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Iron (II) Ions Induced Oxidation of Ascorbic Acid and Glucose

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Lipid peroxidation (LPO) of polyunsaturated fatty acids (PUFAs) is suspected to be involved in the generation of chronic diseases. A model reaction for LPO is the air oxidation of PUFAs initiated by Fe^{2+} and ascorbic acid. In the course of such model reactions glycolaldehyde (GLA) was detected as main aldehydic product. Since it is difficult to explain the generation of GLA by oxidation of PUFAs, it was suspected that GLA might be derived by oxidation of ascorbic acid. This assumption was verified by treatment of ascorbic acid with Fe^{2+}.

Produced aldehydic compounds were trapped by addition of pentafluorobenzylhydroxylamine hydrochloride (PFBHA-HCl), trimethylsilylated and finally identified by gas chromatography/mass spectrometry (GC/MS). Oxidation of ascorbic acid with O_2 in presence of iron ions produced not only glycolaldehyde (GLA), but also glyceraldehyde (GA), dihydroxyacetone (DA) and formaldehyde. Glyoxal (GO) and malondialdehyde (MDA) were detected as trace compounds.

The yield of the aldehydic compounds was increased by addition of lipid hydroperoxides (LOOH) or H_2O_2. The buffer influenced the reaction considerably: Iron ions react with Tris buffer by producing dihydroxyacetone (DA). Since ascorbic acid is present in biological systems and Fe^{2+} ions are obviously generated by cell damaging processes, the production of GLA and other aldehydic components might add to the damaging effects of LPO.

Glucose suffers also oxidation to short-chain aldehydic compounds in aqueous solution, but this reaction requires addition of equimolar amounts of Fe^{2+} together with equimolar amounts of H_2O_2 or 13-hydroperoxy-9-cis-11-trans-octadecadienoic acid (13-HPODE). Therefore this reaction, also influenced by the buffer system, seems to be not of biological relevance.

Keywords: Fe^{2+} induced oxidation, ascorbic acid oxidation, glucose oxidation, glycolaldehyde, glyceraldehyde

Abbreviations: BHT, 2,6-di-tert.-butyl-4-methyl-phenol; DA, dihydroxyacetone; DHA, dehydroascorbic acid; DMF, N,N-dimethylformamide; EDTA-Na_2, ethylenediamine-tetraacetic acid disodium salt dihydrate; EI, electron impact; GA, glyceraldehyde; GC, gas chromatography; GC/MS, gas chromatography/mass spectrometry; GLA, glycolaldehyde; GO, glyoxal; LDL, low density lipoprotein; LOOH, lipid hydroperoxide(s); LPO, lipid peroxidation; MDA, malondialdehyde; MSTFA, N-methyl-N-trimethylsilyl-trifluoroacetamide; PFBHA-HCl, pentafluorobenzylhydroxylamine hydrochloride; PFBO, pentafluorobenzyl-oxime, PUFA, polyunsaturated fatty acid; TMS, trimethylsilyl; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; phosphate buffer, 0.1 M KH_2PO_4/0.1 M Na_2HPO_4 = 19.7:80.3; 13-HPODE, 13-hydroperoxy-9-cis-11-trans-octadecadienoic acid

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INTRODUCTION

The oxidation of LDL is assumed to be an essential step in the generation of atherosclerosis.[1–6] This reaction is commonly mimicked by the metal catalyzed oxidation of LDL.[7] LDL contains unsaturated fatty acids, these are obviously oxidized in this process.[8,9]

The oxidation of unsaturated acids in LDL, especially arachidonic acid, is often simulated by their air oxidation in presence of iron ions and ascorbic acid.[10,11] In the course of such reactions aldehydes are produced,[12–14] some of these are toxic.[15,16]

We detected recently that besides well known aldehydic compounds also α-hydroxyaldehydes are generated,[13,17,18] which cause an oxidative burst in stimulated macrophages.[19]

After oxidation of arachidonic acid we detected glycolaldehyde (GLA) as main hydroxy aldehydic compound. It exceeded the amount of 4-hydroxynonenal—the most prominent hydroxyaldehyde produced by degradation of arachidonic acid—by a factor of 20.[13]

Since it seemed impossible that GLA derived in such quantities from an unsaturated acid, we checked the possibility that it may have been produced from another source, e.g. a compound with the preformed group -HC-CH(OH)-CH_2OH. This structural element is present in ascorbic acid, which is used in the model oxidation reactions of PUFAs to reduce Fe^{3+} ions. Ascorbic acid is oxidized in this process to dehydroascorbic acid (DHA).[20] In aqueous solutions DHA is easily hydrolyzed to 2,3-diketo-L-gulonic acid,[21] which is also rather unstable even in neutral solution[22] and further degraded by cleavage between C-2 and C-3 to give threonic acid and glyoxylic acid or by cleavage of the C-1/C-2 bond to generate oxalic acid and glycerinic acid.[23] Also threose was observed as oxidation product.[23,24] But in this connection the formation of glycolaldehyde had not been reported before. Therefore we subjected ascorbic acid—and further glucose—to reaction with Fe^{2+} only. Here we report on the results of this investigation.

MATERIALS AND METHODS

Materials

N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) was obtained from Macherey & Nagel (Düren, Germany), dihydroxyacetone from Sigma (Deisenhofen, Germany) and dehydroascorbic acid from Aldrich (Steinheim, Germany). All other chemicals were purchased from Fluka (Neu Ulm, Germany). Solvents, obtained from Merck (Darmstadt, Germany), were distilled before use. TLC was performed with home made 0.75 mm PF_{254} silica gel 60 (Merck, Darmstadt, Germany) plates. Arachidonic acid was stored at –18°C under argon. 1-Hydroxy-2-tridecanone, used as standard, was synthesized according to the method of Meyer.[25] 13-Hydroxy-9-cis,11-trans-octadecadienoic acid was prepared as described previously.[26,27]

Autoxidation of Ascorbic Acid (Sodium Salt) and Glucose by Addition of Fe^{2+} According to Toyoda[10,28]

3.8 ml of 20 mM aqueous ascorbate or glucose solution were added to a mixture of 23 ml of 0.1 M Tris/HCl buffer (pH = 7.4) and 45 ml of 0.15 M aqueous KCl solution containing 3.8 ml of 0.8 mM Fe(II)sulfate. The reactions were carried out for 12 h at room temperature.

In a second experiment 15 mg of arachidonic acid were treated with the above described mixture of ascorbate/Fe^{2+} in Tris buffer. The oxidation reaction was stopped after 0 min, 10 min, 60 min, 3 h, 12 h, 24 h and 48 h by addition of 80 µl BHT (2% in methanol) and 1,2 ml EDTA-Na_2 (1% in bidistilled water)[29] to investigate the kinetics of GLA formation.

Autoxidation of Ascorbic Acid (Sodium Salt), Glucose and their Methylated Derivatives with Equimolar Amounts of Fe^{2+}, H_2O_2 or 13-HPODE

In following experiments either 30 µmol of the sodium salt of ascorbic acid, its 2,3-dimethoxy

derivative or its 2,3,5,6-tetramethoxy derivative were reacted with 30 μmol of $Fe\,SO_4$ together with 30 μmol H_2O_2 or 30 μmol 13-HPODE in a solution of 23 ml 0.1 M aqueous Tris/HCl-buffer (pH = 7.4) and 45 ml aqueous KCl (0.15 M).

Similar experiments were carried out with a solution of 30 μmol glucose or its pentamethylated derivative. All solutions were incubated for 24 h at room temperature. The above described reactions were performed also in 0.1 M aqueous phosphate buffered solution (0.1 M KH_2PO_4/0.1 M Na_2HPO_4 = 19.7:80.3) at pH = 7.4.

Autoxidation of GLA

Autoxidation was done as already described[10,28] with 3 mg GLA dissolved in 23 ml of 0.1 M aqueous phosphate buffer and 45 ml 0.15 M aqueous KCl (44 μM $FeSO_4$; 1 mM Na-ascorbate). The solution was incubated for 24 h at room temperature.

Autoxidation of Dehydroascorbic Acid

Autoxidation of dehydroascorbic acid was carried out either as described above in presence of 44 μM Fe^{2+} or additionally with equimolar amounts of Fe^{2+} in phosphate buffer for 24 h as described for ascorbate.

Preparation of 5,6-Di-(trimethylsilyloxy)-2,3-dimethoxy Derivative of Ascorbate

The methanolic solution of 30 mg ascorbic acid was treated with etheric diazomethane solution to convert the enolic hydroxy groups in position 2 and 3 into methoxy derivatives.

The 2,3-dimethoxy derivative was separated from other products by TLC in cyclohexane/ethylacetate/methanol 3:4:1. Detection was performed by measuring the UV absorption at 254 nm.

The fraction (R_f = 0.65–0.77) containing the 2,3-dimethoxy derivative of ascorbic acid was eluted with ethylacetate. The compound was identified after trimethylsilylation by GC/MS:

$$GC\,(DB-1)\text{: } R_i = 1783/1820.$$

MS [relative intensity (%)]: 73(100), 117(78), 147(82), 173(20), 205(82), 216(90), 217(30), 229(24), 258(10), 303(7), 333(38), 348(2).

Preparation of 2,3,5,6-Tetramethoxy Derivative of Ascorbic Acid

The pure 2,3-dimethoxy derivative of ascorbic acid obtained after TLC was further subjected to derivatisation with methyl iodide/silver oxide following the method of Kuhn[30] in order to convert also the alcoholic hydroxy groups in position 5 and 6 to methoxy groups.

$$GC(DB-1)\text{: } R_i = 1440/1495.$$

MS [relative intensity (%)]: 43(41), 58(18), 86(25), 99(100), 115(10), 129(30), 185(12), 200(32), 217(5), 232(2).

Preparation of Pentamethylated Derivative of Glucose

40 mg of D(+)-glucose monohydrate were dissolved in DMF and treated with CH_3I/Ag_2O as described by Kuhn.[30] The MS was in agreement with that described by Heyns[31] and Kochetkov.[32]

Preparation of PFBO-Derivatives and Quantification

The carbonyl groups of oxidation products were transformed to pentafluorobenzyloxime derivatives by reaction with PFBHA-HCl as described earlier.[33] Quantification of GLA was carried out by addition of 0.2 mg 1-hydroxy-2-tridecanone (prepared as described previously[25]) as internal standard. Quantification of MDA and glyoxal was achieved by addition of 0.2 mg PFBO-derivative of acetylacetone[13] as internal standard. The PFBO-derivatives were extracted with $CHCl_3$ and

trimethylsilylated with MSTFA followed by identification with GC/MS.[13] All quantification analysis represented in figures 2–5 were carried out in dublicate. The data represented in figures 1 and 6 are the mean values of three determinations.

Preparation of Comparison Compounds of the PFB-oxime TMS-ether Derivatives of Dihydroxyacetone, DL-Glyceraldehyde, Glycolaldehyde and Formaldehyde

The syntheses were achieved by addition of 0.5 ml PFBHA-HCl solution (0.05 M in Methanol) to a solution of 0.3 mmol of the aldehydic compounds in 10 ml Tris/HCl buffer (pH=7.4).

PFB-oxime TMS-ether Derivative of Dihydroxyacetone

$$GC\,(DB-1)\text{: } R_i = 1703$$

MS [relative intensity (%)]: 73 (100), 103 (65), 147 (40), 158 (17), 181 (67), 218 (30), 248 (15), 324 (17), 339 (40), 414 (38), 429 (2).

PFB-oxime TMS-ether Derivative of DL-Glyceraldehyde

$$GC\,(DB-1)\text{: } R_i = 1671/1690$$

MS [relative intensity (%)]: 73 (100), 103 (79), 147 (50), 181 (33), 218 (68), 248 (18), 326 (15), 339 (2), 414 (10), 429(1).

PFB-oxime TMS-ether Derivative of Glycolaldehyde

$$GC\,(DB-1)\text{: } R_i = 1419/1430$$

MS [relative intensity (%)]: 73 (20), 75 (25), 84 (50), 114 (60), 130 (10), 150 (12), 181 (100), 198 (23), 255 (20), 312 (22), 327 (1).

PFB-oxime Derivative of Formaldehyde

$$GC\,(DB-1)\text{: } R_i = 985$$

MS [relative intensity (%)]: 117(4), 131(3), 161(12), 167(9), 181(100), 195(24), 225(2).

Gas Chromatography/Mass Spectrometry

GC was carried out with a Carlo Erba HRGC 5160 Mega Series chromatograph equipped with a flame ionisation detector, using a DB-1 fused-silica glass capillary column (30 m × 0.32 mm i.d.) and a temperature programme from 80°C to 280°C at 3°C/min. The temperature of the injector and detector was kept at 270°C and 290°C respectively. The carrier gas was hydrogen and the splitting ratio was 1:30. Peak area integration was achieved with a Merck D-2500 integrator.

GC/MS was performed on a Finnigan MAT 95 mass spectrometer connected to a MAT-ICIS data system. EI mass spectra were recorded at an ionisation energy of 70 eV. A HP 5980 series II gas chromatograph with a 30 m × 0.3 mm (i.d.) DB-1 fused-silica column was used for sample separation. The carrier gas was hydrogen and the temperature programme was the same as used for GC.

RESULTS

Oxidation experiments were interrupted after 12 h and 24 h respectively by addition of BHT. The oxidation products were trapped by PFBHA-HCl and the samples were subjected to GC/MS investigation after trimethylsilylation as reported recently.[13] Derivatized α-hydroxyaldehydes were recognized by measuring the ion currents of specific α-fragments (m/z 326, m/z 181) or other typical ions.[13,14,36] Quantification of GLA was achieved by measuring the ion current of the typical M^+-15 fragment in comparison to those of the internal standard. In the case of MDA and glyoxal (GO) mass spectrometric ion tracing of the fragment M^+-197 was used for quantification.[13] Besides quantified aldehydes also other aldehydic compounds (glyceraldehyde, dihy-

droxyacetone, formaldehyde) were found in traces, but quantification was not possible due to lack of a suitable internal standard.

First Fe^{2+} ions were added in 44 μM concentrations to several oxidation samples in Tris buffered solution: After oxidation of arachidonic acid with ascorbate in presence of Fe^{2+} by use of Tris buffer, the amount of GLA produced surmounted those of GO and MDA by a factor of about 50 and 200 respectively (Figure 1A). Nearly the same amount of GLA was detected in absence of arachidonic acid (Figure 1C). By repeating the oxidation reaction with arachidonic acid and iron ions, but in absence of ascorbic acid, less than 1/10 of the amount of GLA was detected compared to the preceeding experiments (figure 1B). Nevertheless this amount was still 10 times higher than that of GO and MDA (Figure 1B).

Therefore GLA formed during incubation of arachidonic acid with Fe^{2+}/ascorbate mainly derives from ascorbic acid and not from arachidonic acid.

Interestingly the addition of glucose to oxidation samples influenced the yield of GLA: If arachidonic acid was oxidized in presence of iron ions and glucose but without addition of ascorbic acid (Figure 1D), the yield of GLA as well as the yield of MDA and GO decreased compared to the experiment carried out without adding glucose (figure 1B): Glucose obviously inhibited the reaction.

The yield of GLA obtained by treatment of ascorbic acid with Fe^{2+} is not increased by increasing the Fe^{2+} concentration (Figure 1C, Figure 2A).

Ascorbic acid produces nearly the same amounts of GLA in presence of a 44 μM Fe^{2+} solution (76 μmol substrate)—(Figure 1C) as well as in presence of equimolar amounts of iron ions (Figure 2A), but addition of 13-

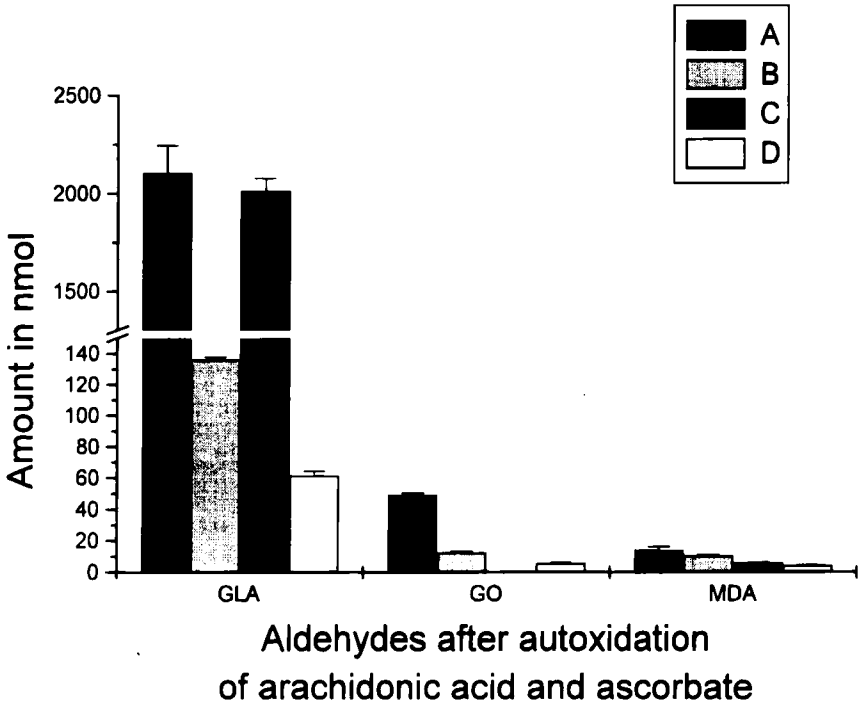


FIGURE 1 Oxidation products (GLA, GO, MDA) obtained after Tris buffered autoxidation (12 h) of: A) arachidonic acid in 44μM Fe^{2+}—and 1 mM sodium ascorbate solution, B) arachidonic acid in 44μM Fe^{2+} solution, C) sodium ascorbate in 44μM Fe^{2+} solution, D) arachidonic acid in 44μM Fe^{2+}—and 1mM glucose solution, Data are shown as mean values ± SD (n = 3).

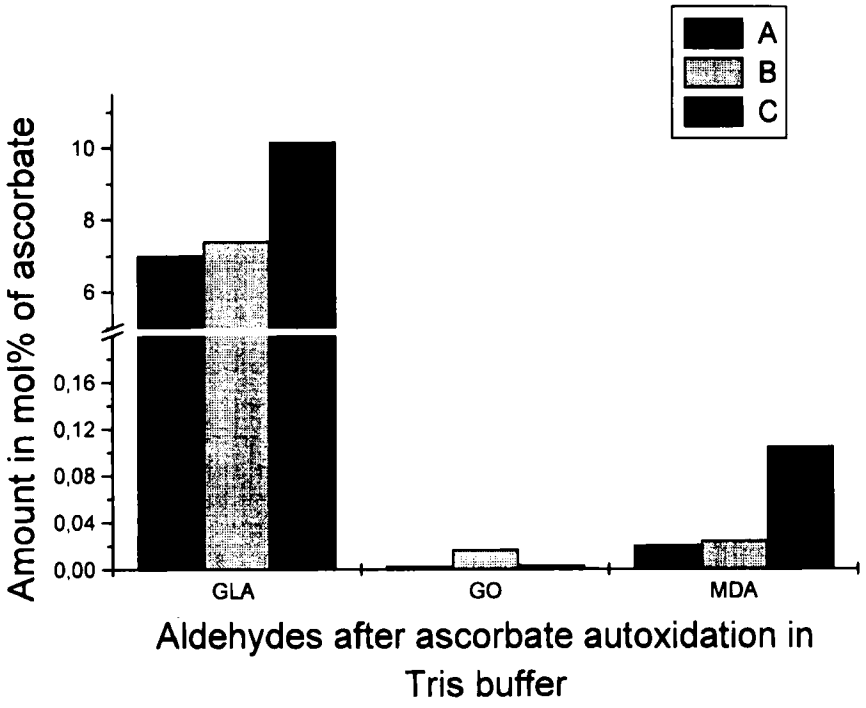


FIGURE 2 Oxidation products (GLA, GO, MDA) obtained after autoxidation of 30 µmol ascorbate in Tris buffer (24 h) by addition of: A) equimolar amounts of Fe^{2+}, B) equimolar amounts of Fe^{2+} and 13-HPODE, C) equimolar amounts of Fe^{2+} and H_2O_2, Data represents mean values of dublicate determinations.

HPODE (figure 2B) and particularly of H_2O_2 (Figure 2C) to the equimolar oxidation samples increased the yield. In this connection only traces of GO were obtained and the amount of MDA increased slightly after addition of H_2O_2 (Figure 2C).

The influence of phosphate buffer on the oxidation of ascorbic acid is shown in figure 3. The yield of GLA obtained by oxidation of ascorbic acid in presence of phosphate buffer with equimolar amounts of Fe^{2+} (Figure 3A) reached only 5% of the amount obtained by use of Tris buffer (Figure 2A). The highest yield of GLA in a phosphate buffer system was produced after addition of equimolar amounts of 13-HPODE (Figure 3B), but even then the yield of GLA was still reduced by a factor of about 3 compared to the Tris buffer system (Figure 2B). Addition of H_2O_2 to the oxidation sample in phosphate buffer (Figure 3C) produced GLA in lower yield than addition of 13-HPODE did (Figure 3B). GO and MDA were nearly absent in the phosphate buffer system.

Oxidation of glucose in Tris buffer and in presence of equimolar amounts of Fe^{2+} ions obviously produced only negligible amounts of GO (Figure 4A). In this connection MDA was produced in just detectable amounts and GLA was formed in 1/40 of the yield compared to ascorbate oxidation with equimolar amounts of Fe^{2+} (Figure 4A and 2A). Addition of 13-HPODE or H_2O_2 increased the yield of GLA for about 50% to 60%, but only H_2O_2 addition caused an increase of MDA by a factor of approximately 10 (Figure 4C).

If glucose was oxidized in phosphate buffer instead of Tris buffer (Figure 5), the yield of GLA was increased by a factor of 3.5 (Figure 4A and 5A). Addition of 13-HPODE to the autoxidation sample led to an increase of the yield for only a few points (Figure 5B) while addition of H_2O_2

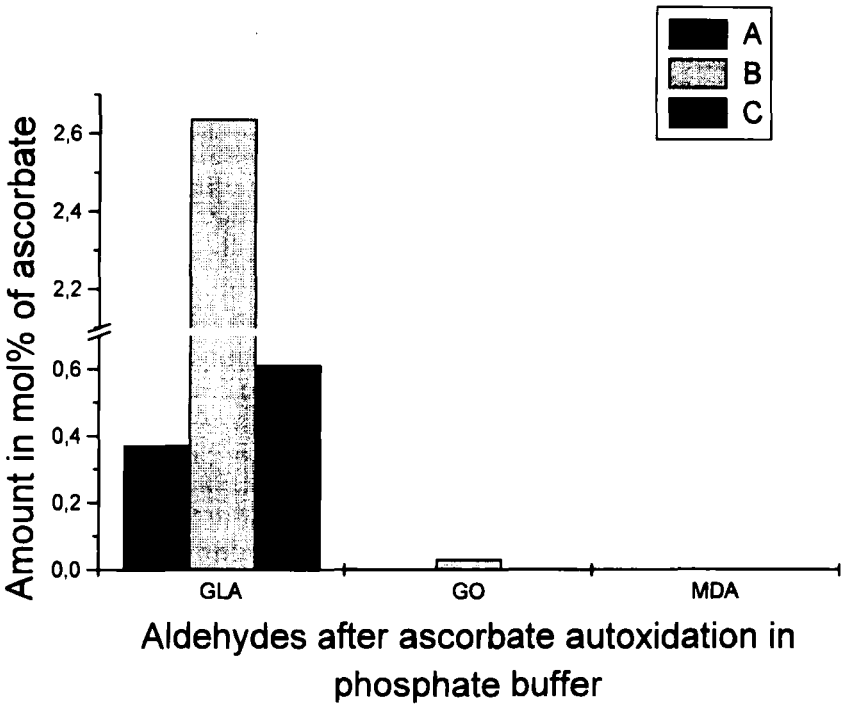


FIGURE 3 Oxidation products (GLA, GO, MDA) obtained after autoxidation of 30 µmol ascorbate in phosphate buffer (24 h) by addition of: A) equimolar amounts of Fe^{2+}, B) equimolar amounts of Fe^{2+} and 13-HPODE, C) equimolar amounts of Fe^{2+} and H_2O_2, Data represents mean values of duplicate determinations.

caused an increase by a factor of more than 8 (Figure 5C). In conclusion autoxidation of ascorbic acid was accelerated by Tris buffer, while the autoxidation of glucose was accelerated by the phosphate buffer system.

In all oxidations experiments carried out in Tris buffer, dihydroxyacetone (DA) was detected in amounts exceeding those of GLA, but if phosphate buffer was used, DA was just detectable. Therefore we checked if Tris reacted with iron ions and in fact DA was obtained in a yield surmounting all other aldehydic products. Use of Tris buffer caused an increase of DA by a factor of approximately more than 500 (estimation of GC measurement). The amount of formed DA correlated with the amount of Tris but was not quantified. This indicates that DA is a product of Tris oxidation.

Oxidation of glucose and ascorbate with equimolar amounts of iron ions, H_2O_2 or 13-HPODE produced also pentoses, tetroses, GA and formaldehyde. Quantities of these products were not determined.

Treatment of pure GA with equimolar amounts of Fe^{2+} ions for 24h and derivatization of the reaction products with PFBHA-HCl and MSTFA yielded the PFB-oxime TMS-ether derivative of GLA. This finding indicates that GA is degraded to GLA. In contrast dihydroxyacetone (DA) is degraded to GLA only in just detectable amounts.

In order to reveal the mechanism of GLA production from ascorbate the enolic OH-groups of ascorbic acid were blocked by treatment with etheric diazomethane solution. After autoxidation of the 2,3-dimethoxy derivative of ascorbic acid using equimolar amounts of Fe^{2+} no GA was detected but extreme large amounts of GLA (99.4 mol% of substrate) exceeding those after oxidation of ascorbic acid. Compared to GLA the

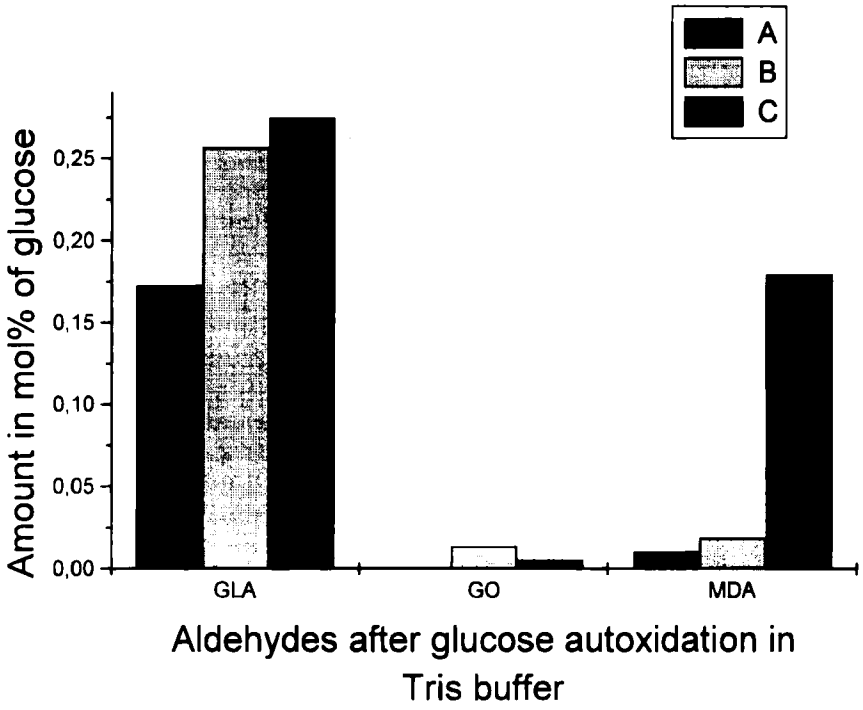


FIGURE 4 Oxidation products (GLA, GO, MDA) obtained after autoxidation of 30 µmol glucose in Tris buffer (24 h) by addition of A) equimolar amounts of Fe^{2+}, B) equimolar amounts of Fe^{2+} and 13-HPODE, C) equimolar amounts of Fe^{2+} and H_2O_2, Data represents mean values of duplicate determinations.

dicarbonyl compounds GO and MDA were produced only in amounts of less than 0.5%. If all OH-functions of ascorbic acid were methylated, no aldehydic products were obtained at all.

GO was described as lipid peroxidation product.[13,14,18,36] It is also a known product of autoxidation of glucose[37,38] and of GA[39] but the production of MDA from glucose (Figure 4) was previously unknown.

The kinetic of GLA production by autoxidation of arachidonic acid in the Fe^{2+}/ascorbate system was studied too (Figure 6). As shown in a previous paper[13] the amounts of hydroxyaldehydes increased steadily with time. In contrast to this behaviour GO production ceased after a short sharp increase but increased again after lag phase.[13] Since GLA showed the same sharp increase followed by a decrease immediately after the start of the reaction as observed for glyoxal,[13] the genesis of both products seems to be linked.

Indeed 0.5% (by mol) of glyoxal were detected after subjection of 0.7 mM GLA to autoxidation in Fe^{2+}/ascorbate system (44 µM $FeSO_4$, 1 mM Na-ascorbate).

DISCUSSION

Previous investigations on the oxidation of ascorbic acid were mainly focussed on the generation of 1,2-dicarbonyl compounds[40] which were shown to react with amines and peptides in a similar way as sugars in the course of a Maillard reaction.[24,41] This Maillard reaction seems to play an essential role in the glycation of lens proteins[42,43] causing the production of fluorescent pentosidine.[44] A similar role in the denaturation of peptides may be attributed to glycolaldehyde. The experiments outlined above show that glycolaldehyde is generated by oxidation of ascorbic acid.

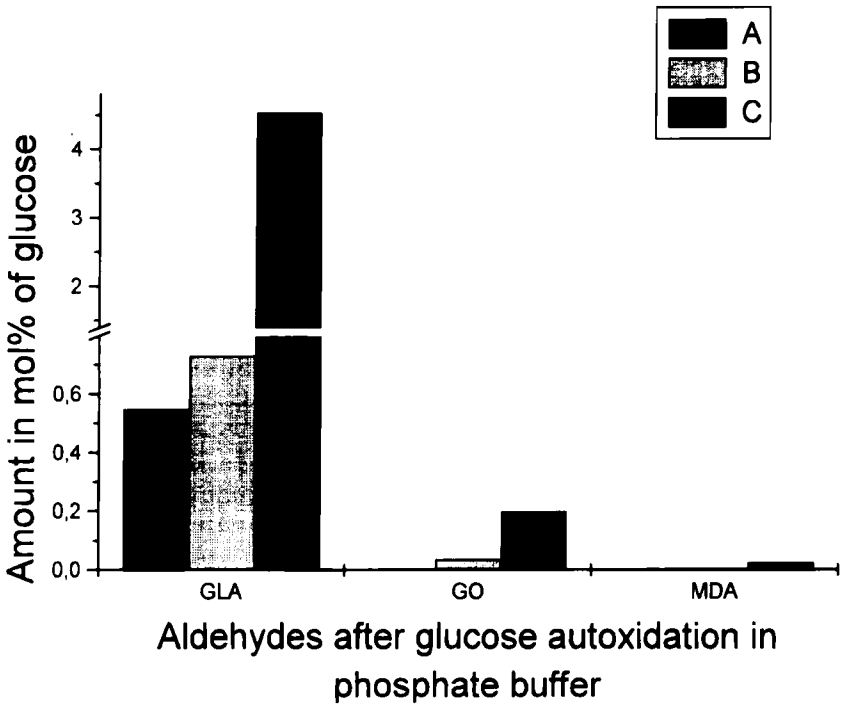


FIGURE 5 Oxidation products (GLA, GO, MDA) obtained after autoxidation of 30 µmol glucose in phosphate buffer (24 h) by addition of: A) equimolar amounts of Fe^{2+}, B) equimolar amounts of Fe^{2+} and 13-HPODE, C) equimolar amounts of Fe^{2+} and H_2O_2, Data represents mean values of duplicate determinations.

GLA is the most simple aliphatic α-hydroxyaldehyde. Aliphatic α-hydroxyaldehydes with 4 and 7 carbon atoms are obtained as products of LPO of PUFAs.[13,17,18] Those with a chain length of 16 and 18 carbon atoms are generated from plasmalogens epoxides.[45–47] They all were shown to induce an oxidative burst.[19]

Glycolaldehyde carries the same functional groups as the physiological active α-hydroxyaldehydes mentioned above. The yield of glycolaldehyde, obtained after model reactions carried out with O_2/Fe^{2+}/ascorbate, surmounted that of 4-hydroxynonenal and α-hydroxyheptanal—the most prominant hydroxyaldehydes derived by oxidation of ω-6 acids[13,17,18]—by a factor of at least 10. Therefore GLA may also contribute to an oxidative burst.

Although the oxidation of ascorbic acid had been studied in detail,[20,23,24] to our knowledge glycolaldehyde had not been described before as its oxidation product, probably since small aldehydic alcohols can be detected only with difficulties. In this connection "in situ" trapping of α-hydroxyaldehydes with PFBHA-HCl had proven to be very helpful.[17,18,36]

Obviously the oxidation of ascorbic acid **1** is induced by ·OH or ·OR radicals produced in a Fenton type reaction from HO-OH or LO-OH.[20] Such radicals are able to remove hydrogen from other molecules.[48,49] Homolytic hydrogen abstraction from ascorbic acid is principally possible either from an enolic O-H bond, an alcoholic O-H bond or a C-H bond. Usually C-H bonds and enolic O-H bonds are more prone to hydrogen abstraction than the others,[24,50] as shown e.g. by the fast conversion of coniferylalcohol to lignin.[51]

The genesis of glycolaldehyde from DHA **2** and in even higher amounts from the dimethoxy derivative of ascorbic acid can be visualized as

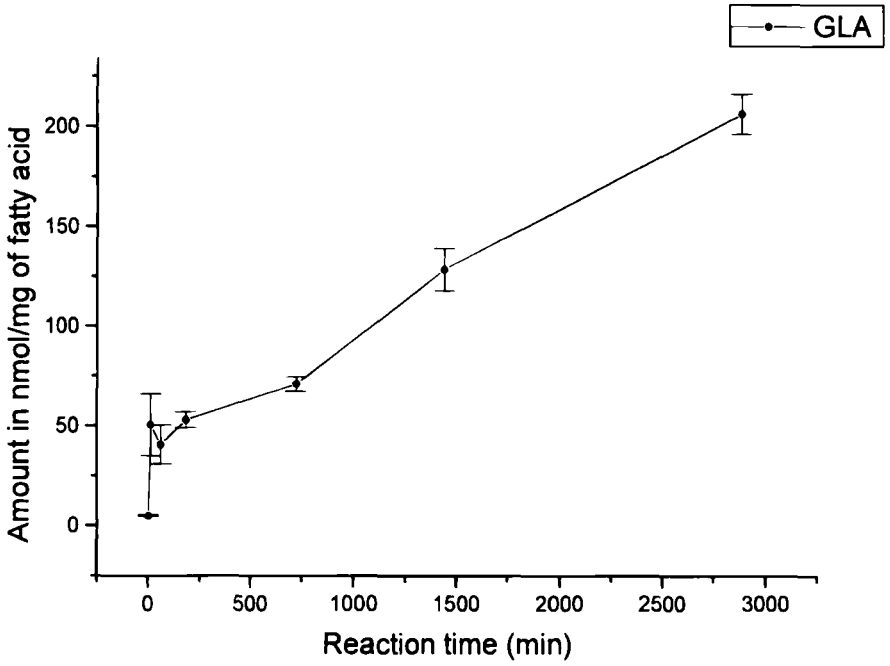


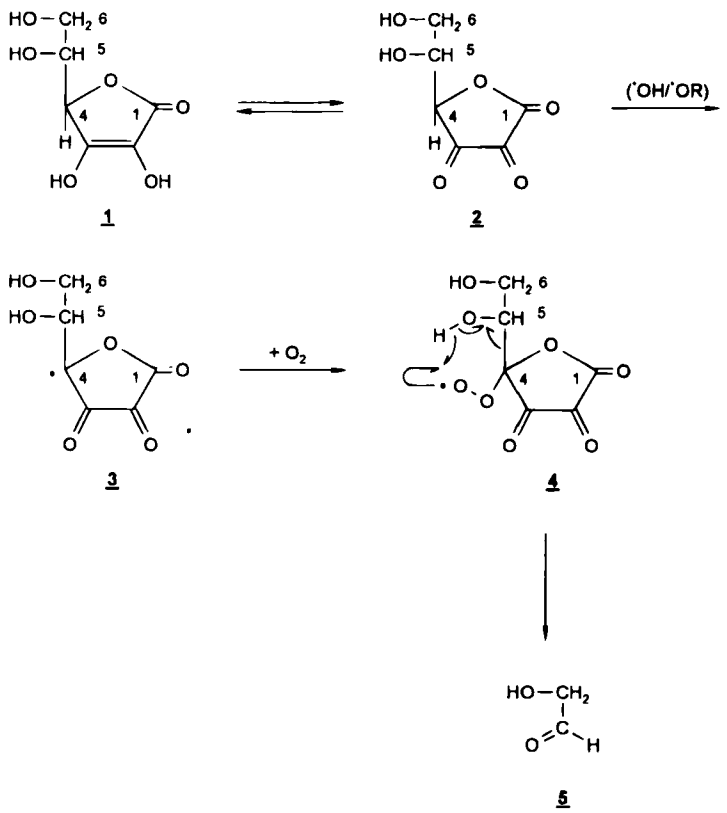
FIGURE 6 Formation of glycolaldehyde (GLA) with time during the autoxidation of arachidonic acid. Data are shown as mean values ± SD (n = 3).

shown in scheme 1. The proposed pathway follows the well investigated radical induced degradation of sugars.[50,52,53] Removal of the hydrogen from the C-H bond at carbon atom 4 would produce the radical *3*. Addition of oxygen leads to the peroxyl radical *4*. A postulated hydrogen shift from the hydroxy group at C-5 to the peroxyl radical site would cause the expulsion of glycolaldehyde *5* (Scheme 1).

Our experiments revealed that glyceraldehyde is easily degraded to glycolaldehyde. This reaction could follow a mechanism outlined by Thornalley *et al.*[39,54] and Steenken *et al.*[55] If glyceraldehyde is supposed to be an intermediate product of ascorbic acid oxidation, its generation from DHA *2* is less easy explicable: GA contains certainly the carbon atoms 4, 5 and 6 of DHA. The hydrogen at C-4 is still present in GA. As a consequence any mechanistic explanation for the genesis of glyceraldehyde requires fragmentation between C-3 and C-4 as well as opening of the lactone ring. Having this in mind we consider that such a reaction might start from 2,3-diketogulonic acid *6*, an already known product of DHA hydrolysis.[21] *6* was reported to suffer further degradation by decarboxylation,[23] leading obviously to the α-oxo-aldehyde *7*.

This aldehyde *7* may loose in analogy to a mechanism postulated for glyceraldehyde[55] in its hydrated form *8* a hydrogen atom to produce the intermediate radical *9*. After elimination of water from *9* and step by step expulsion of two CO molecules, the radical *12* may be produced, perhaps via the intermediates *10* and *11*. Reaction with oxygen would lead to the peroxyl radical *13* which, in accordance with a mechanism postulated by von Sonntag,[50,52,53] could finally produce glyceraldehyde *14* by loss of $\cdot O_2H$ (Scheme 2).

Alternatively we propose another degradation mechanism (scheme 3). Oxidation of ascorbic acid did occur in presence of Fe^{2+} without addition of H_2O_2 or LOOH (Figure 2A).[40] To

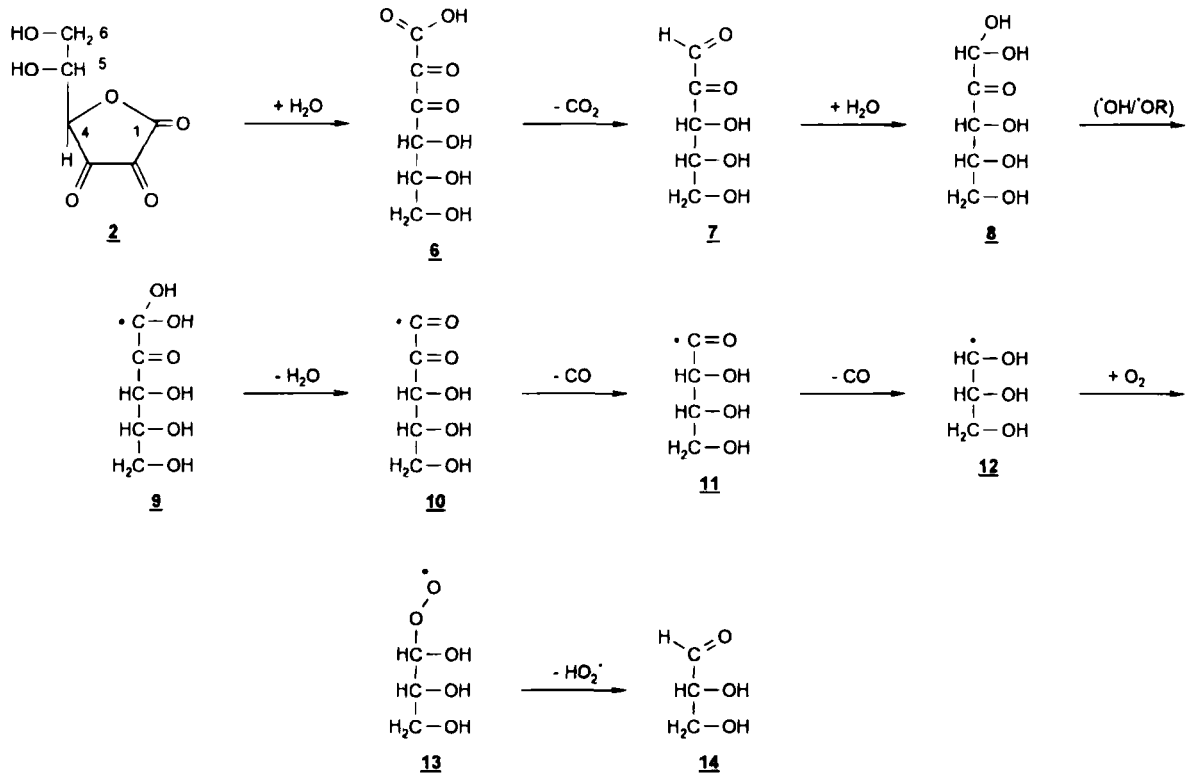


SCHEME 1 Possible mechanism for the direct generation of glycolaldehyde (GLA) from dehydroascorbic acid.

explain this reaction a pathway including a metal-ascorbate complex was postulated by Taqui Khan and Martell.[40] With reference to this assumption we speculate that a ·OH or ·OR radical could attack also carbon atom 1 from DHA producing radical ***15***. This intermediate has the radical site at the oxygen atom which might be stabilized by an iron complex. Its cleavage could directly produce glyceraldehyde ***14*** (Scheme 3).

The observation that dihydroxyacetone (DA) was found by use of Tris 16, but only in just detectable amounts by use of phosphate buffer indicated that DA might be derived from Tris and indeed this was confirmed experimentally. Thus radicals, obtained by Fe^{2+} induced oxidation reactions, do not only attack ascorbic acid but also Tris. This fact could be explained by removal of hydrogen to produce the radical 17 which decomposes to the enamine 18 and hydrolyzes in the iminoform 19 to dihydroxyacetone 20 (Scheme 4).

Since ascorbic acid is a sugar derivative we investigated whether or not Fe^{2+} ions do react with glucose. Oxidation of sugars by free radicals can be imitated by radiation. The influence of radiation on glucose in presence of oxygen was studied carefully.[50] It revealed the generation of a very great number of degradation products including GA, GO and formaldehyde,[52] but again glycolaldehyde had obviously not been detected so far. Our investigations on the oxidation of glucose revealed a much higher stability of glucose against Fe^{2+} induced oxidation compared to the oxidation of ascorbic acid and DHA. As expected, GLA is also a main

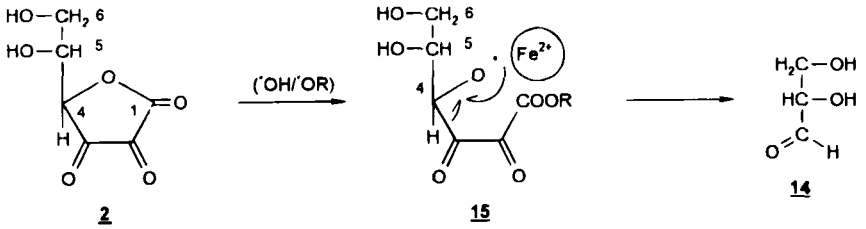


SCHEME 2 Putative pathway for the formation of glyceraldehyde (GA) from dehydroascorbic acid via degradation of 2,3-diketo-L-gulonic acid.

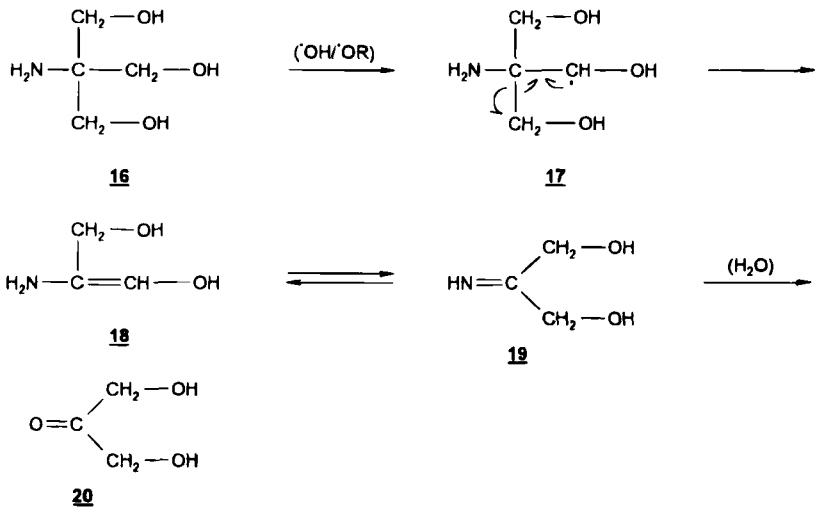
aldehydic oxidation product of glucose, but its generation requires the use of equimolar amounts of Fe^{2+}.

Since such high Fe^{2+} concentration are not observed in biological processes—even if cells are damaged—the generation of glycolaldehyde from glucose seems to contribute much less to biological events than perhaps the degradation of ascorbic acid might do.

During cell damaging processes Fe^{2+} ions are liberated from iron containing peptides.[56] In this investigation they were shown to react with ascorbic acid to glycolaldehyde. GLA is an α-hydroxyaldehyde, these aldehydes are involved in physiological reactions, e.g. an oxidative burst.[19] Therefore it might be that GLA is involved in reactions observed in biological processes connected with cell destruction.



SCHEME 3 Direct generation of glyceraldehyde (GA) from dehydroascorbic acid via an intermediate iron complex.



SCHEME 4 Possible mechanism for the formation of dihydroxyacetone (DA) from Tris.

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