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Glyoxal formation by Fenton-induced degradation of carbohydrates and related compounds

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Abstract—In this paper, we provide a systematic analysis of glyoxal (**1**) formation from a range of monosaccharides and related compounds, to determine their potential role as sources of this α-oxoaldehyde in vivo. Substrates were reacted with the Fenton reagent (Fe^{2+}/EDTA/H_2O_2) and the mixtures were analyzed by HPLC using the 6-hydroxy-2,4,5-triaminopyrimidine fluorimetric assay. The rank order of hexoses and their derivatives as glyoxal sources was found to be fructose > glucose = mannose = galactose > glucose-6-phosphate > mannitol. Within the pentose group, arabinose and ribose gave the higher yields of **1** followed by deoxyribose and its adenine N-glycosides and ribulose. Among the tested substrates, three-carbon compounds, that is, trioses and glycerol, but not glyceraldehyde-3-phosphate, were by far the most effective sources of **1**. The effects of H_2O_2 and Fe^{2+}/EDTA concentrations as well as of other metal ions were also investigated.

Keywords: Glyoxal; Hydroxyl radical; Carbohydrates; Oxidative stress; Free radicals

1. Introduction

Glyoxal (**1**) is a physiological α-oxoaldehyde metabolite, which can elicit a range of cytotoxic and genotoxic responses, including apoptosis and cell growth arrest,[1] because of its ability to form irreversible adducts with nucleic acids[2] and proteins, for example, in the generation of advanced glycation end products (AGEs).[3,4]

The modification of nucleic acids and protein by **1** has been implicated in the development of diabetic complications, for example, retinopathy, neuropathy, nephropathy, as well as in atherosclerosis, aging and uremia, all characterized by an advanced oxidative stress condition.[5–8] Recently, elevated plasma levels of **1** have also been demonstrated in rare genetic disorders with high cancer proneness, such as Fanconi's anaemia[9] and Werner's syndrome.[10] **1**-Modified proteins can undergo receptor-mediated endocytosis and lysosomal degradation in monocytes and macrophages, and can induce adhesion molecule expression, cytokine synthesis and secretion, and in some cases apoptosis.[11] In physiological systems, **1** is produced mainly by the slow spontaneous oxidative degradation of glucose and glycated proteins, through a retroaldol reaction followed by oxidation of the resulting glycolaldehyde.[8,12] Exposure of DNA or deoxynucleosides to a Fenton-type hydroxyl radical-forming system can also generate substantial amounts of **1**,[13,14] while a minor source is lipid peroxidation.[15] Although oxidative breakdown of carbohydrates provides an important contribution to the production of **1** in vivo,[16,17] and several aspects of this chemistry have been clarified,[18–20] a comparative investigation of the various sugars as potential sources of **1** under oxidative conditions of physiological relevance is lacking. A better understanding of the mechanisms of formation of this α-oxoaldehyde from carbohydrate oxidation would be desirable for strategies directed to the prevention and control of clinical complications of disease states

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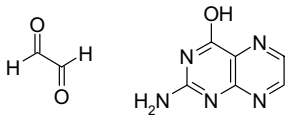
associated with oxidative stress and aberrant carbohydrate metabolism.



1 2

In this paper, we report the formation of **1** by oxidative degradation of a number of representative monosaccharides, as well as of some phosphate ester derivatives, glycitols, nucleosides and nucleotides. As oxidizing system, the Fenton reagent (Fe^{2+}/EDTA/H_2O_2) was chosen as a convenient model for free radical oxidations of (patho)physiological relevance. Specific aim of the study was to assess the relative influence of certain structural features of the substrate, such as the carbonyl function (aldehyde vs ketone), the length of the carbon chain and the nature of the functional groups, and the relative configuration of stereogenic centres, on the yields of formation of **1**.

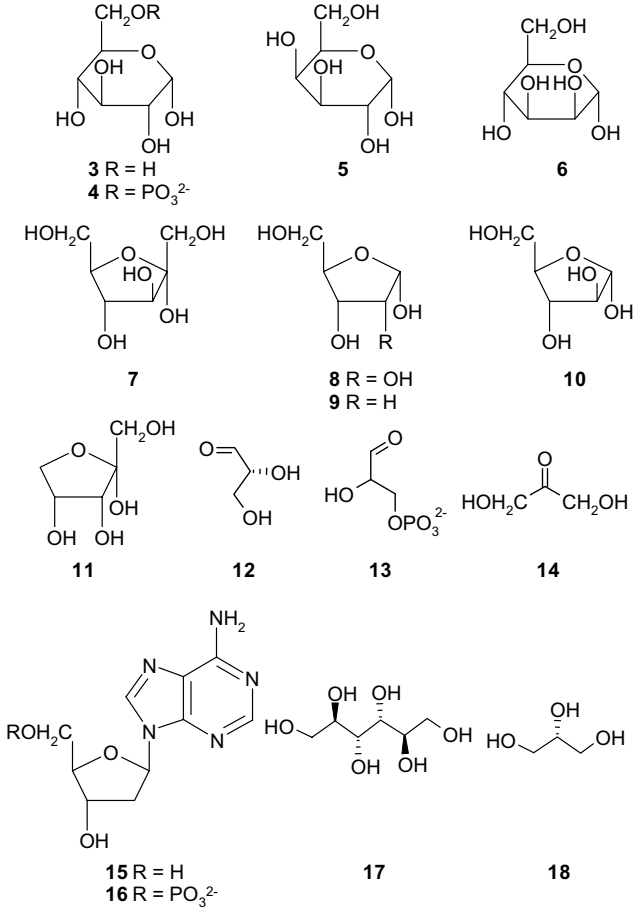
2. Results

Oxidative degradation of carbohydrates was carried out with the Fenton reagent in 0.1 M phosphate buffer, pH 7.4, at 37 °C. Typically, substrate concentration was 1 mM, Fe^{2+}/EDTA complex (1:1) was 100 μM, and H_2O_2 was 1 mM. Determination of **1** was carried out using 6-hydroxy-2,4,5-triaminopyrimidine as the derivatizing reagent, according to a reported procedure.[21] This reagent converts **1** into pterin (**2**), a highly fluorescent derivative ($\lambda_{ex} = 352$ nm, $\lambda_{em} = 447$ nm), which could be conveniently analyzed by high pressure liquid chromatography with fluorescence detection (HPLC-FD).

This method was chosen in view of its sensitivity (fluorimetric detection of the aldehyde derivative at picomole levels) and operational simplicity. Under the elution conditions adopted,[21] **2** was separated from pterins derived from other biological α-oxoaldehydes such as methylglyoxal, and could be unambiguously identified and quantified. Prior to analysis, treatment of oxidation mixtures with a small amount of $NaBH_4$ proved necessary to remove excess H_2O_2 and/or other materials interfering with the assay. This treatment ensured reproducible analyses with satisfactory chromatographic traces without affecting the determination of **1**, as checked in control experiments with pure standards. Preliminarily, the time course of the oxidation of glucose was followed by HPLC-FD analysis. At 15 min the maximum yields of **1** were reached after which time no significant increase was observed over 2 h. Accordingly, a reaction time of 30 min was adopted throughout this study.

The substrates investigated in the present study included hexoses (D-glucose (**3**), D-glucose 6-phosphate (**4**), D-galactose (**5**), D-mannose (**6**) and D-fructose (**7**)), pentoses (D-ribose (**8**), 2-deoxy-D-ribose (**9**), D-arabinose (**10**) and D-ribulose (**11**)), trioses (D-glyceraldehyde (**12**), D,L-glyceraldehyde 3-phosphate (**13**) and 1,3-dihydroxyacetone (**14**)), nucleosides (2′-deoxyadenosine (**15**)), nucleotides (2′-deoxyadenosine-5′-monophosphate (**16**)) and glycitols (mannitol (**17**), glycerol (**18**)).

The yields of formation of **1** by degradation of six-, five- and three-carbon monosaccharides and derivatives are reported in Table 1. Within each of these groups, **7**, **8/10** and **14** gave the highest yields of **1**, in that order. Analysis of the elutographic profiles of the oxidation mixture revealed the presence of 7-methylpterin derived from methylglyoxal. The estimated yield of methylglyoxal for **13** was 400 μM (40%), whereas for all other compounds the yields were much lower, typically below 1 μM, and did not show significant differences.



3 R = H
4 R = PO_3^{2-}
5
6
7
8 R = OH
9 R = H
10
11
12
13
14
15 R = H
16 R = PO_3^{2-}
17
18

Comparative analysis of formation yields of **1** from aldose/ketose couples, such as glucose/fructose, ribose/ribulose and glyceraldehyde/dihydroxyacetone, ruled out any influence of the carbonyl function. Moreover,

Table 1. Formation yields of **1** by Fenton-induced oxidation of compounds **3**–**18**

Compound	Yields of **1**[a] (%)	Compound	Yields of **1**[a] (%)
3	4.5 ± 0.5	**11**	2.5 ± 0.4
4	3.7 ± 0.5	**12**	12.6 ± 0.5
5	4.2 ± 0.5	**13**	4.3 ± 0.5
6	4.6 ± 0.5	**14**	13.6 ± 0.6
7	5.8 ± 0.5	**15**	4.7 ± 0.5
8	7.4 ± 0.5	**16**	3.6 ± 0.5
9	4.7 ± 0.5	**17**	3.2 ± 0.4
10	7.3 ± 0.5	**18**	10.9 ± 0.8

[a] Shown are the means of three experiments ± SD.

within the aldohexose and aldopentose groups, formation of **1** was not affected by changes in configuration at C-2 and C-4.

Glycitols were relatively poor sources of **1** compared to the corresponding sugars. 2′-Deoxyribose (**9**) and 2′-deoxyadenosine (**15**) gave similar yields of **1**, but lower than those from ribose (**8**). Control experiments (not shown) indicated negligible formation of **1** from adenine free base. The presence of a phosphate group on the terminal sugar carbon inhibited the formation of **1** compared to the unsubstituted derivative, though the effect was evident for glyceraldehyde-3-phosphate, lesser for 2′-deoxyadenosine-5′-monophosphate and statistically not significant for glucose 6-phosphate.

In another series of experiments, the formation of **1** from glucose was investigated under different oxidation conditions. The data reported in Figure 1 showed that the Fenton reagent was the most effective in inducing the formation of **1** from glucose, whereas the Cu^{2+}-based variant was significantly less active. Notably, EDTA enhanced the oxidation promoted by the Fe^{2+}/H_2O_2 couple, but substantially decreased that of the Cu^{2+}/H_2O_2 system.

The effect of Fe^{2+}/EDTA concentration on the yields of **1** was also investigated. As shown in Figure 2, product formation increased with increasing complex concentration and reached a plateau at a Fe^{2+}/EDTA value of 1 mM, that is at a substrate/oxidant molar ratio of 1.

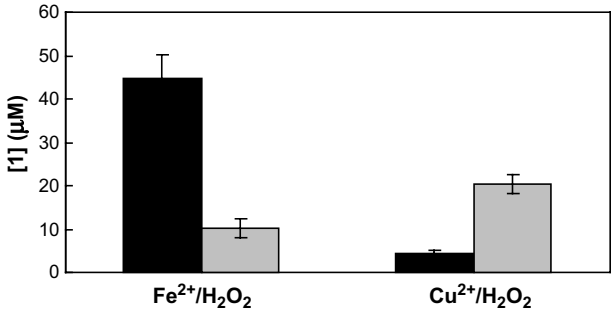


Figure 1. Effects of metal cations on the oxidation of **3** by the Fenton system in the presence (black bars) or in the absence (white bars) of EDTA. Shown are the means of three experiments ± SD.

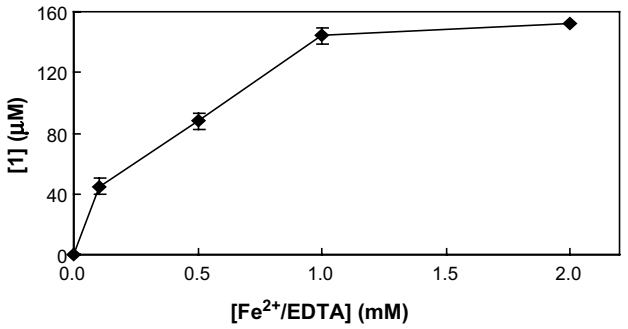


Figure 2. Effect of the concentration of the Fe^{2+}/EDTA complex on the Fenton-induced oxidation of **3**. Shown are the means of three experiments ± SD.

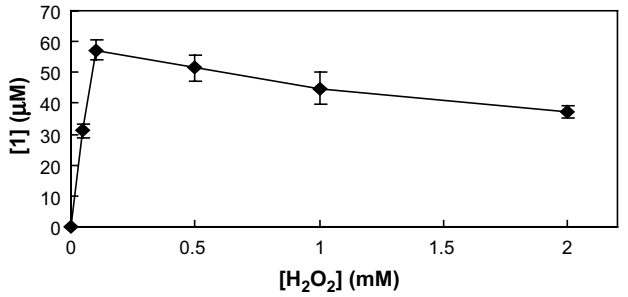


Figure 3. Effect of hydrogen peroxide concentration on the Fenton-induced oxidation of **3**. Shown are the means of three experiments ± SD.

Figure 3 shows the effect of H_2O_2 concentration on the formation of **1** from D-glucose treated with 100 μM of Fe^{2+}/EDTA. Yields of **1** increase with H_2O_2 concentration up to a 1:1 oxidant/iron complex molar ratio, and then decrease with excess of hydrogen peroxide.

With all systems examined, careful exclusion of oxygen from the reaction mixtures resulted in a marked decrease in product formation.

3. Discussion

Carbohydrate oxidation by the Fenton reagent was previously shown by ESR analysis to proceed via hydroxyl radical-mediated H-atom abstraction to produce carbon-centred radicals rather than alkoxyl radicals.[18,19,22,23] In air-saturated medium, carbon-centred radicals can react with oxygen at rates of $10^9\ M^{-1}\ s^{-1}$ to give the corresponding peroxyl radicals,[24] which may decompose with loss of $HOO^{\cdot}$ to give glucosones[18] (this however would hardly be a feasible route to **1**) or may be involved in H-atom abstraction to form the corresponding hydroperoxides. This latter, in turn, can follow two main pathways: (a) Criegee rearrangement with ring expansion, leading to a lactone derivative;[25] (b) reduction by Fe^{2+}

ions, in a Fenton-like reaction, to give an alkoxyl radical with subsequent β-fragmentation.[26] Other decomposition routes of peroxyl radicals, for example, dimerization via labile tetroxide species,[27,28] can be disregarded as being of minor importance.

In the present study, 1 M equiv of hydrogen peroxide with respect to the substrate, with a defect of the Fe^{2+}/EDTA complex, was used to ensure efficient H-atom abstraction and free radical formation from the substrate. Under such conditions, it can be assumed that the Fenton reagent generates a carbon-centred radical triggering the oxidative process, and that subsequent steps, including intermediate degradation, do not involve Fenton-type hydroxyl radical-mediated chemistry.

Straightforward application of the two productive degradation mechanisms, that is, the Criegee and the reduction route, to the six possible hydroperoxides deriving from glucose would indicate that only those at C-3 and C-4 can give rise to **1**. On the other hand, hydroperoxides at C-1, C-2, C-5 and C-6 would decompose through apparently less relevant channels: for example, hydroperoxides at C-1 and C-6 would give rise mainly to formic acid, that at C-2 to glyoxylic acid and that at C-5 to glycolic acid.[18]

The lower ability of deoxyribose (**9**) to yield **1** with respect to ribose (**8**) can be attributed to the different energies needed for H-atom abstraction from the various positions: in particular, the energy of the C–H bond for C-2 is higher (3–4 kcal/mol) than that of C-1, C-3 and C-4 due to the lack of the hydroxyl group, so abstraction from C-2 carbon is less favourable.[29] As a result, **1** can be produced only via radicals at C-3 and C-4, whereas in the case of **8**, **1** can arise from radicals C-2, C-3 and C-4, viz. a higher number of productive fragmentation routes are available to the latter sugar.

The lack of significant differences in the reactivity of epimeric aldohexoses or aldopentoses would rule out any significant influence of the configuration at C-2 and C-4 and hence of axial/equatorial orientation of C–H bonds. As a relevant remark, previous studies reported similar hyperfine splittings in the ESR spectra for radicals at C-4 produced by Ti(III)/H_2O_2 oxidation of β-D-glucose and β-D-galactose, entailing similar structures and, by implication, similar evolution routes.[30] The lower yield of **1** from sugar-phosphates compared to the unsubstituted derivatives could be explained by assuming that the bulky phosphate group hinders in part the CH_2 residue from $HO^{\cdot}$ attack. The different formation yield ratios of **1** determined for aldose/ketose couples belonging to six-, five- and three-carbon monosaccharides can be rationalized, considering the different partitioning of hexoses and pentose between the pyranose and furanose forms, whereas cyclic hemiacetal forms are precluded to trioses. Another noteworthy point about the proposed mechanisms relates to previous ESR experiments showing that for pyranose derivatives $OH^{\cdot}$ attack at C–H bonds is little or not selective,[18,30] but is rather selective in the case of furanose forms, occurring preferentially at the C-5 carbon bearing the alicyclic oxygen.[31]

Oxidation of glycitols (mannitol and glycerol) would likewise yield for the most part glycolaldehyde (CH_2OH–CHO) that would be progressively oxidized to **1**.

Three-carbon substrates, that is, glyceraldehyde (**12**) and dihydroxyacetone (**14**), were by far the best potential sources of **1**. In the case of **12**, a plausible route to **1** would involve Criegee rearrangement of the hydroperoxide at C-3 (formation of the hydroperoxide at C-2 would be unfruitful) (Fig. 4a), whereas for **14** routes of formation of **1** would rely on β-fragmentation of the alkoxyl radicals produced by Fe^{2+}-induced reduction of the primary hydroperoxides (Fig. 4b).

Finally, the superior ability of the Fenton reagent to cause the formation of **1** from glucose oxidation compared to the Cu^{2+} variant, and the divergent effects of EDTA can be explained in terms of changes in reduction potentials after chelation. Whereas EDTA decreases the reduction potential of the couple Fe^{3+}/Fe^{2+} from +0.77 to +0.12 eV,[32] it increases that of the Cu^{2+}/Cu^{+} couple from +0.16 to +5.71 eV (this latter potential has been obtained by CNDO calculations).[33] This implies that the formation of **1** depends on reductive activation of H_2O_2, consistent with operation of Fenton-type chemistry. When an excess of H_2O_2 was used, the yields of **1**

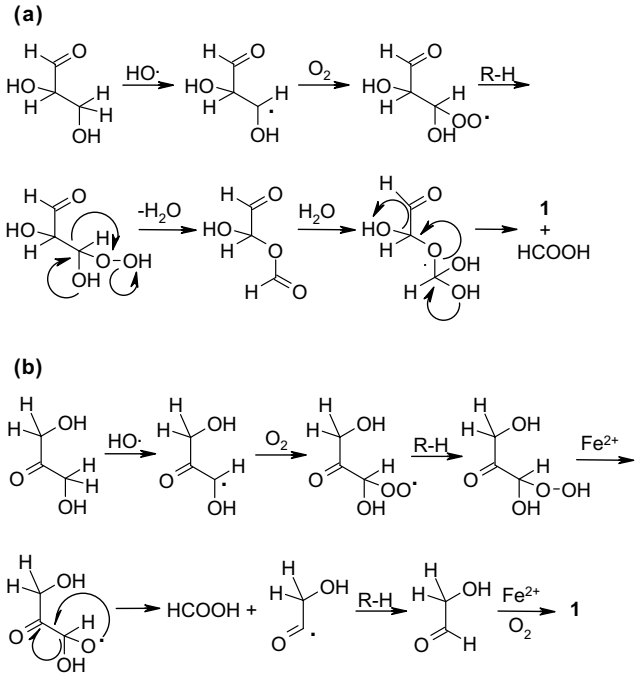


Figure 4. Mechanism proposed for the formation of **1** from the C-2 hydroperoxide of compound **12** (a) and from the C-1 hydroperoxide of compound **14** (b).

decreased quite linearly suggesting that unreacted hydrogen peroxide can act as an OH radical scavenger. In fact, OH radicals react with hydrogen peroxide with a rate constant of 2.7×10^7 M^{-1} s^{-1}.[34,35]

In conclusion, this is the first study providing a comparative analysis of the relative abilities of a number of carbohydrates and related compounds to yield **1** upon oxidation by the Fenton reagent. Within the hexose, pentose and triose groups, fructose, ribose and dihydroxyacetone gave the highest yields of **1**, in that order, with trioses far exceeding higher homologues as potential sources of **1**. This latter finding points to novel interesting links between oxidative stress, altered triose metabolism and AGE-related cyto-/genotoxicity.

4. Experimental

4.1. Materials and methods

D-Glucose, D-glucose-6-phosphate, D-galactose, D-mannose, D-fructose, D-ribose, 2-deoxy-D-ribose, D-arabinose, 1,3-dihydroxyacetone and 2′-deoxyadenosine were purchased from Aldrich. D-Ribulose, D,L-glyceraldehyde-3-phosphate and 2′-deoxyadenosine-5′-monophosphate were from Sigma. D-Glyceraldehyde, 6-hydroxy-2,4,5-triaminopyrimidine, ethylenediaminotetraacetic acid (EDTA) and $CuSO_4 \times 5H_2O$ were from Fluka. Hydrogen peroxide (30% solution in water) and $Fe(NH_4)_2(SO_4)_2 \times 6H_2O$ were from Carlo Erba. Glyoxal (hydrate, in trimeric form) and glycerol were purchased from Merck and mannitol was from ICN. Pterin was synthesized according to the procedure reported in the literature.[21] All buffer solutions were prepared in bidistilled water and were freed from possible metal impurities by passage through a Chelex 100 resin column. High pressure liquid chromatography was carried out on a Gilson instrument equipped with a model 305 pump. A Jasco FP-110 fluorescence detector with excitation set at 365 nm and emission at 447 nm was used for detection. Analyses were carried out on a 250×4.60 mm Sphereclone ODS(2) 5 μm column using 0.02 M sodium acetate buffer (pH 4.0)/acetonitrile (99:1 v/v) as the eluant at a flow rate of 1.3 mL/min.

4.2. Glyoxal assay

Glyoxal determinations were carried out according to a procedure reported in the literature with slight modifications.[21] In brief, 200 μL aliquots of the oxidation mixture were placed in a 3 mL vial and treated with 8 μL of a 25 mM solution of sodium borohydride in water. Three-hundred microlitres of a 7.02×10^{-3} M solution of 6-hydroxy-2,4,5-triaminopyrimidine, 200 μL of a 0.02 M sodium acetate buffer solution (pH 4.0) and deionized water were added up to a 2 mL final volume. The samples were heated at 60 °C for 45 min. Aliquots of the reaction mixture (100 μL) were withdrawn and subjected to HPLC-FD analysis. Under the conditions described above, pterin is eluted at 9.2 min. Quantitation of glyoxal was carried out by comparing integrated peak areas with calibration curves obtained with authentic samples subjected to the derivatization procedure described above.

4.3. Oxidative degradation of monosaccharides: general procedure

Substrate solutions (1.0 mM) in 0.1 M phosphate buffer (pH 7.4) in a water bath thermostated at 37 °C were treated with the Fe^{2+}/EDTA complex (100 μM) and with H_2O_2 (1.0 mM), in order. After 30 min, aliquots of the reaction mixture were withdrawn and subjected to derivatization with 6-hydroxy-2,4,5-triaminopyrimidine followed by HPLC-FD analysis. Similar experiments were performed as follows: (a) varying the amounts of the Fe^{2+}/EDTA complex (0.1, 0.5, 1.0, 2.0 mM); (b) varying the amounts of H_2O_2 (0.05, 0.1, 0.5, 1.0, 2.0 mM); (c) using Cu^{2+}/EDTA/H_2O_2 as the oxidant; (d) without the addition of EDTA and (e) withdrawing aliquots of the oxidation mixture at different time intervals over 2 h. All experiments were run at least in triplicate. In all experiments, the possible artifactual formation of some glyoxal by thermal degradation of the mixture during the derivatization procedure was taken into account by subtracting the values measured at zero time from those determined during oxidation. Usually, zero time levels were low and did not exceed 10% of the values determined after 30 min oxidative degradation.

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