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Variation in Ascorbic Acid Oxidation Routes in H_2O_2 and Cupric Ion Solution As Determined by GC/MS

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Recent reports have suggested that ascorbic acid protects low-density lipoprotein from peroxide-induced oxidation, but does not protect and may actually function as a prooxidant in the presence of cupric ions. However, dehydroascorbic acid, (the first oxidation product of ascorbic acid) has been shown to protect low-density lipoprotein from cupric ion oxidation but not peroxide-induced oxidation. We have examined the degradation of ascorbic acid, uniformly labeled [$^{13}C_6$]ascorbic acid, and [6,6-$^2H_2$]ascorbic acid in hydrogen peroxide and cupric ion solutions using gas chromatography/mass spectrometry to determine products and routes of oxidation using different oxidant sources. We have found that hydrogen peroxide leads to the formation of a six-carbon product with a mass increment of 32 (a double oxygen addition) relative to ascorbic acid, consistent with the oxidation sequence of ascorbic acid (mass 176) going to dehydroascorbic acid (mass 174) to 2,3-diketogulonic acid (mass 192) to 2,3-diketo-4,5,5,6-tetrahydroxyhexanoic acid (mass 208). Cupric ion solutions, on the other hand, do not appear to induce significant amounts of 2,3-diketo-4,5,5,6-tetrahydroxyhexanoic acid but rather lead to the formation of a *threo*-hexa-2,4-dienoic acid lactone (mass 174) as the major six-carbon species. These data suggest that different oxidation stresses lead to solutions containing different ascorbic acid oxidation products. These ascorbic acid-derived species could, in turn, interact differently with other substances in the aqueous environment, including free metal ions and low-density lipoprotein. This may help explain previous reports showing divergent protective effects of ascorbic acid and dehydroascorbic acid on low-density lipoprotein when different oxidation methods are used.

Ascorbic acid (AA) is a carbohydrate which is essential in the diet of humans.[1-3] Besides functioning as a cofactor in a variety of enzymatic processes, AA is also well recognized to be an excellent antioxidant in vitro and in vivo.[1-7] When exposed to oxidant stress, AA is reversibly converted, in a two-step process to dehydroascorbic acid (DHA).[1-8] This process provides the antioxidant effect attributed to AA. When continued oxidation is applied, DHA is irreversibly degraded to 2,3-diketogulonic acid and then to over 50 species containing five or less carbons.[1,8-14]

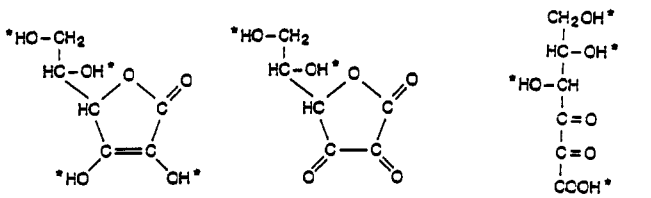

Figure 1. Structures of AA, DHA, and 2,3-diketogulonic acid. AA is reversibly oxidized to DHA. DHA can be reduced to AA or irreversibly oxidized to 2,3-diketogulonic acid. TBDMS-derivatizable sites are marked with an asterisks.

However, the role of AA as an in vivo antioxidant has been confused by data suggesting that AA also functions as a free-radical catalyst in the presence of transition metal ions, generating hydrogen peroxide and hydroxyl radicals through the Fenton reaction.[8,15]

Chronic oxidation injury has been associated with a variety of morbid conditions.[16-22] Recent work has focused on the oxidation of low-density lipoprotein as a precursor of atherosclerotic plaque and cardiovascular disease.[21-23] It appears that oxidant stress leads to the formation of reactive aldehydes from unsaturated fatty acids, which in turn conjugate with free ϵ-amines of apolipoprotein-B.[21-23] This conjugated product, oxidized low-density lipoprotein, is recognized by a macrophage receptor and is incorporated into the blood vessel

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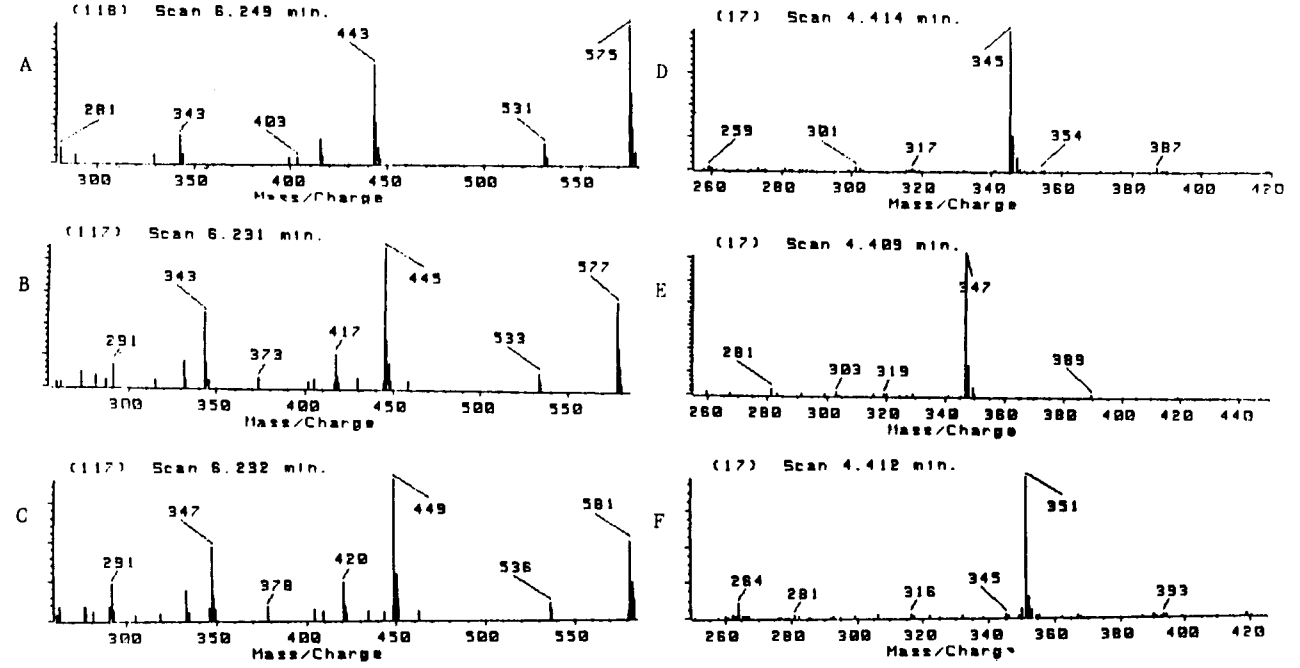

Figure 2. Spectra and retention times for TBDMS-derivatized (A) AA, (B) [6,6-$^2H_2$]AA, (C) [$^{13}C_6$]AA, (D) DHA, (E) [6,6-$^2H_2$]DHA, and (F) [$^{13}C_6$]DHA.

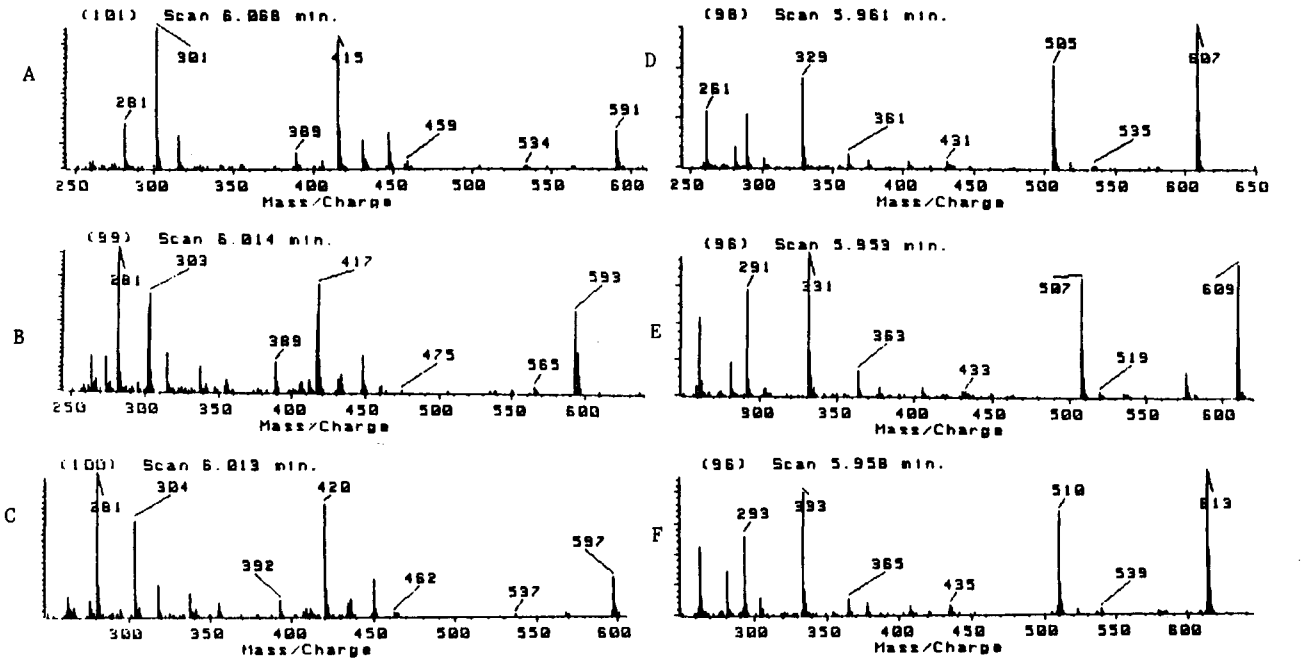

Figure 3. Spectra of compounds arising in AA solution exposed to oxygen. The predicted $[M - 57]^+$ ion for TBDMS-derivatized 2,3-diketogulonic acid is m/z 591. Proposed compounds are (A) 2,3-diketogulonic acid, (B) [6,6-$^2H_2$]2,3-diketogulonic acid, and (C) [$^{13}C_6$]2,3-diketogulonic acid. An additional compound arose with an $[M - 57]^+$ of m/z 607. The spectra of this compound from solutions of (D) AA, (E) [6,6-$^2H_2$]AA, and (F) [$^{13}C_6$]AA are shown. The proposed identity is 2,3-diketo-4,5,5,6-tetrahydroxyhexanoic acid.

wall.[21-23] To prevent this from happening in vivo, it appears that low-density lipoprotein has an antioxidant system consisting of lipid-associated vitamin E (tocopherol), which interrupts the free-radical chain reaction.[23-25] However, in vitro studies have shown that oxidation of vitamin E and other products of peroxide-induced lipid peroxidation does not appear until ascorbate is depleted from the experimental system.[5,7] Of most importance, vitamin E, when oxidized, is then regenerated through reduction by AA.[8,24]

Recently, the relation of AA to the oxidation of low-density lipoprotein has been explored in detail by Retsky et al.[23] Of interest, they found that AA, unlike DHA, protected low-density lipoprotein from peroxide-induced oxidation.[23] In contrast, DHA but not AA prevented cupric ion-induced low-density lipoprotein oxidation.[23] These data are particularly intriguing since AA is well-known to be oxidized in the presence of both hydrogen peroxide[26] and transition metal ions,[27,28] and the first step in AA oxidation is the formation of DHA.[1,8]

These data suggested to us that the oxidation of AA likely proceeds through two or more pathways, depending on the source of oxidant stress, and these different pathways would

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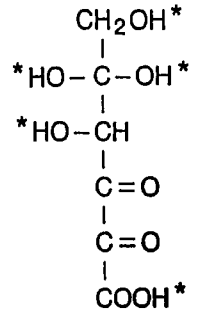

Figure 4. Proposed structure derived from the spectra in Figures 3D–F (2,3-diketo-4,5,5,6-tetrahydroxyhexanoic acid).

Table 1. Ascorbic Acid-Derived Products in Aqueous Solution[a]

compd [M − 57]$^+$ (*m/z*)	retentn time (min)	starting mater no exposures	ratios[b] Ar + dark	Ar + light	O_2 + dark	O_2 + light
345	4.41	0.002	0.27	0.20	8.50	50.00
591	6.06	0	0	0	0.80	3.65
607	5.96	0	0	0	0.18	1.33

[a] Ascorbate solution, 100 μM. [b] The ratios of AA-derived oxidative products to the remaining AA (*m/z* 575 at retention time of 6.25 min) after 4 h in light or dark with either oxygen or argon. The ions represent DHA (*m/z* 345), 2,3-diketogulonic acid (*m/z* 591), and the proposed 2,3-diketo-4,5,5,6-tetrahydroxyhexanoic acid (*m/z* 607).

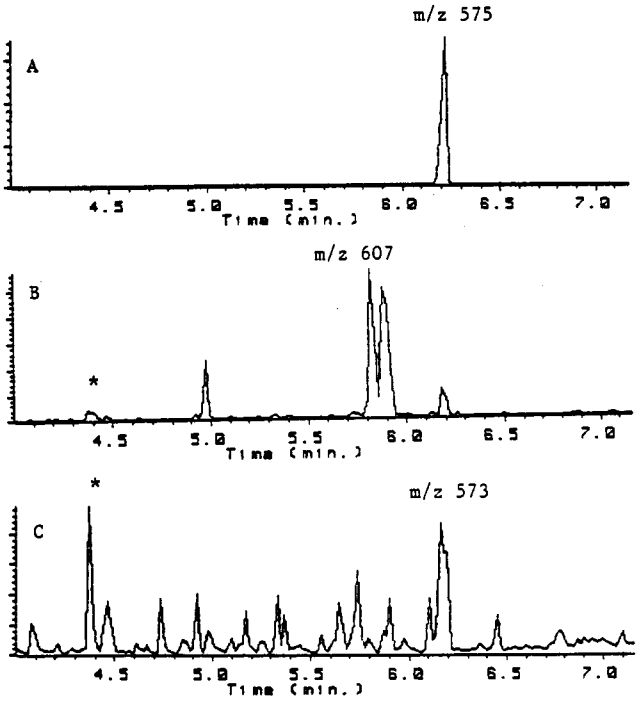

Figure 5. Total ion chromatograms of TBDMS-derivatized solutions of (A) AA, (B) AA exposed to 5% hydrogen peroxide for 2 h, and (C) AA exposed to 20 μM cupric ion solution for 2 h. The [M − 57]$^+$ ion from the main peaks are reported. DHA is marked with an asterisk.

lead to the formation of different AA-derived products. Furthermore, these different downstream oxidation products of AA may account for the reported differences in peroxide versus transition metal-induced oxidation of low-density lipoprotein.[23] We therefore applied gas chromatography/mass spectrometry to examine the oxidation of AA, uniformly labeled [$^{13}C_6$]AA and [6,6-$^2H_2$]AA by different oxidation stresses for downstream products of degradation. We have found profound differences in the AA products produced, depending on the source of oxidant stress. This may help

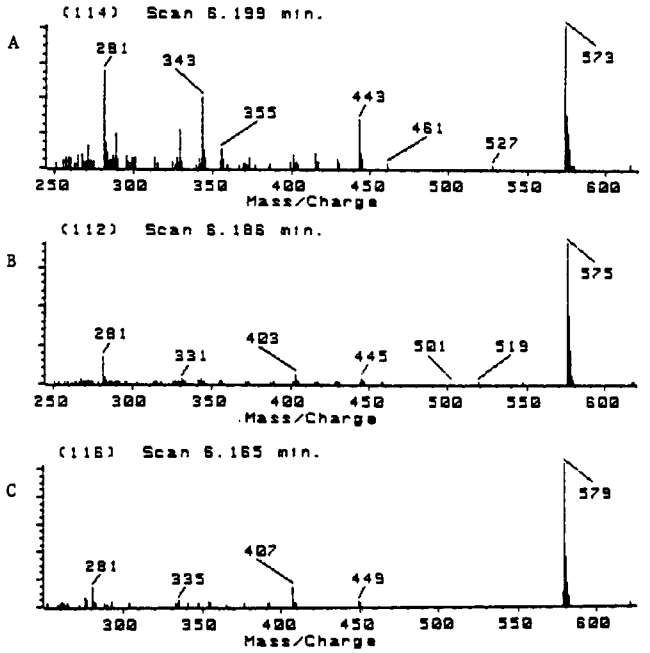

Figure 6. Spectra of the species formed during incubation with cupric ion as derived from (A) AA, (B) [6,6-$^2H_2$]AA, and (C) [$^{13}C_6$]AA. The proposed identity of this compound is a *threo*-hex-2,4-dienoic acid lactone.

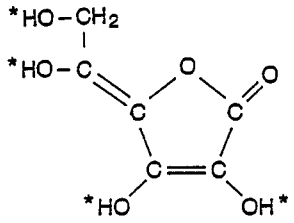

Figure 7. Proposed structure derived from the spectra in Figure 6A–C (a *threo*-hexa-2,4-dienoic acid lactone).

explain the differences observed in AA–low-density lipoprotein interactions in different oxidant systems and may provide the identification of important compounds which can be used to further study oxidation of biologically important substrates.

EXPERIMENTAL SECTION

AA and other reagents of highest grade were obtained from Sigma Chemicals, St. Louis, MO; Aldrich Chemicals, Milwaukee, WI; and Kodak Chemicals, Rochester, NY. *N*-Methyl-*N*-[(*tert*-butyldimethyl)silyl]trifluoroacetamide (TBDMS) was obtained from Regis Chemicals, Morton Grove, IL. [$^{13}C_6$]AA (98% ^{13}C) and [6,6-$^2H_2$]AA (98.5% ^2H) were obtained from MSD Isotopes, Montreal, Quebec, Canada.

Oxygen and argon exposures were carried out in 3-mL volumes in borosilicate tubes while all other reactions were carried out in small volumes (5 μL) in glass autosampler vials containing 5.7 mM solutions of AA, [$^{13}C_6$]AA, or [6,6-$^2H_2$]-AA. Where indicated, oxygen and argon were bubbled through the solutions at 2 L/min for 1–6 h. During gas exposure, solutions were kept in the dark or exposed to a 50-W light source at a 20-cm distance. Other studies were carried out in ambient light. Where indicated, varying amounts of hydrogen peroxide (to final concentrations of 0.005–5%, v/v) and cupric sulfate (final concentration 100 nM to 200 μM) were added and these samples were incubated for 5–120 min

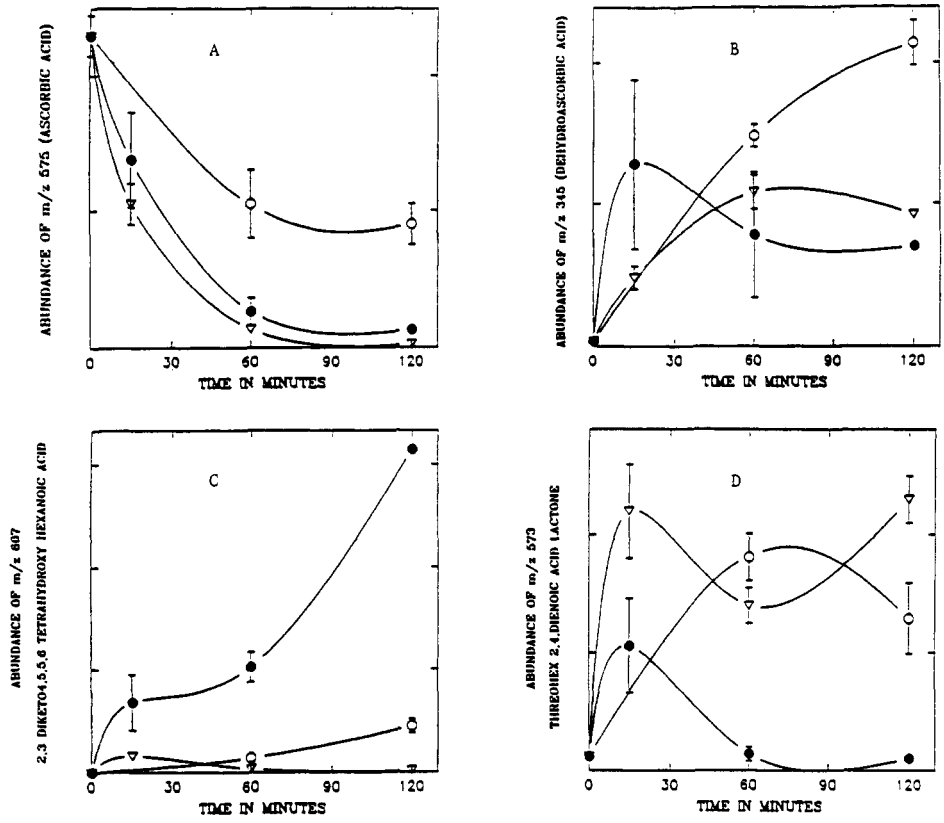

Figure 8. Ion abundance for (A) *m/z* 575 (AA), (B) 345 (DHA), (C) 607 (2,3-diketo-4,5,5,6-tetrahydroxyhexanoic acid), and (D) 573 (a *threo*-hexa-2,3-dienoic acid lactone) over time when solutions of AA are exposed to 0.5% hydrogen peroxide (open circles), 5% hydrogen peroxide (closed circles), and 20 μM cupric sulfate (open triangles). The mean values and standard deviations for triplicate experiments are shown (the 2-h closed circle was a single data point).

at 22 °C. Reactions to which hydrogen peroxide or cupric sulfate had been added were incubated for the indicated times, following which the metal chelator diethylenetriaminepentaacetic acid was added to each sample to a final concentration of 40 μM, and volatile substances (hydrogen peroxide and water) were immediately removed (due to the small volume of reaction) by drying in a Savant (Farmingdale, NY) vacuum centrifuge.

The dried aliquots were derivatized by adding 30 μL of a 1:2 mixture of TBDMS/acetonitrile for 2 h at 60 °C, and 2-μL aliquots were applied to a Hewlett Packard (Avondale, PA) 5890 gas chromatograph. Gas chromatography was carried out through a Supelco (Bellfonte, PA) 10-m fused silica capillary column (i.d. 0.25 mm) using a temperature ramp of 30 °C/min from 80 to 300 °C with helium as a carrier, and mass spectrometry was done on a Hewlett Packard 5971A mass spectrometer. The scan mode was used to obtain full spectra (including the $[M - 57]^+$ ion) and appropriate retention times. Analysis was carried out by scanning and by selected ion monitoring at the respective $[M - 57]^+$ ions. The electron multiplier was at 1350–2000 V.

Experiments were done in triplicate from freshly prepared stock solutions, with comparisons done on solutions of AA, $[^{13}C_6]$AA, and $[6,6\text{-}^2H_2]$AA to determine proposed structures of the AA-related products. The mean values and standard deviations were determined on relative ion abundances where indicated. Significance was defined as $p < 0.05$ on a two-tailed Student's *T*-test.

RESULTS AND DISCUSSION

AA is reversibly oxidized to DHA and then irreversibly to 2,3-diketogulonic acid[1] as shown in Figure 1. Derivatization of these species occurs by substituting (within steric allowances) a (*tert*-butyldimethyl)silyl group at hydroxyl and carboxyl hydrogens, increasing the mass by 114 units per derivatized site.[29-31] Characteristically, when TBDMS-derivatized compounds are ionized, a major ion in the spectrum is formed by removal of a *tert*-butyl group from one of the derivatized sites.[29-31] This is referred to as the $[M - 57]^+$ ion. To provide structural information, we carried out experiments in parallel using AA, $[^{13}C_6]$AA, and $[6,6\text{-}^2H_2]$AA. Full spectra and the $[M - 57]^+$ ions for TBDMS-derivatized AA, $[6,6\text{-}^2H_2]$AA, $[^{13}C_6]$AA, DHA, $[6,6\text{-}^2H_2]$DHA, and $[^{13}C_6]$-DHA had been previously determined[31] and are shown in Figure 2. The predicted major ion $[M - 57]^+$ for 2,3-diketogulonic acid is *m/z* 591, for $[6,6\text{-}^2H_2]$2,3-diketogulonic acid is *m/z* 593, and for $[^{13}C_6]$2,3-diketogulonic acid is *m/z* 597.

Following exposure of AA and $[^{13}C_6]$AA solutions to oxygen and 50-W light, the relative abundance of DHA (based on *m/z* 345) and $[^{13}C_6]$DHA (*m/z* 351) greatly increased over time, following which there was an increase of a product containing *m/z* 591/597 at two retention times consistent with the formation of two isomers of 2,3-diketogulonic acid. Full spectra (including the similar compound formed from $[6,6\text{-}^2H_2]$AA) are shown in Figure 3A–C. In addition, another six-carbon product was formed, following oxygen and light exposure of AA solutions, which had an apparent $[M - 57]^+$ of 16 mass units greater (*m/z* 607/613) than 2,3-diketogulonic

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acid. Full spectra (including a similar compound formed from [6,6-$^2H_2$]AA) are shown in Figure 3D–F, and the proposed structure is shown in Figure 4 (2,3-diketo-4,5,5,6-tetrahydroxyhexanoic acid). Confirmation of the proposed structure using mass spectrometry/mass spectrometry was performed by Dr. Xianlin Han in the laboratory of Dr. Richard Gross in the Division of Bioorganic Chemistry and Molecular Pharmacology at Washington University School of Medicine. The formation of these products after 4 h in oxygen compared to argon and light compared to dark (based on the ratio of the major ions to the remaining $[M - 57]^+$ ion of AA) are shown in Table 1.

Solutions of AA, [$^{13}C_6$]AA, and [6,6-$^2H_2$]AA were then exposed to either hydrogen peroxide (0.005–5%) or cupric ions (100 nM to 20 μM). Following incubation ranging from 5 to 120 min, diethylenetriaminepentaacetic acid was added to all solutions, and they were immediately dried by vacuum centrifugation. Controls were done with and without diethylenetriaminepentaacetic acid additions. Figure 5A shows the total ion chromatogram from control AA with diethylenetriaminepentaacetic acid (which is identical to controls without the chelator), compared to the total ion chromatogram following 2-h exposure of AA to 5% hydrogen peroxide (Figure 5B) and 20 μM cupric sulfate (Figure 5C). The major species (the split peak based on two potential derivatization-induced isomers) formed in hydrogen peroxide (Figure 5B) had an $[M - 57]^+$ ion of m/z 607/613/609 coming from AA, [$^{13}C_6$]AA, and [6,6-$^2H_2$]AA and spectra identical to that shown in Figure 3D–F. However, only trace amounts of this species was formed during cupric ion-induced oxidation. Rather a new six-carbon species arose, having an $[M - 57]^+$ of 573/579/575 (marked on the ion chromatogram in Figure 5C). Spectra of this compound derived from AA, [$^{13}C_6$]AA, and [6,6-$^2H_2$]AA containing solutions are shown in Figure 6A–C, respectively. A proposed structure for this compound (a *threo*-hexa-2,4-dienoic acid lactone) is shown in Figure 7. In neither the hydrogen peroxide nor the cupric ion-exposed solutions of AA did more than trace amounts of m/z 591/597/593 (2,3-diketogulonic acid) ever accrue.

Figure 8 shows the relative abundances of ions (at the appropriate retention times for (A) m/z 575 (AA), (B) 345 (DHA), (C) 607 (2,3-diketo-4,5,5,6-tetrahydroxyhexanoic acid), and (D) 573 (a *threo*-hexa-2,4-dienoic acid lactone) following exposure to two levels of hydrogen peroxide (open circles 0.5%, closed circles 5%) and cupric ions (20 μM) over 120 min. Based on Figure 8A, AA degradation proceeded to a similar extent in 5% hydrogen peroxide and 20 μM cupric ion solutions. On the basis of the m/z 345 content in the lower hydrogen peroxide (0.5%) incubation in Figure 8B, it appeared that 5% hydrogen peroxide and 20 μM cupric ion solutions were forming and degrading DHA. Figure 8C shows that hydrogen peroxide incubation resulted in the formation of m/z 607 (2,3-diketo-4,5,5,6-tetrahydroxyhexanoic acid) to a much greater extent than cuproic ions, while the data in Figure 8D suggest that m/z 573 (a *threo*-hexa-2,4-dienoic acid lactone) is produced and destroyed following hydrogen peroxide exposure but is relatively stable in the presence of cupric ion.

Figure 9 shows the ratio of (A) m/z 607 (2,3-diketo-4,5,5,6-tetrahydroxyhexanoic acid) to 575 (the remaining AA) and

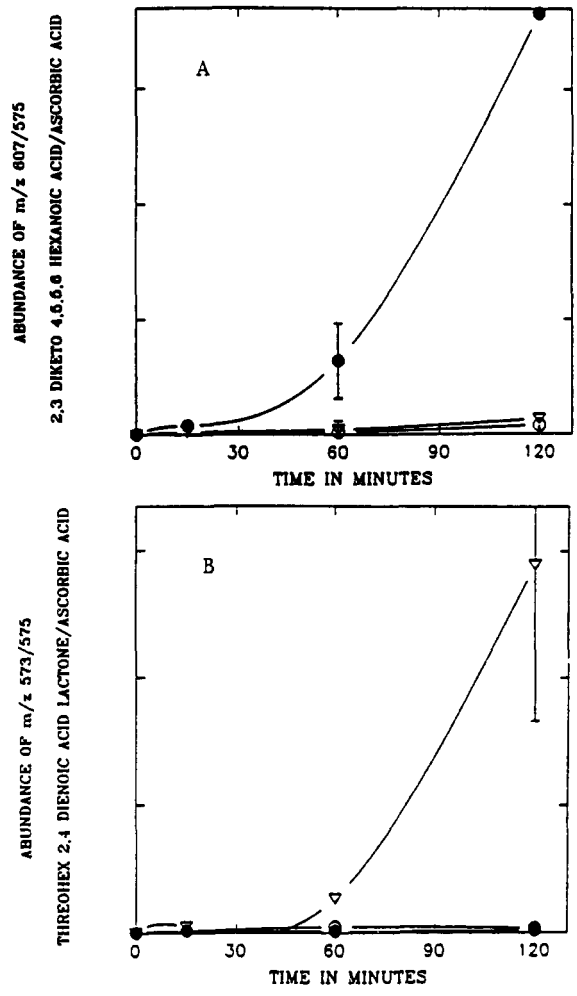

Figure 9. Rise of (A) m/z 607 (2,3-diketo-4,5,5,6-tetrahydroxybenzoic acid) and (B) 573 (a *threo*-hexa-2,4-dienoic acid lactone) relative to the loss of m/z 575 (AA) over a 2-h interval in 0.5% hydrogen peroxide (open circles), 5% hydrogen peroxide (closed circles), and 20 μM cupric sulfate (open triangles).

(B) m/z 573 (a *threo*-hexa-2,4-dienoic acid lactone) to 575 from 0.5% and 5% hydrogen peroxide, and 20 μM cupric ion solutions. As shown in Figure 9A, hydrogen peroxide-induced destruction of AA results in the relative formation of large quantities of m/z 607, while conversely Figure 9B shows that cupric ion exposure results in a relative increase in m/z 573.

These data show profound differences in the products formed and accumulated, depending on the source of oxidant stress. Although evidence of oxidative stress from exposure to either hydrogen peroxide or cupric ions was present based on the relative loss of AA (m/z 575) and the formation of DHA (m/z 345), the products resulting from further oxidation diverge, depending on the source of oxidation. The hydrogen peroxide-induced oxidation resulted in the formation of large quantities of a derivatized product having a mass which was 32 mass units greater than AA ($[M - 57]^+$ of m/z 607) and 16 mass units greater than 2,3-diketogulonic acid. The position 6 hydrogen labels were not disturbed. This compound had four derivatized sites (hydroxyl groups) and on the basis of the double peak elution, consisted of (at least) two isomers. These data are consistent with, but not absolute proof of, the formation of a diol on carbon 5 of 2,3-diketogulonic acid. Although five potential derivatizable sites are present on this structure, steric hindrance could prevent the addition of two

TBDMS groups to carbon 5 hydroxyls and would generate isomers which could have different retention times.

Cupric ion, on the other hand, formed only trace amounts of the m/z 607 product. Over time, cupric ion (but not hydrogen peroxide) formed relatively large amounts of a six-carbon species with four derivatizable sights, a single retention time, and no disturbance of the hydrogens on the carbon at position 6.

CONCLUSIONS

Data in this paper provide at least a partial explanation of the interesting results reported recently by researchers studying the relation of AA to the oxidation of low-density lipoprotein.[23] Since different AA-derived products accumulate, depending on the source of oxidative stress, it is possible that one of these products is responsible for the differences observed when oxidation is carried out in a transition metal- versus peroxide-based system. Studies can now be expanded to examine the effects of other AA-derived products, particularly these newly identified products (m/z 607 and 573 related compounds) in redox reactions of biologically important substances, such as in the oxidation of low-density lipoprotein or nucleic acid polymers.

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