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FORMATION OF GUANOSINE ADDUCTS FROM L-ASCORBIC ACID UNDER OXIDATIVE CONDITIONS

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Abstract: L-Ascorbic acid (AA) was incubated with guanosine under physiological conditions and the reaction was monitored by HPLC-DAD. Four reaction products were isolated and identified as two pairs of diastereomers of N^2-(1-Carboxyethyl)-guanosine and N^2-(1-Carboxy-3-hydroxypropyl)-guanosine, respectively. They were formed from AA in the presence of oxygen as well as from its degradation products L-dehydroascorbic acid and L-xylosone. © 1997 Elsevier Science Ltd.

Introduction. The role of L-ascorbic acid (AA) in biological systems has been controversially discussed in the past. Except of its well-documented anti-carcinogenic properties,[1] AA can also show mutagenic effects. For instance exposure of Chinese hamster ovary cells to AA resulted in the induction of somatic mutations[2] and exchanges of sister chromatides.[3] Furthermore in the presence of oxygen AA induces DNA fragmentation of cultured mammalian cells,[4,5] single strand breaks in DNA[6], and is genotoxic for phages.[7] Since some of these effects are potentiated by the addition of transition metal ions[3] or inhibited in the presence of catalase[2,8] it was suggested that they are caused by the prooxidative activity of AA. In the presence of Cu (II) or Fe (III) autoxidation of AA produces H_2O_2 and in a Fenton reaction free radicals are formed. Both species can be responsible for oxidative damage of DNA.[2,4,9,10]

It must be assumed, however, that besides of oxidation reactions other mechanisms and reactions play a role in DNA modifications caused by AA.[11] Under aerobic conditions solutions of AA show even in the absence of metal ions considerable genotoxic activity. When AA was exposed to oxygen prior to reactions, this effect could be evoked even under anaerobic conditions and without addition of metal ions. These observations indicate mutagenic activity of degradation products of AA.

Recently it was found, that AA binds covalently to alkyl amines or to lysine side chains of proteins[12], resulting in the formation of e.g. 3-deoxy-3-alkylamino-ascorbic acid,[13] 2-desoxy-2-alkylamino-ascorbic acid and oxalic acid mono- and bisamide.[14] The formation of AA derived modifications of amines is particularly favoured under oxidative conditions.[15] It can be assumed, that AA is first oxidized to L-dehydroascorbic acid (DHA) ,or other reactive degradation products which react in a further step with amino groups of proteins.

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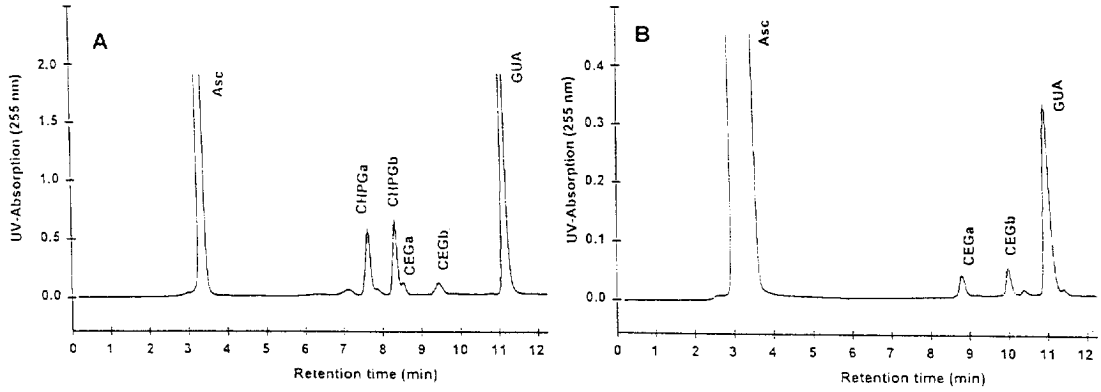
Thus it can be assumed, that AA can also react with nucleophilic groups of DNA resulting in the formation of covalently bound products. This reaction would be of particular importance under oxidative stress. It has been shown that especially the amino group at C-3 of guanosine is prone to electrophilic attack on DNA, indicating a possible centre for the reaction with AA.[16]

Therefore we investigated if AA or its degradation products can bind covalently to guanosine, a reaction which can result in the formation of DNA modifications. Guanosine was reacted with AA under physiological or model reaction conditions, products were isolated, and their structures elucidated. Furthermore the role of oxygen in this reaction was investigated.

Results

When L-ascorbic acid is incubated with guanosine in nearly neutral (pH 7.4), aqueous solution at 37 °C under aerobic (Fig. 1, panel A) or anaerobic conditions (Fig. 1, panel B) several reaction products can be separated and detected using HPLC with diode array detection[17] (Fig. 1).

Figure 1

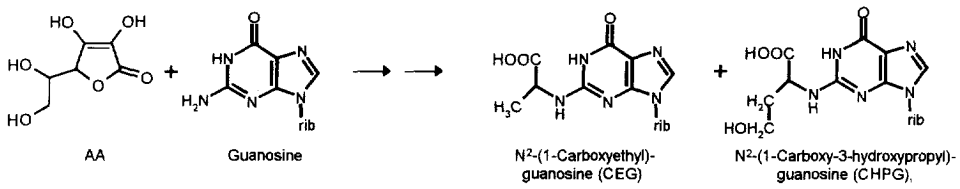


The main products show UV- maxima which are slightly shifted towards higher wavelengths (λ_{max} = 256 nm), compared to that of guanosine (λ_{max} = 255 nm). This is typical for N^2-alkyl substituted guanosine derivatives.[18]

Raising the reaction temperatures resulted in increased yields of the four main products so that they could be isolated and their structures elucidated by spectroscopic data. The AA derived guanosine modifications were identified as the two diastereomers of N^2-(1-Carboxyethyl)-guanosine (CEG)[19] and N^2-(1-Carboxy-3-hydroxypropyl)-guanosine (CHPG),[20] respectively.

CHPG represents a new guanosine adduct which has not been described so far. CEG which was previously isolated from reaction mixtures of glucose and guanosine was identified for the first time as an ascorbylation product of guanosine (Fig. 2).

Figure 2



For isolation of CEG, AA and guanosine were heated for 12 hours in phosphate buffer (pH 7.0) at 100 °C. CHPG was obtained in highest yield when DHA was heated with guanosine in phosphate buffer (pH 7.0) for three days at 100 °C. The guanosine derivatives were obtained as pure compounds by injecting the crude reaction mixtures into a preparative HPLC [column: LiChrospher™ RP 18, 250 × 25 mm i.d., 10 µm particle size, eluent: mixtures of ammonium formate buffer (5mM) and methanol (for CHPG: 98 : 2; for CEG 96 : 4)].

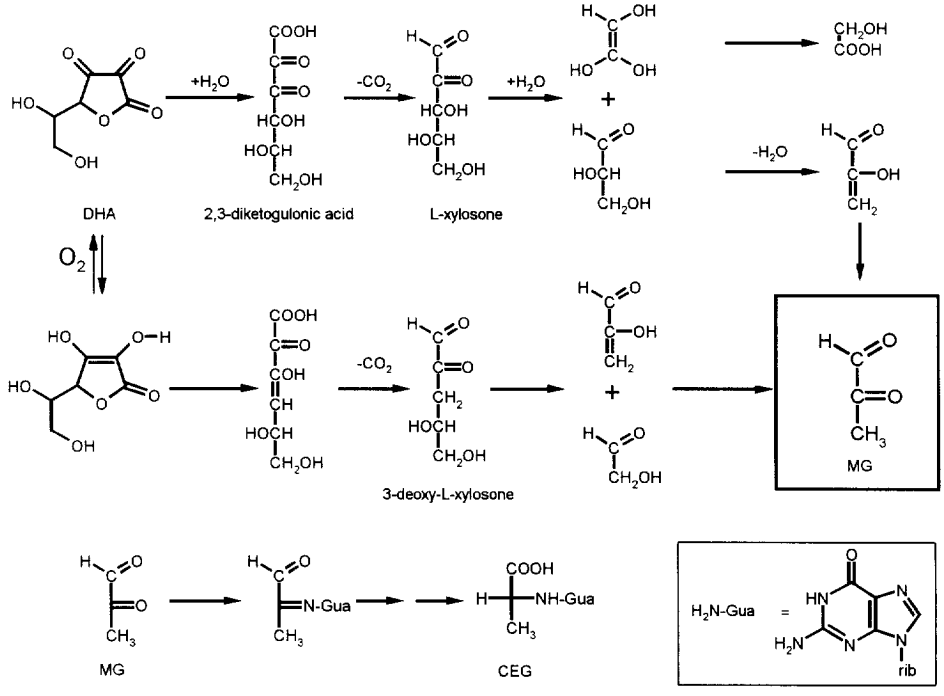
To elucidate the reaction mechanisms which lead to CEG and CHPG besides AA its degradation products L-dehydroascorbic acid and L-xylosone were used as educts. In another experiment AA was incubated under anaerobic conditions and in the presence of a transition metal scavenger (diethylentriamine-pentaacetic acid) to investigate if an oxidation step is necessary for the formation of CEG and CHPG, respectively.

CEG is formed when guanosine is reacted with AA, DHA or L-xylosone. Starting from AA CEG can be detected both under aerobic conditions and in the absence of oxygen. The yield is much higher if the reaction is carried out under oxidative conditions. These results support the assumption that two pathways may lead from AA to the reactive dicarbonyl compound methylglyoxal (MG), which reacts further with guanosine to give CEG (Fig. 3). Under anaerobic conditions ring opening of AA and decarboxylation afford 3-desoxy-L-xylosone which gives after retro-aldol-cleavage MG. On the other hand in the presence of oxygen L-dehydroascorbic acid is formed as primary degradation product. After hydrolysis of the lactone and decarboxylation L-xylosone may undergo retro-aldol-cleavage to give glyceraldehyde which eliminates water and forms MG. CEG was previously detected in reaction mixtures of glucose, glucose-6-phosphate or amadori product and guanosine.[16,21] In these cases MG was considered to be the reactive intermediate as well.

In contrast to CEG, CHPG is not formed when AA is incubated with guanosine in the absence of oxygen. Oxidation of AA to DHA and degradation to L-xylosone seems to be necessary for the formation of CHPG. Therefore another reaction mechanism must be envisaged (Fig. 4). L-Xylosone eliminates water and gives the hemiacetal of a 1,2,3-tricarbonyl compound (**X**). The aldehyde group is hydrated and formic acid eliminated. The

resulting C-4 compound (the hemiacetal of a 3-deoxy-tetroson) forms a Schiff base with the primary amino group of guanosine and intramolecular rearrangement gives a lactone. After ring-opening N^2-(1-carboxy-3-hydroxypropyl)-guanosine, an α-amino acid is formed. The analogous 2,4-dihydroxybutanoic acid has previously been isolated as a degradation product of AA in the absence of amine.[22]

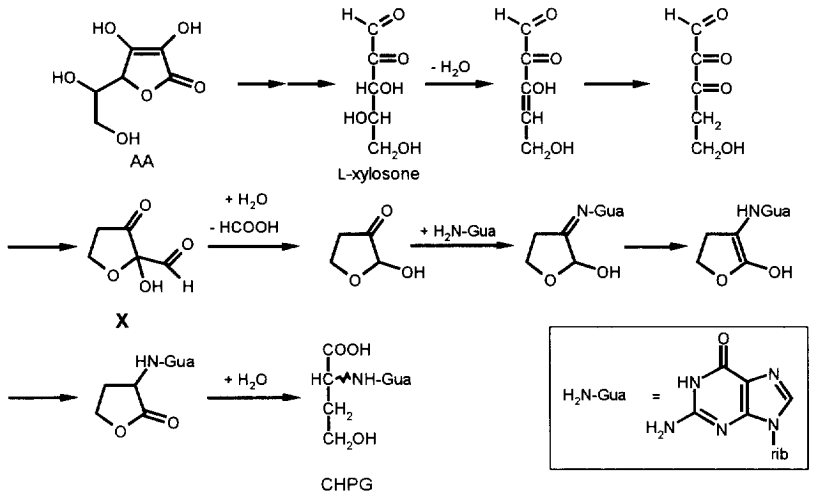
Figure 3



Since CHPG is also formed from L-xylosone it can be assumed that other osones react in the similar way resulting in analogous products. Glucosone, for example, which is formed by autoxidation of glucose or amadori product is an important intermediate with high glycation activity.[23,24] Therefore it can be assumed, that under aerobic conditions glucose is oxidized to give glucosone, which is able to react with the primary amino group of guanosine in a similar way as L-xylosone.

To summarize the results it can be said that CHPG is the main product if AA is incubated with guanosine under aerobic conditions or if DHA or L-xylosone are used as educts. In this case CEG is formed only in minor amounts. In contrast with AA as educt and anaerobic conditions CEG is the major product, whereas CHPG can not be found.

Figure 4



It can be concluded that N^2-(1-carboxyethyl)-guanosine and particularly N^2-(1-carboxy-3-hydroxypropyl)-guanosine are major advanced glycation end products of guanosine, which are derived from AA under oxidative stress. It can be assumed that they are responsible for DNA modifications and therefore age-dependent changes in the genetic material. Increased tumorigenesis, DNA strand breaks, chromosomal aberrations as well as decreases in transcription, replication and DNA repair can be the consequences. However it is not clear so far if covalent modifications of DNA induced by AA under oxidative stress occur in vivo. It must be considered that several protection mechanisms exist including enzymatic and non-enzymatic glutathione dependent reduction of reactive dicarbonyl compounds. Therefore further investigations will be necessary to quantify the occurrence of CEG and CHPG in vivo.

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17. Analytical HPLC was performed with a Merck L-7100 gradient pump fitted with a 20 µl sample loop, and a L-7450 diode array detector including Merck-Hitachi Model D-7000 Chromatography Data Station software. A column packed with LiChrospher™ (RP 18, 280 x 4 mm i.d., 5µm particle size) was used. The column was protected with a guard cartridge (25 x 4 mm) packed with the same material as the column. Eluent: gradient elution starting with ammonium formate buffer (5 mM), ending within 25 min with 25 : 75 buffer-methanol at a flow rate of 0.8 mL/min. The substances were detected with a diode array detector from 225 nm to 400 nm.
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19. Spectral data for CEG (diastereomer a): ^{1}H NMR (D_2O, COSY, 400 MHz): δ 1.41 (d, J = 7.2 Hz, 3H, CH_3), 3.86 (m, 2H, H-5 rib), 4.13 (m, 1H, H-4 rib), 4.17 (q, J = 7.2 Hz, 1H, N^2CH), 4.39 (t, J = 5.4 Hz, 1H, H-3 rib), 5.02 (t, J = 5.4 Hz, 1H, H-2 rib), 5.83 (d, J = 5.4 Hz, 1H, H-1 rib), 7.90 (s, 1H, H-8 gua). ^{13}C NMR (D_2O, COSY, DEPT, 100 MHz): δ 20.2 (CH_3), 55.5 (CHN^2), 64.4 (rib-5), 73.0 (rib-3) 74.8 (rib-2), 87.4 (rib-4), 91.7 (rib-1), 119.8 (gua-5), 142.4 (gua-8), 154.4 (gua-4), 154.8 (gua-2), 162.1 (gua-6), 184.0 (COO^-). HRFAB-MS: m/z (MH^+) calc.: 356.1206, found: 356.1215; UV: λ_{max} = 256 nm.
20. Spectral data for CHPG (diastereomer a): ^{1}H NMR (D_2O, COSY, 400 MHz): δ 2.00 (m, 1H, CHaHb-CH_2OH), 2.10 (m, 1H, CHaHb-CH_2OH), 3.79 (m, 2H, CHaHb-CH_2OH), 3.90 (m, 2H, H-5 rib), 4.13 (m, 1H, H-4 rib), 4.36 (dd, J = 8.9 Hz + 4.5 Hz, 1H, N^2CH), 4.47 (t, J = 5.4 Hz, 1H, H-3 rib), 5.01 (t, J = 5.4 Hz, 1H, H-2 rib), 5.91 (d, J = 5.4 Hz, 1H, H-1 rib), 7.93 (s, 1H, H-8 gua). ^{13}C NMR (D_2O, COSY, DEPT, 100 MHz): δ 36.2 (CH_2-CH_2OH), 56.9 (CHN^2), 61.1 (CH_2-CH_2OH), 63.9 (rib-5), 72.5 (rib-3) 74.6 (rib-2), 86.8 (rib-4), 91.2 (rib-1), 119.2 (gua-5), 141.6 (gua-8), 153.8 (gua-4), 155.6 (gua-2), 161.3 (gua-6), 182.2 (COO^-). HRFAB-MS: m/z (MH^+) calc.: 386.1311, found: 386.1312; UV: λ_{max} = 256 nm.
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