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THE OXIDATION OF SOME POLYSACCHARIDES BY THE HYDROXYL RADICAL: AN E.S.R. INVESTIGATION*

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ABSTRACT

E.s.r. experiments employing a flow system in conjunction with the Ti^{III}–H_2O_2 couple show that dextrans react with the hydroxyl radical (HO·) *via* indiscriminate attack (except that abstraction of hydrogen atoms from carbons which are both linked by glycosidic bonds and included in the pyranose ring may be inhibited, possibly for steric reasons). Acid- and base-catalysed transformations of first-formed radicals have been demonstrated; the suggestion that such reactions can lead to glycosidic cleavage is supported by viscosity studies which confirm the pH-dependence of radical-initiated degradation. For galacturonan and related compounds, e.s.r. results indicate that reaction with HO· proceeds preferentially *via* abstraction of the hydrogen on the carbon adjacent to the carboxyl group. The crucial step in the subsequent degradation pathway probably involves a pH-independent rearrangement.

INTRODUCTION

The *in vivo* reaction of radicals with polysaccharides can have serious consequences: for example, a loss of integrity of structural carbohydrates (*e.g.*, hyaluronic acid[2]) results from their exposure to radicals, and radiation can cause[3] radical-induced cleavage of the sugar phosphate backbone of DNA. Radical oxidation of certain plant polysaccharides is also important, since it can alter the solubility, solution viscosity, and gel-forming properties of polymers employed in the food industry.

The hydroxyl radical (HO·) has been implicated in many of these processes. The methods employed to generate this radical for mechanistic investigation include radiolysis[4], the use of Fenton's reagent (Fe^{II}–H_2O_2)[5], and systems based on oxygen, ascorbate, and metal ions (see, for example, ref. 6). All of these methods lead to depolymerisation of the polysaccharide (as shown by a fall in solution viscosity) and an increase in the number of oxidised (*i.e.*, carbonyl and carboxyl)

*Radical Reactions of Carbohydrates, Part 5. For Part 4, see ref. 1.
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functional groups (see, for example, ref. 5). The extent of degradation generally increases with the concentration of initiating species and with temperature, and decreases if radical scavengers are added. Little *direct* evidence has been obtained, however, concerning the precise nature of the radical species involved in the degradations; for example, the few reported applications of e.s.r. spectroscopy to the study of the reaction of HO· with polymeric carbohydrates have usually given spectra too complex to analyse[7] (but see also ref. 8).

We now report the results of an e.s.r. investigation of the oxidation of some polysaccharides by the hydroxyl radical. The study employed a flow system and the Ti^{III}–H_2O_2 couple (reaction *1*), and is an extension of previous investigations involving mono- and di-saccharides[1,9–11]. It was anticipated that analysis of spectra from polysaccharides (as with some disaccharides[10]) would be most readily achieved for substrates for which the spectra from the constituent monosaccharides (*e.g.*, D-glucose) can be fully rationalised[9–11]*. We have therefore studied dextrans, since these are composed of D-glucosyl residues, are freely soluble in water, and are available in well-defined ranges of molecular weight, and galacturonan and its derivatives (pectins), which are examples of acidic polysaccharides used in the food industry because of their gel-forming properties. Experiments were performed in the pH range 1–10 in an attempt to delineate possible acid- and base-catalysed transformations of the radicals formed by HO· attack.

$$Ti^{III} + H_2O_2 \rightarrow Ti^{IV} + HO\cdot + HO^- \qquad (1)$$

Viscosity studies of solutions of the polymers were carried out before and after reaction with HO·, in an attempt to elucidate the extent of any radical-induced degradation and the possible effects of such factors as the concentration of reagents and the pH.

RESULTS AND DISCUSSION

E.s.r. studies. — (*a*) *The reaction of HO· with dextran.* Dextrans, which are (1→6)-α-D-glucans containing short side-chains attached by α-(1→3) linkages, are available in a pure state in fractions having a low heterogeneity index ($\bar{M}_w/\bar{M}_n \sim 2$) and weight-average molecular weights ranging from 10,000 (T-10) to 2,000,000 (T-2000). Samples in the (T-10)–(T-500) range were studied.

The e.s.r. spectra obtained from the reaction of HO· [generated by means of the Ti^{III}–H_2O_2 couple (reaction *1*)]** with dextran T-10 were closely similar to those obtained[10] from α-D-glucopyranose under the same conditions, except that the signals from the polysaccharide had a greater line-width than those from the

*In an e.s.r. examination[7] of the reaction of HO· with starch, dextrin, and maltose, the similarity of the (unanalysed) spectra obtained was attributed to the common structural feature of the substrates

**For reactions carried out at pH >2, it was necessary to add EDTA to sequester titanium.

TABLE I

E.S.R PARAMETERS OF RADICALS FORMED FROM THE REACTION OF HO· WITH DEXTRAN T-10 (AT pH 4)

Position of abstraction[a]	*Hyperfine splittings*[b] *(mT)*		g-*Value*[c]
	a *(α-H)*	a *(β-H)*	
C-2		1.13(1), 3.03(1)	2.0031
C-3		2.80(1), 2.99(1)	2.0031
C-4		4.83(1 +1)[d]	2.0031
C-5		3.3(1)[e,f], 1.0(1)[e,g], 0.7(1)[e,g]	2.0031
C-6	1.85(1)	0.56(1)	2.0032

[a]No distinction could be made between radicals formed at equivalent positions in the polymer chains. [b]±0.01 mT, unless otherwise stated. [c]±0.0001. [d]Only the sum of the two β-proton couplings could be measured for this species. [e]Peaks largely obscured; only approximate measurements could be made, ±0 1 mT. [f]From H-4. [g]From the diastereotopic C-6 protons.

monomer [reflecting, at least in part, the increased size and slower rotational correlation time, τ_c, (and a corresponding contribution to line-broadening from anisotropic hyperfine interactions[12]) for the larger radical]. Presumably, a contribution to broadening also arises from the fact that radicals formed at different, but equivalent, positions in the polymer chains [*e.g.*, at C-2 in the various D-glucosyl residues) should have very similar but not identical spectra. Table I contains the spectral

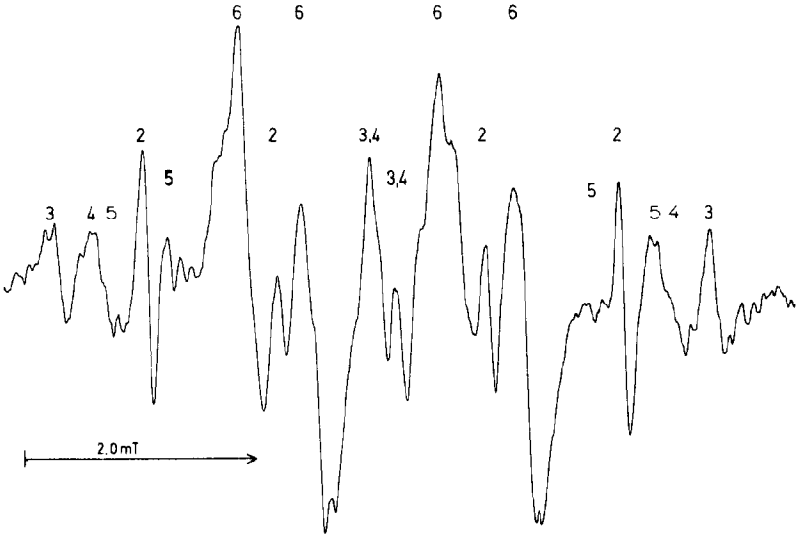
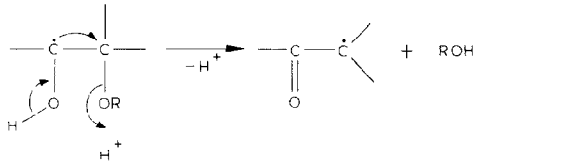


Fig. 1. E.s.r. spectrum obtained from the reaction of HO· with dextran T-10 at pH 4 (positions of hydrogens abstracted are indicated).

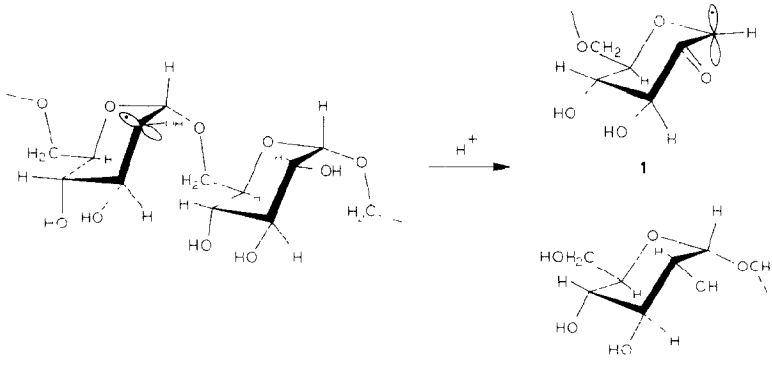
parameters of the radicals detected from dextran T-10 at pH 4, and a typical spectrum is shown in Fig. 1.

Radicals formed at all positions except C-1 were identified and assigned by comparison with the spectra from α-D-glucose: the parameters indicate that the polymer-derived radicals must, like those from D-glucose, be present predominantly in chair conformations (with, for example, effective hyperconjugative interaction and hence large β-splittings of ~3 mT from *axial* β-protons, which subtend a small angle with the orbital of the unpaired electron). The C-1-derived species was either not formed in a concentration high enough to be detected (possibly for steric reasons), or was rapidly removed by oxidation (as might be expected[13] for a dioxygen-conjugated radical). Spectra recorded under conditions of high modulation show that the peaks assigned to the radical formed by abstraction of hydrogen from C-6 are approximately twice as intense as those from each of the other species. This is indicative of relatively unselective attack by HO· and suggests that, unlike C-1, C-6 is not hindered by the glycosidic bond.

As the pH was lowered, significant changes were observed. Signals from all the first-formed radicals were removed by pH 0.5, those from the C-6-derived species being the last to disappear. Two new sets of signals could be discerned as long as the experiments were conducted in the absence of EDTA*: one had g 2.0049, a(1 H) 1.42 and a(2 H) 0.51 mT, and the other g 2.0045, a(1 H) 1.81 and a(1 H) 3.76 mT, with further long-range couplings that could not be measured unambiguously. Both signals are associated with carbonyl-conjugated radicals, presumably derived *via* the acid-catalysed rearrangement of the first-formed species (reaction *2*, see also refs. 9 and 10). By comparison with spectra obtained from α-D-



(2)



(3)

*Carbonyl-conjugated radicals are rapidly reduced[10] by Ti^{III}–EDTA.

glucose at pH 1, the former is assigned to the radical (**1**) derived from the C-2 primary species (reaction *3*), whereas the latter is derived by rearrangement of either (or both) the C-3 or C-4 radicals. Reaction *3* represents a pathway to radical-induced glycosidic cleavage in polysaccharides (as noted previously[10] for such related compounds as methyl α-D-glucopyranoside and trehalose). Similarly, rearrangement of the C-4-derived radical may lead to cleavage of chain-branches starting from C-3. Radicals formed by fragmentation of the polymer chain in this way will be smaller, and hence more mobile, than their precursors, and it is anticipated that their spectra would have narrower peaks. However, no reliable information about the bulk of the rearranged species could be obtained, because high modulation had to be employed in order to obtain spectra of reasonable intensity.

When the pH was raised from ~4, the signals from the first-formed radicals were again removed, but no new signals were observed. It is believed that primary radicals undergo *base*-catalysed transformations, analogous to reaction *2*, to give carbonyl-conjugated species, which, as at low pH in the presence of EDTA, are rapidly reduced[11] by Ti^{III}. Semidiones [RC(O·) = C(O^-)H] akin to those previously identified[11] were not detected, but these would only be expected to result from the reducing-sugar residue in the dextran chains, so that only a low concentration of, and very weak signals from, such species would be anticipated.

Dextrans T-70, T-110, T-250, and T-500 were also studied. Each fraction gave e.s.r. spectra which were virtually identical to those from dextran T-10; the only significant difference was an increase in line-width with increasing molecular weight of the fraction, which presumably reflects the increase in rotational correlation time with molecular size.

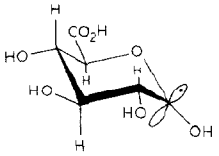
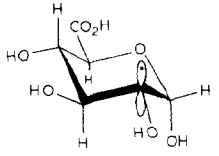
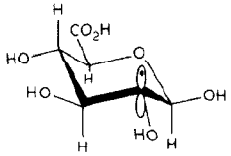
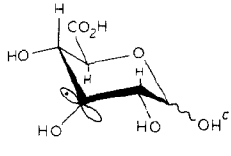
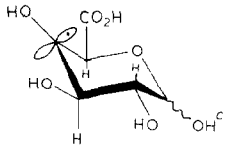
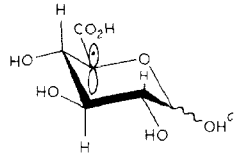
(*b*) *The oxidation of pectins and some uronic acids by the hydroxyl radical.* Similar experiments were carried out with pectins [(1→4)-α-D-galactopyranuronans having some residues present as methyl esters], galacturonan, α-D-galacturonic acid, and D-glucuronic acid.

(*i*) D-*Glucuronic acid.* The e.s.r. spectrum (Fig. 2) was obtained at pH ~4 from the reaction of HO· with a solution of D-glucuronic acid (recorded ~30 min after the dissolution of sodium β-D-glucuronate). One set of signals (with peaks marked □) has *g* 2.0031, *a*(1 H) 2.950, *a*(1 H) 1.350, and *a*(1 H) 0.170 mT, parameters which are closely similar to those of the C-2-derived radical from α-D-glucose, rather than any radical from the β anomer[10]. This finding suggests that the species observed is the α-C-2 radical (**3**, see Table II). The mutarotation of a freshly prepared solution of sodium β-D-glucuronate was virtually complete within 30 min of dissolution at 20°: ^{13}C-n.m.r. spectroscopy confirmed the rapid establishment of equilibrium and showed that, at 25° in ^2H_2O, the proportions of α- and β-pyranoses at equilibrium are 43% and 57%, respectively. The observed e.s.r. spectra must therefore be analysed in terms of the reaction of an *ca.*, 1:1 mixture of the two pyranose forms.

By comparison of Fig. 2 with spectra obtained from α- and β-D-glucose under similar conditions, signals may be assigned to radicals formed by abstraction of hy-

TABLE II

E S R. PARAMETERS OF RADICALS DERIVED FROM REACTION OF HO· WITH D-GLUCURONIC ACID AT pH 4

	Hyperfine splittings[a,b] *(mT)*	
	a *(β-H)*	a *(other)*
 2	2.070(1)	0.180(1) 0.085(1)
 3	2.950(1) 1.350(1)	0.170(1)
 4	3.000(1) 2.515(1)	
 5	2.92(1)[d] 2.72(1)[d]	
 6	2.09(1)[d] 1.20(1)[d]	
 7	1.425(1)	

[a] ±0.005 mT, unless otherwise stated. [b] All radicals have *g* 2.0031 ±0.0001, except for the C-5-derived species (7) which has *g* 2.0049 ±0.0001. [c] No distinction between reaction of the two anomeric forms could be made. [d] ±0.01 mT

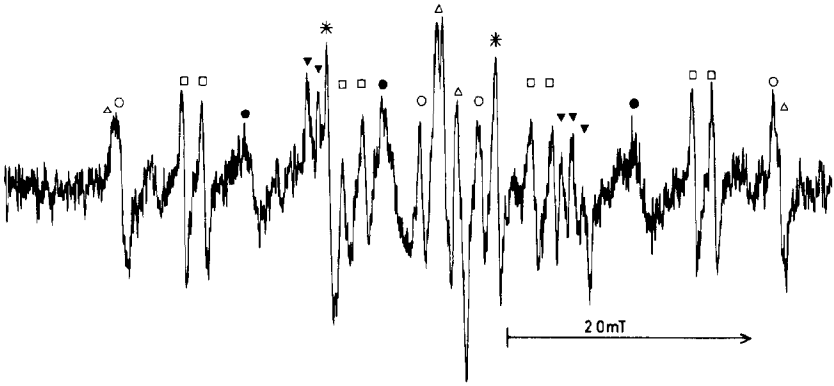
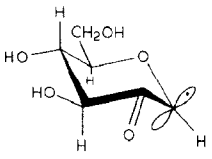


Fig. 2. E.s.r. spectrum obtained from HO· and D-glucuronic acid at pH 4. Signals are assigned to radicals formed by hydrogen-atom abstraction at positions as follows: ▼, C-1; □, C-2α; ○, C-2β; △, C-3α,β; ●, C-4α,β; *, C-5α,β.

drogen from C-1 and from C-2 in both anomers of D-glucuronic acid (**2–4**, respectively; Table II). A C-3-derived radical (**5**) was also recognised, although it is not clear whether this is from both, or only one, of the anomeric forms (the line-width of the peaks suggests the former, and that two radicals with closely similar spectra are present). The two other potential first-formed radicals [*i.e.*, the C-4 and C-5-derived species (**6** and **7**)] could not be detected by reference to the spectra from D-glucose. However, oxidation of D-glucuronic acid at C-4 should give a radical (**6**) identical with that formed by oxidation of D-galacturonic acid at the same position, and comparison of the spectra from the two compounds (see below) shows that certain peaks (marked in Fig. 2) are also present in the spectrum from D-galacturonic acid (they are slightly broader in the spectra from D-glucuronic acid, possibly because this acid is a mixture of two anomers, whereas the D-galacturonic acid is predominantly α). The splittings of ~2.1 and 1.2 mT for the formally axial β-protons in this spectrum are remarkably low and are indicative (*cf.* ref. 9) of dihedral angles of ~45° and 55°. This suggests that the ring is flattened about C-4 in **6**, a feature attributed to the effect of the adjacent carboxyl group (see below).

The only set of signals left to be assigned in Fig. 2, namely, the doublet with a(1 H) 1.425 mT and g 2.0049 (marked *), is attributed to the remaining first-formed radical **7**, on the basis of its g-value (which is significantly greater than those associated with radicals possessing a single α-oxygen substituent), despite the fact that its value is even higher than might be expected[14] for a radical of this type and is closer to that of species which are conjugated to both oxy- and keto-functions (*e.g.*, radical **8** from α-D-glucose[10]). A possible explanation is that intramolecular hydrogen-bonding between the acid group and HO-4 occurs; this would be expected to increase the effective −M effect of the carboxyl group (see **9**) and the extent of delocalisation of the unpaired electron onto carbonyl oxygen. Such hydrogen bonding may also account for the low magnitude of the β-proton coupling in

this radical and in the C-4-derived species (**6**), since the pyranose ring is then constrained to adopt a more nearly planar conformation about the C-4 and C-5 positions. Experiments designed to demonstrate that intramolecular hydrogen-bonding occurs (such as the addition of species to the system which may disrupt the hydrogen bonds, including Mg^{2+} and Al^{3+}) were inconclusive.



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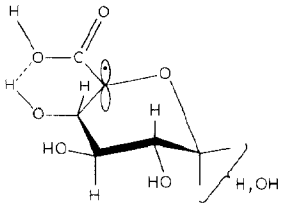

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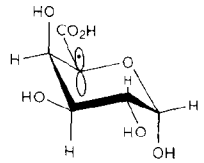
When the pH was lowered, signals from all the first-formed radicals, with the exception of the C-5-derived species (**7**), disappeared. The order of removal was **3**, then **2**, then **5** and **4**, and finally **6**; these observations are consistent with rearrangement of these radicals *via* reaction *2* and the stereoelectronic requirement (as observed[9] for radicals derived from *myo*-inositol) that the reaction occurs most rapidly when the leaving group can eclipse the orbital of the unpaired electron. The proposed hydrogen bonding in **6** may account for rearrangement of this radical being particularly slow, as the β-OH group may be inhibited from achieving a position where it is in the same plane as the orbital of the unpaired electron. The reluctance of the carboxyl-conjugated radical **7** to rearrange is probably due to delocalisation of the unpaired electron in this species.

At low pH, only very weak signals (which could not be unambiguously analysed) were observed to replace those from the first-formed radicals. It may be significant that D-glucuronic acid exists in solution in equilibrium with both anomers of the 6,3-lactone; the latter are more prevalent[15] at low pH, so that a complex mixture of signals would be expected.

(*ii*) α-D-*Galacturonic acid.* The e.s.r. spectra from the reaction of α-D-galactopyranuronic acid with HO· were dependent upon the age of the solution, evidently as a result of mutarotation. Fig. 3 shows the spectrum obtained at pH 4 from a solution that had been kept for ~30 min at room temperature. The dominant doublet has g 2.0037, which is typical[14] of a radical formed by abstraction of hydrogen from a position adjacent to both oxy- and carboxyl-functions; assignment is made to the C-5-derived radical **10***. Compared with signals from fresh solutions, the intensity of the spectrum from **10** has slightly diminished and a much weaker set of similar signals (marked X) has appeared. The new signals are presumably due to the radical formed at C-5 in the β anomer, the extra splitting being derived from

*Intramolecular hydrogen-bonding analogous to that suggested for the C-5-derived radical from D-glucuronic acid cannot occur in **10**, and an abnormal g-value is therefore not to be expected.

the proton at C-1, which is in the same plane as the orbital of the unpaired electron (similar large couplings across oxygen are known[10]). Together, the two carboxyl-conjugated radicals appear to account for ~50% of the signals in Fig. 3. Now it appears[16] that radicals formed by α-H abstraction from α-hydroxy acids have slightly lower rates of bimolecular termination than simple hydroxy-conjugated radicals (an effect that may be either electronic or steric in origin and becomes much more pronounced at higher pH, when the radicals are deprotonated, and for polyfunctional species); the dominance here of the carboxyl-conjugated species appears to be too great to be accounted for simply in terms of their being relatively long-lived and it seems likely that there is some preference for reaction at C-5. This might be a result of the stabilisation of the transition state preceding radical formation by the combined stereoelectronic effects of the adjacent ring-oxygen atom (*cf.* selectivity in the reaction of furanose sugars[1]), the carboxyl group, and the β-hydroxyl group which is in the same plane as the incipient orbital of the unpaired electron.



10

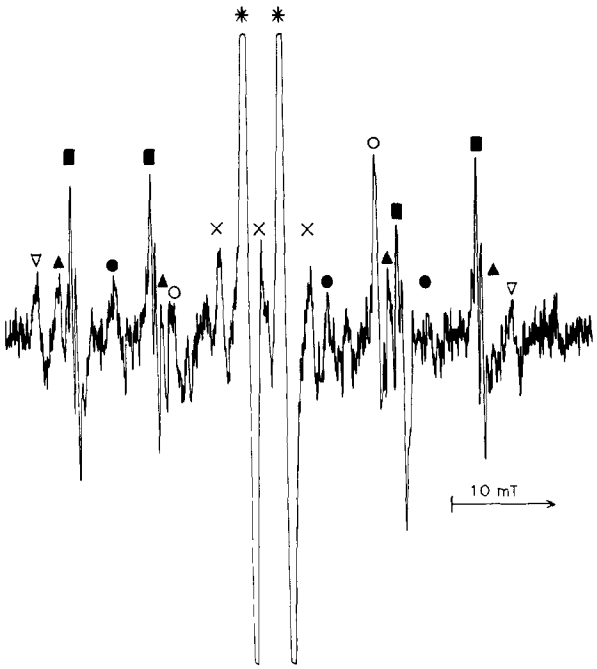
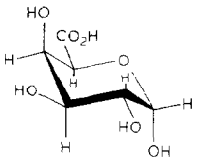
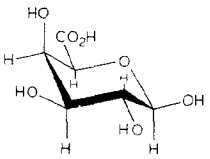


Fig. 3. E.s.r. spectrum obtained from HO· and D-galacturonic acid at pH 4. Position of hydrogen-abstraction, α anomer: ○, C-1; ■, C-2; ▲, C-3; ●, C-4; *, C-5; β anomer: ▽, C-2; ×, C-5.

TABLE III

E S R PARAMETERS OF RADICALS FORMED FROM THE REACTION OF HO· WITH D-GALACTURONIC ACID AT pH ~4

	Position of abstraction	*Hyperfine splittings*[a,b] *(mT)*	
		a *(β-H)*	a *(other)*
 α Anomer	C-1	2.05(1)[c]	
	C-2 [d]	0.785(1) 3.243(1)	0.045(3)
	C-3 [d]	3.15(1)[c] 1.12(1)[c]	
	C-4	2.09(1)[c] 1.20(1)[c]	
	C-5	0.360(1)	
 β Anomer	[f] C-2 C-5	4.750(1 + 1)[g] 0.480(1)	0.405(1)

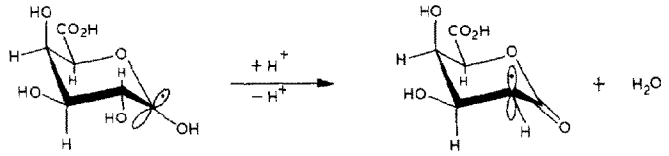
[a] ±0.005 mT, unless otherwise stated. [b] All radicals have *g* 2.0031 ±0.0001, except for the C-5-derived species which have *g* 2.0037 ±0.0001. [c] ±0.01 mT, peaks partially obscured. [d] The spectra of these radicals cannot be unambiguously distinguished. [e] Further long-range couplings which could not be measured. [f] Signals from other radicals from the β anomer could not be unambiguously detected. [g] Only the outer peaks of this spectrum could be clearly discerned.

Several other signals also present are assigned (by comparison with the spectra from related compounds, and from the typical angular-dependence of β-splitting constants) to the radicals formed by abstraction of hydrogen from the remaining positions in the carbon skeleton (see Table III). The C-4-derived radical is identical to the corresponding species derived from glucuronic acid.

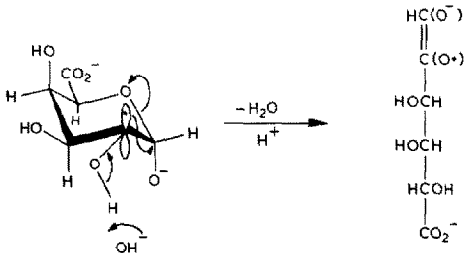
When the pH was lowered, the signals from all of the first-formed radicals disappeared, except those of the radicals derived by attack at C-5, which may well be stabilised by delocalisation of the unpaired electron as discussed above. Several new signals were detected; these had *g*-values typical of carbonyl-conjugated radicals, presumably formed *via* acid-catalysed rearrangement of the primary species (reaction *2*). One had *a*(1 H) 2.015, *a*(1 H) 4.110, *a*(1 H) 0.065 mT, and *g* 2.0035, which is characteristic of the radical **11** formed by rearrangement of the C-1-derived species (reaction *4*). Spectra from two other carbonyl-conjugated radicals had *a*(1 H) 1.880, *a*(1 H) 3.170, *a*(1 H) 0.100 mT, and *g* 2.0045, and *a*(1 H) 1.820, *a*(1 H)

3.930, a(1 H) 0.100 mT, and g 2.0045. Assignment to the radicals formed by rearrangement of the C-3- and C-4-derived species is suggested, though no unambiguous distinction could be made.

Oxidation of D-galacturonic acid (and D-glucuronic acid) at high pH led to the detection of a ring-opened semidione in each case, evidently *via* a base-catalysed reaction of the first-formed C-2-derived species (see, for example, reaction 5 for D-galacturonic acid, and ref. 11).

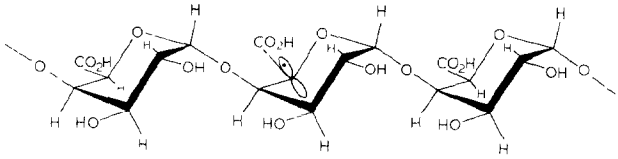


11 (4)



(5)

(*iii*) *Galacturonan.* Spectra obtained from galacturonan and HO· at pH ~4 were dominated by an intense doublet with g 2.0037 and a(1 H) 0.13 mT. This is assigned to the radical(s) formed by abstraction of hydrogen from the carbon adjacent to the carboxyl group (**12**) (*cf.* **10** from α-D-galacturonic acid). Double integration of the spectrum showed that **12** accounts for ~80% of the total radical concentration. As with the observed dominance in the spectra from D-galacturonic acid of the carboxyl-conjugated radicals derived from each of the anomeric forms, this probably reflects not only the longevity of these radicals but also a preference (of stereoelectronic origin) for their formation. The enhanced intensity of the signals from **12** compared to those of the corresponding species from the monomer presumably also reflects steric hindrance at other sites in the polymer. Under conditions of high modulation, other weak signals could be discerned. Two, with g 2.0031, a(1 H) 3.36 and a(1 H) 1.20 mT, and g 2.0031, a(1 H) 3.30, a(1 H) 0.81, and a(1 H) 0.23 mT, are characteristic of radicals possessing one axial and one equatorial β-proton. Assignment is therefore made to the C-2- and C-3-derived species, although no distinction between these two is possible (*cf.* the analogous radicals from α-D-galacturonic acid, Table III). The two remaining first-formed radicals (*i.e.*, those formed by attack at C-1 and C-4) could not be identified.



12

At high pH, the signals from **12** were slightly reduced in intensity, but there was no evidence for the occurrence of any base-catalysed rearrangement. In contrast, the other first-formed radicals disappeared (though no trace of any new species could be discerned): for these radicals, loss of a β-OH^- group (*cf.* reaction *2*) presumably occurs, to give carbonyl-conjugated radicals which are rapidly reduced[10] by Ti^{III}–EDTA.

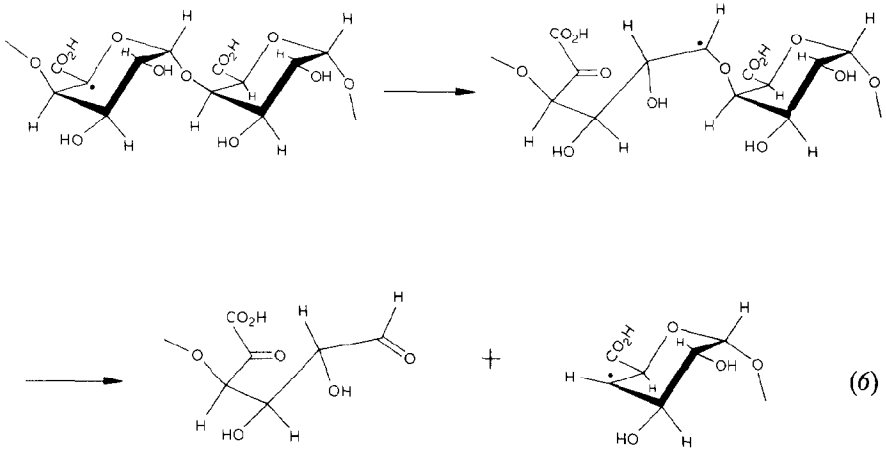
When the pH was lowered to ~3, solutions of the polymer gelled, which prevented any information being obtained on acid-catalysed transformations of the first-formed radicals.

(*iv*) *Methyl esters of galacturonan.* The spectra obtained from the reaction of HO· with some methyl esters of galacturonan (differing in the degree of esterification) were very similar to those obtained from galacturonan and are analysed in terms of dominant signals from carboxyl-conjugated radicals. No evidence was obtained for attack by HO· on the methyl groups; this is not entirely unexpected, since such sites are less reactive to attack by the electrophilic hydroxyl-radical than those adjacent to simple oxy-functions, and, further, the maximum degree of esterification was only 40%. At high and low pH, the esters appeared to react exactly as the free acid, except that the higher the degree of esterification, the lower the pH at which gelation occurred: all had gelled by pH ~2.

(*c*) *Summary.* The e.s.r. results suggest that neutral polysaccharides react with HO· *via* indiscriminate attack (as with monosaccharides), with the exception that abstraction of hydrogen atoms on carbons in the pyranose ring and linked by glycosidic bonds may be inhibited (probably for steric reasons). The first-formed radicals rearrange on lowering or raising the pH, and at least one route which leads to glycosidic cleavage, and hence degradation, has been observed.

For the galacturonan and D-galacturonic acid itself, the spectra are dominated by signals from radicals formed by abstraction of the hydrogen from the carbon adjacent to the carboxyl group. This selectivity, which is more marked than for D-glucuronic acid, is attributed to the longevity of these radicals and a preference for attack at C-5 due to steric and to stereoelectronic factors. The carboxyl-conjugated radicals also appear to be resistant to base-catalysed transformation, and it is assumed that they are also relatively stable in acid (since the corresponding species from their separate monomer units were not observed to rearrange on lowering the pH). It may be significant that the extensive depolymerisation of glycuronans on oxidation by HO· has been rationalised[17] principally in terms of a pH-independent rearrangement of the carboxyl-conjugated radicals (reaction *6*). Though

reactions of this type have not been observed in the e.s.r. experiments described here, the favoured formation and relative stability to changes in pH of the carboxyl-conjugated radicals from the *acidic* sugars are consistent with this reaction being an important pathway in the radical-induced degradation of such substrates.



(6)

Viscosity studies. — On the basis of the e.s.r. results, radical-induced degradation of neutral (though not necessarily of acidic) polysaccharides should be most efficient at high and low pH. To confirm this, and to monitor the efficacy of the Ti^{III}–H_2O_2 system in inducing polysaccharide degradation, the viscosities of polysaccharide solutions in the presence and the absence of the Ti^{III}–H_2O_2 couple were compared. We determined the specific viscosity η_{sp} (equation 7, where t_s is the time for a fixed volume of the solution to pass through a capillary, and t_w is the time for the same volume of water to pass through the same capillary), as a function of pH at 25° for solutions of the polymer alone and for solutions to which different concentrations of titanium(III) and/or hydrogen peroxide had been added. Values of the reduced viscosity (η_{sp}/c where c is the concentration of organic substrate) were then estimated.*

$$\eta_{sp} = \frac{t_s - t_w}{t_w} \tag{7}$$

The viscosity of solutions containing polysaccharide and *both* H_2O_2 and Ti^{III} decreased significantly with time, the greatest fall occurring before the first measurement could be made (~3 min). The change, after 3 min, in η_{sp}/c for 1% solutions of dextran T-250 after treatment with different concentrations of titanium(III) at fixed concentrations of hydrogen peroxide at pH ~2 is shown in

*Ideally, intrinsic viscosities should have been taken. At low concentrations, however, the substrate does not scavenge HO·, so [η] measured is not a realistic measure of the extent of degradation.

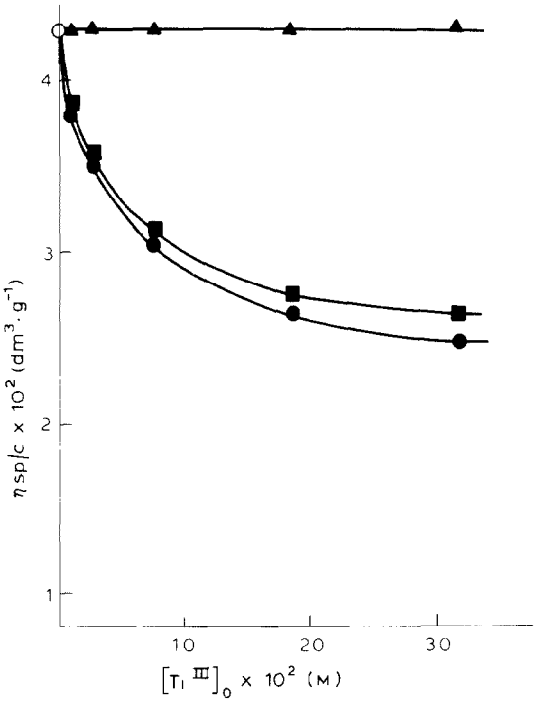


Fig. 4. Variation in reduced viscosity of a 1% (w/v) aqueous solution of dextran T-250 following reaction at 25° and pH 2 with $[Ti^{III}]_0$, at fixed concentrations of H_2O_2 (●, 1.74M; ■, 0.35M; ▲, no peroxide).

Fig. 4. Similar plots were obtained at other pH values, and in experiments where $[H_2O_2]_0$ was varied and $[Ti^{III}]_0$ kept constant. The first point to note is that solution viscosity fell significantly only when *both* reagents were present, supporting the view that this results from a radical-induced degradation*. A second feature is the approximately exponential increase in degradation with increase in the concentration of reagents, presumably because, although the extent of depolymerisation increases with increase in the concentration of the hydroxyl radical, at high concentrations of the reagents, side reactions (*e.g.*, reactions *8* and *9*) become important.

$$Ti^{III} + HO\cdot \rightarrow Ti^{IV} + HO^- \qquad (8)$$

$$H_2O_2 + HO\cdot \rightarrow HO_2\cdot + H_2O \qquad (9)$$

Fig. 5 shows the variation with pH of the reduced viscosity of oxidised solutions of dextran T-250, viscosities being expressed as a percentage of the values of unreacted solutions at the same pH. Experiments were carried out on thoroughly

*Much smaller, slower changes in η_{sp} with time were observed. These presumably reflect heterolytic depolymerisation.

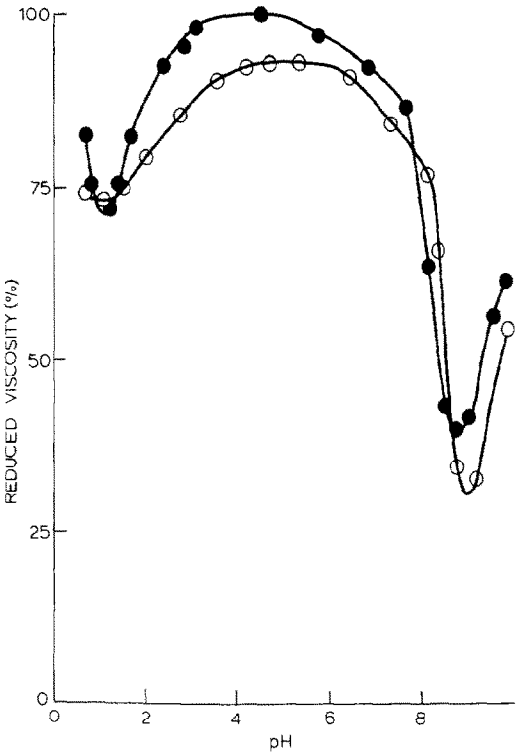


Fig. 5. Variation with pH of the reduced viscosity of a 1% (w/v) aqueous solution of dextran T-250 subject to oxidation by the Ti^{III}–H_2O_2 couple (25°; $[Ti^{III}]_0$ 16mM, $[H_2O_2]_0$ 0.88M; ● deoxygenated conditions, ○ oxygenated conditions).

deoxygenated solutions and on solutions that had been saturated with oxygen. The concentrations of reagents were chosen to produce a high degree of depolymerisation with a minimum of side reactions as discussed above.

Both plots in Fig. 5 indicate that the extent of degradation of dextran by HO· is pH-dependent. When oxygen is absent, little or no degradation is observed in the region pH 3–5 where e.s.r. experiments show the maximum concentration of first-formed radicals and where no transformations of these species can be characterised. Presumably, the first-formed species decay primarily by non-degradative pathways (*e.g.*, radical "repair" by hydrogen abstraction) in this pH region. As the pH is lowered from ~3, or raised from ~5, the extent of degradation increases markedly. This again agrees with e.s.r. findings of acid- and base-catalysed transformations of primary radicals occurring under these conditions. Basification of the solution leads to the greatest fall in viscosity, reflecting, at least in part, the fact that the rates of the base-catalysed processes are faster than those of the acid-catalysed reactions[18].

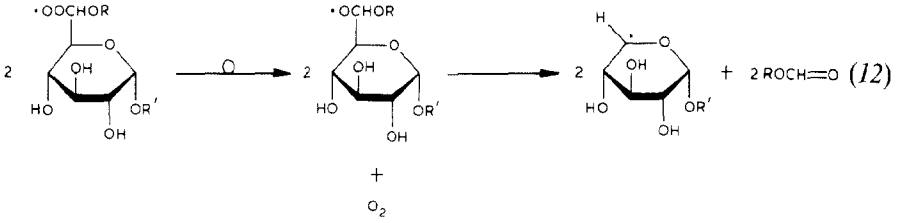
More detailed interpretation is not justified, as other factors may contribute to changes in solution viscosity. For instance, the base-catalysed decomposition of

hydrogen peroxide (reaction *10*) reduces the concentration of this reagent as well as retarding the flow of solutions through the capillary by producing oxygen. Ionisation or protonation of HO· at extremes of pH will lead to species which might be anticipated to react very differently to HO· whereas hydrolysis and oxidation of Ti^{III} is particularly prevalent at high pH. Some or all of these factors may account for the reversal at pH ~1 and ~9 in the trend of the change in viscosity.

$$2H_2O_2 \rightarrow O_2 + 2H_2O \qquad (10)$$

The results in the presence of oxygen are, strictly, not comparable to those from the experiments described above, because some Ti^{III} is oxidised to Ti^{IV} by oxygen. The concentration of HO· produced must be lower than in the absence of O_2. However, the extent of degradation is still greater; depolymerisation occurs at all pH values and again is most marked at high and low pH. Two types of reaction probably contribute to this behaviour. The first is rapid addition of oxygen to first-formed radicals to give peroxyl radicals (reaction *11*), thus preventing radical repair*. The peroxyl radicals can fragment *via* bimolecular processes which are not catalysed by acid or base[19] (reaction *12*). The second is the occurrence of the acid- or base-catalysed rearrangement of the first-formed species, as described above. The latter processes are important only when their rates are similar to, or greater than, that of reaction *11*, a condition which appears to be met outside the pH region 3–6.

(11)



(12)

A similar series of experiments was carried out with galacturonan. Gelation was minimised by employing a highly methylated polymer; even so, the solution viscosity was very dependent upon pH and upon the presence of titanium(III) ions. A further problem is that the pH falls during the oxidation, a feature also noted[20] in product studies of the reaction of HO· with smaller carbohydrate molecules, so

*In e.s.r. experiments, the presence of oxygen is sufficient to lower significantly the intensity of signals from first-formed radicals and, in some cases, to lead to the detection of weak, broad signals (g ~2.015) from peroxyl radicals.

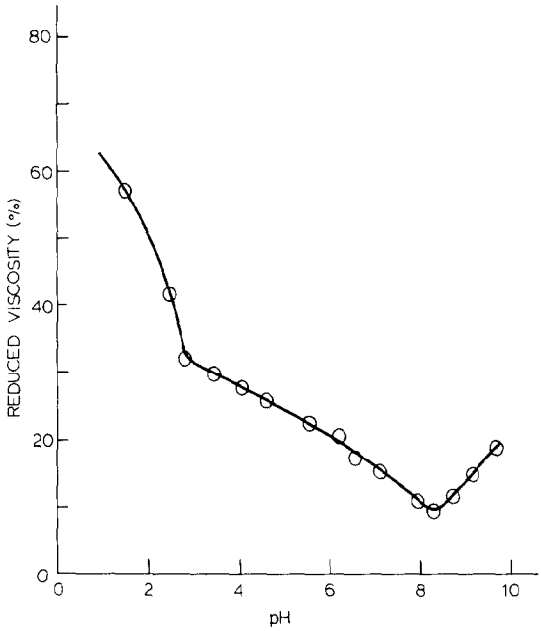


Fig. 6. Variation with pH of the reduced viscosity of a 0.5% (w/v) deoxygenated, aqueous solution of galacturonan methyl ester subject to oxidation by the Ti^{III}–H_2O_2 couple (conditions as for Fig. 5).

that reaction occurs at one pH, but the viscosity is measured at a different value. Hence, the reduced viscosity of the degraded solution was compared with that of the unreacted polymer at the final pH of the reaction. The reduced viscosity of oxidised solutions of galacturonan methyl ester is plotted against the pH of the reaction in Fig. 6. Although gelation, particularly below pH 3, renders the results less reliable than those obtained for dextran, it is clear that degradation is much more extensive than for the neutral polysaccharide, a finding supported by the observation[21] that dextran is much more resistant to radical-induced depolymerisation than glycuronans. Degradation of galacturonan methyl ester does not show the same pH-dependence as does that of dextran. Thus, for pectin, viscosity decreased only slightly as the pH of the reaction was raised. Above pH ~8, it increased again, although this may be due to an artefact of the system (see above). The results agree with those from the e.s.r. studies of the reaction of HO· with pectin where predominant attack occurs at the carbon adjacent to the carboxyl group, to give a radical apparently resistant to the effects of base.

EXPERIMENTAL

E.s.r. spectra were recorded with a Varian E-104 spectrometer equipped with X-band klystron and 100-kHz modulation. Hyperfine splittings were measured to within 0.005 mT (except where peaks were partially obscured, for which

the accuracy is estimated as ±0.01 mT or ±0.1 mT) from the spectrometer field-scan which itself was calibrated using the spectrum of Fremy's salt [a(N) 1.309 mT[22]; *g*-values were measured by comparison with the same standard[23] (*g* 2.0055).

All the rapid-flow experiments utilised a flattened, aqueous-sample cell and a mixing chamber which allowed three reagent streams to be mixed simultaneously. The flow was maintained by a Watson–Marlowe HR Flow-Inducer positioned upstream of the sample cell; the flow rate was adjusted so that the mixing time was ~50 ms. The pH of the reaction was measured to within 0.05 unit by means of a Pye–Unicam PW 9410 digital pH-meter coupled to a Russell pH Ltd. glass electrode, inserted into the effluent stream immediately above the cavity of the spectrometer. Three streams of the flow system contained, typically, (*i*) 7–8mM titanium(III) [added as aqueous 12.5% (w/v) titanium(III) chloride], (*ii*) 50mM hydrogen peroxide (added as 100-volume hydrogen peroxide), and (*iii*) the carbohydrate at the required concentration. Stream (*i*) also included conc. sulphuric acid or 0.880 ammonia solution to give the required pH; the disodium salt of EDTA (3–4 g/L) was added to sequester the titanium(III) when a pH of >2.5 was required. The solutions were made up in water deoxygenated with a nitrogen purge and were held under a nitrogen atmosphere during use.

The viscosities of polysaccharide solutions subject to oxidation by the hydroxyl radical were measured by means of an Ostwald bulb viscometer. An aliquot of a standard solution (containing EDTA) of the polysaccharide under study was equilibrated in a thermostatically controlled water-bath at 25° (and, as with the other solutions, oxygenated or deoxygenated as required). Titanium(III) chloride solution was added with rapid stirring and the pH adjusted, to that required, with conc. sulphuric acid or 0.880 ammonia solution. Distilled water was then added so that addition of an aliquot of aqueous hydrogen peroxide gave the required volume. The solution then contained carbohydrate, 0.5–1% (w/v); titanium(III) (and an equivalent concentration of EDTA), 0–32mM; and hydrogen peroxide 0–1.75M. After rapid stirring, the solution was transferred to the viscometer (which was contained in a water bath at 25°) and its viscosity determined.

All of the substrates were commercial materials of the highest available purity, and were used without further purification. Samples of pectins were obtained from Bulmer's, dextrans having M_w/M_n ~2 were obtained from Pharmacia, and dextran Grade A (mol. wt. range 200,000–275,000) was supplied by B.D.H. Ltd. Polarimetry was used to determine the configuration of the sample of sodium β-D-glucuronate employed; the $[\alpha]_{589}^{20}$ value of a freshly prepared 0.01M solution of the sugar (determined with a Perkin–Elmer 141 polarimeter) changed rapidly from ~ +11.7 to +36.3°, and it was evident that equilibrium had been established within 30–40 min. ^{13}C-N.m.r. spectra for a solution of sodium β-D-glucuronate in ^2H_2O were recorded at 25° with a JEOL FX-90 Q Fourier Transform spectrometer. Spectra were recorded at 2-min intervals after dissolution, thus allowing resonances from the β-pyranose (prominent for freshly prepared solutions) to be distinguished from those of the α-pyranose, which was evidently the only other form pre-

sent at equilibrium. Integration of the spectrum recorded after equilibrium had been established (and with a time interval of ~30 s between pulses) gave an α:β ratio of 43:57 (which, in view of the similarity of the compounds concerned, is considered to be a reliable measure of the relative concentrations of the two anomers). The spectrum contained the following signals: δ 177.75 (C-6α), 176.89 (C-6β), 96.49 (C-1β), 92.86 (C-1α), 76.83, 76.28, 74.21, and 72.55 (C-2,3,4,5 in the β anomer; no distinction can be made) and 73.36, 72.76, 72.55, and 71.95 (C-2,3,4,5 in the α anomer; again, no distinction can be made).

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