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Kinetic Evidence for Inter-residue Hemiacetal Formation during the Oxidation of Amylose by Periodate Ion

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When amylose, having a viscosity-average degree of polymerisation of 2700, was oxidised in 5 mM sodium metaperiodate at 20°, the second-order rate-constant decreased sharply as reaction progressed, and became constant at about 4 % of its initial value after the consumption of 0.62 ± 0.01 mole of oxidant per glucose residue. At this stage, Barry degradation indicated the virtual absence of contiguous, unoxidised glucose residues in the chains.

At any stage in the oxidation, reduction of the substrate with sodium borohydride restored the second-order rate-constant to its initial value, and, when carried out after the consumption of 0.62 mole of periodate per glucose residue, it permitted completion of the reaction constantly at that rate.

On the assumption that the chains are attacked randomly, one unit at a time, and that after oxidation of any unit, the reactivity of one of its unoxidised, nearest neighbours is greatly diminished, it was calculated that the rate of reaction should become constant after the consumption of 0.635 mole of oxidant per glucose residue.

It was concluded that oxidation of a glucose residue is followed by the rapid establishment of an equilibrium, in which one aldehyde group exists mainly as an inter-residue hemiacetal, formed with the closest hydroxyl group on an adjacent, unoxidised residue, while the other more readily forms an intra-residue hemiacetal with the hydroxyl group at C(6) of the same unit.

The extreme departure from second-order kinetics that is shown when alginates[1,2] and homopolymeric, β-1,4-linked xylans[3] are oxidised with periodate has been traced to the formation of 6-membered hemiacetal rings between the aldehyde groups of oxidised monosaccharidic units and the closest hydroxyl groups on adjacent, unoxidised units in the chains.[2,3] Evidence was obtained that oxidation of these materials proceeds initially in an approximately random manner, and that, once an aldehyde group is liberated, an equilibrium is rapidly established between the open-chain aldehydic and the cyclic, inter-residue, hemiacetal forms.[2,3]

In the case of alginate, the equilibrium lies so far on the side of the inter-residue hemiacetal form that the oxidised material gives no significant reac-

tion with Schiff's reagent, and the reaction virtually ceases when every unoxidised unit has at least one oxidised unit as a nearest neighbour.[2]

In the case of xylan, hemiacetal formation is less complete, and the rate of oxidation becomes constant at about 2 % of its initial value when every unoxidised unit has both its nearest neighbours in the oxidised state.[3]

The strong tendency of these two substrates to form inter-residue hemiacetal structures was suggested[3] to be due in part to the fact that they both lack a hydroxyl group at position 6. Such a hydroxyl group would be able to form intra-residue hemiacetal structures[4-6] competitively with the inter-residue forms, and might thereby displace the equilibrium away from the latter, permitting a more independent oxidation of the separate monomeric units.

In a 1,4-linked hexoglycan, however, an oxidised unit contains two aldehyde groups, but only one remaining hydroxyl group, namely, that at position 6. On average, therefore, at least one aldehyde group should still be free to form an inter-residue hemiacetal, and the oxidation could still be expected to exhibit some departure from second-order kinetics.

To test these ideas, a kinetic study of the periodate oxidation of amylose was undertaken, and is now described. It is, of course, very well known that amylose readily consumes one mole of periodate for every non-terminal glucose residue, and periodate oxidation is an established, routine method for determination of the degree of branching in starches and glycogens.[7,8] Such uses, however, require only that the final consumption of oxidant should correspond to the cleavage of all the *vic*-diol groups initially present, and an interpretation of the form of the reaction curve in mechanistic terms has not hitherto been attempted.

THEORY

In a previous study of the periodate oxidation of xylan, a simple, recursive formula was derived, permitting calculation of the limiting consumption of oxidant when the chains are randomly attacked, one unit at a time, with instantaneous and complete protection of both nearest neighbours of the oxidised units from future oxidation.[3] Although, in practice, protection of nearest neighbours in the xylan was not complete, as had been found in the case of alginate, it was sufficiently so that good agreement was observed between the calculated oxidation-limit and the degree of oxidation at which the rate of oxidation became virtually constant.[3]

In the present work, it is of interest to calculate another kind of theoretical oxidation-limit, and to use it in a similar way. In this case, it is again assumed that oxidative attack is random, and that only one unit in a given chain is oxidised at a time, but it is now supposed that only one nearest neighbour of an oxidised unit is protected, such protection again being complete. It is further assumed that, in a given chain, protected units always have the same relationship to the oxidised neighbours that protect them; that is to say, they always lie on either the right or the left when the chain is scanned in a defined direction. This condition will clearly permit a limit-oxidised chain to contain contiguous, oxidised units, but not contiguous, unoxidised units.

Proceeding as before,[3] the condition of random attack is imposed by dividing a hypothetical population of chains, all N units in length, into N equal subgroups, each consisting of chains that suffered their first oxidative attack in the same position relative to the terminal units. The two terminal units in any chain are regarded as distinguishable, and, beginning with one of these, the units are numbered consecutively from left to right, with protected units lying always to the right of the oxidised units that protect them.

In the subgroup whose chains were all initially attacked on the first unit, the second unit in every chain will be protected, so that the mean degree of oxidation for the first pair of units in these chains is 1/2. The mean degree of oxidation of the remaining sequence of $(N-2)$ contiguous units must be identical with that (D_{N-2}) of a complete population of chains, $(N-2)$ units in length. The overall oxidation-limit of this subgroup can then be written:

$$(d_N)_1 = (2/N)(1/2) + [(N-2)/N]D_{N-2}$$

In the subgroup whose chains were first attacked on the second unit, the third unit will be protected, while the first unit must be oxidised. The mean degree of oxidation of this first triplet is therefore 2/3, while that of the remaining $(N-3)$ units can be written as D_{N-3}. The overall oxidation-limit of this subgroup is then:

$$(d_N)_2 = (3/N)(2/3) + [(N-3)/N]D_{N-3}$$

Continuing in this manner, it is found that the oxidation-limit of the nth subgroup can be written:

$$(d_N)_n = (2/N)(1/2) + [(n-1)/N]D_{n-1} + [(N-n-1)/N]D_{N-n-1}$$

where $1 < n < N$.

For the last subgroup, the oxidation-limit is clearly:

$$(d_N)_N = [(N-1)/N]D_{N-1} + (1/N)(1)$$

The overall oxidation-limit of the whole population is now given by the arithmetic mean of those of the N subgroups:

$$D_N = (1/N)^2\left[N + \sum_{n=2}^{N}(n-1)D_{n-1} + \sum_{n=1}^{N-2}(N-n-1)D_{N-n-1}\right]$$

By priming this expression with the value of D_1, which is clearly unity,* the oxidation-limit for chains of any length can be arrived at in a stepwise manner. This is readily accomplished in the digital computer, and a selection of values is given in Table 1. It is seen that the oxidation-limit approaches a constant value of 0.635 as N increases.

EXPERIMENTAL

Material and methods. The amylose was a specially purified sample of amylose V, obtained from Avebe A/G, Veendam, Holland. Its intrinsic viscosity in anhydrous dimethyl sulphoxide at 20° was kindly measured by Dr. O. Smidsrød, and was 1.0 dl/g.

* The additional consumption of periodate by terminal units is not considered here because the amylose was of sufficiently high molecular weight that correction for this was unnecessary. Such correction, if required, could be made as described earlier for xylan.[3]

Table 1. Theoretical oxidation-limits, expressed as moles of periodate consumed per monosaccharidic unit, and calculated on the assumption of instantaneous and complete protection of one nearest neighbour of oxidised units, the protection being unidirectional. N is the number of units per chain, and D_N is the oxidation-limit for chains of that length. No correction is made for any additional consumption of oxidant by terminal units.

N	D_N	N	D_N
2	0.750	20	0.645
3	0.722	25	0.643
4	0.698	30	0.641
5	0.685	40	0.639
6	0.676	50	0.637
7	0.670	60	0.637
8	0.665	70	0.636
9	0.661	80	0.635
10	0.659	90	0.635
15	0.650	100	0.635

From published figures [9] for the constant and exponent in the Staudinger equation, this corresponds to a viscosity-average degree of polymerisation of 2.7×10^3. The iodine blue value [10] of the material was 1.28, and its ash content 0.5 %. Samples were measured by weighing, correction being made for ash and moisture content (which was usually about 10 %).

Solutions of amylose were prepared by shaking samples (1.000 g, on a dry, ash-free basis) with 2 N sodium hydroxide (20 ml) under nitrogen for 1 h. The clear solutions were then brought to pH 6.0 with N acetic acid, and diluted immediately to 100 ml with water. These solutions were prepared just before use and were stable for several hours before the onset of retrogradation.

All reagents were Merck analytical grade, and solutions were prepared just before use. Sodium thiosulphate (0.01 M) was freshly prepared and standardised at 12 h intervals during the experiments. Glassware was cleaned with chromic acid just before use. Reference is made to some observations concerning accuracy in an earlier paper.[3] Barry degradation and chromatography were also carried out as described elsewhere.[2,3]

Analytical oxidations. These were carried out at $20.1 \pm 0.1°$. Oxidation of amylose was studied in two separate experiments, A and B, designed to study the full range and the early stages of the reaction, respectively.

In experiment A, 25 ml of the stock solution of amylose (1.000 % in 0.4 M sodium acetate at pH 6.0) was diluted to 250 ml with water, and brought to 20°. Aqueous sodium metaperiodate (10 mM; 250 ml) was similarly brought to 20°, and reaction was initiated by rapid mixing of the two solutions. Light was excluded from the reaction vessel by covering it with aluminium foil. At intervals, samples (10 ml) were withdrawn and pipetted rapidly into a mixture of phosphate buffer (0.5 M, pH 7.0; 10 ml) and aqueous potassium iodide (15 % w/v; 1 ml). The liberated iodine was titrated with 0.01 M sodium thiosulphate, with starch as the indicator. A blank experiment was similarly performed to correct for spontaneous decomposition of the periodate, which, however, was negligible.

In experiment B, the conditions were the same as in A, except that two glass beakers were used to facilitate very rapid mixing of the two solutions of reactants. Nine Erlenmeyer flasks (100 ml), all containing phosphate buffer (25 ml) and aqueous potassium iodide (1 ml), were prepared beforehand, and nine numbered, empty Erlenmeyer flasks (150 ml) were set out close at hand. Immediately after initiation of the reaction, one investigator began transferring samples (25 ml) to the empty flasks without regard to time, every transfer taking an average of about 40 sec. One minute after initiation, a second investigator rapidly poured one of the portions of buffer-iodide mixture into one of the samples of reaction mixture with vigorous mixing. This operation required about 2 sec, and the flask was then stoppered to prevent evaporation of iodine, and set aside for later titration. Eight further samples were similarly treated at one-minute intervals, and titration of each was carried out after the last sample had been taken.

Oxidation of the sample of amylose that had previously been oxidised to 64 % completion, and of the similar sample that had been treated with sodium borohydride, was carried out as in experiment A, with 695 mg (dry, ash-free basis) of substrate in 500 ml of 5 mM periodate (*i.e.*, 3 mM with respect to unoxidised glucose residues).

Preparative oxidations. These were carried out on 1 g samples of amylose, under conditions identical with those used in analytical oxidations, the amounts of all components being increased by a factor of 4. Oxidation was stopped, by the addition of ethan-1,2-diol (10 ml), at the desired time, read from the analytical curves. The solution was then concentrated to 500 ml under diminished pressure at 30°, dialysed exhaustively against distilled water, concentrated further to 100 ml, and then freeze-dried. The yield was quantitative. The 64 % oxidised product contained 0.3 % ash, had $[\alpha]_D^{22} = +77°$ ($c=4$, in water), and dissolved freely in water upon gentle warming.

Reduction of partially oxidised amylose. This was carried out as described elsewhere [2] for limit-oxidised alginate, and the yield of freeze-dried material after exhaustive dialysis was quantitative. The product obtained from the 64 % oxidised amylose contained 0.2 % ash, had $[\alpha]_D^{22} = +100°$ ($c=2.4$, in water), and was freely soluble in water.

Limits of error. Twenty control titrations permitted the calculation that, in experiment A and the other experiments performed under similar conditions, the probable error in a single reading was ±0.5 % of the titre corresponding to complete oxidation throughout. In experiment B, it was about ±0.2 % of the maximum consumption, corresponding to ±1.2 % of the range of consumption studied (0—14 %).

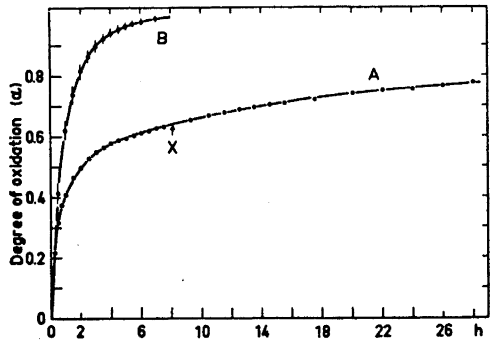

Fig. 1. Curve A: oxidation of amylose (3 mM) in sodium metaperiodate (5 mM) at 20°. Curve B: theoretical curve for a second-order reaction with the same rate as the initial rate of the experimental curve (A).

RESULTS

Fig. 1 (curve A) shows the oxidation of amylose in 5 mM periodate at 20°. The concentration of substrate was 3 mM with respect to *vic*-diol groups. These concentrations are lower than those normally used in structural analysis, and were chosen to permit accurate measurement of the initial rate, and of the changes in rate in the early stages of the reaction. Only the first 78 % of the reaction is shown, but it proceeded to completion as expected, the degree of oxidation (α) of the glucose residues being 0.93 after 120 h and 0.98 after 240 h.

The initial rate of the reaction was measured from the initial slope of the curve, and, expressed as a second-order rate-constant, it was 250 ± 30 l mole^{-1} h^{-1}. Curve B in Fig. 1 is a theoretical one for a bimolecular reaction with the

same rate-constant, and shows how the reaction should have proceeded, had second-order kinetics been obeyed throughout. The points on the curve are also theoretical, and are inserted to show the maximum possible effect of the limits of error in measuring the initial slope of the experimental curve. Thus, a bimolecular reaction with a rate-constant of 220 l $mole^{-1}$ h^{-1} would give a curve passing through the lower limits of the points, and one with a rate-constant of 280 l $mole^{-1}$ h^{-1} would give a curve passing through the upper limits.

When the amylose was oxidised under the same conditions, but in the presence of 5 mM sodium iodate, the curve obtained was identical with curve A in Fig. 1. The departure from second-order kinetics was not, therefore, caused by any inhibition of oxidation by the iodate that was formed from the periodate during the reaction. A similar result was obtained earlier, in studies of the periodate oxidation of alginate.[2]

Although iodate is the only known product when periodate is reduced by a *vic*-diol near neutral pH, the possibility that the oxidation was being inhibited by some other reduction product of periodate was nevertheless investigated. This was done by isolating 64 % oxidised amylose from the reaction mixture (at point X in Fig. 1), and dialysing it free from all low molecular-weight material.

This product was then further oxidised in fresh 5 mM periodate, its concentration in the reaction mixture being adjusted to make the solution 3 mM with respect to *unoxidised* glucose residues. In Fig. 2 (curve C) the results are plotted on the same time-scale as that used in Fig. 1, with the ordinates in this case referring to the degree of oxidation of the unoxidised glucose residues present in the substrate at the start of the experiment. The initial slope of this curve may therefore be directly compared with the initial slope of curve A in Fig. 1.

Since it was clear that the shape of curve A in Fig. 1 could not be explained in terms of inhibition by a reduction product of periodate, the alternative possibility, namely, inhibition by oxidised glucose residues, was next in-

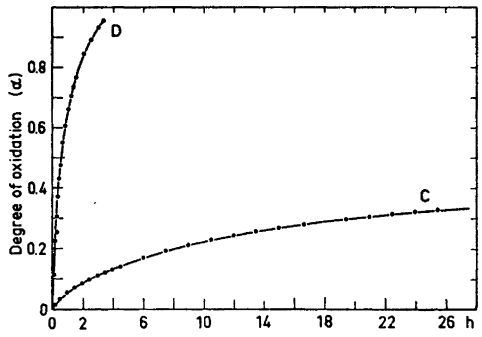

Fig. 2. Curve C: further oxidation of 64 % oxidised amylose, with the initial concentrations of *vic*-diol groups and of periodate readjusted to 3 mM and 5 mM, respectively. Curve D: further oxidation of 64 % oxidised amylose, after treatment with sodium borohydride, under the same conditions. The temperature was 20° in both experiments.

vestigated. The 64 % oxidised amylose was therefore treated with sodium borohydride to reduce all the aldehyde groups to primary alcohols, and the product was then further oxidised in 5 mM periodate, the concentration of unoxidised glucose residues again being adjusted to 3 mM. The resultant curve (Fig. 2, curve D) is seen to resemble closely the theoretical curve (B) in Fig. 1.

The significance of these results in quantitative terms is more clearly shown in Fig. 3, where curves A, C, and D are re-plotted according to second-order kinetics. This is done in the conventional way, by plotting $[1/(a-b)]\ln[(b/a)(a-x)/(b-x)]$ against time, where a and b are the initial concentrations of periodate and *vic*-diol groups (0.005 M and 0.003 M, respectively), and $x=b\alpha$, α being the degree of oxidation, expressed as a fraction of the *vic*-diol groups initially present in each substrate. According to this scheme, a second-order reaction should give a straight line, with a slope equal to the rate-constant.

It is thus seen that, from point X onwards, amylose is oxidised in good accordance with second-order kinetics. The slope of curve C is virtually identical with that of the linear part of curve A, and they both correspond to a second-order rate-constant of 9 ± 1 l mole^{-1}h^{-1}. On the other hand, the slope of curve D is virtually identical with the initial slope of curve A, and corresponds to a second-order rate-constant of 285 ± 15 l mole^{-1}h^{-1}.

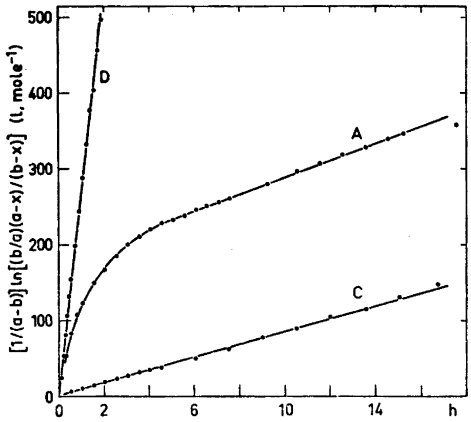

Fig. 3. Experimental data from Figs. 1 and 2, re-plotted according to second-order kinetics.

From curve A in Fig. 3, the slopes of tangents were measured throughout, and in Fig. 4, the second-order rate-constants so obtained are plotted as a function of the degree of oxidation (α). It is seen that the degree of oxidation at which they become constant can be rather accurately located, at $\alpha=0.62\pm0.01$.

Treatment of partially oxidised amylose with borohydride at degrees of oxidation other than 0.64 also restored the second-order rate-constant to approximately its initial value, but further oxidation thereafter obeyed

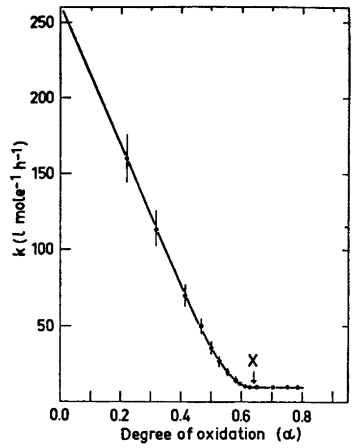

Fig. 4. The slope (k) of curve A in Fig. 3, plotted against the degree of oxidation (α).

second-order kinetics only when the first stage of the oxidation had been allowed to reach 64 % oxidation or more. After reduction of less highly oxidised material, further oxidation was again characterised by a declining rate.

The course of the oxidation was also studied in a qualitative way, by thin-layer chromatography of the phenylosazones liberated from the substrate by Barry degradation (*cf.* Refs. 2 and 3). Up to a degree of oxidation of about 0.6, this showed the phenylosazones of glucose and the polymer-homologous series of maltodextrins. As the degree of oxidation increased above 0.6, however, the oligosaccharide phenylosazones rapidly disappeared, and, at $\alpha = 0.64$, only a relatively small amount of maltose phenylosazone could be detected in addition to glucose phenylosazone, erythrose phenylosazone, and glyoxal bisphenylhydrazone. When α had reached 0.70, oligosaccharide phenylosazones could no longer be detected at all.

In an attempt to obtain some more direct evidence concerning the number of unoxidised glucose residues whose reactivities are modified by one oxidised glucose residue, a special study was made of the early stages of the reaction. The first 14 % of the reaction was studied as accurately as possible, and the curve obtained is shown in Fig. 5.

It was reasoned that, in this phase of the reaction, the small fraction of oxidised glucose residues would, on an average, be well separated from one another in the chains, and virtually all of them would have unoxidised residues in both neighbouring positions. The number (P) of additional, unoxidised glucose residues that are effectively rendered unreactive for every residue oxidised should therefore be initially constant.

This condition permits integration of the expected relationship, $d\alpha/dt = 10^{-3}\, k_p(5-3\alpha)[1-(P+1)\alpha]$, to give:

$$[10^3/(2+5P)]\ln[(5-3\alpha)/(1-\alpha-P\alpha)] = k_p t + \text{constant}$$

A plot of the left-hand side of this equation against time should therefore give, initially, a straight line of slope k_p when the correct value of P has been

chosen. Fig. 6 shows the curves obtained for chosen values of P of 0.0, 1.0, 1.5 and 2.0, with the intercepts arbitrarily displaced to prevent crowding of the points, and thus to facilitate comparison. The initial slope of all four curves corresponded to a value of k_P of 225 ± 12 l mole^{-1}h^{-1}. By plotting these and other, similar curves on a large scale, it was estimated that P had a value of 1.25 ± 0.25.

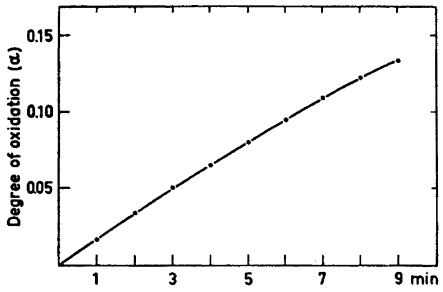

Fig. 5. Early stages of the oxidation of amylose (3 mM) by sodium metaperiodate (5 mM) at 20°.

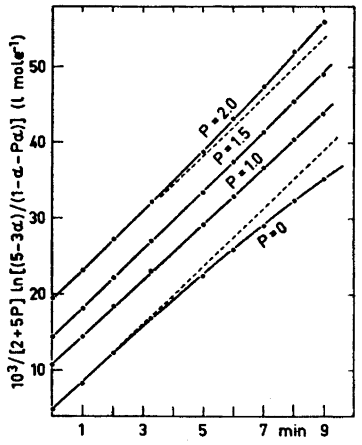

Fig. 6. Data from Fig. 5, re-plotted according to different rate equations, in which P is the number of unoxidised glucose residues protected by every oxidative attack. Linearity implies obedience to the rate-equation.

An indication as to how this total amount of inhibition is distributed between the two nearest neighbours of the oxidised residues was sought from a consideration of this result, together with the amount of inhibition shown in the final stage of the reaction, when every unoxidised glucose residue has both nearest neighbours in the oxidised state.

This was done by writing X_L and X_R for the reactivities of the unoxidised residues lying immediately to the left and right, respectively, of the oxidised residues, all chains being scanned in the same direction. If these reactivities are expressed as fractions of the reactivity of uninhibited glucose residues, then $X_L + X_R = (2-P)$. Furthermore, provided that X_L and X_R do not change as reaction proceeds, it is possible to write $R = (X_L)(X_R)$, where R is the ratio of the final to the initial rate of oxidation.

These two equations could then be solved with the measured values of P and R. The value of R was known with reasonable accuracy, and was 0.040 ± 0.005 (Figs. 3 and 4). Together with the estimated value of P, this gave $X_L = 0.07 \pm 0.03$, and $X_R = 0.70 \pm 0.30$.

DISCUSSION

Significance of the kinetic data. No part of the deviation from second-order kinetics can be attributed to inhibition by reduction products of periodate. Furthermore, the fact that second-order kinetics are closely obeyed during the last 36 – 38 % of the reaction, proves that the deviation observed initially cannot be attributed to some unexpected rate-law, in which the rate is proportional to some power of the periodate concentration other than unity. The departure from second-order kinetics is therefore solely due to inhibition of the oxidation of unoxidised glucose residues by oxidised glucose residues.

The fact that the rate becomes constant when contiguous, unoxidised residues are no longer present in the chains suggests that oxidised residues inhibit the oxidation of adjacent, unoxidised residues only, and, since the inhibition is completely removed by reduction with borohydride, it must be caused by some kind of interaction between the aldehyde groups of oxidised residues and the hydroxyl groups on unoxidised neighbours, leading to a reduction in their reactivity.

The inhibitory reaction is non-competitive; that is to say, the amount of inhibition is independent of changes in the periodate concentration occurring under the conditions of the experiment. With initial concentrations of periodate much higher than those used in the present work, it is possible that the inhibition would be demonstrably competitive, but the reaction would then be so fast that special techniques would be required to study it.

The degree of oxidation at which the rate becomes constant corresponds closely to that calculated on the assumption that an oxidised unit diminishes the reactivity of only one adjacent, unoxidised unit, and that this effect is unidirectional along the chain. It would, however, require a much more detailed theoretical analysis than that undertaken here to justify the conclusion that there is not a smaller amount of inhibition of the other nearest neighbour. The degree of oxidation corresponding to maximal inhibition may be insensitive to a relatively small effect upon the second neighbour.

The experiment illustrated in Figs. 5 and 6 did not provide any more precise information on this question, and merely supported the conclusion that the inhibitory effect is considerably greater upon one neighbour than upon the other. The low accuracy in the estimated value of P can be understood when it is considered that, when α is, for example, 0.1, the rate of oxidation would be 80 % of the initial rate for $P=1$, and 75 % for $P=1.5$. It is impossible to distinguish between such small differences in rate by ordinary titrimetric methods of analysis.

This difficulty points to the need for an advance in theory, or in computer simulation-techniques, that would permit the complete reaction curve to be analysed in integral form. With the help of a colleague, Dr. Smidsrød, work on this problem is now in progress, and should allow a more detailed analysis of the present data to be reported later.

Mechanistic interpretation. The extensive studies that have been carried out in other laboratories on the structure of periodate oxycellulose and periodate oxystarch have been comprehensively reviewed by Guthrie.[11] Most of this work has been done on fully or highly oxidised ("dialdehyde") starch

and cellulose, and it has shown that, in these materials, one of the aldehyde groups is much more reactive than the other in a wide range of reactions, including oxidation, reduction, condensation, and substitution.

It has been generally assumed that this is primarily because the other aldehyde group preferentially forms a hemiacetal with the primary hydroxyl group in the same unit, and this interpretation is strongly supported by the isolation and characterisation of such hemiacetals as products of the periodate oxidation of simple glycosides.[4-6]

Attempts to determine which of the two aldehyde groups preferentially forms the intra-residue hemiacetal have led to conflicting reports,[11] but the view that it is the aldehyde group at position 2 that does this, and the one at position 3 that is the more reactive, appears to have the stronger experimental support.

It seems reasonable to assume that the aldehyde group that is the more reactive in fully oxidised starch is the same one that, in partially oxidised amylose, inhibits the oxidation of a neighbouring, unoxidised glucose residue. In the light of the evidence obtained earlier with alginate[1,2] and xylan,[3] it is suggested that this inhibition is due to inter-residue hemiacetal formation. A methylation study of partially oxidised amylose, similar to those carried out on the previously studied substrates,[2,3] is now in progress and will, it is hoped, both confirm the presence of the inter-residue hemiacetals and indicate which aldehyde group is mainly implicated in these.

Since the oxidation of amylose, like that of xylan,[3] proceeds ultimately to completion, it must again be assumed that an equilibrium exists between the inter-residue hemiacetal and other possible forms, including the free and solvated aldehydic forms, hemialdal forms, and intra-residue hemiacetal forms. A good test of this hypothesis would be to study the kinetics of oxidation in some solvent other than water, in which the positions of the equilibria could be expected to be different because of a change in the capacity of the solvent to solvate the free aldehydic form.

Such a study has, in fact, already been effectively made by Zitko and Bishop,[12] who, in connection with an investigation of the oxidation of polysaccharides by lead tetra-acetate in dimethyl sulphoxide, published numerous reaction curves for many different polysaccharides, including amylose and xylan. Many of these curves exhibited such an extreme deviation from second-order kinetics that the authors were prompted to extrapolate the very slow, secondary phase of the oxidation to zero time, implying the existence of a well-defined, anomalous oxidation-limit.

These observations subsequently led Yu and Bishop[13] to suspect not the different oxidant, but the solvent, and in an investigation of the oxidation of simple glycosides and dextran by periodic acid in dimethyl sulphoxide, they elegantly demonstrated that the "anomalous" results were due to strong stabilisation by the solvent of intra-residue hemiacetals. Thus, methyl pentopyranosides and a 1,6-linked dextran consumed only one molar equivalent of oxidant, after which one of the liberated aldehyde groups formed a stable, 6-membered hemiacetal ring with the remaining hydroxyl group, preventing further oxidation.

In the light of Yu and Bishop's findings, and of the evidence now available [1-3] for the existence of inter-residue hemiacetals, it is possible to attempt a further interpretation of the valuable data published by Zitko and Bishop.[12] In Fig. 5 of their paper,[12] a curve is shown for the oxidation of amylose, from which it is apparent that the final rate of oxidation must be slower than the initial rate by several orders of magnitude. The degree of oxidation corresponding to completion of the initial, rapid phase of the oxidation can be measured from the authors' own extrapolation of the slow phase, and it is very close to 0.65.

In Fig. 1 of the same paper,[12] Zitko and Bishop have shown the oxidation of a xylan in both dimethyl sulphoxide and water, and in the former solvent, the ratio of the initial to the final rate is so large that the inter-residue hemiacetal formation must be regarded as virtually complete. In water, it is only about 85 % complete.[3] The limiting degree of oxidation of Zitko and Bishop's xylan was about 0.48, and corresponds to that calculated, on the assumption of complete inhibition of both nearest neighbours of oxidised units, for chains having a degree of polymerisation of 75 units.[3]

In Fig. 6 of their paper,[12] Zitko and Bishop showed some results obtained with carboxyl-reduced pectins in dimethyl sulphoxide. Again, the ratios of the initial to the final rates were so large that the anomalous oxidation-limits could reasonably be regarded as absolute. A substrate containing only 0.7 % of galacturonic-acid residues, and which was therefore almost a pure, α-1,4-linked galactan, showed an oxidation-limit of about 0.65, while materials with progressively increasing contents of hexuronic acid showed progressively decreasing oxidation-limits. The exceptionally low oxidation-limit (0.2) reported for unreduced pectates cannot, however, be explained in terms of inter-residue hemiacetal formation, and may, as the authors recognised, have been due to incomplete dissolution of these materials in the solvent.

Conclusions. The suggestion made earlier,[2,3] that the extreme departure from second-order kinetics shown by alginate and xylan is due to the absence of a hydroxyl group at position 6, is only partly correct. This is because the effect of such a hydroxyl group is mainly upon only one of the two equilibria that are set up between the two aldehyde groups and their corresponding, inter-residue hemiacetal forms.

Such a unilateral withdrawal of protection raises the degree of oxidation corresponding to maximal inhibition (for infinitely long chains, from 0.435 to 0.635), but still permits a large difference between the initial and final rates of oxidation. It does, nevertheless, decrease this difference to some extent, because it removes the cumulative effect of double protection [3] of a single, unoxidised unit by two oxidised neighbours.

It is not very meaningful to distinguish sharply between 1,4-linked polysaccharides that oxidise "normally" and those that do not. Under the conditions that were chosen for study, the final rate was 4 % of the initial rate for amylose, for xylan [3] it was 2 %, and for alginate it was not measurable, but was quite possibly 0.1 %. A small change in the positions of the equilibria, such as could be brought about by a change in solvent or temperature, could easily make these differences smaller still.

The distinction is therefore subjective, and depends upon how long the experimenter continues to observe the consumption of oxidant. When the polysaccharide is liable to overoxidation, the rate of the last stages of Malapradian oxidation may be comparable to that of the overoxidation reaction, and it may be impossible to detect any oxidation "limit" other than the "anomalous" one corresponding to the establishment of maximal hemiacetal formation.

All such difficulties in the use of periodate oxidation for the structural analysis of polysaccharides can, however, be easily overcome by the use of borohydride reduction in the way proposed earlier,[2] and moreover, the ability to recognise inter-residue hemiacetal formation when it occurs could well increase the amount of structural information obtainable from a periodate-oxidation experiment.

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