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Monosaccharide–H_2O_2 reactions as a source of glycolate and their stimulation by hydroxyl radicals

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Abstract—An analysis of the H_2O_2-induced breakdown and transformation of different keto-monosaccharides at physiological concentrations reveals that glycolate and other short-chained carbohydrates and organic acids are produced. Depletion of monosaccharides and glycolate synthesis occurs at increased rates as the length of the carbohydrate chain is decreased, and is significantly increased in the presence of trace amounts of Fe^{2+} ions (10 μM). Rates of monosaccharide depletion (initial concentration of 3 mM) observed were up to 1.55 mmol h^{-1} in the case of fructose, and 2.59 mmol h^{-1} in the case of dihydroxyacetone, depending upon pH, H_2O_2 concentration, temperature and the presence or absence of catalytic amounts of Fe^{2+}. Glycolate was produced by dihydroxyacetone cleavage at rates up to 0.45 mmol h^{-1} in the absence, and up to 1.88 mmol h^{-1} in the presence of Fe^{2+} ions (pH 8). Besides glycolate, other sugars (ribose, glyceraldehyde, glucose), glucitol (sorbitol) and organic acids (formic and 2-oxogluconic acid) were produced in such H_2O_2-induced reactions with fructose or dihydroxyacetone. EPR measurements demonstrated the participation of the $^{\bullet}OH$ radical, especially at higher pH. Presence of metal ions at higher pH values, resulting in increased glycolate synthesis, was accompanied by enhanced hydroxyl radical generation. Observed changes in intensity of DEPMPO-OH signals recorded from dihydroxyacetone and fructose reactions demonstrate a strong correlation with changes in glycolate yield, suggesting that $^{\bullet}OH$ radical formation enhances glycolate synthesis. The results presented suggest that different mechanisms are responsible for the cleavage or other reactions (isomerisation, auto- or free-radical-mediated oxidation) of keto-monosaccharides depending of experimental conditions.

Keywords: Fenton reaction; Dihydroxyacetone; Glycolate; Hydrogen peroxide; Hydroxyl radical; Monosaccharides

1. Introduction

Basic organic chemistry stipulates that strongly alkaline conditions make sugars more susceptible to oxidation, hydrogen peroxide interactions and carbon chain fragmentations.[1] Simple monosaccharides have been shown to be capable of glycolate production under nonphysiological conditions, such as extreme peroxide concentrations, pH, temperatures and the presence of complex catalysts.[2–4] Also, synthesis of carboxylic acids (including glycolate) from fructose and dihydroxyacetone has been shown using high concentrations of hydrogen peroxide and 24-hour-long incubations.[5,6] In our previous reports, we tested the ability of different monosaccharides to produce glycolate in the reaction with hydrogen peroxide under conditions close to those of the physiological state and have shown that keto forms can produce glycolate, as opposed to the corresponding aldo forms, especially at higher pH values (pH $\geqslant$8).[7] Presence of metal ions in trace amounts stimulated this reaction, indicating the involvement of free radicals from a Fenton-type reaction. However, those results were obtained using the classical Calkins colorimetric assay and liquid ion-exchange chromatography,[8] a procedure with a number of drawbacks and lack of specificity.

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The aim of this work was to test the ability of hydrogen peroxide to cleave keto-monosaccharides of different chain length under conditions close to physiological and analyse the products of such a reaction (especially glycolate). In order to bypass various non-specific photometric derivatisation methods prone to formation of artifacts, quantification of products was carried out by anion-exchange HPLC with electrochemical and UV detection, its sensitivity being such that no derivatisation is required. Also, we wanted to determine if and what oxygen-centred free-radical species participate in such a reaction, in the presence and absence of trace metals and chelators, using a selective electron spin resonance trapping technique.

2. Results and discussion

Preliminary skim tests demonstrated that keto forms of simple monosaccharides in reaction with hydrogen peroxide produce glycolate, as opposed to aldo isomers. Those findings are supported by earlier reports in which a stepwise degradation mechanism of aldoses is proposed, yielding formic acid as final product.[9] Tested ketoses (dihydroxyacetone, erythrulose and fructose) produced glycolate as a characteristic product, as described in previous investigations employing higher alkaline conditions and concentrations of reactants.[10] Glycolate yield increased with decreasing carbon chain length (0.11 mmol h^{-1} at 0.75 mM H_2O_2 for dihydroxyacetone in comparison to 0.012 mmol h^{-1} from fructose), these results being in line with our recently published results, as well as older data obtained using Calkins colorimetric assay.[11,12]

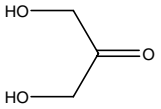
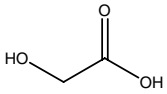
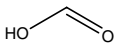
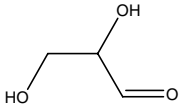
2.1. HPLC analysis of reaction

Dihydroxyacetone and fructose were used for a detailed analysis of the reaction with H_2O_2 at two pH values (4 and 8) and in the absence or presence of Fe^{2+}, the relative share of the products after 30 min of incubation at 40 °C being presented in Tables 1 and 2. In the dihydroxyacetone–H_2O_2 reaction, in the case of the absence of Fe^{2+} ions, dihydroxyacetone decomposed to a greater degree at lower pH 4. Addition of Fe^{2+} ions greatly enhanced the cleavage of the monosaccharide, and the pH effect was reversed, that is, dihydroxyacetone decomposed to a greater degree at higher pH 8. The main product of H_2O_2-induced decomposition in the presence of Fe^{2+} ions, as well as in the absence of iron at low pH, was glycolate with formate as the main byproduct. Glyceraldehyde became the predominant product in the absence of metal ions at pH 8.

In the fructose–H_2O_2 reaction, the main products were 2-oxogluconic acid, glucitol (sorbitol) and a small amount of glycolate in the reaction without metal ions. Addition of 10 μM $FeSO_4$ did not alter the product distribution, with the exception of ribose, which appeared as the fragmentation product (Table 2).

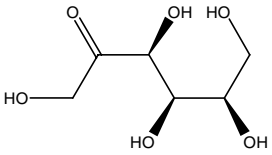
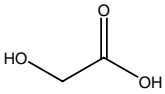
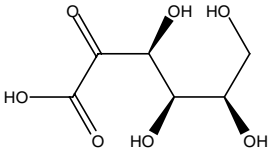
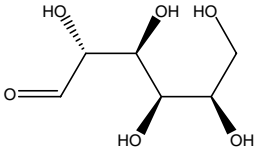
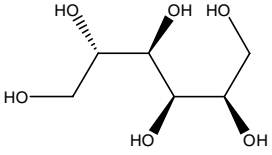
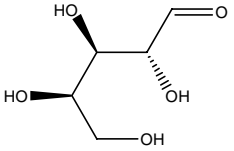
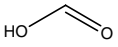
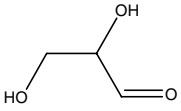
The observed differences in glycolate yield between dihydroxyacetone and fructose could be explained by different levels of enolisation of those two ketoses.[13] The susceptibility of a monosaccharide to enolisation is inversely proportional to the extent of formation of

Table 1. Relative share (in %) in overall chromatogram area of dihydroxyacetone/H_2O_2 reaction products, in the absence or presence of 10 μM $FeSO_4$[a]

Reactant/product	Structure	DHA/H_2O_2 reaction		DHA/H_2O_2 + $FeSO_4$	
		pH 4	pH 8	pH 4	pH 8
Dihydroxyacetone		70.05	77.39	49.92	31.06
Glycolic acid		18.93	6.58	23.96	39.74
Formic acid		2.68	1.04	15.14	18.81
Glyceraldehyde		2.49	10.11	1.23	2.76
Unidentified	—	≈6	≈5	≈9.5	≈7.5

[a] Reaction mixtures contained 3 mM dihydroxyacetone, 0.75 mM H_2O_2 and 5 mM phosphate buffer at pH 8, the reaction being carried out for 30 min at 40 °C.

Table 2. Relative share (in %) in overall chromatogram area of fructose/H_2O_2 reaction products in the absence or presence of 10 μM $FeSO_4$[a]

Reactant/product	Structure	Fructose/H_2O_2 reaction		Fructose/H_2O_2 + $FeSO_4$	
		pH 4	pH 8	pH 4	pH 8
Fructose		87.32	95.52	68.20	73.50
Glycolic acid		3.45	0.27	7.92	0.85
2-Oxogluconic acid		6.62	0.14	10.47	3.04
Glucose		n.d.	2.65	n.d.	n.d.
Sorbitol		0.35	n.d.	1.33	6.54
Ribose		n.d.	n.d.	12.47	0.13
Formic acid		n.d.	n.d.	n.d.	n.d.
Glyceraldehyde		n.d.	n.d.	n.d.	n.d.
Unidentified	—	≈2.5	≈1.5	—	≈16

[a] The mixtures contained 3 mM fructose, 0.75 mM H_2O_2 at pH 4 or 8 adjusted with 5 mM phosphate buffer, the reaction time being 30 min at 40 °C.

furanose or pyranose rings (i.e., C-chain length); thus the presence of an overt carbonyl group in dihydroxyacetone (that exists in the open-chain form) could be the main reason for its higher reactivity towards H_2O_2.[14] Additionally, the enediol intermediate was shown to possess different properties, depending on the pH.[15] Lower pH stimulates the conversion of the enediol to an α-oxoaldehyde intermediate,[16] which through further oxidation and decarboxylation could produce glycolate, as we have indeed observed in metal-free reactions of dihydroxyacetone and fructose at pH 4. Also, unlike aldoses, in alkaline solutions ketoses could be degraded to glycolic acid via nucleophilic attack of the perhydroxyl anion to the carbonyl group, the mechanism being similar to that described that involves an enediolate intermediate.[10,17]

2.2. Concentration dependence

The depletion of monosaccharides and quantity of glycolate produced as a function of hydrogen peroxide concentration is shown in Figure 1A and C. At all H_2O_2

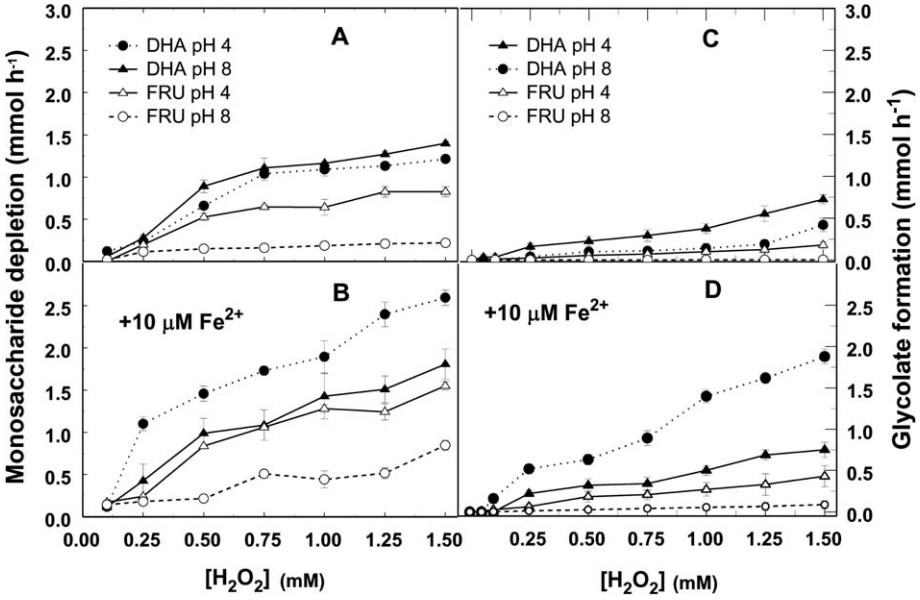


Figure 1. Monosaccharide decomposition and glycolate formation during 30 min of incubation (at 40 °C) of 3 mM dihydroxyacetone or fructose with different concentrations of H_2O_2 in the absence (A–C) and presence (B–D) of 10 μM $FeSO_4$, at pH 4 or pH 8.

concentrations and both pH values, dihydroxyacetone reacted more rapidly than fructose. There was not much difference in the depletion of dihydroxyacetone at pH 4 (1.22 mmol h^{-1} at 0.75 mM H_2O_2) compared to pH 8 (1.04 mmol h^{-1} at 0.75 mM H_2O_2). In the case of fructose, acidic pH significantly increased the rate of monosaccharide depletion, compared to alkaline pH. The fact that similar rates of dihydroxyacetone breakdown were observed at either pH value of the reaction medium, while glycolate yield differed (being lower in alkaline conditions), is an indication of the existence of reaction routes other than glycolate synthesis at higher pH. The most probable mechanisms would be auto-oxidation and enediol polymerisation, these having been shown to be stimulated under similar reaction conditions.[18] Auto-oxidation is also initiated by the formation of an enediol intermediate, which by oxidation with O_2 or H_2O_2 subsequently forms various other intermediates,[19] glycolate not necessarily being among them. Thus, alkaline conditions could favour the transition of a newly formed enediol intermediate in the presence of H_2O_2 into two directions: formation of glycolate by hydroxyl radicals or by auto-oxidation.

In order to study the stimulating effect of free radicals on the monosaccharide–H_2O_2 reaction,[20,21] we employed the Fenton reaction as a simple method for obtaining ·OH radicals.[22] Catalytic amounts of metal ions added to the reaction medium exhibited a differential effect on the monosaccharide reaction at low and high pH (Fig. 1B and D). Higher pH stimulated the rate of breakdown of dihydroxyacetone, whereas lower pH stimulated the rate of fructose decomposition. Thus, the depletion of dihydroxyacetone was almost three times as rapid at pH 8 compared to pH 4 at 0.75 mM H_2O_2, the stimulation induced by the trace metal presence decreasing with increasing hydrogen peroxide concentrations. A fourfold increase in glycolate yield was observed in the reaction of hydrogen peroxide with dihydroxyacetone compared to fructose (0.29 vs 0.08 mmol h^{-1}, respectively) at pH 4 and 0.75 mM H_2O_2, almost negligible quantities of glycolate being released from fructose at higher pH (0.012 mmol h^{-1}). At low pH (4), the rate of glycolate synthesis from dihydroxyacetone was slightly enhanced (from 0.29 to 0.34 mmol h^{-1} at 0.75 mM H_2O_2), with similar tendency throughout the whole range of hydrogen peroxide concentrations used. A striking difference was observed at weakly alkaline pH (pH 7.5–8.5), the rate of glycolate production increasing from 0.11 to 0.89 mmol h^{-1} at 0.75 mM H_2O_2 (pH 8) in the presence of metal ions.

Increased reactivity of dihydroxyacetone can again be explained by its open-chain form and greater susceptibility to free-radical attack, probably by ·OH radical-mediated decarboxylation.[20,23] Also, it is known that the enediol oxidation mechanism that leads to monosaccharide fragmentation at higher pH is enhanced by the Fenton reaction.[17,24] In the case of fructose, at low pH a small increase of glycolate synthesis (from 0.07 to 0.2 mmol h^{-1} at 0.75 mM H_2O_2) could be observed. However, alkaline pH (8) did not exhibit such an effect as observed in the case of dihydroxyacetone, the low rate production of glycolate being monotonous (0.04 mmol h^{-1}at 0.75 mM H_2O_2). In reactions proceeding with relevant rates, first-order reaction kinetics are not supported by the log-derived plots of presented data.

2.3. pH and temperature dependence

In the case of fructose, acidification of the medium increased the yield of glycolate, higher pH values slowly decreasing the yield. Dihydroxyacetone exhibited a more complex dependence with two distinct regions of stimulation, one strong in acidified solution in the region of pH < 5 (0.51 mmol h^{-1} at 0.75 mM H_2O_2) and another weaker at pH 8 (0.21 mmol h^{-1} at 0.75 mM H_2O_2). In the presence of metal ions, a pronounced increase in glycolate yield occurred in the case dihydroxyacetone at higher pH values, a maximum being observed at pH 8 (Fig. 2).

Differences in dihydroxyacetone depletion to glycolate yield ratio at various pH values argue in favour of a change in the reaction mechanism—either the formation of intermediates capable of quenching the ·OH radicals produced by the Fenton reaction, or the formation of ·OH radicals participating in dihydroxyacetone cleavage and glycolate synthesis. The decrease in glycolate yield from dihydroxyacetone at pH $\leqslant 7$, despite the expected stimulation of the Fenton reaction, can be explained by the formation of a hydroxyalkyl radical intermediate capable of quenching ·OH radicals.[25] It should be mentioned that hydroxyalkyl radicals formed in such a reaction could, through decarboxylation, produce glycolate, but at much slower rate. Our HPLC analysis confirmed the presence of smaller quantities of glycolate among the products at lower pH, accompanied by a slight stimulation of the reaction by $FeSO_4$.

Temperature dependence of glycolate production in the monosaccharide–H_2O_2 reaction (at pH 8 for dihydroxyacetone and pH 4 for fructose) again demonstrated that an increase in temperature resulted in a significant increase of glycolate synthesis in the case of dihydroxyacetone, both in the presence or absence of metal ions. Arrhenius plots and similar activation energies calculated for Fe^{2+}-catalysed reactions supports hypothesis that activation of the ·OH radical is the rate-limiting step for both keto-monosaccharides. The stimulation of glycolate synthesis observed at higher temperatures supports the proposed free-radical mechanism, since it has been shown that the reactivity of ·OH radicals rapidly declines at temperatures below 30 °C and significantly increases above 40 °C (Fig. 3).[26]

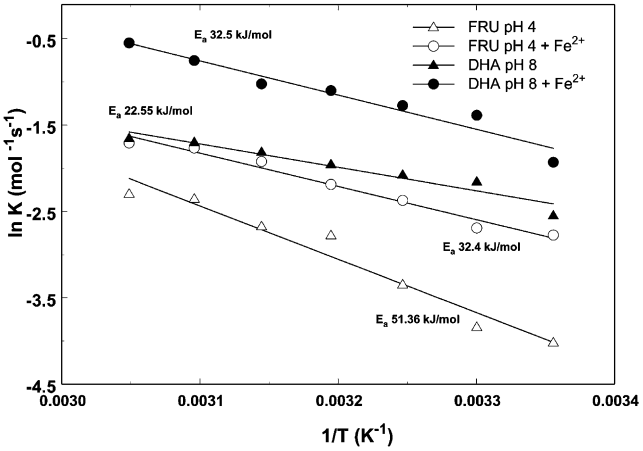


Figure 3. Arrhenius plots of dihydroxyacetone and fructose reactions after 30 min of incubation of 3 mM dihydroxyacetone or fructose with 0.75 mM H_2O_2 in the absence and presence of 10 μM $FeSO_4$.

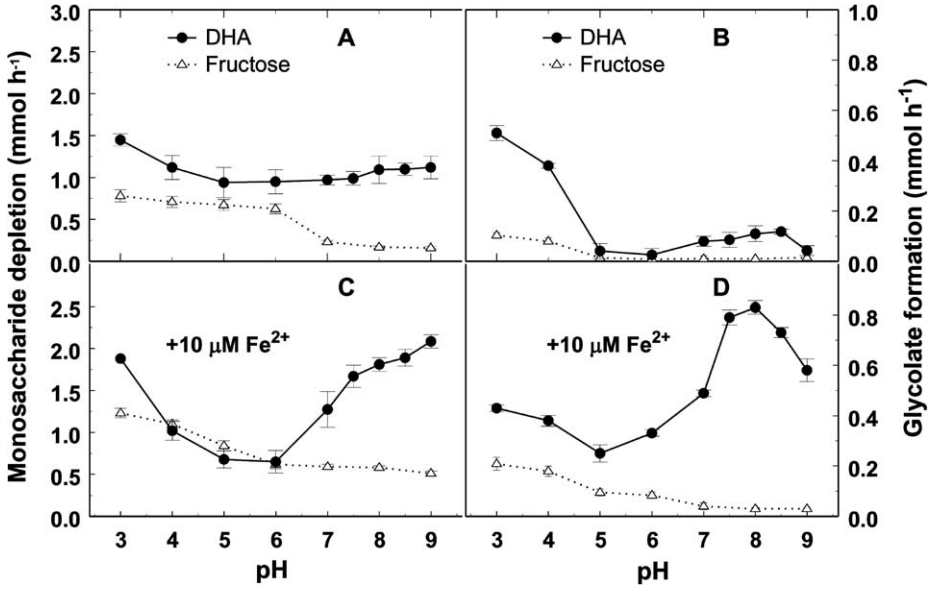


Figure 2. Effect of pH on monosaccharide decomposition and glycolate production during 30 min of incubation (at 40 °C) of 3 mM dihydroxyacetone or fructose with 0.75 mM H_2O_2 in the absence (A–B) and presence (C–D) of 10 μM $FeSO_4$.

2.4. Effect of EDTA and DETAPAC on glycolate synthesis

To test the stimulatory effect of metal ions on glycolate synthesis through the presumed formation of reactive oxygen species, changes in glycolate yield were monitored upon addition of chelators EDTA and DETAPAC. DETAPAC is known to be a very potent inhibitor of the formation of highly reactive species through metal–hydrogen peroxide interactions,[27] while EDTA was shown to be inefficient in quenching hydroxyl radicals,[28] even being capable of $^{\bullet}OH$ formation by EDTA–iron complexes.[29,30] Various $FeSO_4$–chelator concentration ratios were used in order to avoid problems described elsewhere, regarding the influence of different chelator concentrations on free-radical production and possible competition for the spin trap.[31] A strong suppression of glycolate synthesis ($\geqslant$93%) was present at all DETAPAC/$FeSO_4$ ratios with minor differences between experimental variations (Table 3), while the presence of EDTA did not inhibit to such a degree the glycolate production, no inhibition being observed for dihydroxyacetone at pH 8.

2.5. EPR spectroscopy

DEPMPO is a spin trap capable of forming different adducts with $^{\bullet}OH$ and $^{\bullet}O_2$ radicals,[32] thus differentiating between different oxygen-centred free-radical species present. EPR spectra of DEPMPO shown in Figure 4, obtained in the reaction of dihydroxyacetone with H_2O_2 at pH 8, in the absence or presence of metal ions, showed the existence of a DEPMPO-OH adduct, no other oxygen centred radical species being observed. In the spectra shown in Figure 4, no difference in the measured signals could be observed in the case of N_2-purged samples from those kept in air, even after repeated substitution of the two. We have recently demonstrated that it is possible to discern between free-radical species dependent on external molecular oxygen, from endogenously generated, oxygen-centred free radicals, by use of gas-permeable Teflon tubes to contain measured samples.[33] Control measurements using DEPMPO in buffered water solution were preformed showing that the system is free of possible artifacts. Thus, the DEPMPO adducts detected in our measurements were due to endogenously generated, oxygen-centred free-radical species. This is in line with our O_2 polarographic measurements, where no change in oxygen concentration could be observed in any of the samples (data not shown), arguing that the cleavage or transformation of monosaccharides is an anaerobic reaction.

Indeed, our EPR measurements (Figs. 4 and 5) show that in the presence of EDTA a DEPMPO-OH adduct signal can be observed, accompanied by an incomplete inhibition of glycolate production in the dihydroxyace-

Table 3. Inhibition of glycolate synthesis after addition of DETAPAC or EDTA to dihydroxyacetone or fructose at different pH values[a,b]

	DHA		Fructose	
	pH 4	pH 8	pH 4	pH 8
10 μM Fe^{2+} 50 μM DETAPAC	98.95 ± 0.79	96.82 ± 1.85	96.35 ± 0.45	99.02 ± 0.25
30 μM Fe^{2+} 150 μM DETAPAC	97.47 ± 1.16	93.91 ± 3.59	97.47 ± 0.16	99.21 ± 0.18
10 μM Fe^{2+} 50 μM EDTA	14.49 ± 1.67	11.46 ± 1.20	26.12 ± 4.43	23.25 ± 8.67
30 μM Fe^{2+} 150 μM EDTA	9.42 ± 1.23	0.86 ± 0.38	19.50 ± 3.64	14.76 ± 4.16

[a] Expressed as % of control in the same reaction without chelator added.
[b] Reaction mixtures contained 3 mM monosaccharide, 0.75 mM H_2O_2 at pH 4 or 8 adjusted with 5 mM phosphate buffer, the reactions being carried out for 30 min at 40 °C.

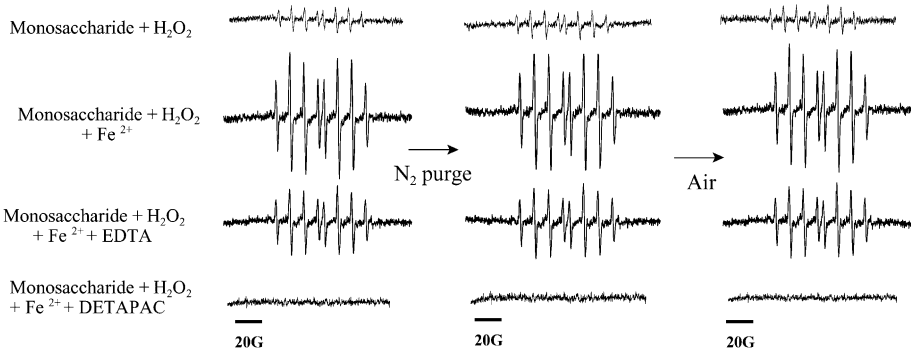


Figure 4. EPR measurements of the DEPMPO spin trap signal after 15 min of incubation in N_2 or O_2 atmosphere of the reaction of 3 mM dihydroxyacetone with 1 mM H_2O_2, in the absence or presence of 10 μmol $FeSO_4$, 0.5 mM EDTA or 0.5 mM DETAPAC (at 40 °C and pH 8, adjusted with 5 mM phosphate buffer).

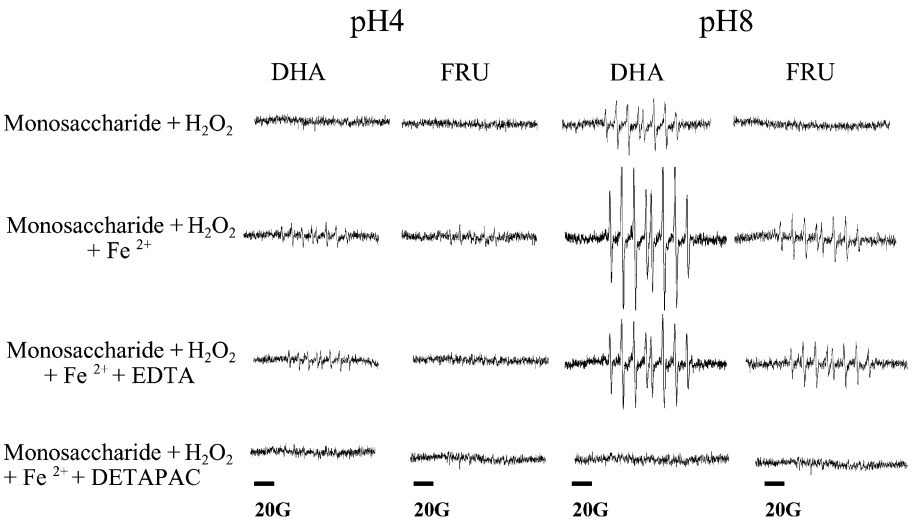


Figure 5. EPR measurements of the DEPMPO spin trap signal after 15 min of incubation of 3 mM dihydroxyacetone or fructose with 1 mM H_2O_2, in the absence or presence of 10 μmol $FeSO_4$, 0.5 mM EDTA or 0.5 mM DETAPAC (at 40 °C and pH 8, adjusted with 5 mM phosphate buffer).

tone–H_2O_2 reaction, suggesting that ·OH radical formation was not prevented. Nearly complete inhibition of glycolate production, following the addition of DETAPAC, as well as the disappearance of the DEPMPO-OH adduct signal, additionally proves that ·OH radicals are required for efficient glycolate synthesis. The absence of a DEPMPO-hydroxyalkyl radical adduct signal can be explained by the competition between ·OH radicals produced by the Fenton reaction and a low concentration of unstable hydroxyalkyl radicals.[34]

Figure 5 shows the EPR spectra of DEPMPO adducts in the reaction of dihydroxyacetone and fructose with hydrogen peroxide in the presence and absence of chelators and at two different proton concentrations of the reaction medium.

Presence of Fe^{2+} ions in the reaction markedly increased the signal, showing only the presence of the DEPMPO-OH adduct, confirmed by comparison of recorded signals with computed simulation of the DEPMPO-OH signal. An important difference could be observed between dihydroxyacetone and fructose at pH 8 (without metal present), where dihydroxyacetone exhibited a DEPMPO-OH signal and fructose did not. No indications of the presence of ·OH radicals were observed at lower pH in the case of dihydroxyacetone or any experiments with fructose. The absence of a DEPMPO-OH adduct signal at pH 4 in the case of dihydroxyacetone is a possible outcome of ·OH quenching by the present enediol in its more stable unionised form, leading to the formation of hydroxyalkyl radicals by the reaction of addition. At higher pH dihydroxyacetone enolisation is more rapid, leading to the formation of an unstable enediolate anion that rapidly reduces hydrogen peroxide, thus forming small amounts of ·OH radicals, even in the absence of metal ions.[35] Correlation of low glycolate yield from dihydroxyacetone with a weak DEPMPO-OH signal supports our previous hypothesis that in the absence of Fe^{2+}, the free-radical mechanism of glycolate synthesis is predominant at higher pH.

Thus, EPR measurements clearly demonstrate that hydroxyl radicals are involved in the reaction of H_2O_2-induced cleavage of monosaccharides, especially at high pH and with the shorter chain sugar, dihydroxyacetone. Furthermore, the observed formation of a DEPMPO-OH adduct signal at higher pH exhibited a positive correlation with enhanced glycolate formation from the dihydroxyacetone–H_2O_2 reaction, resulting in maximal amplitudes of the DEPMPO-OH adduct signal recorded. Observed anaerobic properties and absence of any adducts except the DEPMPO-OH signal argues in favour of a Fenton-like generation of ·OH radicals as being the sole source of radicals, excluding the superoxide forming Haber–Weiss reaction.

2.6. Physiological relevance

A number of simple sugars capable of tautomerising to enediols, such as those present in various biochemical pathways, are possible targets for H_2O_2 and free-radical action.[36,37] The results presented above demonstrate that different products of such action on monosaccharides (auto-oxidation, cleavage, isomerisation, free-radical formation) can be expected under physiological conditions, depending upon the microenvironment (pH, presence and concentration of H_2O_2 and metal ions, or hydroxyl radicals produced by other reactions), which will affect the direction of the reaction. Also, it is obvious that at the concentrations of monosaccharides and reactive oxygen species that can be expected within the cell or its organelles, especially under stress, a significant quantity of biochemical intermediates can be diverted to other biochemical cycles.

Glycolate, first isolated from plant tissue, is usually identified with the process of photorespiration[38] in photosynthetic cells, but it has been found in other tissues, for example, cerebrospinal fluid and kidney in animals,[39,40] often associated with urolithiasis or liver disorders.[41,42] In the case of photorespiration, enzymatic synthesis of glycolate via the oxidation of the transketolase reaction intermediate with H_2O_2,[43] or oxygenase action of Rubisco are considered to be the two mechanisms responsible for the phenomenon.[38] However, they are not able to explain the stoichiometry and outburst of glycolate synthesis with the rise in temperature, high light intensities and (drought) stress.[44] Direct oxygenation of monosaccharides with H_2O_2, studied previously under physiological conditions did not show rates efficient for the estimated glycolate production.[45,46] In those reports, investigators did not monitor the whole pH range of the reaction, with concomitant problems concerning quantification of reaction products as well as sugar depletion with the methods employed.

3. Experimental

3.1. Chemicals and reagents

All sugar standards, DETAPAC and EDTA were obtained from Sigma–Aldrich (Sigma Co. St. Louis, MO). We tested all of the sugars and standards for traces of all possible reaction intermediates and products, especially glycolate and formate. Standard and control solutions were prepared daily by dissolving in 18 MΩ redistilled and deionised water (Millipore, Bedford, MA) or in the mobile phase, which were additionally filtered and degassed through a 0.22-μm filter (Pall Gelman Lab. MI). For the preparation of 200 mM NaOH solution, 10.5 mL of sodium hydroxide solution (50% w/w, low carbonate, J.T. Baker, Deventer, Holland) was diluted in 1 L of previously vacuum-degassed deionised water. For organic acid analysis, 5 mM sulfuric acid was prepared daily by dissolving of 5 mL of filtered 1 M acid in 1 L of 18 MΩ deionised water.

3.2. Reaction parameters

If it not otherwise specified, reaction conditions in all experiments were carried out for 30 min at pH 4 or 8 (adjusted with 5 mM phosphate buffer), 40 °C, with the concentration of dihydroxyacetone and fructose being 3 mM and hydrogen peroxide 0.1–1.5 mM. The concentration of metals ($FeSO_4$), when added, was 10 μM and chelators were at 500 μM. Reactions were stopped by freezing in liquid nitrogen and stored at −20 °C prior to HPLC analysis.

3.3. HPLC determination of reaction products

Separations were performed on a Waters Breeze chromatographic system (Waters, Milford, MA) containing binary pumps system, a thermostated column compartment and a model 2465 Waters electrochemical detector. Injections were made manually with a Rheodyne 7725*i* valve (Rheodyne, Rohnert Park, CA), with injection-data start signal triggered through a 20 μL sample loop. The 2465 EC detector was equipped with flow cell body made of moulded polyurethane and a 3-mm gold working electrode with a hydrogen reference electrode. The 2465 detector was connected to the system and data acquisition devices by a BUS-SATIN module, as an external channel. Data acquisition and evaluation were carried out by Waters Breeze software.

3.3.1. HPLC determination of sugar utilisation. Separation of sugars was performed on CarboPac PA1 (Dionex, Sunnyvale, CA) 250 × 4 mm column equipped with corresponding CarboPac PA1 guard column. Sugars were eluted for 20 min at a flow rate of 1.0 mL min^{-1} at a constant temperature of 30 °C. Signals were detected at in the pulse mode with the waveform: $E_1 = +0.1$ V for 280 ms; $E_2 = +0.75$ V for 150 ms; $E_3 = -0.85$ V for 150 ms and within 80 ms of integration time. The filter timescale was of 0.2 s, and the range was 200–500 nA for the full mV scale.

3.3.2. HPLC analysis of organic acids. Separation of organic acids was performed on 250 × 4 mm Aminex HPX-87H (Bio-Rad Laboratories, CA) anion-exchange column with 5 mM H_2SO_4 as the mobile phase. Elution used was isocratic with a flow of 0.6 mL min^{-1} at 30 °C with two different HPLC systems: (a) a model 2465 Waters electrochemical detector adjusted for the pulse mode with the waveform: $E_1 = +0.3$ V for 280 ms; $E_2 = +1.4$ V for 150 ms; $E_3 = -0.4$ V for 280 ms and 40 ms integration time; (b) a Hewlett–Packard HP1100 series chromatograph (Palo, Alto, CA) composed of an inline degasser, an autosampler, a thermostated compartment and an HP 1100 photo diode array detector adjusted at 191 and 210 nm, with a reference signal at 600 nm. Additional peak confirmations were made with peak spectral evaluation via HP Chemstation chromatographic software, also used for data acquisition and method/run control.

3.4. EPR measurements

Spin-trapping EPR measurements were performed using a Varian EPR spectrometer, model E 104A, operating at X-band frequency (9.5 GHz). Measuring conditions ($H = 3410$ G, SW = 200 G, MA = 2 G, $P = 10$ mW) were the same in all EPR measurements,

using the spin-trap DEPMPO (5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-*N*-oxide) (Alexis Biochemicals, Switzerland) in either oxygen-permeable Teflon tubes (Zeus industries, Raritan, NJ) or in glass capillaries. We tested all of the reaction mixtures and buffers for occurrence of free radicals, and we did not detect any except the ones described in our results. Computer simulations of EPR spectra were performed using the computer program, WINEPR SimFonia (Bruker Analytische Messtechnik GmbH). Distilled and deionised 18 MΩ water (Millipore) was used in all experiments.

4. Conclusions

Our results for the first time demonstrate that keto-monosaccharides could under appropriate micro-environmental physiological conditions and oxidative stress be transformed nonenzymatically into a number of different intermediates including glycolate, but also 2-oxogluconic acid, glucose, ribose, glucitol (sorbitol), glyceraldehyde, formate and others, depending upon the monosaccharide. The results presented suggest that different mechanisms are responsible for the cleavage or other reactions (isomerisation, auto- or free-radical-mediated oxidation) of keto-monosaccharides depending on the keto-monosaccharide carbon chain length and presence of metal ions. Dihydroxyacetone derivatives could especially be a source of glycolate in a cleavage reaction with H_2O_2, especially at higher temperatures and pH value of the solution. At pH 8, the presence of metal ions stimulates the reaction by a Fenton-type of mechanism, with the participation of only $^{\bullet}OH$ radicals. Addition of DETAPAC to the reaction medium suppressed glycolate formation and prevented $^{\bullet}OH$ radical formation, supporting our hypothesis that a Fenton-type reaction is necessary for efficient glycolate production from keto-monosaccharides. These results could explain some of the discrepancies in the photorespiration-related literature.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carres.2006.06.023.

References

1. Moody, G. J. *Adv. Carbohydr. Chem.* **1964**, *19*, 149–179.
2. Friedmann, T. E. *J. Biol. Chem.* **1927**, *73*, 331–335.
3. Varela, O. *Adv. Carbohydr. Chem. Biochem.* **2003**, *58*, 307–369.
4. Arts, S.; Mombarg, E.; van Bekkum, H.; Sheldon, R. *Synthesis* **1997**, *6*, 597–613.
5. Dubois, M. F.; Rigal, L.; Gaset, A. *Can. J. Chem.* **1995**, *73*, 1582–1590.
6. Rodio, R. T.; Pereira, E. M.; Tavares, M. F. M.; Ferreira, A. M. D. *Carbohydr. Res.* **1999**, *315*, 319–329.
7. Maksimović, V.; Sladić, D.; Vučinić, Ž. *Yugoslav. Physiol. Pharmacol. Acta* **1998**, *34*, 135–144.
8. Calkins, V. P. *Anal. Chem.* **1943**, *15*, 762–763.
9. Isbell, H. S.; Frush, H. L.; Martin, E. T. *Carbohydr. Res.* **1973**, *26*, 287–295.
10. Isbell, H. S.; Frush, H. L. *Carbohydr. Res.* **1973**, *28*, 295–301.
11. Maksimović, V.; Mojović, M.; Neumann, G.; Vučinić, Ž. *Ann. N.Y. Acad. Sci.* **2005**, *1048*, 461–465.
12. Maksimović, V.; Vučinić, Ž. In *Photosynthesis: Mechanisms and Effects*; Garab, G., Ed.; Kluwer Academic: Dordecht–Boston–London, 1998; Vol. 5, pp 3719–3722.
13. De Wit, G.; KIeboom, A. P. G.; Van Bekkum, H. *Carbohydr. Res.* **1979**, *74*, 157–175.
14. Isbell, H. *Adv. Chem. Ser.* **1973**, *117*, 70–87.
15. Crabbe, M. J. C.; Goode, D. *Prog. Ret. E. Res.* **1998**, *17*, 313–383.
16. Stanek, J.; Gerny, M.; Kocourek, J.; Pacak, J. In *The Monosaccharides*; Academic Press: New York, 1963; pp 139–164.
17. Shalaby, M. A.; Isbell, H. S.; Elkhadem, H. S. *J. Carbohydr. Chem.* **1995**, *14*, 429–437.
18. Thornalley, P. J. *Environ. Health Persp.* **1985**, *64*, 297–307.
19. Mashino, T.; Fridovich, I. *Arch. Biochem. Biophys.* **1987**, *254*, 547–551.
20. Isbell, H. S.; Czubarow, P. *Carbohydr. Res.* **1990**, *203*, 287–289.
21. Isbell, H. S.; Frush, H. L.; Parks, E. W. *Carbohydr. Res.* **1976**, *51*, C5–C9.
22. Fenton, H. J. H. *J. Chem. Soc.* **1894**, *65*, 899.
23. Isbell, H. S.; Frush, H. L. *Carbohydr. Res.* **1977**, *59*, C25–C31.
24. Wolff, S. P.; Dean, R. T. *Bioelectrochem. Bioenerg.* **1987**, *18*, 283–293.
25. Davis, G.; Thornalley, P. J. *Biochim. Biophys. Acta—Bioenergetics* **1983**, *724*, 456–464.
26. Willson, R. L. *Chem. Ind.* **1977**, *1*, 183–192.
27. Buettner, G. R.; Doherty, T. P.; Patterson, L. K. *FEBS Lett.* **1983**, *158*, 143–146.
28. Mc Cord, J. M.; Day, E. D., Jr. *FEBS Lett.* **1978**, *86*, 139–142.
29. Elstner, E. F.; Osswald, W.; Konze, J. R. *FEBS Lett.* **1980**, *121*, 219–221.
30. Halliwell, B. *FEBS Lett.* **1978**, *92*, 321–326.
31. Burkitt, M. J. *Free Radical Res. Commun.* **1993**, *18*, 43–57.
32. Frejaville, C.; Karoui, H.; Tuccio, B.; Le Moigne, F.; Culcasi, M.; Pietri, S. L.; Tordo, P. *J. Med. Chem.* **1995**, *38*, 258–265.
33. Mojović, M.; Vuletić, M.; Bačić, G.; Vučinić, Ž. *J. Exp. Bot.* **2004**, *55*, 2523–2531.
34. Welch, K. D.; Davis, T. Z.; Aust, S. D. *Arch. Biochem. Biophys.* **2002**, *397*, 360–369.

35. Wolff, S. P.; Crabbe, M. J.; Thornalley, P. J. *Experientia* **1984**, *40*, 244–246.
36. Weiss, M. F.; Erhard, P.; Kader-Attia, F. A.; Wu, Y. C.; Deoreo, P. B.; Araki, A.; Glomb, M. A.; Monnier, V. M. *Kidney Int.* **2000**, *57*, 2571–2585.
37. Exner, M.; Hermann, M.; Hofbauer, R.; Kapiotis, S.; Speiser, W.; Held, I.; Seelos, C.; Gmeiner, B. M. K. *FEBS Lett.* **2000**, *470*, 47–50.
38. Andrews, T. J.; Lorimer, G. H.; Tolbert, N. E. *Biochemistry* **1971**, *10*, 4777–4782.
39. Hoffmann, G. F.; Meieraugenstein, W.; Stockler, S.; Surtees, R.; Rating, D.; Nyhan, W. L. *J. Inher. Metab. Dis.* **1993**, *16*, 648–669.
40. Lederer, F.; Belmouden, A.; Gondry, M. *Biochem. Soc. Trans.* **1996**, *24*, 77–83.
41. Baker, P. W.; Rofe, A. M.; Bais, R. *Crit. Rev. Clin. Lab. Sci.* **1996**, *33*, 39–82.
42. Yanagawa, M.; Maedanakai, E.; Yamakawa, K.; Yamamoto, I.; Kawamura, J.; Tada, S.; Ichiyama, A. *Biochim. Biophys. Acta* **1990**, *1036*, 24–33.
43. Wilson, T. A.; Calvin, M. J. *J. Am. Chem. Soc.* **1955**, *77*, 5948–5953.
44. Vučinić, Ž.; Grodzinski, B. *Fiziol. Biokhim. Kulturnih Rastenii* **1986**, *18*, 1–23 (in Russian).
45. Parnik, T.; Keerberg, O. *J. Exp. Bot.* **1995**, *46*, 1439–1447.
46. Oliver, J. D. *Plant Sci. Lett.* **1979**, *15*, 35–40.