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THE PRODUCTION OF FREE RADICALS DURING THE AUTOXIDATION OF MONOSACCHARIDES BY BUFFER IONS

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ABSTRACT

The production of free radicals during the autoxidation of simple monosaccharides at 37° has been studied by the electron spin resonance (e.s.r.) technique of spin trapping. In the presence of the spin trap 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), monosaccharides undergoing autoxidation produced hydroxyl and 1-hydroxyalkyl radical-derived spin adducts, indicating that hydroxyl and hydroxyalkyl free-radicals are involved in the autoxidation of monosaccharides. The pH profile for the production of free radicals from monosaccharides undergoing autoxidation revealed the formation of both hydroxyl and hydroxyalkyl radicals at relatively high pH, whereas at low pH, only the formation of hydroxyalkyl radicals was observed; the transition between these routes for the production of free radicals occurred at pH 8.0–8.5. Glycolaldehyde, glyceraldehyde, dihydroxyacetone, and erythrose are relatively rapidly enolised (to an ene-diol) and autoxidised with the concomitant production of free radicals. Ribose and glucose enolise and autoxidise very slowly without detectable production of free radicals. A comparison of the pH profiles of the rates of enolisation and the pH dependence of the production of free radicals from glyceraldehyde during autoxidation suggests that a change in reaction mechanism occurs at pH 8.2. Below pH 8.2, the rates of enolisation and autoxidation increase with increasing pH, with a concomitant increase in the formation of hydroxyalkyl spin-adducts. Above pH 8.2, glyceraldehyde undergoing autoxidation shows a much higher rate of enolisation than of autoxidation and, although the formation of hydroxyalkyl radicals is decreased, the production of hydroxyl radicals is also observed. A free-radical mechanism for the autoxidation of monosaccharides is proposed, to account for the pH-dependent characteristics of the production of free radicals and the relationships between the production of free radicals, autoxidation, and enolisation of the monosaccharides.

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INTRODUCTION

The autoxidation of monosaccharides occurs relatively rapidly under alkaline conditions[1]. Such reactions are important in biological systems and in many technological and industrial operations[2]. Recently, the autoxidation of monosaccharides has been demonstrated under physiological conditions. The oxygen-derived reactive intermediates thereby produced have been implicated in the cleavage of nucleic acids and the inactivation of viruses and bacteriophages[3,4]

Although the mechanism of the autoxidation of monosaccharides is still largely unclear, several ionic and free radical-mediated mechanisms have been proposed[2,5]. The enolisation of monosaccharides to ene-diol intermediates appears to be an obligatory first step[1].

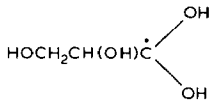
During a study of glyceraldehyde-induced effects in red blood cells and in homogenates from the lens tissue of rats, the autoxidation of glyceraldehyde to hydroxypyruvaldehyde was found to be an important fate of the monosaccharide, particularly in phosphate-buffered systems[6]. Further investigations[7] disclosed that the autoxidation reactions of several simple monosaccharides occur at a significant rate, such that oxygen-consumption kinetics, the production of free radicals, and the formation of ketoaldehydes can be quantified.

We now report on the pH dependence of the production of free radicals from monosaccharides undergoing autoxidation together with the pH dependence of the relationships between monosaccharide enolisation, autoxidation, and the production of free radicals. The formation of free radicals from monosaccharides undergoing autoxidation was detected by e.s.r. using the spin-trapping technique[8].

RESULTS AND DISCUSSION

Production of free radicals from autoxidation of DL-*glyceraldehyde.* — *(a) Characterisation of the spin-trapped intermediates.* Fig. 1a shows the e.s.r. spectrum obtained from 10mM DL-glyceraldehyde in 100mM sodium phosphate buffer (pH 8.5) in the presence of 100mM DMPO spin-trap (under these conditions, DL-glyceraldehyde is autoxidised to form hydroxypyruvaldehyde and hydrogen peroxide[6,7]). The spectrum may be assigned to two component e.s.r. spin-adduct spectra: namely, an OH component, characterised by the parameters g = 2.0051, $a_N = a_H = 14.9$G; and an $R_{Glyceraldehyde}$ component characterised by the parameters g = 2.0055, $a_N = 15.8$G, and $a_H = 22.8$G. The parameters of the OH component are identical to those previously assigned[8] to the hydroxyl-radical spin adduct [5,5-dimethyl-2-hydroxypyrrolidino-1-oxyl] of DMPO. The $R_{Glyceraldehyde}$ component has spectral parameters similar to those previously assigned to 1-hydroxyalkyl (carbon-centred) radical-derived spin adducts, DMPO-C(OH)RR[1], *e.g.*, the DMPO-1-hydroxyethyl spin adduct[8]. From radiolysis studies[9,10], the major carbon-centred free radical produced from the reaction of hydroxyl radicals with glyceraldehyde is

1 ($R_{Glyceraldehyde}$) formed by abstraction of a hydrogen atom from the acetal C–H bond of glyceraldehyde hydrate.



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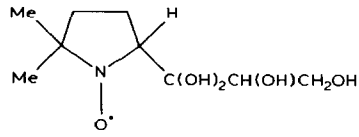

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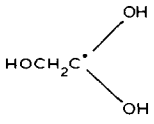
Therefore, the formation of DMPO-OH and DMPO-R spin adducts may be used to follow the involvement of free radicals in the autoxidation of monosaccharides.

Figs. 1b and 1c shows the e.s.r. spectra recorded under incubation conditions similar to those associated with Fig. 1a, but using 10mM glycolaldehyde and 10mM dihydroxyacetone, respectively. Glycolaldehyde undergoing autoxidation shows an e.s.r. spectrum consistent with the formation of two spin-adducts, namely, DMPO-OH and DMPO-$R_{Glycolaldehyde}$, the latter being characterised by $g_{Glycolaldehyde}$ = 2.0055, a_N = 15.8G, and a_H = 22.8G. Similar analysis of the results of radiation chemistry has shown that H abstraction from the acetal C–H bond (→**3**) yields the major free radical in the reaction of hydroxyl radicals with glycolaldehyde (hydrate).



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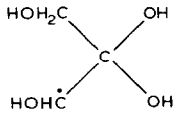

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Dihydroxyacetone undergoing autoxidation exhibited only the production of DMPO-OH, the e.s.r. spectrum of which is shown in Fig. 1c. That **1** is formed by the attack of hydroxyl radicals on glyceraldehyde was confirmed by generating hydroxyl radicals from a Fenton reaction (ferrous chloride + hydrogen peroxide) in the presence of 10mM glyceraldehyde and 100mM DMPO. The e.s.r. spectrum observed 30 s after mixing at 25° is given in Fig. 1d which corresponds to Fig. 1a, indicating production of the same spin adduct in the two systems. The relative formation of DMPO-OH and DMPO-$R_{Glyceraldehyde}$ (**2**) in the Fenton system could be controlled by varying the ratio of glyceraldehyde and DMPO concentrations. With a constant 100mM DMPO, increase of the glyceraldehyde concentration increased the formation of **2** and decreased that of DMPO-OH, as expected for simple kinetic competition by glyceraldehyde and DMPO for hydroxyl radicals. The relative ratio of DMPO-OH to **2** in the autoxidising glyceraldehyde system could be controlled by changing the glyceraldehyde concentration. A decrease from 50 to 5mM maintained the production of DMPO-OH, but decreased the production of **2** below the limits of detection (data not shown). This result is to be expected if hydroxyl

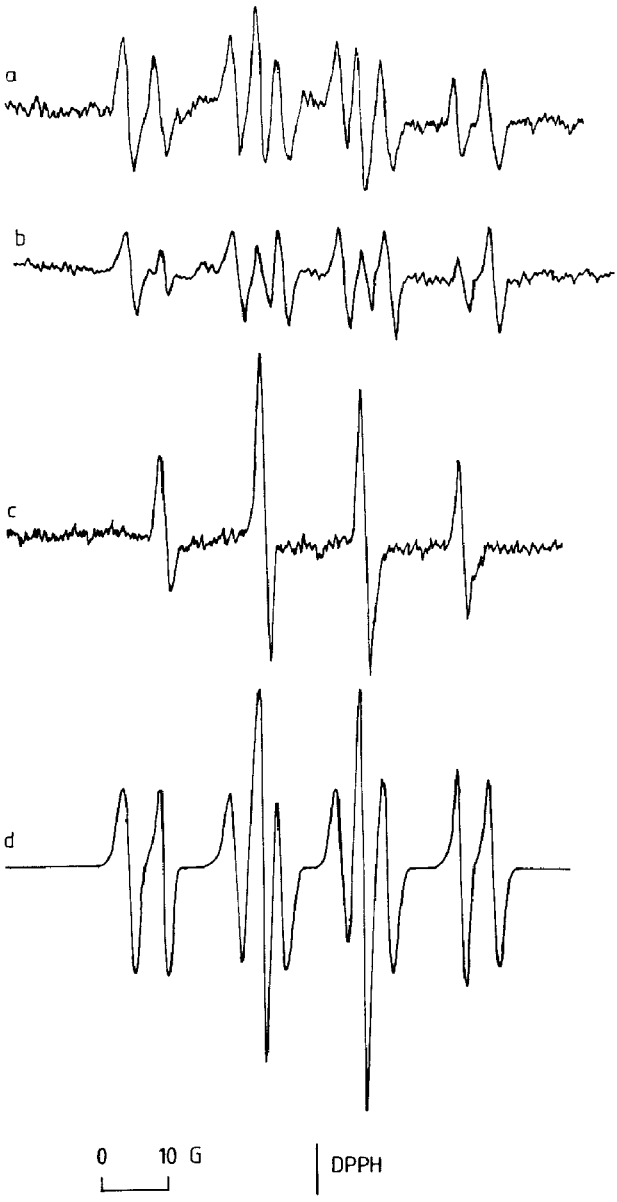


Fig. 1. Spin-trapped intermediates from monosaccharide autoxidation at pH 9.0 and 37°. Reaction mixtures contained (*a*) 10mM DL-glyceraldehyde, (*b*) 10mM glycolaldehyde, and (*c*) 10mM dihydroxyacetone in 100mM sodium pyrophosphate buffer (pH 9.0) with 100mM DMPO (solutions were kept at 37° for 10 min and the e.s.r. spectrum was then recorded), (*d*) 10mM DL-glyceraldehyde and 100mM DMPO at 25° (the e.s.r. spectrum was recorded immediately after mixing). E.s.r. details: field set, 3390G; field scan, 100G; modulation frequency, 100 kHz; modulation amplitude, 0.5G; time constant, 1 s; scan time, 4 min; receiver gain, 5×10^2 (*a* − *c*), 2×10^3 (*d*); microwave power, 20 mW, microwave frequency, 9.510 GHz.

radicals, produced during the autoxidation of glyceraldehyde, are quenched by excess of glyceraldehyde in the autoxidation reaction mixture.

At pH 7.5, the production of free radicals during the autoxidation of DL-glyceraldehyde, glycolaldehyde, and dihydroxyacetone, observed by spin-trapping with DMPO, is that shown in Figs. 2a–c. With 10mM monosaccharide incubated for 10 min in 100mM sodium phosphate (pH 7.4) at 37°, in the presence of 100mM DMPO, intense e.s.r. spectra of **2** (Fig. 2a) and DMPO-$R_{Glycolaldehyde}$ were observed during the autoxidation of DL-glyceraldehyde and glycolaldehyde, respectively.

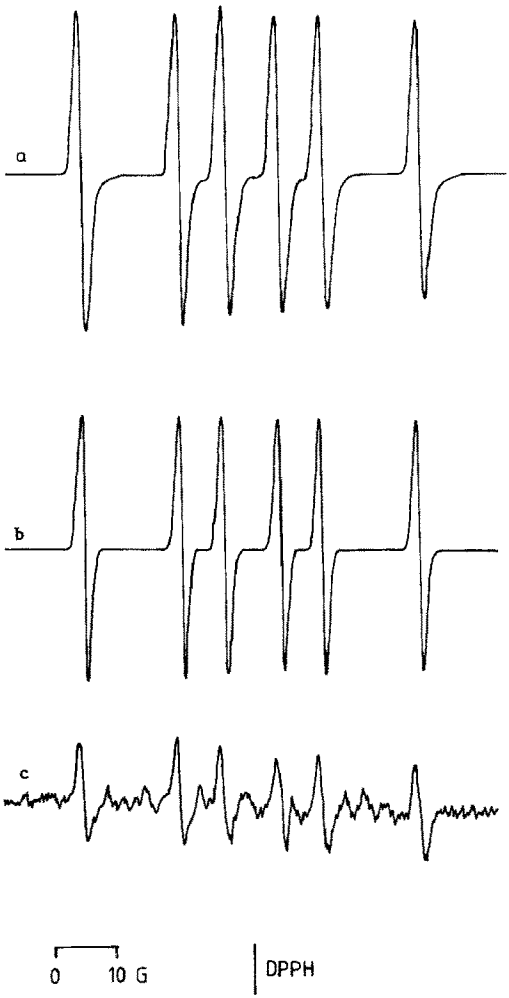


Fig. 2. Spin-trapped intermediates from monosaccharide autoxidation at pH 7.4 and 37°. Reaction mixtures contained (*a*) 50mM DL-glyceraldehyde, (*b*) 50mM glycolaldehyde, and (*c*) 10mM dihydroxyacetone in 100mM sodium phosphate buffer (pH 7.4) and 37°, with 100mM DMPO. E.s.r. spectra were recorded after 10 min. Instrumental details as in Fig. 1, except for receiver gains which were 2×10^3 (*a* and *b*) and 1×10^4 (*c*).

However, the e.s.r. spectrum of the dihydroxyacetone autoxidation system showed a signal of relatively low intensity assigned to DMPO-$R_{Dihydroxyacetone}$, where $g_{Dihydroxyacetone} = 2.0055$, $a_N = 15.8G$, and $a_H = 22.8G$. From previously reported radiolysis and e.s.r. observations[9,10], the structure **4** can be assigned to $R_{Dihydroxyacetone}$ (presumably formed by attack of hydroxyl radicals on the hydrate).

(b) The time course and concentration-dependence of the formation of spin adducts. Fig. 3a shows the production of spin adduct **2** from 10 and 50mM DL-glyceraldehyde incubated in 100mM sodium phosphate buffer (pH 7.4) at 37° in the presence of 100mM DMPO. The signal height of the low-field peak in the 1st derivative e.s.r. spectrum of **2** was followed with time. Incubations containing 50mM DL-glyceraldehyde showed a relatively rapid increase in the intensity of the signal for **2** during the autoxidation reaction. The intensities of signals for incubations containing 5–50mM DL-glyceraldehyde, after 10 min at 37° in 100mM sodium phosphate buffer (pH 7.4) containing 100mM DMPO, are shown in Fig. 3b. This demonstrates that the intensity of the signal for **2** is proportional to the concentration of DL-glyceraldehyde. The incubations containing 50mM DL-glyceraldehyde in closed cells became anaerobic after 12 min (data not shown). In Fig. 3a, the intensity of the signal for **2** in the 50mM DL-glyceraldehyde incubation continued to increase after 20–30 min, suggesting that **2** may be produced both in the aerobic

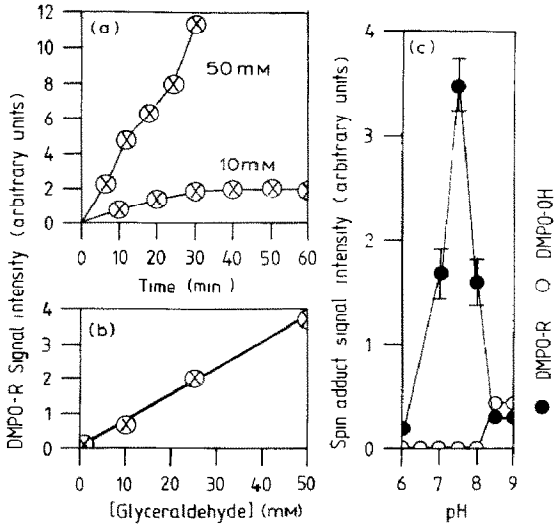


Fig. 3. Time course of reaction, concentration dependence, and pH profile for the production of spin adducts during the autoxidation of DL-glyceraldehyde. (*a*) Time course for 10 and 50mM substrate [reaction mixtures at 37° also contained 100mM DMPO, 100mM sodium phosphate buffer (pH 7.4)]; (*b*) concentration dependence [reaction mixtures at 37° contained 5, 10, 25, and 50mM DL-glyceraldehyde, 100mM DMPO, and 100mM sodium phosphate buffer (pH 7.4), and were kept for 10 min]; (*c*) pH profile [reaction mixtures at 37° contained 50mM DL-glyceraldehyde, 100mM DMPO, and 100mM sodium phosphate buffer (pH 6.0–8.0) or sodium pyrophosphate buffer (pH 8.5–9.0), and were kept for 10 min]. Data shown are the peak heights of the low-field peak in the 1st derivative e.s.r. spectrum of DMPO-$R_{Glyceraldehyde}$ (Fig. 2a).

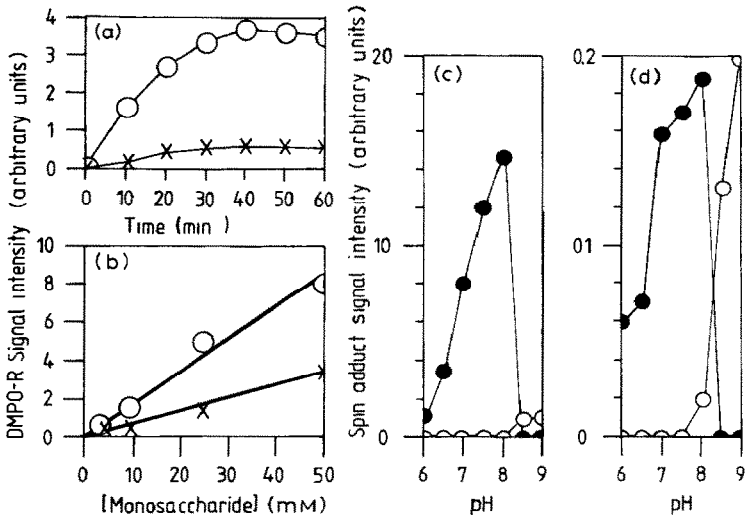
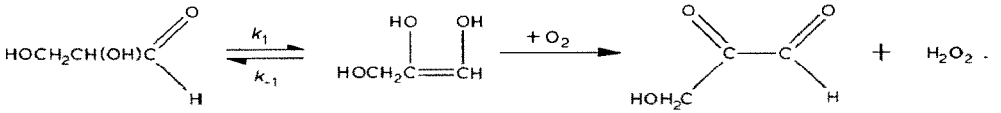


Fig. 4. Time course of reaction, concentration dependence, and pH profile for the formation of spin adducts during the autoxidation of glycolaldehyde and dihydroxyacetone. (*a*) Time course for 10mM glycolaldehyde (—○—) and 10mM dihydroxyacetone (—×—) [reaction mixtures at 37° also contained 100mM sodium phosphate buffer (pH 7.4) and 100mM DMPO]; (*b*) concentration dependence [reaction mixtures at 37° contained 5, 10, 25, and 50mM glycolaldehyde (—○—) or dihydroxyacetone (—×—) with 100mM DMPO in 100mM sodium phosphate buffer (pH 7.4), and were kept for 10 min]; (*c*) pH profile [reaction mixtures at 37° contained 50mM glycolaldehyde, 100mM DMPO in 100mM sodium phosphate buffer (pH 6.0–8.0) or sodium pyrophosphate buffer (pH 8.5–9.0), and were kept for 10 min]; (*d*) pH profile [reaction mixtures contained 10mM dihydroxyacetone with the other parameters as in (*c*)]. Data presented are peak heights of the down-field peak in the 1st derivative e.s.r. spectrum of DMPO-$R_{Monosaccharide}$.

and anaerobic phases of the incubation reaction. Figs. 4a and 4b show time-course and concentration-dependence data for the intensity of the signals of the spin adducts produced during the autoxidation of 10mM glycolaldehyde and 10mM dihydroxyacetone. The intensity of the signals of DMPO-$R_{Glycolaldehyde}$ and $R_{Dihydroxyacetone}$ appeared to reach a maximum after incubation for 40 min (Fig. 4a); the intensities of the signals of these spin adducts appeared to be proportional to the initial concentration of the monosaccharide (Fig. 4b).

(c) The dependence of the production of spin adducts on pH. Fig. 3c shows the pH dependence of the formation of **2** and DMPO-OH from 10mM DL-glyceraldehyde in 100mM sodium phosphate/pyrophosphate buffer after incubation for 10 min at 37° in the presence of 100mM DMPO. The production of **2** appeared to be a maximum between pH 7.0 and 8.0. Between pH 8.0 and 8.5, the formation of **2** fell as the formation of DMPO-OH was first detected. Similar events occurred in incubations with glycolaldehyde (Fig. 4c) and dihydroxyacetone (Fig. 4d) except that, for dihydroxyacetone, the formation of DMPO-OH was detected first at a pH slightly lower than that for glyceraldehyde and glycolaldehyde. The sharp change in the production of free radicals between pH 8.0 and 8.5 may indicate a change in reaction mechanism.

(d) The relationship between autoxidation, enolisation, and the production of free radicals. The kinetics of glyceraldehyde autoxidation, previously reported[6], were consistent with a two-step reaction, namely, enolisation of the monosaccharide and subsequent oxidation to hydroxypyruvaldehyde. Under normal conditions, the enolisation step was rate-determining. Fig. 5 shows the dependence on pH of oxygen consumption (autoxidation) and iodine uptake (enolisation) by 10mM DL-glyceraldehyde in 100mM sodium phosphate buffer (sodium pyrophosphate was used at pH 9.0). In the pH range 6–7, the rate of uptake of iodine was slightly higher than the rate of consumption of oxygen by 10mM DL-glyceraldehyde. This finding is consistent with the autoxidation of DL-glyceraldehyde proceeding *via* enolisation and autoxidation of the ene-diol intermediate[6,7].



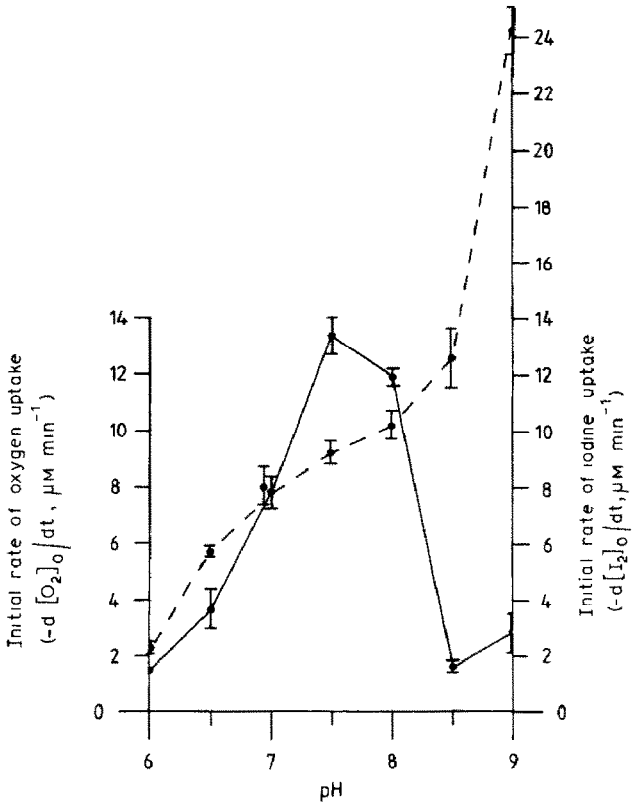
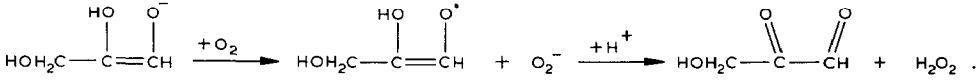


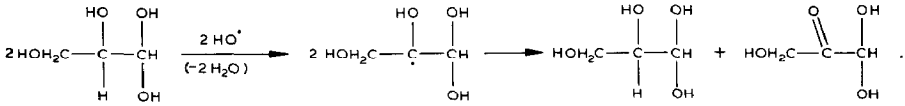
Fig. 5. The pH dependence of the rate of oxygen consumption and iodine uptake by glyceraldehyde undergoing autoxidation at 37°. Reaction mixtures contained 10mM DL-glyceraldehyde in 100mM sodium phosphate (pH 6.0–8.5) or sodium pyrophosphate buffer (pH 9.0) and, for iodine uptake experiments, 20–40μM iodine and 50mM potassium iodide. Data presented are the means ±standard deviation of four estimations.

Over this pH range, the production of free radicals (DMPO-$R_{Glyceraldehyde}$) also increases (Fig. 3c). In the pH range 7.0–8.0, the rate of oxygen consumption exceeds the rate of iodine uptake by DL-glyceraldehyde; the production of free radicals also reaches a maximum (Fig. 3c). At this stage, the production of free radicals during the autoxidation of the ene-diol may have become so rapid that some free radicals initiate further autoxidations and the process becomes a chain reaction.

In the pH range 8.0–9.0, the rate of iodine uptake by 10mM DL-glyceraldehyde rapidly increases, whereas oxygen consumption and the production of free radicals both decrease (Figs. 5 and 3c). Furthermore, DMPO-OH was observed in the e.s.r. spectrum of the autoxidation mixtures despite the lower consumption of oxygen (and also of hydrogen peroxide, the precursor to hydroxyl radicals)[6,7]. An explanation proposed for this apparent transition in reaction mechanism is based on the pK_a of the ene-diol in the region pH 8.0–8.5. The anionic form of the ene-diol would be expected to be very unstable towards autoxidation:



The neutral form of the ene-diol, however, would be metastable and is also an excellent free-radical scavenger[11]. Therefore, it is proposed that hydroxyl radicals are scavenged by both glyceraldehyde hydrate (see previous discussion) and the ene-diol of glyceraldehyde below pH 8:



Above pH 8, only glyceraldehyde hydrate scavenges hydroxyl radicals; the ene-diol anion becomes so unstable with respect to autoxidation that its concentration is very low despite a high rate of enolisation. The rate of oxygen consumption rapidly decreases at high pH and is much lower than the rate of enolisation. This suggests that fates other than autoxidation for the ene-diol, *e.g.*, reduction of the aldehyde tautomer (which is in large excess) to glycerol or polymerisation of ene-diols and aldehydes, become important.

The production of free radicals from other monosaccharides. — Erythrose, ribose, and glucose show much lower rates of enolisation and autoxidation than glycolaldehyde, glyceraldehyde, and dihydroxyacetone (Table I). However, erythrose autoxidation mixtures gave an intense e.s.r. spectrum in the presence of 100mM DMPO (Fig. 6a). The spectrum is assigned to DMPO-$R_{Erythrose}$ where $g_{Erythrose}$ = 2.0056, a_N = 15.8G, and a_H = 22.7G. By analogy, $R_{Erythrose}$ would be

TABLE I

RELATIVE RATES OF AUTOXIDATION AND ENOLISATION OF SOME MONOSACCHARIDES

	Iodine uptake[a] $-d[I_2]/dt$	*Oxygen consumption*[b] $-d[O_2]/dt$
Dihydroxyacetone	120	120
Glyceraldehyde	100	100
Glycolaldehyde	80	75
D-Erythrose	38	30
D-Ribose	13	4
D-Glucose	2	1

[a]Measured at pH 7.4 and 37° [reaction mixtures contained 50mM monosaccharide, 50mM KI, and 40μM I_2 in 100mM sodium phosphate (pH 7.4)]. [b]Measured at pH 7.4 and 37° [reaction mixtures contained 50mM monosaccharide in 100mM sodium phosphate (pH 7.4)].

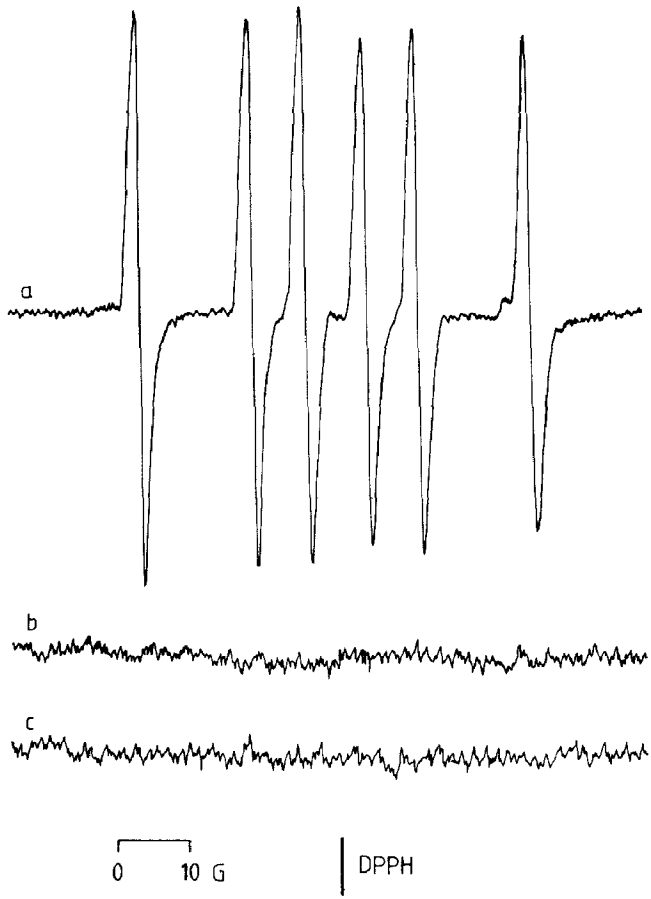
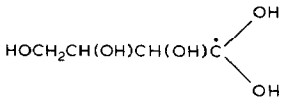


Fig. 6. Production of free radicals during the autoxidation of D-erythrose, D-ribose, and D-glucose. Reaction mixtures at 37° contained (*a*) 50mM D-erythrose, (*b*) 50mM D-ribose, and (*c*) 50mM D-glucose in 100mM sodium phosphate buffer (pH 7.4). Incubation conditions and instrumental details are as in Fig. 1, except for receiver gains which were 2×10^3 (*a*) and 1×10^5 (*b* and *c*).

expected to be the free radical **5** formed by abstraction of the acetal hydrogen from erythrose hydrate.



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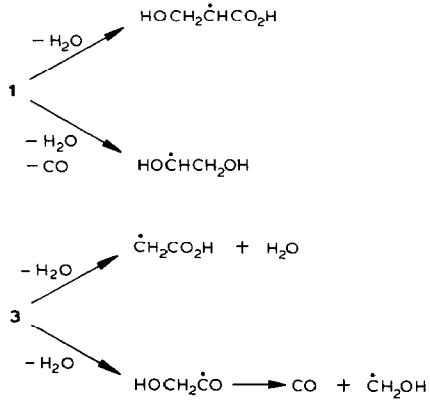
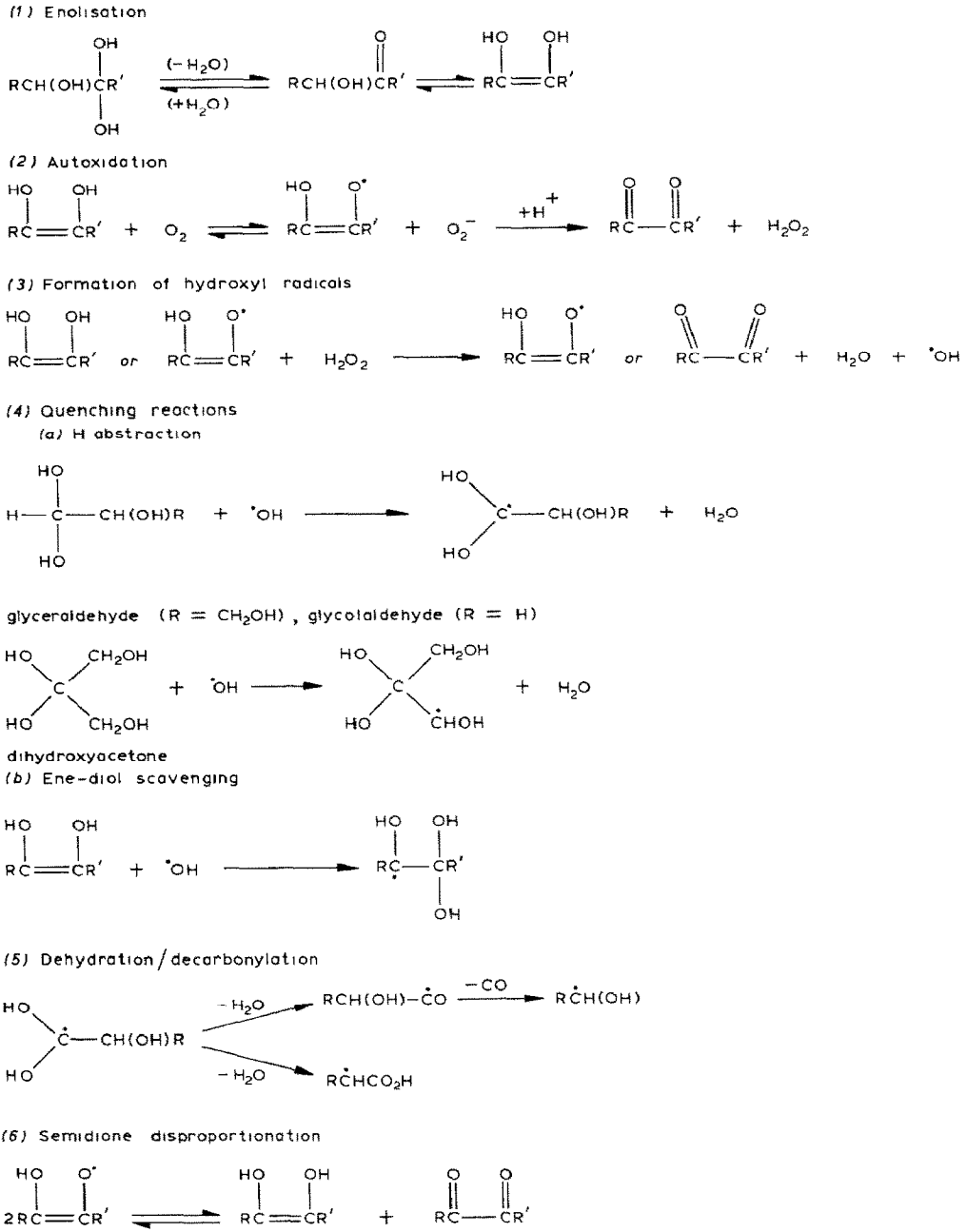
The production of free radicals from ribose and glucose was not detected. Since the formation of an ene-diol is necessary for monosaccharide autoxidation[6], the production of free radicals and α-ketoaldehydes may be dependent on the extent to which the parent monosaccharide exists in the aldehyde form because the furanose and pyranose forms are not susceptible to autoxidation. It is, therefore, not surprising that, in the series glycolaldehyde, glyceraldehyde, erythrose, ribose, and glucose, the susceptibility to autoxidation decreases sharply beyond erythrose in parallel with the fall in the percentage of aldehyde/aldehyde hydrate forms in aqueous solution.

Mechanism of the autoxidation of monosaccharides. — From reports on the autoxidation of monosaccharides[5,6] and radiolysis studies[9,10] coupled with the spin-trapping studies described above, a general mechanism for the autoxidation of monosaccharides can now be constructed (Scheme 1) involving (*1*) enolisation, (*2*) reaction with oxygen (autoxidation) to give α-ketoaldehyde and hydrogen peroxide, and (*3*) reaction of the ene-diol/ene-dioloxy radical with hydrogen peroxide to produce hydroxyl radicals. Further reactions then depend on the particular monosaccharide and pH (for ene-diol scavenging of the hydroxyl radicals), and examples are given in *4(a)* and *4(b)* of Scheme 1. The hydroxyalkyl radicals formed in reaction *4* may decompose by dehydration and decarbonylation reactions (*5* in Scheme 1). For $R_{Glyceraldehyde}$ (**1**) and $R_{Glycolaldehyde}$ (**3**), Steenken and Schulte-Frohlinde[9] suggested the following mechanisms:

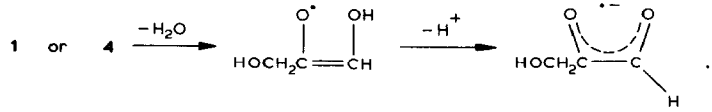


$R_{Dihydroxyacetone}$ (4) may decay by disproportionation to give hydroxypyruvaldehyde and dihydroxyacetone.



Scheme 1. A mechanism for the autoxidation of monosaccharides. (The ene-diol and semidione free-radical may be metal-ion coordinated. The distribution of geometric isomers of the ene-diol is not known.)

The effect of pH on the formation of monosaccharide radicals ($R_{Monosaccharide}$) from the reaction of hydroxyl radicals with monosaccharides has not been fully investigated. Kuwabara *et al.*[12] used a nitroso spin-trap to observe the free radicals produced from hexoses with radiolytically generated hydroxyl radicals, but were restricted to acid and neutral pH because of the instability of the spin adduct. The work of Gilbert and co-workers[13] on free radicals produced by the reaction of hydroxyl radicals with hexoses relates only to pH $\leqslant$ 4. The monosaccharide free-radicals formed are a mixture of all possible hydroxyalkyl radicals. Steenken and Schulte-Frohlinde[9] suggested that the dehydration and decarbonylation reactions occur throughout the pH range, although dehydration to $RCHCO_2H$ would be favoured where pH <1 and >10 and the dehydration–decarbonylation reaction would be favoured by an increase in temperature. The report by West *et al.*[14] provides an independent observation of free-radical processes occurring in monosaccharide solutions at alkaline pH. At pH 8, semidione e.s.r. spectra were observed and the semidiones were suggested to be formed by rearrangement of the hydroxyalkyl radicals. For example:



Therefore, the decreased formation of DMPO-$R_{Monosaccharide}$ and the formation of DMPO-OH at pH > 8 may be explained by rearrangement of $R_{Monosaccharide}$ to the semidione (or ene-dioloxy radical), which then reduces hydrogen peroxide to hydroxyl radicals (Scheme 1, reaction *4*). Ene-dioloxy radicals may also decay by disproportionation (Scheme 1, reaction *6*).

EXPERIMENTAL

Spin-trapping experiments were performed using purified[15] 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO). E.s.r. spectra were recorded with a Varian E109 dual cavity, X-band, e.s.r. spectrometer. Oxygen consumption measurements were made using a Clark-type oxygen electrode (YSI Model 53), and iodine uptake was measured by following the disappearance of I_3 at 351 nm in 20–40 μM I_2 and 50mM potassium iodine, where[16] E_{351} is 26500 $M^{-1}.cm^{-1}$.

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