The Anomalous Periodate Oxidation Limit of Guaran

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Guaran, containing residues of D-galactose and D-mannose in the ratio 36:64, was oxidised in 12.5 mM sodium metaperiodate at 20°, and the consumption of oxidant and the liberation of formic acid were measured until constant values were obtained. At intervals during the reaction, samples of substrate were isolated and reduced with borohydride; they were then re-oxidised, with measurement of the consumption of additional periodate and of the formaldehyde liberated.

From these data, curves were obtained showing, separately, the consumption of periodate and the liberation of formic acid by the galactose residues, and the consumption of periodate by the mannan backbone. The galactose residues behaved similarly to methyl α-D-galactopyranoside, oxidised under the same conditions, and ultimately consumed 2 mol of periodate with liberation of 1 mol of formic acid.

Only 58 % of the mannose residues in the main chain were directly oxidisable. The remainder resisted oxidation, even in 60 mM periodate. They became freely oxidisable, however, after the 58 % oxidised chain had been reduced with borohydride.

From these and other data, it was concluded that, when a mannose residue carrying a galactose residue at position 6 is oxidised, the liberated aldehyde groups immediately form stable hemiacetals with the closest hydroxyl groups on the two adjacent mannose residues, thus preventing their subsequent oxidation.

The anomalous periodate-oxidation limit of sodium alginate (0.44 mol of periodate per non-terminal hexuronic-acid residue) has been traced to the formation of stable, six-membered hemiacetal rings between the aldehyde groups of oxidised hexuronic-acid residues and the closest hydroxyl groups on adjacent, unoxidised hexuronic-acid residues in the 1,4'-linked polyuronide chains.[1] The methyl ester of alginic acid behaved similarly, and therefore the extreme stability of these inter-unit hemiacetal rings could not be attributed to some special property of the carboxyl groups.[1]

It was therefore suggested that the stability arose mainly because of the absence of a primary hydroxyl group at position 6 of the oxidised residues. Such a group, if present, could be expected to give rise to intra-residue

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hemiacetals,[2] formed competitively with the inter-residue forms, thus shifting the position of the equilibrium away from the latter.

To test this idea, it was necessary to find a β-1,4'-linked mannan in which the hydroxyl groups at C(6) were blocked or replaced by some group other than a carboxyl group. However, native mannans are so insoluble that it seemed an impossible task to modify them chemically in a systematic manner, to yield a suitable, water-soluble derivative.

This difficulty prompted the present re-investigation of the periodate oxidation of guaran. Although only about 50 % of the mannose residues in the main chain of guaran are substituted at position 6 with α-D-galactopyranosyl groups, a clearly anomalous periodate-oxidation limit should still be demonstrable if the reasoning just described is correct. The galactose side-groups also consume periodate, but a way was found to correct for this, and to study the oxidation of the mannan chain separately.

Although standard text-books [3,4] state that guaran, containing residues of galactose and mannose in the usual ratio of 1:2, shows the expected, Malapradian oxidation-limit of 1.33 mol of periodate per hexose residue, they do not record the full historical facts. Before the correct structure of guaran had been established by methylation [5,6] and partial hydrolysis,[7,8] its oxidation by periodate had been studied by two groups of workers.[9,10] Both reported oxidation-limits close to 1.0 mol of periodate per hexose residue, and from this result, they inevitably drew erroneous conclusions about the structure of guaran.[9,10]

The results reported here are in good agreement with those of the early workers,[9,10] and it is not understood why later workers [5,6] were able to report the theoretical oxidation-limit.

EXPERIMENTAL

Materials. The sample of guaran was Meypro rein Guarin, supplied by Meyhall Chemical AG, Kreuzlingen, Switzerland. It contained 10.1 % moisture and 1.2 % ash, which were corrected for in subsequent analyses, and 0.2 % nitrogen (Kjeldahl). By hydrolysis and gas-liquid chromatography of the derived alditol acetates,[11] it was found to contain residues of galactose and mannose in the ratio 36:64.

The methyl α-D-galactopyranoside was purchased from Koch-Light Laboratories, Colnbrook, England, as its monohydrate, having m.p. 110° and $[\alpha]_D^{20} = +178°$ (c, 1.0 in water). Meso-inositol, m.p. $224-226°$, was purchased from the same company. The other reagents were of Merck analytical grade.

Analytical oxidations. Reference is made to earlier papers [1,12,13] for detailed information concerning procedure and accuracy. Guaran (600 mg) was oxidised in unbuffered, 12.5 mM sodium metaperiodate (800 ml) at 20° in the dark. The consumption of periodate was followed by withdrawing portions (10 ml), adding them to an ice-cold mixture of 0.5 M sodium phosphate buffer (pH 7.0; 20 ml) and aqueous potassium iodide (30 % w/v; 3 ml), and titrating the liberated iodine with 0.01 M sodium thiosulphate. The liberation of formic acid was followed by pipetting portions (10 ml) of reaction mixture into aqueous ethanediol (50 % v/v; 3 ml), waiting 10 min, and titrating with 0.005 N sodium hydroxide, with methyl red as the indicator. A blank solution was prepared and used to correct the titres in the usual way.

In another experiment, guaran (150 mg) was oxidised in 60 mM periodate (200 ml). In this case, portions (10 ml) of reaction mixture were added to 50 ml of the ice-cold buffer-potassium iodide mixture, and the sodium thiosulphate solution used for titration was 0.05 M.

Methyl α-D-galactopyranoside monohydrate (58 mg) was oxidised in 11.25 mM periodate (200 ml). The conditions and methods of measurement were otherwise identical with those used for oxidation of guaran in 12.5 mM periodate.

Preparative oxidations. Eleven portions of guaran (750 mg each) were dissolved separately in water (950 ml). The solutions were brought to 20°, and oxidation was started by adding 0.25 M sodium metaperiodate (50 ml) to each. The solutions were kept in the dark at 20°, and after chosen times (35 min, 1.5 h, 3.5 h, 5 h, 7 h, 9 h, 11 h, 13.5 h, 20 h, 24 h, and 30 h, respectively), reaction was stopped by adding ethanediol (50 ml). The resultant solutions were concentrated separately in the rotary evaporator at 30° to 100 ml, and then dialysed against distilled water until the dialysates gave no colour with an acidified mixture of starch and potassium iodide. They were then concentrated again to 50 ml, and sodium borohydride (1 g) was dissolved in each. After 12 h, they were brought to pH 6 with glacial acetic acid, and dialysed thoroughly against distilled water. They were then centrifuged for 1 h at 40 000 g, and freeze-dried. The yields were almost quantitative. The conditions of dialysis required to give ash-free products were ascertained by carrying out ash determinations on several samples. The samples were stored in a desiccator.

Re-oxidation of the partially oxidised and reduced samples of guaran. These experiments were carried out on 150 mg samples in 200 ml of reaction mixture at 20° in the dark. The initial concentration of periodate was adjusted in each case so as to correspond as closely as possible to the concentration of periodate remaining in the reaction mixture at the time when the first part of the oxidation was stopped. This was done by pipetting the following volumes (given in ml) of 0.25 M periodate into the eleven reaction mixtures, respectively, before diluting them up to 200 ml: 7.81, 7.47, 7.22, 7.13, 7.05, 6.98, 6.92, 6.84, 6.63, 6.56, and 6.51. Blank solutions containing the same amounts of periodate were also prepared and titrated. The consumption of periodate was followed by withdrawing 10 ml samples and titrating them as before.

After the titres became constant, the amount of formaldehyde that had been liberated was measured as follows. A portion (25 ml) of the reaction mixture was treated with a solution of meso-inositol (250 mg) in water (10 ml). After 1 h, aqueous sodium sulphite (26 % w/v; 1.25 ml) was added, and the solution was diluted to 50 ml with water. As a standard, erythritol (27 mg) was oxidised in 12.5 mM periodate (200 ml) at 20° for 45 min, after which titration of a portion indicated liberation of a theoretical yield of formic acid. A portion (25 ml) was then withdrawn and treated in the same way as the test samples. As a blank, 12.5 mM periodate (25 ml) was treated similarly. Portions (0.5 ml) were then taken in triplicate from each solution for the assay of formaldehyde by the chromotropic acid method.[14]

RESULTS

Figs. 1 and 2 show the consumption of periodate (P) and the liberation of formic acid (F) by guaran in 12.5 mM periodate at 20°. Because of the very slow rate of the terminal phase of the oxidation, the full range of the reaction is shown by plotting the results on different time-scales in the two figures. The final consumption of periodate is 1.10 mol per hexose residue, and the final yield of formic acid is 0.36 mol. Also shown is a curve, calculated from the two others, according to the formula, $P - 2F$. This is clearly the fraction of hexose residues that have suffered a single oxidative attack at any time. As expected, it rises to a maximum, and then decreases again, as the singly oxidised galactose residues are replaced by doubly oxidised galactose residues.

The final value of $P - 2F$ is 0.38. At that time, all the galactose residues had been attacked twice, as is shown by the liberation of a theoretical yield of formic acid. The singly oxidised residues that remained must therefore have been mannose residues. However, the fraction of mannose residues in the guaran was 0.64, and hence only 0.38/0.64, or 59 % of them, were oxidisable under the conditions of the experiment.
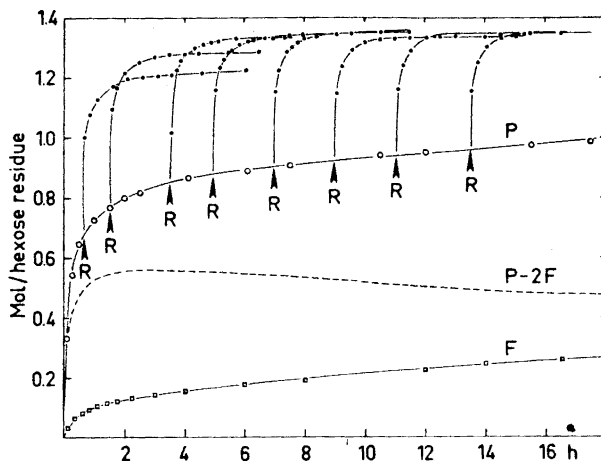
Fig. 1. Oxidation of guaran in 12.5 mM periodate at 20°. The concentration of substrate was 4 mM with respect to hexose residues. P is the periodate consumed and F is the formic acid liberated. The broken curve is calculated from $P - 2F$, and shows the fraction of singly oxidised hexose residues present at any time. At the points marked R, samples of substrate were reduced with borohydride, and re-oxidised, with measurement of the additional periodate consumed, as shown.
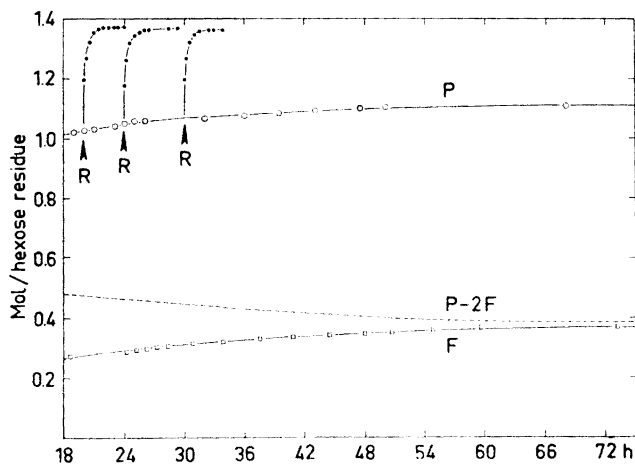

Fig. 2. Continuation of the experiment shown in Fig. 1. The time-scale is shortened to one-third of that in Fig. 1.

The effect of reduction of the substrate with borohydride at different times is also shown in the figures. The conditions were chosen such that the curves give an accurate impression of what would have happened, had it been possible to reduce the substrate instantaneously during the original oxidation,

without interrupting the reaction. Apart from the first two samples, and possibly also the third, which can be expected still to contain contiguous, unoxidised mannose residues and hence to give rise to a second, anomalous oxidation-limit, it is seen that in every case, the theoretical, Malapradian oxidation-limit of 1.36 mol of periodate per hexose residue is quickly reached.

The measurement of formaldehyde in the samples that were reduced and re-oxidised was undertaken, since this would give a direct measure of the fraction of singly oxidised galactose residues present at any time. If subtracted from $P - 2F$, this would then give the fraction of singly oxidised mannose residues present at any time, thus permitting construction of a curve, showing the oxidation of the mannan chain alone.

Table 1 shows the yields of formaldehyde (A) from the eleven samples, the corresponding values of $P - 2F$, the differences ($P - 2F - A$), and the differences expressed as percentages of the total mannan. The limits of error

Table 1. Calculation of the degree of oxidation of the mannan chain in guaran after different times of oxidation in 12.5 mM periodate at 20°. P is the periodate consumed by the whole guaran, F is the formic acid liberated from the galactose residues, and A is the formaldehyde liberated after reduction of the substrate with borohydride, and re-oxidation with periodate. All yields are given in moles per hexose residue. The guaran contained 64 % mannose.

Time	A	$P - 2F$	$P - 2F - A$	$(P - 2F - A)/0.64$ %
35 min	0.258	0.480	0.222	34.6
1.5 h	0.255	0.539	0.284	44.3
3.5 h	0.192	0.555	0.363	56.6
5.0 h	0.190	0.550	0.360	56.2
7.0 h	0.176	0.537	0.361	56.3
9.0 h	0.141	0.520	0.379	59.1
11.0 h	0.117	0.507	0.390	60.8
13.5 h	0.112	0.488	0.376	58.7
20.0 h	0.103	0.475	0.372	58.0
24.0 h	0.071	0.471	0.400	62.4
30.0 h	0.071	0.449	0.378	59.0

in this last column are such that the degree of oxidation of the mannan chain should be regarded as reaching a constant value of 58 ± 2 % from 3.5 h onwards.

In an attempt to determine whether the use of more-concentrated periodate would increase the oxidation limit, guaran was oxidised in five times the concentration of periodate (60 mM). The molar excess of periodate in the system was then about 12-fold. The results are shown in Table 2. The limiting consumption in this case was 1.02 mol, which was substantially lower than that (1.10 mol) in 12.5 mM periodate.

An experiment was next carried out to investigate the possibility that the anomalous oxidation limit of the mannan could be due to the formation of hemiacetals between the aldehyde groups of singly oxidised galactose residues

Table 2. Oxidation of guaran in 60 mM periodate at 20°. The concentration of substrate was 4 mM with respect to hexose residues.

Time (h)	Periodate consumed (mol/hexose unit)
0.25	0.594
1.00	0.715
2.00	0.795
3.00	0.850
5.00	0.895
17.0	0.968
25.0	0.986
29.0	1.02
100	1.02

and the hydroxyl groups of unoxidised mannose residues. It was reasoned that, if this happened, the kinetics of the oxidation of the galactose residues in guaran should be different from those of the oxidation of methyl α-D-galactopyranoside under the same conditions.

Methyl α-D-galactopyranoside was therefore oxidised in a solution that was equimolar, in galactose residues, with the solution of guaran studied earlier. To correct as far as possible for the amount of periodate consumed by the mannan chain, the initial concentration of periodate in this experiment was 11.25 mM. This introduced a maximum possible error of 10 % into the measured consumption of periodate at any time.

Fig. 3 shows the consumption of periodate (P') and the liberation of formic acid (F') by the methyl galactoside, with the data for the release of formic acid by guaran included for comparison. In this figure, all the quantities are expressed as moles per galactose residue.
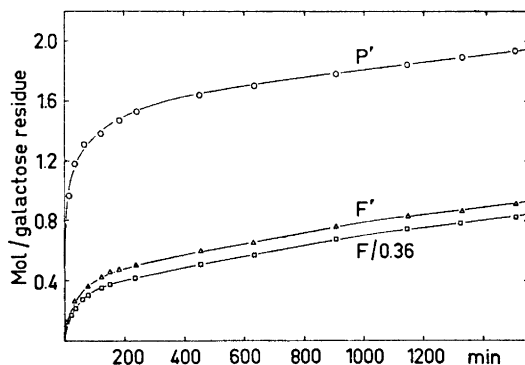

Fig. 3. Oxidation of methyl α-D-galactopyranoside (1.4 mM) in 11.25 mM periodate at 20°. P' is the periodate consumed, and F' is the formic acid liberated. For comparison with F', values of F for guaran, taken from Fig. 1, are plotted as $F/0.36$.

In Fig. 4, the points are values of $(P-2F-A)$, taken from Table 1. The curve in the figure, however, was drawn from calculated values of $P-0.36P'$, where P is expressed in moles per hexose residue, and P' is expressed in moles per galactose residue.
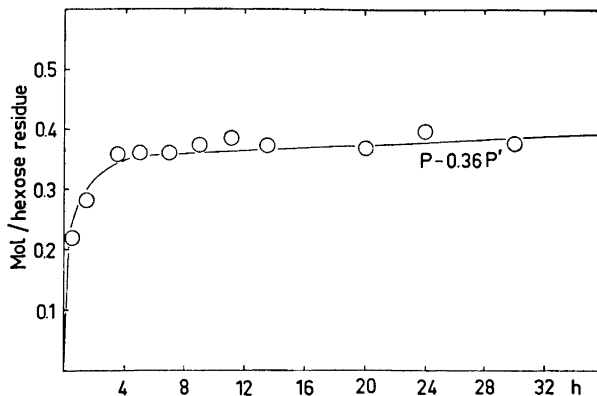

Fig. 4. The open circles are values of $P-2F-A$, taken from Table 1, and show the oxidation of the mannan chain when guaran is oxidised in 12.5 mM periodate at 20°. The curve is drawn from calculated values of $P-0.36P'$, where P is taken from Fig. 1, and P' from Fig. 3.

The possibility that mannose residues could be protected from oxidation by forming hemiacetals with the aldehyde groups of doubly oxidised galactose residues was discounted, because the yield of formic acid after 3.5 h (Fig. 1) showed that only about one-third of the galactose residues had been doubly oxidised, whereas the mannan chain had been fully oxidised to its anomalous limit of 58 % after that time (Table 1 and Fig. 4).

DISCUSSION

Insofar as the consumption of periodate by guaran becomes immeasurably slow before the theoretical, Malapradian oxidation-limit is reached, the oxidation of this polysaccharide is just as anomalous as that of alginate.[1] Indeed, the anomaly is more easily demonstrable with guaran, because the attachment of galactose residues to position 6 of mannose residues in the main chain prevents overoxidation.

An increase in the concentration of periodate in the system (from 12.5 mM to 60 mM) does not increase the oxidation-limit, but lowers it. This result is startling, but a similar effect has been observed with other polysaccharides, and is now under investigation. It appears that, at concentrations above 25 mM, the periodate ion is able to stabilise the inter-residue hemiacetals, thus inhibiting its own action. The effect of increasing the concentration of periodate still further has not been investigated, but any increase in con-

sumption resulting from this would be more likely to arise from non-specific oxidation than from Malapradian oxidation.

There is little doubt that the anomalous oxidation limit is a fundamental property of the β-1,4'-linked mannan chain itself, and that it does not arise from any direct chemical interaction between the galactose and mannose residues. The galactose residues and the mannan chain appear to oxidise independently of one another (Figs. 3 and 4). This is to be expected, since the smallest hemiacetal ring that could be formed between an aldehyde group in an oxidised galactose residue and a hydroxyl group in an unoxidised mannose residue would be eight-membered. It is very unlikely that a stable structure of this kind would be formed, in competition with the six-membered, intra-residue hemiacetals that could be formed in both singly [15] and doubly [2] oxidised galactose residues.[16]

In the light of earlier work,[1,12,13,16] the effect of borohydride reduction in permitting the rapid completion of Malapradian oxidation is here accepted as adequate evidence that the anomaly is due to the formation of highly stable, six-membered hemiacetal rings between the aldehyde groups of oxidised mannose residues and the closest hydroxyl groups on adjacent, unoxidised mannose residues in the main chain.

The question remains as to which kind of mannose residues are involved in these stable hemiacetal structures. The early work of Moe et al.[9] is helpful in this respect. These workers not only oxidised guaran, with results very similar to those reported here, but they also examined Honey locust (*Gleditschia tricanthos*, L.) gum, which contained only 18 % of galactose, and locust-bean (*Ceratonia siliqua*, L.) gum, which contained only 20 – 25 % of galactose. These other galactomannans showed oxidation limits of 1.04 and 1.06 mol of periodate per hexose residue, respectively, from which it may be calculated that their mannan chains contained only 20 – 25 % of periodate-resistant residues. The oxidation-limit of the mannan chain therefore increases as the galactose content of the galactomannan decreases, and hence unsubstituted mannose residues cannot, when oxidised, completely protect other unsubstituted mannose residues from oxidation. A similar conclusion was reached in an earlier study of partially carboxyl-reduced alginates.[1]

Finally, it may be noticed that, in all three galactomannans, the percentage of periodate-resistant mannose residues is about the same as the percentage of galactose-bearing mannose residues. Because of the statistical pattern of the attack of periodate on the chains, this could only come about if the substituted mannose residues, when oxidised, are able to protect both their nearest neighbours from oxidation, regardless of whether or not these neighbours are themselves substituted.

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