup>25</sup> Methoxyl was determined by a modified procedure<sup>26</sup> that gives accurate results with hemiacetal as well as ether methoxyl. Because of the difficulty in drying sodium alginate and its fragments (B and C), their concentration in solution was determined by titration with cetylpyridinium chloride.<sup>27</sup> The cetylpyridinium chloride was standardised by volumetric assay of the chloride ion (Volhard titration). The equivalent weight of alginate during periodate oxidation was also determined by titration with cetylpyridinium chloride, after the necessary<sup>6</sup> reduction of excess periodate with ethylene glycol.

Gel-permeation chromatography was carried out on a column (2.6  $\times$  170 cm) of Sephadex G-50, with 250 mg samples of material and 0.1 M sodium sulphate as the eluent; fractions (2 ml) were collected at the rate of 5 per hour, and analysed for total carbohydrate after appropriate dilution with 0.1 M sodium sulphate. Paper chromatography was carried out with Schleicher and Schüll No. 2043 paper, irrigated in the ascending direction with pyridine-ethyl acetate-acetic acid-water (5:5:1:3 v/v); the spots were located with the aniline hydrogen phthalate reagent.<sup>28</sup> Thin-layer chromatography of phenylosazones was carried out on strips of Eastman-Kodak silica gel-coated plastic, with ethyl acetate-acetic acid-water (2:1:3 v/v, freshly mixed, organic layer).

Unoxidised sugar residues in periodate-oxidised polysaccharides were identified by reduction of the sample with aqueous sodium borohydride,<sup>29</sup> followed by hydrolysis for 12 h at 100° in 0.2 N sulphuric acid, neutralisation with barium carbonate, filtration, concentration of the filtrate, and paper chromatography.

Schiff's reagent was purchased in a form ready for use from Merck AG, Darmstadt, Germany. The buffers were either sodium acetate—acetic acid (0.2 M) or sodium hydrogen phosphate (0.1 M). Solutions were concentrated on a rotary evaporator under diminished pressure at 30°. Optical rotations were measured at 24°. Infra-red absorption spectra were recorded on a Perkin-Elmer Model 257 Grating Spectrophotometer.

*Preparation of partially carboxyl-reduced alginates.* Samples (1 g) of sodium alginate were dispersed in water (20 ml) and hydrochloric acid (0.5 N; 20 ml) was added. The precipitated alginic acid was collected by vacuum filtration and pressed free from excess of the acidic supernatant. Three such samples of alginic acid, obtained as hydrated gels, were suspended separately in propylene oxide (20 ml) and kept at room temperature for 4, 9, and 24 h, respectively. At the ends of these separate periods, the mixtures were triturated with an excess of ethanol—diethyl ether (2:1 v/v), and the residues were collected by filtration, washed with the same mixture of solvents, and dried. They were then dissolved separately in water (20 ml), and potassium borohydride (0.5 g) was added to each solution. After 5 h at room temperature, the solutions were acidified and dialysed, first against 0.1 N hydrochloric acid, and then against water. The precipitated products (a, b, and c) were dehydrated by trituration with ethanol, then collected by filtration, washed with ethanol—diethyl ether, and dried to constant weight *in vacuo*. Their equivalent weights, determined by dissolution of weighed samples in excess of standard alkali, followed by back-titration, were 286, 400 and 574 respectively.

*Preparation of partially acetylated alginates.* This was accomplished by controlled, partial saponification of a sample of acetylated alginate<sup>23</sup> as follows. A sample of acetylated alginate (sodium salt; 104 mg) was dissolved in water (10 ml). Aqueous sodium hydroxide (0.0975 N; 10 ml) was added, and the mixture was kept 30 min at room temperature. Acetone (25 ml) was then added, and the mixture was shaken for 10 min, and then titrated with 0.1 N oxalic acid, with phenolphthalein as the indicator. The titre (4.05 ml) corresponded to a degree of acetylation of 1.41 in the starting material. Four further portions (104 mg) of the same material were then treated, respectively, with 3.5, 4.0, 4.5, and 5.0 ml of 0.0975 N sodium hydroxide, and the solutions were kept 12 h, after which the pH of each had fallen to 7. The four partially saponified samples, having calculated degrees of substitution of 0.453, 0.346, 0.238 and 0.135, respectively, were isolated by precipitation with acetone in the usual way.

*Analytical oxidations.* These were normally carried out in standard volumetric flasks, covered with aluminium foil, and kept at  $20.6 \pm 0.2^\circ$  in a thermostatic water-bath. The same stock solution (0.8 % w/v) of sodium alginate was used for most of the experiments. In a typical experiment, 50 ml of this solution was mixed with 40 ml of the required aqueous salt or buffer solution, and reaction was initiated by addition of freshly prepared, 0.25 M sodium metaperiodate (10 ml). Samples (5 ml) were withdrawn at intervals, and pipetted rapidly into a mixture of phosphate buffer (0.5 M; pH 7.0; 5 ml) and freshly prepared aqueous potassium iodide (30 % w/v; 1 ml). The liberated iodine was then titrated with 0.01 M sodium thiosulphate, using starch as the indicator. Where no additional salt or buffer was used in the reaction mixture, aqueous propan-1-ol (25 % v/v) was used as the diluent. Variations in the various quantities were made as indicated in the text and figures.

*Preparation of limit-oxidised alginate.* Sodium alginate (8 g) was dissolved in water (1.3 l) and mixed with propan-1-ol (200 ml) and aqueous sodium metaperiodate (2 % w/v; 500 ml). After 24 h in the dark at room temperature, the mixture was treated with ethylene glycol (10 ml). Sodium chloride (40 g) was then dissolved in it, and it was poured with stirring into ethanol (4 l). The precipitate was collected by filtration, dissolved in water (400 ml), and dialysed against distilled water until a sample of the dialysate gave no colour with acidified starch—potassium iodide solution. Sodium chloride (10 g) was then added to the solution of non-diffusible material, which was then poured with stirring into ethanol (1 l). The precipitate was collected by filtration, washed well with methanol, and dried at room temperature in the vacuum desiccator. The yield was 7.2 g.

*Reduction of limit-oxidised alginate.* Sodium borohydride (1 g) was added to a solution of the material (1 g) in water (50 ml), and after 1 h, glacial acetic acid was added dropwise until the pH came to 6. The solution was then dialysed thoroughly against distilled water, and the product was isolated by freeze-drying. The yield of highly hygroscopic polymer was essentially quantitative.

*Further oxidation and reduction of reduced, limit-oxidised alginate.* These were carried out essentially as in the first oxidation and reduction, the amount of oxidant being diminished proportionately with the amount of unoxidised residues remaining, in order to minimise non-specific oxidation. The products were isolated by freeze-drying of the solutions after exhaustive dialysis against water, and the yields throughout were almost quantitative.

*Methylation of limit-oxidised alginate.* Addition of aqueous silver nitrate (4 % w/v; 50 ml) to a solution of the title substance (2.1 g) in water (50 ml) precipitated the silver salt, which was isolated, washed with ethanol and ether, and dried, to yield 3.11 g of pale yellow solid. This was suspended in a mixture of freshly distilled dimethylformamide (25 ml) and methyl iodide (10 ml), and dry, freshly precipitated silver oxide (5 g) was added portionwise with simultaneous cooling. This mixture was mechanically shaken for 24 h at room temperature in a sealed flask covered with aluminium foil. Further portions of methyl iodide (10 ml) and silver oxide (5 g) were then added, and shaking was continued for 48 h. The mixture was then filtered, and the silver residue was washed with chloroform. The washings were combined with the filtrate, and this mixture was diluted to 300 ml with chloroform, and kept overnight in the refrigerator. The silver salt that separated was removed by filtration, and the filtrate was washed with aqueous sodium cyanide (5 % w/v; 200 ml), followed by cold 0.01 N sulphuric acid, and then by water. The organic layer was dried over sodium sulphate, after which evaporation of the solvent afforded 1.2 g of pale yellow syrup. This was further methylated with methyl iodide (10 ml) and silver oxide (5 g) in the dark for 7 days at room temperature. The product (1.2 g) had  $[\alpha]_D = +75^\circ$  (c. 3.0 in chloroform), and in the infra-red it showed no absorption in the hydroxyl region, but a strong carbonyl-stretching peak at  $1750\text{ cm}^{-1}$ . (Found:  $\text{OCH}_3$ , 31.3; saponification number, 206. Calc. for  $[(\text{C}_5\text{H}_5\text{O}_2)(\text{CO}_2\text{CH}_3)(\text{OCH}_3)_2]_{0.55} [(\text{C}_5\text{H}_5\text{O}_4)(\text{CO}_2\text{CH}_3)]_{0.45}$ :  $\text{OCH}_3$ , 32.0; saponification number, 204.6).

A portion (0.5 g) of the product was dissolved in 90 % (w/w) aqueous formic acid (6 ml) and heated under reflux at  $100^\circ$  for 12 h. The solution was then concentrated to dryness, and the residue was heated with water (10 ml) at  $100^\circ$  for 15 min. This solution was then evaporated to dryness, and the residue was twice more heated with water with intermediate evaporation in the same way. It was then heated in 0.5 N sulphuric acid at  $100^\circ$  for 12 h. The resultant mixture was neutralised with barium carbonate and filtered. The filtrate and washings were concentrated to yield a syrup (250 mg), paper chromatography of which indicated the presence of mainly mannuronic acid, a possible trace of guluronic acid, mannuronolactone, and a streak of slow-moving, polymeric material. Removal of barium ions by treatment of an aqueous solution with Dowex-50 ( $\text{H}^+$  form) cation-exchange resin afforded an acidic syrup suitable for analysis. [Found:  $\text{OCH}_3$ , 3.9. Calc. (for theory, see text):  $\text{OCH}_3$ , 3.86].

A further portion (0.5 g) of the methylated product was dissolved in aqueous acetone (50 % v/v; 50 ml) and saponified by addition of a calculated amount of 2 N sodium hydroxide. After 2 h at room temperature, the pH reached 7, and the acetone was removed by distillation. The resultant aqueous solution did not reduce Fehling's solution, gave no colour with Schiff's reagent, and formed no precipitate upon addition of phenylhydrazine. Evaporation of the water yielded a yellow syrup, absorbing in the infra-red at 1410 and  $1610\text{ cm}^{-1}$ , but showing no significant peak at  $1750\text{ cm}^{-1}$ . (Found:  $\text{OCH}_3$ , 16.0. Calc. for  $[(\text{C}_5\text{H}_5\text{O}_4\text{Na})(\text{OCH}_3)_2]_{0.55} [(\text{C}_5\text{H}_5\text{O}_6\text{Na})]_{0.45}$ :  $\text{OCH}_3$ , 16.19).

## RESULTS

*Relevance of the copolymeric nature of alginate to the anomalous oxidation-limit.* It has been shown<sup>21,30</sup> that alginates are block copolymers, in which long sequences of contiguous D-mannuronic-acid residues and long sequences of contiguous L-guluronic-acid residues are joined through long sequences in which the two monomers are arranged in a largely alternating fashion.

Ideas<sup>5,7</sup> that the anomalous oxidation-limit is due to a large difference in the rates of oxidation of the two types of unit have hitherto been largely speculative, because of a lack of suitable model substances for test.

It was possible in the present work to obtain a definitive answer to this question, because previous work<sup>21</sup> has made available three fragments (A, B, and C) of alginate, which are much enriched, respectively, in the alternating sequences, the polymannuronic-acid sequences, and the polyguluronic-acid sequences.

Fig. 1. Oxidation at 20° of oligomannuronide (O) and oligogulonide (×) in (a) 5 mM NaIO<sub>4</sub> in 40 mM MgCl<sub>2</sub> and (b) 5 mM NaIO<sub>4</sub> in water. The substrate concentration was 5 mM with respect to anhydrohexuronic acid.

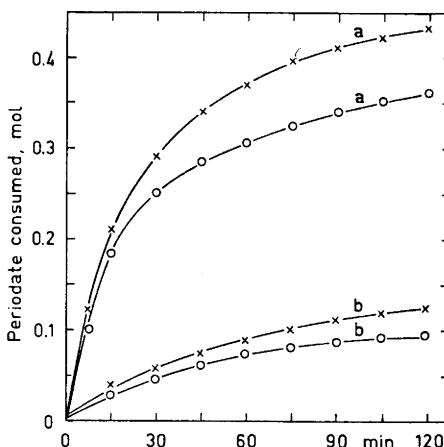


Fig. 1 shows the initial stages of the oxidation of fragments B and C in 5 mM periodate in both the presence and absence of 40 mM magnesium chloride. In both cases, fragment C was initially oxidised about 50 % faster than fragment B. The large effect of the magnesium ions upon the absolute initial rates was clearly due to their capacity to diminish the electrostatic repulsion between the periodate ions and the alginate polyanion, as pointed out by Scott and Harbinson.<sup>6</sup> It is noteworthy, however, that the relative rates of oxidation are independent of the ionic strength of the solution.

This rather small difference in the rates of oxidation of the two monomeric units is clearly incompatible with suggestions<sup>5,7</sup> that such differences could account for the well-defined oxidation-limit of alginate.

With reference to the kinetic model discussed in the theoretical section, it is evident without elaborate calculations that this small departure from the assumption of random attack will have very little effect upon the calculated oxidation-limits in Table 1. Within each long, homopolymeric segment of the polymer chain, the attack should be initially random, and the limit should be the same for both polymannuronic and polyguluronic segments. For the alternating segments, the limiting degree of oxidation should tend to 0.5 as the ratio of the rates tends to infinity.

The alternating segments represent about one third of the total molecule, and therefore, for long chains, the maximum possible effect of non-random attack would be to increase the limit from about 0.44 to 0.46 for two-sided protection, and to decrease it from 0.63 to 0.59 for one-sided protection. Since the ratio of the rates is only 1.5, any effect will clearly be negligibly small.

*Avoidance of overoxidation and depolymerisation during oxidation.* In their original investigation, Drummond, Hirst and Percival<sup>1</sup> noted rapid overoxidation of alginate at 25°, and avoided it in the established manner<sup>31</sup> by working at 2°. In the present work, it was noticed that oxidation was usually accompanied by extensive depolymerisation of the chains. The extent of the depolymerisation varied considerably from sample to sample, as indicated by variations in the intrinsic viscosity of the limit-oxidised product. Depolymerisation appeared to be associated with the presence of condensed phenolic, and possibly other, impurities, as judged by the colour of the sample and its turbidity in solution.

These observations suggested that the depolymerisation was of the free-radical type which has previously been investigated in this laboratory<sup>32,33</sup> and shown to occur when the phenolic impurities undergo autoxidation in air. Periodate is known to react with phenols very readily, giving rise to free radicals.<sup>34</sup> It has been shown<sup>32,33</sup> that certain buffer salts and aliphatic alcohols, by acting as radical scavengers, inhibit this kind of depolymerisation. Fig. 2 shows the oxidation at 20° of an ordinary, cream-coloured sample of *Laminaria digitata* alginate, in the presence and absence of propanol. The limit-oxidised product was isolated in each case, and Dr. Smidsrød kindly measured their intrinsic viscosities in 0.01 M sodium chloride at 20°. The product obtained by oxidation in the presence of propanol had an intrinsic viscosity of 4.0 dl/g, whereas that obtained without the use of propanol had an intrinsic viscosity of 0.17 dl/g.

It was concluded that the high rate of overoxidation of alginate at 20° (cf. Drummond *et al.*, Ref. 1) was due to the depolymerisation, which greatly increased the number of end-groups at which overoxidation could occur. Accurate results could therefore be obtained even at 20°, by inclusion of propanol in the reaction mixture.

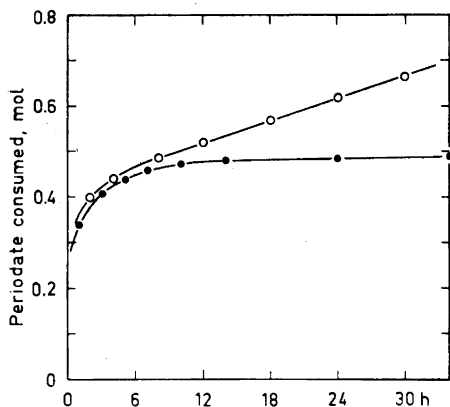


Fig. 2. Oxidation at 20° of *Laminaria digitata* alginate in 0.025 M NaIO<sub>4</sub> in the presence (●) and absence (○) of propanol.

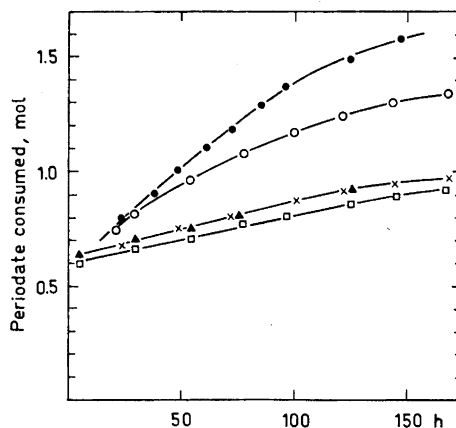


Fig. 3. Oxidation at 2° of oligoguluronide in 0.01 M NaIO<sub>4</sub>, buffered to: pH 3.5 (●); pH 4.3 (▲); pH 5.0 (×); pH 5.9 (□); and pH 6.8 (○).



As could be expected, this device did not prevent overoxidation of fragments A, B, and C, which had a low number-average degree of polymerisation (about 20) at the outset, and it was therefore necessary to work at 2° in investigating the oxidation-limits of these materials. Even at that temperature, overoxidation of the fragments was quite rapid, and it was also necessary to investigate the pH-dependence of the overoxidation in order to obtain good results. Those obtained with the guluronic acid-rich fragment (C) are shown in Fig. 3, and demonstrate that overoxidation is minimal in the pH range from 4.3 to 5.9. This optimum pH is significantly higher than that (pH 3.6) reported<sup>35,36</sup> for neutral sugars (*cf.* Meyer and Rathgeb, Ref. 37).

Fig. 4 shows the oxidation of fragments A, B, and C at pH 5 and 2°, and it is evident that all three show a clear oxidation-limit of 0.55 mol of oxidant per anhydrohexuronic-acid unit. Scott<sup>7</sup> reported that an oligoguluronide similar to fragment C showed no oxidation-limit, and proposed accordingly that it is the mannuronic-acid residues in alginate that are, for conformational reasons, resistant to periodate. The conditions of the experiment were not stated, but Fig. 3 shows that, if the oxidation were carried out at, for example, pH 3.5 or 6.8, the existence of the oxidation-limit could be easily overlooked.

*Position of the oxidation-limit.* Except at extremes of pH, at which alginate is insoluble (low pH) or at which glycol cleavage is impaired (high pH), most reported values<sup>1,5</sup> for the oxidation-limit of high molecular-weight alginates vary between 0.49 and 0.55 mol of periodate per anhydrohexuronic-acid unit. As indicated in the theoretical section, particular attention was paid to the exact position of the oxidation-limit, in the belief that this would provide a clue to the protective mechanism.

The presence of phenolic and other impurities in most ordinary samples of alginate is important in this connection, firstly because they can be expected to consume periodate themselves, and secondly because they can cause depolymerisation. If the depolymerisation is severe, the additional two moles of periodate consumed by the end-groups may significantly increase the measured oxidation-limit.

The theoretical figures in Table 1 are not corrected for the additional consumption of oxidant by the end-groups, but in fragments A, B, and C, for example, the limit would, on the basis of simple Malapradian oxidation only, increase from 0.45 mol (9 oxidised residues in a chain of 20) to 0.55 mol (11 oxidised sites per molecule).

Even when overoxidation appears to have been avoided, an additional increase in the consumption of periodate can be expected when depolymerisation occurs. This could arise from the special kind of overoxidation that, in polyuronides, takes place at the non-reducing terminal unit.<sup>38-41</sup> In this reaction, Malapradian cleavage between C(3) and C(4) is believed to be followed by rapid, non-Malapradian hydroxylation at C(5), and the rate-controlling step for the subsequent peeling is thought to be the oxidation of the resultant hydroxy-aldehyde. Thus, methyl (methyl  $\alpha$ -D-galactopyranosid)uronate showed a break in the reaction curve after consumption of three moles of periodate, instead of the expected two.<sup>41</sup> For other model compounds, the consumption of periodate in excess of that expected for Malapradian oxidation was rather

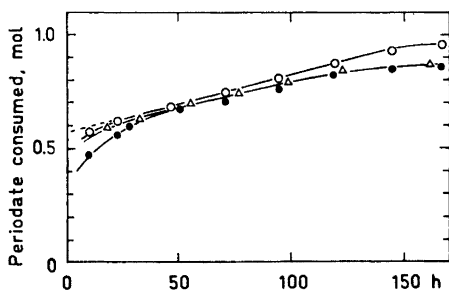


Fig. 4. Oxidation at 2° and pH 5.0 of oligomannuronide (●); oligoguluronide (○); and oligouronide containing alternating sequences of both monomers (△). The periodate concentration was 0.01 M.

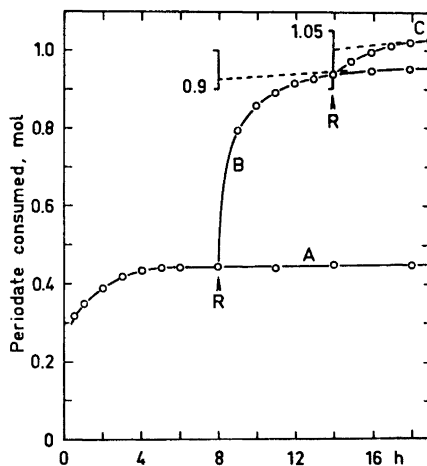


Fig. 5. Oxidation at 20° of highly purified *Laminaria digitata* alginate in 0.025 M NaIO<sub>4</sub> containing propanol. At the points labelled R, samples were reduced with sodium borohydride and then oxidised further (curves B and C).

less than 1 mol,<sup>41</sup> and hence the oxidation-limit for a fragment of 20 units should fall between 0.55 and 0.60 mol (*cf.* Figs. 3 and 4).

Fig. 5 (Curve A) shows the oxidation at 20° of a highly purified sample of alginate in the presence of propanol. The amount of alginate present in the reaction mixture was carefully controlled by titration with cetylpyridinium chloride,<sup>27</sup> and on the basis of this and many similar experiments, it was concluded that the correct oxidation-limit for high-polymeric alginate, oxidised in dilute periodate and in solutions of low ionic strength, is  $0.45 \pm 0.02$  mol.

At intervals throughout the reaction (Fig. 5, curve A), the equivalent weight of the substrate was measured by titration with cetylpyridinium chloride,<sup>6,27</sup> and was found to remain constant at the theoretical value for intact alginate (*ca.* 200). This constancy in titration was additionally useful in showing that there had been no significant peeling of the chains, since monomeric material is not determined by the method. At the end of the reaction, the polymeric product was isolated in almost quantitative yield after exhaustive dialysis against water. It was also totally excluded from a column of Sephadex G-50, and on the basis of experience with unoxidised fragments of alginate of known molecular weight, it was roughly estimated that it contained no chains less than 100 units in length. The material gave no reaction with Schiff's reagent, even when kept in the presence of the reagent in a sealed tube at room temperature for 24 h.

*The second and third oxidation-limits.* The limit-oxidised alginate can be regarded as composed of an average repeating-unit of five unoxidised hexuronic-acid residues and four oxidised ones. On the assumption of two-sided

protection, there would be no contiguous oxidised residues, and the unoxidised residues would be grouped, on average, into three isolated ones and one pair of contiguous ones. If the source of protection were now removed, the three isolated, unoxidised residues should become fully oxidisable, but only one in every group of two should be oxidisable, because, once oxidised, this would protect the second residue. Thus, out of the original repeating unit of nine residues altogether, only one would remain, giving a second oxidation-limit of 0.88 mol.

Following reduction with borohydride under the conditions stated in the experimental section, the limit-oxidised material still had an equivalent weight of 200, and was recovered in quantitative yield after exhaustive dialysis against water. It was also still completely excluded by a column of Sephadex G-50. Fig. 5 (curve B) shows the consumption of periodate by the reduced material in the presence of propanol. The second oxidation limit thus obtained ( $0.92 \pm 0.02$  mol) was less well-defined than the first, but this was probably not due to overoxidation, because the titre with cetylpyridinium chloride again remained constant throughout, and the polymeric product was again isolated in almost quantitative yield after exhaustive dialysis against water. It is therefore possible that the protection giving rise to this limit was a little less complete than that responsible for the first. On the other hand, the product still gave no detectable colour with Schiff's reagent.

Removal of any remaining protection should then permit oxidation to a third limit, corresponding to complete oxidation of every residue. Fig. 5 (curve C) shows the consumption of periodate by the reduced material obtained after oxidation to the second limit. The apparent sequence of events throughout the three phases of the oxidation is illustrated in Fig. 6.

Pre-treatment of intact alginate with sodium borohydride prior to periodate oxidation had no effect upon the position of the first oxidation-limit.

*Evidence for hemiacetal formation from changes in optical rotation.* During periodate oxidation, two asymmetric carbon atoms are lost for every hex-

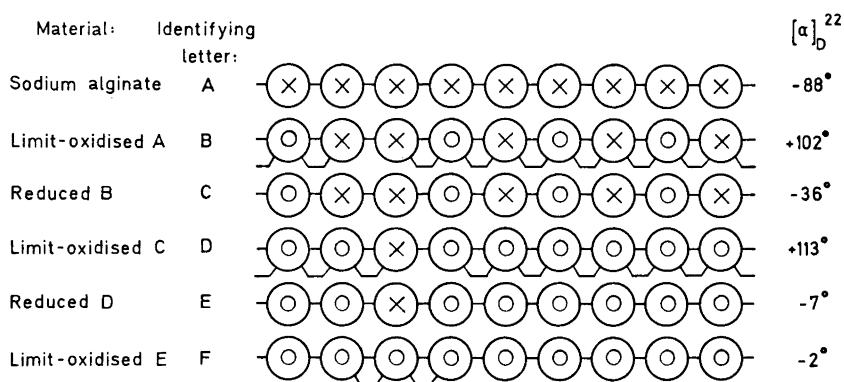


Fig. 6. Sequential periodate oxidation and borohydride reduction of alginate. X represents an unoxidised unit and O an oxidised unit. The straight adjoining lines represent glycosidic linkages and the bent ones represent hemiacetal rings.

uronic-acid residue attacked, and therefore the optical rotation must change during the three oxidative steps, regardless of whether or not hemiacetals are simultaneously formed. If, however, the resultant aldehydic groups, once formed, remained free, reduction with borohydride would convert them into primary alcohols without any concomitant change in the number of asymmetric centres. The changes in optical rotation brought about by reduction at both the first and second oxidation-limits were, in fact, not only large, but were also from positive to negative values, as shown in Fig. 6.

These changes were not caused by epimerisation or depolymerisation under the mildly alkaline conditions of borohydride reduction, because aqueous sodium tetraborate caused no significant change in optical rotation at the same temperature, and over the relevant time-period. The borohydride ion itself is not known to cause epimerisation, and so there must have been a change in the number of asymmetric centres upon reduction. The carboxyl groups were freely titratable throughout, and hence these asymmetric centres must have been present in hemiacetal or hemialdal<sup>42</sup> structures.

*Evidence for the formation of hemiacetals in preference to hemialdals.* The failure of limit-oxidised alginate to give a colour with Schiff's reagent suggests the absence of free aldehydic groups. This implies that the aldehyde groups must either be linked to hydroxyl groups, to form hemiacetals, or that one aldehyde group combines with the hydrated form of another, to give a hemialdal structure.<sup>42</sup>

To investigate this, four different samples of partially acetylated alginate were prepared, which contained one *O*-acetyl group for every two, three, four, and seven hexuronic-acid residues, respectively. After oxidation to their limits, they had consumed 0.25, 0.30, 0.40, and 0.43 mol of periodate per anhydrohexuronic-acid residue, respectively. Aqueous solutions (0.5 %) of these limit-oxidised materials gave in every case a strong reaction with Schiff's reagent, the colour reaching maximum intensity after about 15 min at room temperature in the usual way.

The limit-oxidised products were then saponified by addition of a calculated amount (see experimental section) of dilute sodium hydroxide; they were then again tested with Schiff's reagent, and gave no detectable reaction within 6 h at room temperature. Addition of an excess of alkali was avoided, because this caused depolymerisation of the chains, with liberation of free aldehydic groups.

It was concluded that hemiacetals were formed in preference to hemialdals, and that any hemialdals formed when a hydroxyl group is not available for hemiacetal formation are unstable.

*Evidence for hemiacetal formation from methylation data.* Methylation of alginate after oxidation to its first limit was accomplished with methyl iodide and silver oxide in dimethylformamide,<sup>43</sup> and proceeded with remarkable ease. The yield of chloroform-soluble product (about 50 %) was a little low, probably because the methyl ester groups at C(6) were partly saponified during washing with aqueous sodium cyanide, leading to extraction of some of the polysaccharidic material into the aqueous phase.

After further methylation with Purdie's reagents, the product, isolated as the chloroform-soluble methyl ester, gave no hydroxyl band in the infrared.

After saponification, it was soluble in water and could be titrated with cetylpyridinium chloride. It contained no free aldehydic groups, as indicated by the absence of a band at  $1730\text{ cm}^{-1}$  in the infra-red, and negative reactions with Fehling's solution, Schiff's reagent, and phenylhydrazine.

Its methoxyl content corresponded to the introduction of one methoxyl group for every secondary, alcoholic hydroxyl group in the remaining 55 % of unoxidised hexuronic-acid residues. It was hydrolysed with acid under conditions<sup>44</sup> that are recommended for the hydrolysis of methylated polysaccharides, and that are known to cause very little demethylation of alcoholic hydroxyl groups. The hydrolysate consisted overwhelmingly of unsubstituted mannuronic acid. Although some inaccuracy could be expected to arise from decomposition (dehydration and decarboxylation) of hexuronic acid during hydrolysis, and the gain in weight due to the addition of the elements of water for every glycosidic linkage cleaved, the methoxyl content of the hydrolysate was almost exactly one-fifth of that of the starting material (not counting ester methoxyl). Thus, four-fifths of the methoxyl in the starting material was hemiacetal methoxyl, the remainder ether methoxyl.

It was concluded that, for every aldehydic group formed upon oxidation of alginate, one secondary hydroxyl group is blocked, and is replaced by a hemiacetal hydroxyl group. In the approximate repeating unit of five unoxidised and four oxidised residues, therefore, eight aldehydic groups have reacted with eight secondary hydroxyl groups to give eight hemiacetal hydroxyl groups, and there are two alcoholic hydroxyl groups remaining.

The foregoing results are complemented by those of Rees and Samuel,<sup>4</sup> who methylated limit-oxidised alginate after it had been reduced with borohydride; hydrolysis of the product in that case led to the recovery of the unoxidised hexuronic-acid residues, after reduction at C(6) to facilitate identification, as the acid-stable 2,3-di-*O*-methyl ethers of D-mannose and L-gulose.

*Evidence that the hemiacetal formation is intramolecular.* Alginate showed essentially the same first oxidation-limit when its concentration in the reaction mixture was varied from 1 mM to 80 mM. The limit was also unchanged when alginate was oxidised in the presence of amylose or guaran, the final consumption in each case being the sum of the separately measured consumptions of the two components. If there were any intermolecular cross-linking, oxidation of alginate should cause it to separate out from solution as a gel, but this does not occur.

Whereas the oxidation-limits of physical mixtures of alginate and amylose are strictly additive, with the alginate exhibiting its usual uptake, and the anhydrohexose units in the amylose each consuming the expected 1 mole of periodate, the behaviour was very different when the hexuronic-acid and hexose residues were present in the same chains. Fig. 7 shows the oxidation of three partially carboxyl-reduced alginates (*a*, *b*, and *c*), containing 40, 58, and 71 mol % of hexose (mannose and gulose) residues, respectively.

With these materials, there was apparently a slight overoxidation, which was not prevented by addition of propanol, and this may have been due to prior depolymerisation, by the  $\beta$ -alkoxycarbonyl elimination,<sup>45</sup> of the esterified alginates under the alkaline conditions of borohydride reduction.<sup>9</sup> The ap-

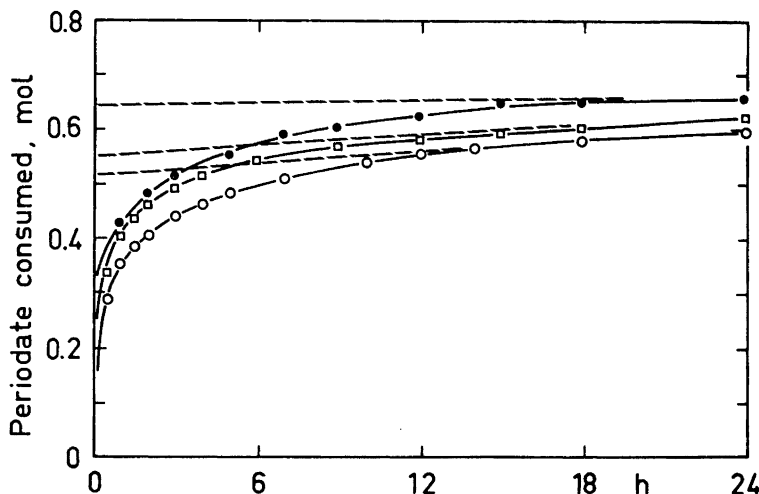


Fig. 7. Oxidation at 20° of carboxyl-reduced alginates containing 40 % (○), 58 % (□), and 71 % (●) of hexose residues, in 0.01 M NaIO<sub>4</sub>.

parent, Malapradian oxidation-limits (Table 2), obtained by extrapolation to zero time in the usual way,\* were nevertheless quite clear, and it is evident that they do not increase, with increasing hexose content, to the extent expected for physical mixtures.

Chromatography of acid-hydrolysates of the limit-oxidised products indicated in each case the presence of mannose and gulose, in addition to mannanuronic and guluronic acids; quantitative colorimetric analysis of the hydrolysates was probably not very accurate because of incomplete recovery of the monomers after acid-hydrolysis, but it indicated that the yield of unoxidised hexose, relative to that of unoxidised hexuronic acid, was very substantial, the ratio being roughly the same as in the starting materials.

It seems useful to attempt to arrive at a rough quantitative interpretation of the small but significant increase in oxidation-limit with increasing hexose content. In general, the oxidation-limit ( $L_F$ ) of partially reduced alginate can be arbitrarily expressed as  $L_0(1-X) + X$ , where  $L_0$  is the oxidation-limit of unreduced alginate. For a physical mixture of alginate and a 1,4'-linked hexosan, it is evident that  $X = F$ , the mole fraction of hexose residues in the mixture. The oxidation-limits that would be expected for  $a$ ,  $b$ , and  $c$  on the basis of this assumption are shown in Table 2, and are seen to be much higher than the experimental values.

\* It may be noted that this widely used method of extrapolation is, strictly speaking, incorrect, because the initial Malapradian oxidation and the ensuing overoxidation are not simultaneous, but consecutive reactions. In general, limits measured in this way will be too low, because the curve for the overoxidation should show an induction period. When the overoxidation is relatively slow, the error incurred is of course very small.

Table 2. Calculated and experimental values of the oxidation-limits of carboxyl-reduced alginates.  $F$  is the mole fraction of reduced units and  $X$  the mole fraction of units that are not subject to protection. The theoretical limits ( $L_F$ ) are calculated from the formula  $L_F = L_0(1 - X) + X$ , with  $L_0$  set equal to 0.435 (see Table 1).

Sample	$F$	Experimental limit	Limit calculated assuming $X = F$	Limit calculated assuming $X = F^3$
<i>a</i>	0.40	0.515	0.661	0.472
<i>b</i>	0.58	0.550	0.763	0.545
<i>c</i>	0.71	0.643	0.836	0.637

Also shown in Table 2 are theoretical oxidation-limits, calculated by putting  $X = F^3$ , and it is seen that the agreement is then much better. It is noteworthy that, to the extent that the carboxyl groups can be assumed to have been reduced in a random manner, the quantity,  $F^3$ , has a simple physical meaning: it is the mole fraction of hexose residues that have two other hexose residues as their nearest neighbours in the chains. Expressed in other terms,  $F^3$  is the mole fraction of hexose residues that do not have a hexuronic-acid residue as a nearest neighbour.

It is therefore useful arbitrarily to regard the chains in the partially reduced alginates as consisting of two kinds of sequence: those in which all units, regardless of identity, have at least one hexuronic-acid residue as a nearest neighbour; and those consisting of contiguous hexose residues not having a hexuronic-acid residue as a nearest neighbour. Although the assumption of random reduction is not proven, and no correction has been made for the finite lengths of the sequences, the data in Table 2 support the idea that sequences of the first type show anomalous oxidation-limits, while the others do not.

*Direct evidence for hemiacetal formation between nearest neighbours.* Apart from the capacity of the assumption of two-sided protection of nearest neighbours to account quantitatively for both oxidation-limits of alginate, evidence of a more direct nature was obtained.

If the aldehyde groups of oxidised hexuronic-acid residues formed hemiacetals with the hydroxyl groups of unoxidised residues that were situated at remote positions in the same chain, then limit-oxidised alginate would be a very compact and rigid molecule. Indeed, to account for the total absence of free aldehyde groups, it would have to coil up into, perhaps, a helix, a process which would require much energy, especially in solutions of low ionic strength. Dr. Smidsrød found, however, that the intrinsic viscosity of limit-oxidised alginate was highly dependent upon the ionic strength of the solution, and considered this to be strong evidence that the molecule was flexible and expanded in dilute solution. Moreover, the flexibility, measured in this way, did not increase very markedly upon reduction with borohydride. The presence of even one cross-linkage in the molecule would very possibly have been revealed by this method.

Barry degradation<sup>46</sup> of limit-oxidised alginate was carried out to determine the size-distribution of the groups of contiguous unoxidised residues. In the

absence of a specific, nearest-neighbour effect, a polydisperse distribution would be expected, and, on a basis of random cross-linking, a degree of oxidation of 0.45 would correspond to an optimum yield of trimer and tetramer.<sup>47,48</sup> On the other hand, the reaction scheme depicted in Fig. 6 requires, for the same degree of oxidation, the formation of monomer and dimer in a molar ratio of 3:1, and the total absence of any higher oligomer.

The results indeed showed the liberation of the phenylosazones of sugars migrating of thin-layer chromatograms at the same speeds as those of D-mannuronic acid and 4-O- $\beta$ -D-mannuronopyranosyl-D-mannuronic acid, and no trace of the phenylosazone of any higher oligomer could be detected. The ratio of the monomeric to the dimeric phenylosazone appeared to vary between wide limits, depending upon conditions, but the former was always the major component, and in some experiments it was difficult to detect the dimeric phenylosazone at all. This lack of reproducibility can be understood, because the ratio is highly sensitive to the degree of oxidation of the limit-oxidised material. For example, at the theoretical degree of oxidation of 0.435 (Table 1), the ratio should be 2.35:1, whereas for a degree of oxidation of 0.475, the ratio would be 10:1.

*Evidence that the hemiacetal rings are 6-membered.* There is a formal, though highly unlikely, possibility that nearest-neighbour protection occurs through the formation of 7-membered hemiacetal rings, the aldehyde group at position 2 of an oxidised residue reacting with the hydroxyl group at position 2 of the corresponding, unoxidised aglycone, and the aldehyde group at position 3 reacting with position 3 of the corresponding, unoxidised glucose unit.

For long chains, the experimental data do not distinguish between the possibilities of protection by 6- or 7-membered hemiacetal rings, but for short ones they do, because, on a basis of 7-membered ring-formation, the end-groups of chains whose penultimate units are oxidised before the ultimate units would consume no additional periodate, and these chains would not be susceptible to overoxidation. The close agreement between the experimentally determined oxidation-limits of fragments A, B, and C (Figs. 3 and 4) with those expected on the assumption that all end-groups consume an additional mole of periodate therefore supports the idea of 6-membered ring-formation.

*Stereochemistry.* With space-filling atomic models, it was possible to construct models of all the structures represented schematically in Fig. 6, on the assumption of 6-membered hemiacetal ring-formation, without any evidence of severe steric clashes. On the other hand, 7-membered ring-formation appeared to be sterically impossible.

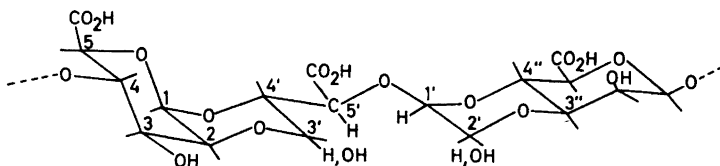


Fig. 8. An oxidised site in an alginate chain.



Fig. 8 depicts an oxidised site at the centre of a long chain of unoxidised residues, and assumes that the hexuronic-acid residues and the dioxan rings are all in the chair conformation. Apart from the aldehydic carbon atoms, which should be free to take up the energetically most favoured configuration, it is noted that both dioxan rings carry a bulky substituent in the axial conformation. For simplicity, D-mannuronic-acid residues are considered throughout.

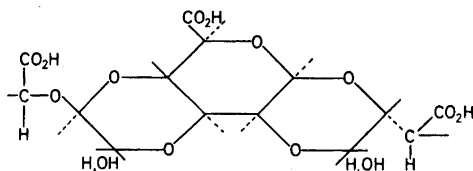


Fig. 9. An unoxidised hexuronic-acid residue with both its nearest neighbours oxidised.

Fig. 9 shows a doubly protected unit, whose two nearest neighbours are both oxidised. Because of the difficulty in drawing this tricyclic system in perspective, the configuration of the carbon atoms is shown according to the convention used in steroid chemistry.

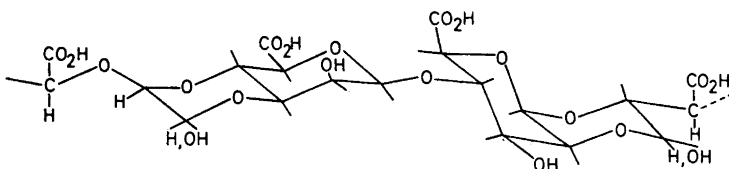


Fig. 10. An unoxidised doublet, with each unit protected by one oxidised neighbour.

Fig. 10 shows an unoxidised doublet in limit-oxidised alginate, each residue being singly protected by an oxidised neighbour. The two free alcoholic hydroxyl groups, one in each residue, are clearly those which retained their methyl groups when fully methylated, limit-oxidised alginate was hydrolysed in dilute acid. The dioxan rings are again arbitrarily shown in chair conformations.

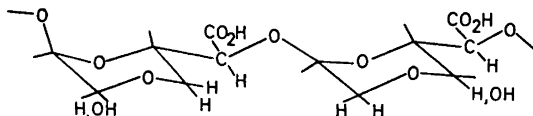


Fig. 11. The principal repeating-unit in alginate after oxidation to its second limit.

Fig. 11 shows the principal repeating-unit in alginate after oxidation to its second limit, following reduction of the material obtained after oxidation to the first limit. These are accompanied in the chains by isolated, unoxidised singlets, of which one half should be singly protected at position 2, and the other half singly protected at position 3; these structures are represented, respectively, by the right- and left-handed bicyclic moieties in Fig. 10, taken separately.

It was also possible to build models of all these structures with the unoxidised residues in the *C-1* conformation as in Figs. 8, 10, and 11, but with the dioxan rings in distorted boat (skew) conformations.<sup>49</sup> It should perhaps not be too readily assumed that the conformations shown are the most stable ones, and in the absence of that information, it may be more correct to draw the structures as in Fig. 9.

A point of interest is that it is not possible to have a residue of either mannuronic acid or guluronic acid in the *I-C* conformation, when it is protected at position 3, because then the substituents at C(3) and C(4) would be *trans*-diaxial. This implies that, even if some of the hexuronic-acid residues in alginate exist in solution in the *I-C* conformation, they must be able to convert very freely into the *C-1* conformation upon oxidation by periodate.

*Unimportance of the iodate liberated during oxidation.* A hypothesis that sought to explain the oxidation-limit in the early stages of this investigation was that the iodate ions formed from the periodate during oxidation were strongly bound by the hexuronic-acid residues that were still unoxidised, thus inhibiting subsequent attack by periodate ions. In fact, addition of sodium iodate to the reaction mixture in amounts several times greater than those which could be formed by reduction of the periodate, did not affect the oxidation-limit. Moreover, addition of sodium iodate did not affect the initial rate of oxidation, provided that sufficient additional salt (*e.g.* 2 M sodium chloride) was added to both test and control samples to neutralise the Donnan effect.<sup>6</sup> In solutions of low ionic strength, addition of sodium iodate increased the rate of oxidation, but not the limit, by virtue of its own ionic strength.

*Effect of esterification of the carboxyl groups in alginate.* The methyl ester of alginate initially underwent very rapid oxidation, probably because the molecule was uncharged, and showed a very sharp oxidation-limit at 0.44 mol of periodate per anhydrohexuronic-acid residue.

## DISCUSSION

The present investigation relates to the periodate oxidation of sodium alginate in dilute solutions of relatively low ionic strength, under conditions that are ordinarily used in structural investigations of polysaccharides. The behaviour under forcing conditions has not been examined.

The results obtained lead immediately to the question as to why alginate, and at least some other<sup>14,15</sup> hexuronic-acid containing polysaccharides should show anomalous oxidation-limits, while the vast majority of other polysaccharides do not. There is no evidence that the carboxylate anion exerts any direct catalytic effect upon hemiacetal formation, and indeed it appears from molecular models to be physically rather remote from the site of reac-

tion. Moreover, the fact that methyl alginate shows the same clear oxidation-limit as does sodium alginate argues against both this and the idea that inductive effects are of key importance.

The vast difference in inductive effect between the carboxylate anion and the unionised carboxyl group or carboxylic ester group can be seen in the relative rates of acid-hydrolysis of hexuronosides in strongly and weakly acidic solutions,<sup>50</sup> and the relative stability to alkali of sodium and methyl pectates<sup>45</sup> and sodium and methyl alginates.<sup>9</sup>

The proposition that a hydroxyaldehyde should form a six-membered hemiacetal ring when it is sterically free to do so should, on general principles, scarcely require justification. It therefore seems more pertinent to enquire, not why inter-residue hemiacetal formation should occur in polyuronides, but why it should not occur in other polysaccharides. The authors suggest that, in fact, it does. This statement is compatible with the known fact that most polysaccharides oxidise "normally", if it is assumed that an equilibrium exists between the cyclic, inter-residue hemiacetal, the open-chain form of the free aldehydic group, and any intra-residue hemiacetal or hemialdal structures that can possibly be formed.

The difference between alginate and other polysaccharides would then be one of degree only; in alginate, the equilibrium lies so far on the side of the inter-residue hemiacetal that protection is virtually complete, whereas in other polysaccharides, oxidation could still proceed after the position of maximum possible inter-residue hemiacetal formation has been reached, albeit at a rate which is reduced to an extent determined by the position of the equilibrium. Experimental evidence in support of this idea has been obtained, and will be published in another paper.

It is, however, still necessary to account for the extreme stability of the inter-residue hemiacetals in the case of alginate. One contributory factor is clearly the absence of a primary hydroxyl group at position 6, which could form intra-residue<sup>51</sup> hemiacetal rings competitively with the hydroxyl groups on neighbouring residues, thus changing the position of the equilibrium. This, however, is also true of xylans, which are reported to oxidise "normally", albeit under rather forcing conditions.<sup>52,53</sup>

The only obvious factor that distinguishes both alginate and its methyl ester from xylan is the bulkiness of the group attached at C(5). Any attempt to understand the stability of the structures shown in Figs. 8—11 in quantitative terms would seem at the present time to be premature, when so little is known even about the conformational stabilities of the sugar rings in unoxidised polysaccharides. However, for the moment it seems reasonable to accept that the stabilising effect of one bulky substituent could be sufficient to account for the quantitative difference between alginate and xylan.

The present results can be seen to cast a shadow of doubt over the results of many structural investigations in which periodate oxidation has been applied to hexuronic acid-containing polysaccharides. This applies not only to analytical oxidations, but also to applications of the Barry<sup>46</sup> and Smith<sup>54</sup> degradations to such materials. However, investigators have rarely based their conclusions upon periodate-oxidation data alone, and in general, such

data have only been reported when they are supported by independent evidence.

Far from diminishing the status of periodate oxidation as a tool in structural polysaccharide chemistry, the present work enhances it, by showing how the problem can be overcome. When a periodate-oxidised polysaccharide fails to consume any additional oxidant after treatment with borohydride, it can be assumed that the correct oxidation-limit has been reached. It seems reasonable to propose that this be adopted as a routine procedure until more information is available about the kinds of sugar residue that are likely to behave anomalously.

So far as the authors have been able to trace, the idea that the aldehyde groups of one oxidised unit in a periodate-oxidised polysaccharide could react with the functional groups in neighbouring units, was first put forward, 17 years ago, by Head.<sup>55</sup> The material considered in that case was fully periodate-oxidised cellulose, and the proposed linkage was of the hemialdal type.

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#### REFERENCES

1. Drummond, D. W., Hirst, E. L. and Percival, E. *J. Chem. Soc.* **1962** 1208.
2. Hirst, E. L., Jones, J. K. N. and Jones, W. O. *J. Chem. Soc.* **1939** 1880.
3. Chanda, S. K., Hirst, E. L., Percival, E. G. V. and Ross, A. G. *J. Chem. Soc.* **1952** 1833; Hirst, E. L. and Rees, D. A. *J. Chem. Soc.* **1965** 1182.
4. Rees, D. A. and Samuel, J. W. B. *J. Chem. Soc. C* **1967** 2295.
5. Szejtli, J. *Acta Chim. Acad. Sci. Hung.* **49** (1966) 205.
6. Scott, J. E. and Harbinson, R. J. *Histochemie* **14** (1968) 215.
7. Scott, J. E. *Biochim. Biophys. Acta* **170** (1968) 471.
8. Nisizawa, K., Fujibayashi, S. and Habe, H. *Proc. VIth Intern. Seaweed Symp.*, Santiago de Compostella 1968.
9. Haug, A., Larsen, B. and Smidsrød, O. *Acta Chem. Scand.* **21** (1967) 2859.
10. Larsen, B., Haug, A. and Painter, T. J. *Acta Chem. Scand.* **20** (1966) 219.
11. Zitko, V. and Bishop, C. T. *Can. J. Chem.* **44** (1966) 1749.
12. Wolfrom, M. L. and Juliano, B. O. *J. Am. Chem. Soc.* **82** (1960) 1673.
13. Toro-Feliciano, E. D. *M.Sc. Thesis*, Ohio State University 1957.
14. Sloneker, J. H., Orentas, D. G., Knutson, C. A., Watson, P. R. and Jeanes, A. *Can. J. Chem.* **46** (1968) 3353.
15. Larsen, B. and Painter, T. J. *Carbohydrate Res.* **10** (1969) 186.
16. Meyer, K. H. and Gonon, W. F. *Helv. Chim. Acta* **34** (1951) 294.
17. Whelan, W. J. and Roberts, P. J. *J. Chem. Soc.* **1953** 1298.
18. Juncosa, M. L. *Random-number generation on the BRL high-speed computing machines*, Rep. No. 855, Ballistics Research Laboratories, Aberdeen Proving Ground, Maryland, U.S.A. 1953.
19. Haug, A. *Methods in Carbohydrate Chemistry*, Academic, New York and London 1965, Vol. 5, p. 69.
20. Haug, A. and Larsen, B. *Acta Chem. Scand.* **16** (1962) 1908.
21. Haug, A., Larsen, B. and Smidsrød, O. *Acta Chem. Scand.* **21** (1967) 691.
22. Lucas, H. J. and Stewart, W. T. *J. Am. Chem. Soc.* **62** (1940) 1070, 1792.
23. Schweiger, R. G. *J. Org. Chem.* **27** (1962) 1786.
24. Bitter, T. and Muir, H. M. *Anal. Biochem.* **4** (1962) 330.
25. Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. and Smith, F. *Anal. Chem.* **28** (1956) 350.

26. Laver, M. and Wolfrom, M. L. *Methods in Carbohydrate Chemistry*, Academic, New York and London 1962, Vol. 1, p. 454.
27. Scott, J. E. *Methods Biochem. Anal.* **8** (1960) 163.
28. Blass, J., Macheboeuf, M. and Nunez, G. *Bull. Soc. Chim. Biol.* **32** (1950) 130.
29. Hay, G. W., Lewis, B. A. and Smith, F. *Methods in Carbohydrate Chemistry*, Academic, New York and London 1965, Vol. 5, p. 381.
30. Larsen, B., Smidsrød, O., Haug, A. and Painter, T. J. *Acta Chem. Scand.* **23** (1969) 2375.
31. Hough, L. *Methods in Carbohydrate Chemistry*, Vol. 5, p. 370, and references therein cited.
32. Smidsrød, O., Haug, A. and Larsen, B. *Acta Chem. Scand.* **17** (1963) 2628.
33. Smidsrød, O., Haug, A. and Larsen, B. *Acta Chem. Scand.* **19** (1965) 143.
34. Sklarz, B. *Quart. Rev. (London)* **21** (1967) 1.
35. Schopf, C. and Wild, H. *Chem. Ber.* **87** (1954) 1571.
36. Hughes, G. and Nevell, T. P. *Trans. Faraday Soc.* **44** (1948) 941.
37. Meyer, K. H. and Rathgeb, P. *Helv. Chim. Acta* **32** (1949) 1102.
38. Huebner, C. F., Lohmar, R., Dimler, R. J., Moore, S. and Link, K. P. *J. Biol. Chem.* **159** (1945) 503.
39. Sprinson, D. B. and Chargaff, E. *J. Biol. Chem.* **164** (1946) 435.
40. Halsall, T. G., Hirst, E. L. and Jones, J. K. N. *J. Chem. Soc.* **1947** 1427.
41. Edington, R. A., Hirst, E. L. and Percival, E. *J. Chem. Soc.* **1955** 2281.
42. Guthrie, R. D. *Advan. Carbohydrate Chem.* **16** (1961) 105.
43. Kuhn, R., Trischmann, H. and Löw, I. *Angew. Chem.* **67** (1955) 32.
44. Bouveng, H. O. and Lindberg, B. *Methods in Carbohydrate Chemistry*, Vol. 5, p. 296.
45. Albersheim, P., Neukom, H. and Deuel, H. *Arch. Biochem. Biophys.* **90** (1960) 46.
46. O'Colla, P. S. *Methods in Carbohydrate Chemistry*, Vol. 5, p. 382.
47. Kuhn, W. *Ber.* **63** (1930) 1503.
48. Painter, T. J. *J. Chem. Soc.* **1963** 779.
49. Bentley, R. J. *Am. Chem. Soc.* **82** (1960) 2811.
50. Capon, B. and Ghosh, B. C. *Chem. Commun.* **1965** 586.
51. Cadotte, J. E., Dutton, G. G. S., Goldstein, I. J., Lewis, B., Smith, F. and Van Cleve, J. W. *J. Am. Chem. Soc.* **79** (1957) 691.
52. Chanda, S. K., Hirst, E. L., Jones, J. K. N. and Percival, E. G. V. *J. Chem. Soc.* **1950** 1289.
53. Ehrenthal, I., Montgomery, R. and Smith, F. *J. Am. Chem. Soc.* **76** (1954) 5509.
54. Goldstein, I. J., Hay, G. W., Lewis, B. A. and Smith, F. *Methods in Carbohydrate Chemistry*, Vol. 5, p. 361.
55. Head, F. S. H. *J. Textile Inst.* **43** (1952) T1.

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