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Characterization and Properties of Degradation Products of Dehydro-L-Ascorbic Acid in Aqueous Solution

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Abstract

An aqueous solution of dehydro-L-ascorbic acid (DHA) was heated for 1 hr at 100°C, and five degraded products, including L-ascorbic acid (AsA), 3-hydroxy-2-pyrone, and 2-furoic acid, were detected. 3-Hydroxy-2-pyrone and 2-furoic acid accounted for about 11% and 0.8% yield of DHA used, respectively. 3, 4-Endiol form of 2, 3-diketo-L-gulono-δ-lactone (3, 4-End DKGL) was formed from DHA under mild deoxygenated conditions in an approximate yield of 10%, and it reduced DHA to AsA. 3, 4-End DKGL was unstable and suppressed the peroxidation of linoleic acid. The oxidized form of 3, 4-End DKGL may catalyse browning reaction.

Introduction

L-Ascorbic acid (AsA) has strong reducing properties. Therefore, it is used not only in beverages, and especially in soft drinks, as an antioxidant[1], anti-browning agent[2], and as a preservative, but also as an inhibitor of the nitration of N-nitroso compounds, which may be carcinogens[3]. However, AsA in neutral or alkaline solution is not very stable. When AsA is oxidized in solution, the products formed begin to accelerate lipid peroxidation[4,5], and cause some deterioration of the lipid. The mechanism of the accelerated lipid peroxidation by the oxidized forms of AsA, dehydro-L-ascorbic acid (DHA) and 2, 3-diketo-L-gulonic acid (DKG), is not clear. We have reported a convenient procedure for the preparation of pure DHA[6,7], which should be useful in studies of the peroxidation of linoleic acid or of the degradation mechanism of oxidative products of AsA. When an aqueous solution of DHA was left at room temperature, several compounds with reducing ability were found.

Here, we deal with the degradation of DHA and the reduction of DHA to AsA by a transient derivative of DHA. We also describe the involvement of oxidation materials in the reduction of DHA.

Materials and Methods

Chemicals. AsA was obtained from Wako Pure Chemical Industries. DHA was prepared from AsA as reported previously[4,5]. The K-salt of DKG was prepared as reported by Kagawa[8] with the slight modification that methanol was used instead of ethanol for the precipitation of the K-salt. Linoleic acid (LA) was purchased from Sigma Chemical Co., diluted to 100 mM with distilled water under N_2 bubbling, and stored at –20°C until use. Dithiothreitol (DTT) was obtained from Wako Pure Chemical and sometimes used for the

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reduction of DHA. 2, 6-Dichlorophenol indophenol (DCIP) was obtained from Nacalai Tesque, and used as a spray reagent for the detection of reducing materials. Other organic and inorganic chemicals were of the purest grade available from commercial sources.

Apparatus. High-performance liquid chromatography (HPLC) was done with an Irica apparatus S-553 as the spectrophotometric detector, E-502 as the amperometric detector, and P-500 as the pump system. The Irica RP-18 column (4 mm i.d. × 250 mm) was eluted with water/MeOH/AcOH (370 : 30 : 4, v/v) at a flow rate of 0.6 ml/min. The sample was monitored photometrically (254 nm) and amperometrically at an applied potential of 0.5 V (*vs.* an Ag/Ag^+ electrode). The UV absorption spectrum was recorded with a Hitachi spectrophotometer model 200-10. Elementary analysis was done with a Yanagimoto CHN-corder MT-1. The IR spectrum was taken with a JASCO IRA-20 on a KBr pellet. Nuclear magnetic resonance (NMR) was measured with a JEOL FX-100 for ^1H and ^{13}C with TMS as the internal standard in $CDCl_3$. TLC was done with a precoated plate of Merck silica gel 60. The solvent for development was CH_3CN/H_2O/acetone/AcOH (80 : 15 : 5 : 1, v/v), and the spray reagent was a 0.2% aqueous solution of DCIP[9].

Analytical methods. Evaporation was done at below 40°C under reduced pressure. DHA was reduced with a 5-fold excess of an aqueous solution of DTT at 37°C. DHA was dissolved in buffer or deionized water, both of which were deoxygenated under nitrogen bubbling. LA peroxidation was analyzed as reported before[4]: a reaction mixture containing 10 mM LA, 20% EtOH, and catalysts in 0.1 M phosphate buffer (pH 7.0) was incubated at 37°C with shaking, then each 0.3-ml portion was withdrawn at specified intervals, and 3 ml of a 60% aqueous solution of EtOH containing 200 ppm of EDTA was added to the solution. The UV absorption spectrum was measured over the range of 225-300 nm. The responses at 233 nm and 265 nm were used for the measurement of the LA hydroperoxide formed and the AsA remaining, respectively.

Results and Discussion

Degraded products of DHA in aqueous solution

DHA (100 mM) dissolved in deionized water was heated for 1 hr at 100°C. The pH of the solution after heating was 3.1. A portion of the heat-treated sample was spotted on a TLC plate. Small amounts of AsA (Rf 0.50) and large amounts of an unknown substance (Rf 0.86) were found (data not shown). AsA was formed from DHA during the heat treatment. A portion (5 μl) of the heat-degraded solution of DHA was analyzed by HPLC (Fig. 1). Five peaks (called **I** – **V**) were obtained. Because the pH of the heat-degraded solution of DHA was 3.1, we used acetate buffer, pH 3.1, to test the degradation of DHA so as to avoid any more side reactions. AsA and DHA are relatively stable around pH 3[10]. Little or no AsA was formed. Only three peaks (**III**, **IV**, and **V**) were observed.

Peak **I** coincided with that of authentic AsA on TLC, and peak **IV** coincided with the substance with an Rf of 0.86. Peaks **II** and **III** were seemed to be intermediate substances, but we do not identify them. For the preparation of **IV** and **V**, we used solvent extraction as follows. The heat-degraded sample (5 g in 30 ml of deionized water kept at 100°C

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for 2 hr) was extracted three times with an equal volume of ice-cooled ethyl acetate. The combined extract was dehydrated with anhydrous Na_2SO_4, and then filtered with a glass filter. After the filtrate had been evaporated to dryness, the residue containing crude crystals was then allowed to sublimate at 60°C. The sublimate was recrystallized with ethyl acetate. The crystalline solution eluted with peak IV of the HPLC (Fig. 1). The peak IV material was obtained at about 11% yield from DHA in crystalline form.

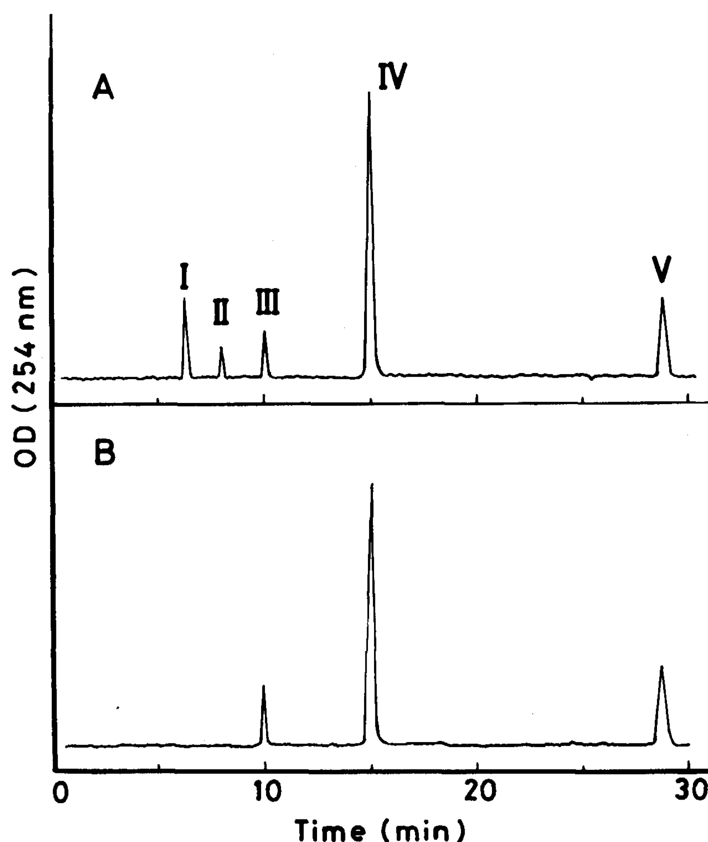

Fig. 1 HPLC of degraded products of DHA in water (A) or acetate buffer (0.2 M, pH 3.1) (B) at 100°C for 1 hr.
First, 100 mM DHA in distilled water or acetate buffer was heated for 1 hr at 100°C. A portion (5 μl) of the reaction mixture was analyzed by HPLC. HPLC was done with a solvent mixture of water/MeOH/AcOH (370:30:4, v/v) at the flow rate of 0.6 ml/min and monitored at 254 nm. **I**; AsA, **IV**; 3-hydroxy-2-pyrone, **V**; 2-furoic acid.

Another portion of the residue prepared from the reaction mixture was dissolved in a small volume of MeOH. To the MeOH solution was added a small amount of silica gel, and the mixture was evaporated to dryness. This sample was put on a silica gel column and eluted stepwise with *n*-hexane, chloroform, and ethyl acetate. The $CHCl_3$ eluting fractions that turned filter paper moistened with DCIP red were collected and evaporated to a small volume. This sample was put on an activated charcoal column and eluted with MeOH aqueous solution. The eluates containing **V** from the 20% MeOH in water were collected and evaporated to dryness. The residue was dissolved in a minimum volume of ethyl acetate and stored in a refrigerator. The crude crystals that formed were recrystal-

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lized from ethyl acetate. The peak **V** material was obtained at about 0.8% yield from the DHA in crystalline form.

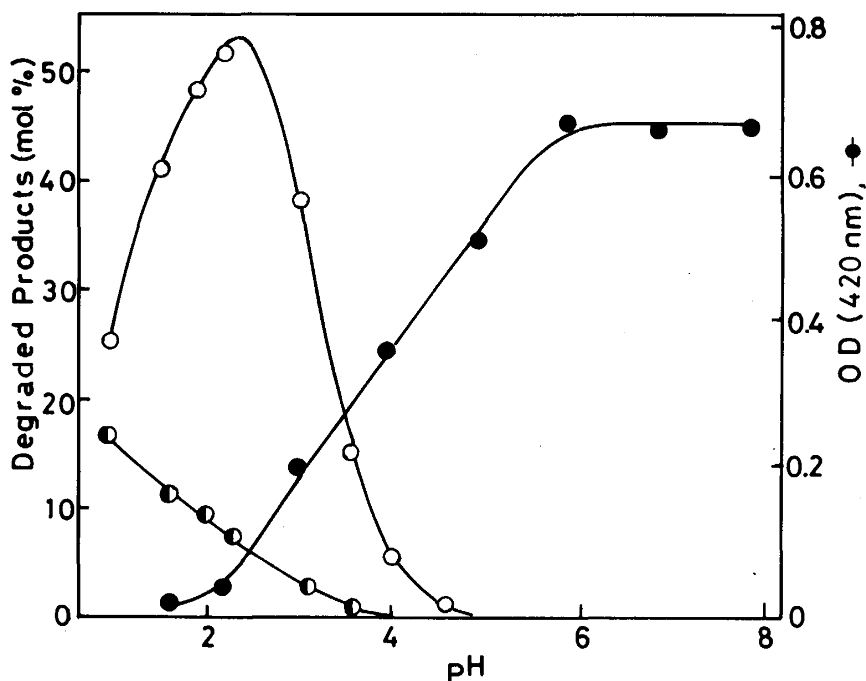

Fig. 2 Effect of pH on the degradation of DHA at 100°C for 1 hr.
DHA (100 mM) dissolved in various buffers was heated at 100°C for 1 hr. A portion of the reaction mixture was analyzed by HPLC as in the legend of Fig. 1. The reaction mixture was measured by the absorbance at 420 nm. •, browning products; ○, **IV** (3-hydroxy-2-pyrone); ◐, **V** (2-furoic acid).

Figure 2 shows the effects of pH on products of DHA degraded by being heated at 100°C for 1 hr. The browning reaction was prominent at above pH 6, and the formation of **IV** was maximum at around pH 2. Peak **V** was formed in the pH range more acidic than 1.5.

Structures of peaks IV and V

Crystallized sample of **IV** and **V** were used for structural investigation. Crystalline **IV** gave the following analytical data: m.p. 90°C, UVλ_{max}(MeOH): 233 nm, 295 nm; Found: C, 52.52; H, 3.31. Calcd. for $C_5H_4O_3$: C, 53.58; H, 3.60; 0, 42.82%. IR ν_{max}(KBr) cm^{-1}: 3050–3400 (OH, CH), 1620 – 1700 (C = 0), 1560 (α-pyran, νC = C), 1285, 1218, 1129, 1060, 760; NMR δ_H ($CDCl_3$): 6.20 (1H, d.d., J = 3.5, 2.6 Hz), 6.65 (1H, d.d., J = 3.5, 0.8 Hz), 7.12 (1H, d.d., J = 2.6, 0.8 Hz); δ_C ($CDCl_3$): 107.2 [C-5, –C(H)=], 114.3 [C-4, –C(H)=], 142.1 [C-6, –C(H)=], 142.5 [C-3, –C(OH)=], 161.6 [C-2, –C(=O)–]. From these data, compound IV was identified as 3-hydroxy-2-pyrone. KURATA *et al.* also found 3-hydroxy-2-pyrone by a preparative GLC from DHA[11].

The crystallized sample **V** gave the following analytical data: m.p. 133°C, UV λ_{max}(MeOH): 247 nm; IR ν_{max}(KBr) cm^{-1}: 3000 (CH), 1680 (COOH), 1580 (νC = C), 1470, 1300, 1195, 1125, 1020(νC = C), 890 (νC = C), 760 (νC = C); NMRδ_H($CDCl_3$): 6.55 (1H, d.d., J = 1.9, 0.8 Hz), 7.30 (1H, d.d., J = 1.9, 0.5 Hz), 7.62 (1H, d.d., J = 0.8, 0.5 Hz). From these data, compound **IV** was identified as 2-

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furoic acid. This 2-furoic acid was reported by KURATA *et al.*[12,13] in a strongly acidic medium (5% sulfuric acid). However, our results showed that both 3-hydroxy-2-pyrone and 2-furoic acid were formed by the heating of DHA in aqueous solution and that both could be easily crystallized as described above without derivatization with the use of phenylhydrazine.

Degraded products derived from mild degradation of DHA

DHA (100 mM) was left at room temperature overnight and a portion of the sample was used in TLC. There was no clear difference in the degradation of DHA and DKG, except that only DKG was formed from DHA at pH 3.7 (Fig. 3). More kinds of degraded

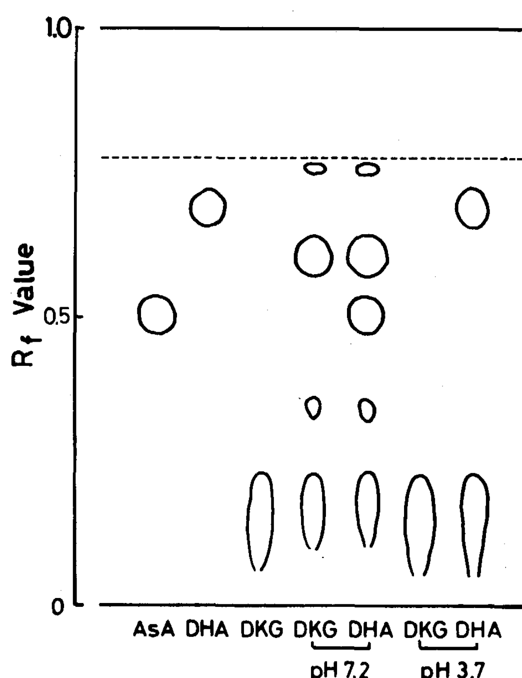

Fig. 3. TLC of the degraded products of DHA and DKG treated at room temperature overnight.

DHA and DKG (100 mM each) dissolved in acetate buffer (0.2 M, pH 3.7) or phosphate buffer (0.2 M, pH 7.2) were left at room temperature overnight. The solvent for development was CH_3CN/H_2O/acetone/acetic acid (80:15:5:1, v/v) and the spray reagent was a 0.2% aqueous solution of DCIP.

products were formed from DHA and DKG at pH 7.2, but AsA was not formed form DKG. This suggests that more intermediate materials were formed in the neutral pH range than in the acidic. This suggests that no production of AsA occurred in the case of DKG, and the coexistence of DHA and some intermediate materials in the reaction mixture might be necessary. The substance with the Rf value of 0.33 was found only in a trace amount. At neutral pH, the substance with an Rf of 0.61 was more abundant than that with an Rf of 0.76. These results mean that DHA might be reduced to AsA by

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its intermediate product. This suggests that there was an easily oxidizable substance in the solution that was involved in an oxidation-reduction reaction. We characterized these intermediate materials.

Preparation of the intermediate compound and its structure

The spot corresponding to Rf 0.61 was developed on preparative TLC plates with a solvent mixture of CH_3CN/H_2O/acetone/AcOH (80 : 15 : 5 : 1, v/v) under an N_2 stream. The band required was scraped off and eluted with the same solvent mixture as for development. The extract was evaporated to dryness and the syrup that formed was used in the following experiment. A portion of the syrup was dissolved in CD_3OD and analyzed by ^1H NMR. The material was identified as being the 3,4-endiol form of 2,3-diketo-L-gulono-δ-lactone (3,4-End DKGL) by the NMR data and by the agreement of the Rf value, the IR data, the fragmentation patterns by GC-MS, and other analytical data reported by Otsuka *et al.*[9].

Some properties of 3,4-End-DKGL

In a preliminary experiment, the lactone ring of DHA was confirmed to be relatively stable in acidic pH (below pH 6.5). DHA remaining in the reaction mixture was reduced to AsA in neutral or weakly alkaline solution, accompanied by the formation of 3,4-End DKGL, which was also produced by the hydrolysis of the lactone ring of DHA. The rate of reduction of DHA to AsA depended on the amount of DHA remaining in the solution and the concentration of 3,4-End DKGL as the intermediate.

Table 1 shows the reduction ratio of AsA formed from 3,4-End DKGL in concentra-

Table 1. Effect of concentration of DHA on the reduction of DHA to AsA under deoxygenated conditions

DHA solution (mM)	AsA produced (mM)	Reduction ratio of DHA (%)
1	–	–
10	0.13	1.3
25	1.19	4.8
50	4.30	8.6
75	7.10	9.5
100	11.58	11.6

DHA was dissolved in 0.5 M phosphate buffer (pH 7.2) and left for 1 hr at 37°C under deoxygenated conditions. A portion of the sample was analyzed by HPLC, with monitoring at 245 nm. The amount of AsA formed was measured by the use of authentic AsA.

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tions of DHA up to 100 mM in 0.5 M phosphate buffer (pH 7.2) at 37°C for 1 hr under deoxygenated conditions. With 50 mM DHA or higher, the reduction was about 10%, but with 25 mM DHA or less, the yield was below 5%. This suggests that the easily

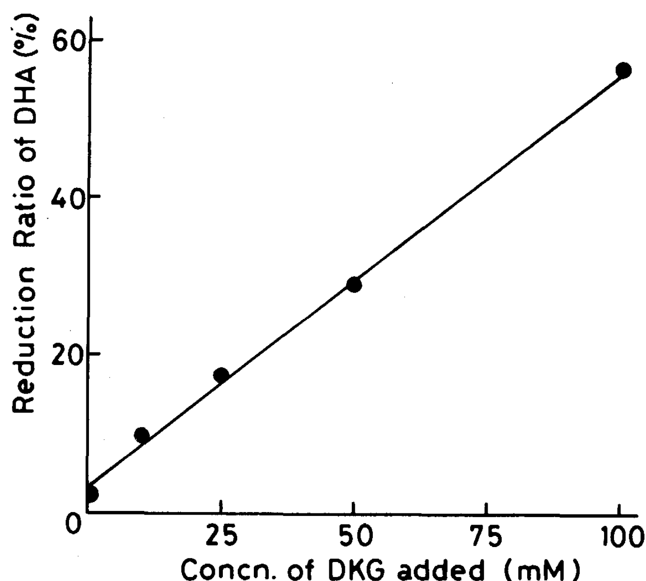

Fig. 4 Effect of concentration of DKG on the reduction of DHA.
DHA was dissolved in 4 ml of phosphate buffer (0.2 M, pH 7.2) to give a final concentration of 10 mM, and DKG was added to make the given concentration under deoxygenated conditions.

oxidizable compound, 3,4-End DKGL, would be consumed by small amounts of oxygen in the reaction mixture.

Figure 4 shows the reduction ratio of DHA *vs.* the concentration of DKG added to the DHA solution at 37°C for 1 hr under deoxygenated conditions. The slope was linear to the concentration of 100 mM DKG, and the reduction ratio was about 60% with 100 mM DKG.

Time course of 3,4-End DKGL formation from DKG in 0.5 M phosphate buffer was tested by photometric and amperometric detection. The amount formed was proportional to the concentration of DKG. The incubation time of 30 min was enough for the formation of 3,4-End DKGL, and the response was proportional in the range of 12.5 to 100 mM DKG tested.

Figure 5 shows the effect of pH on the production of 3,4-End DKGL and on the browning of the reaction mixture with 10 mM DKG in phosphate buffer. Formation of 3,4-End DKGL was maximum around pH 6-7; it was much less at below pH 5. In the alkaline range, the amount of 3,4-End DKGL gradually decreased, and the extent of browning increased with pH, reflecting the instability of 3,4-End DKGL. This result also suggested that 3,4-End DKGL was formed from DKG more rapidly than from DHA.

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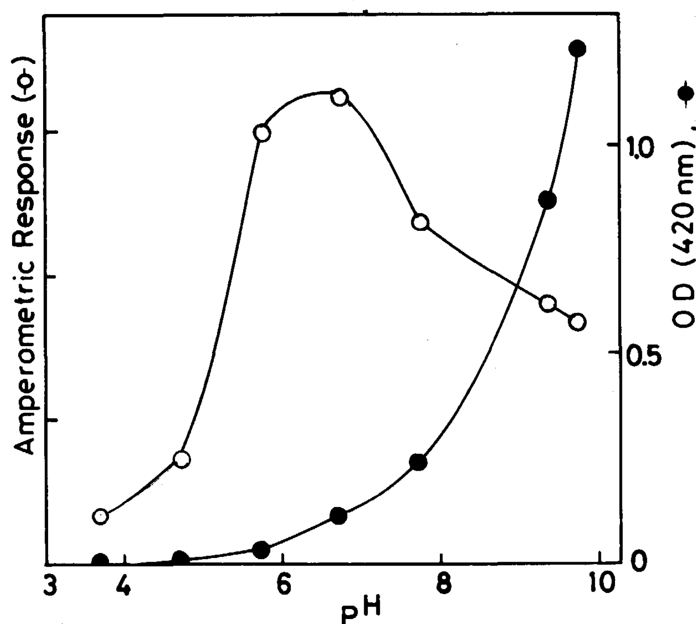

Fig. 5 Effect of pH on the formation of 3,4-End DKGL and browning products.

DKG was dissolved in various kinds of buffer to a final concentration of 10 mM and incubated at 37°C for 1 hr. A portion of the reaction mixture was measured by the absorbance at 420 nm for the estimation of browning products, and another portion was used for HPLC with an amperometric detector for the detection of 3,4-End DKGL.

Effect of degraded products of DHA on LA peroxidation

3-Hydroxy-2-pyrone, 2-furoic acid, and 3,4-End DKGL were tested for LA peroxidation in phosphate buffer (pH 7.0, 0.1 M) containing 20% EtOH and 10 mM LA (data not shown). Neither 3-hydroxy-2-pyrone nor 2-furoic acid (each 135 μM) had much effect on the suppression of LA peroxidation. However, the addition of 3,4-End DKGL (110 μM) suppressed LA peroxidation more than that of AsA. After the suppression of LA peroxidation by 3,4-End DKGL had ended, LA peroxidation was accelerated, as in the autoxidation catalyzed by AsA or DHA[4]. This result suggests that 3,4-End DKGL suppresses LA peroxidation in the same way as AsA, because the two had the same UV spectra, and might have similar keto-enol transition. The formation of an easily oxidizable product is interesting in relationship to the reduction or oxidation of biological fluids and human blood in particular.

In this experiment, 3,4-End DKGL was formed in neutral to slightly alkaline solutions under deoxygenated conditions. As was clear from its structure, 3,4-End DKGL may be unstable, and be easily oxidized. When 3,4-End DKGL was added to a reaction mixture at pH 7 containing LA and 20% ethanol, it suppressed the lipid peroxidation at first, and then gradually accelerated. Therefore, most of the deteriorative effect of AsA is probably due to 3,4-End DKGL or its further degraded products.

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REFERENCES

1) SMOOT, J.M. and NAGY, S. (1980). Effect of storage temperature and duration on total vitamin C content of canned single-strength grapefruit juice. *J. Agric. Food Chem.*, **28**, 417–421.
2) HOROWITZ, I., FABRY, E.M. and GERSON, C.O. (1976). Bioavailability of ascorbic acid in orange juice. *J. Am. Med. Ass.*, **235**, 2624–2625.
3) OHSHIMA, H. and BARTSCH, H. (1981) The influence of vitamin C on the *in vivo* formation of nitrosamines. In "Vitamin C" (Eds. by COUNSELL, J.N. and HORNIG, D.H.). Applied Sci. Publishers, London, 215–224.
4) TAKAGI, M. HIGASHIOKA, H., TAMURA, K. and MORITA, N. (1986). Effect of L-ascorbic acid and dehydro-L-ascorbic acid on the peroxidation of linoleic acid in neutral phosphate buffer containing alcohol. *Agric. Biol. Chem.*, **50**, 41–47.
5) TAKAGI, M., HIGASHIOKA, H. and MORITA, N. (1986). Effects of lipophilic derivatives of L-ascorbic acid and dehydro-L-ascorbic acid on the peroxidation of linoleic acid in neutral phosphate buffer containing alcohol. *J. Vit. Nutr. Sci.*, **32**, 389–394.
6) OHMORI, M. and TAKAGI, M. (1978). A facile preparation of dehydro-L-ascorbic acid-methanol solution and its stability. *Agric. Biol. Chem.*, **42**, 173–174.
7) OHMORI, M., HIGASHIOKA, H. and TAKAGI, M. (1983). Pure dehydro-L-ascorbic acid prepared by O_2-oxidation of L-ascorbic acid with active charcoal as catalyst. *Agric. Biol. Chem.*, **47**, 607–608.
8) KAGAWA, Y. (1962). Enzymatic studies on L-ascorbic acid catabolism in animals. *J. Biochem.*, **51**, 134–144.
9) OTSUKA, M., KURATA, T. and ARAKAWA, N. (1986). Isolation and characterization of a degradation product derived from 2,3-diketo-L-gulonic acid. In "Amino-Carbonyl Reactions in Food and Biological Systems" (Eds. by FUJIMAKI, M., NAMIKI, M. and KATO, H.). Developments in Food Sci. 13, Elsevier, Amsterdam, 77–84.
10) ANMO, T., WASHITAKE, M., HAYASHI, H. and TAKAHASHI, M. (1971). Decompositsition of L-ascorbic acid in the presence of D-arabinoascorbic acid. II. Anerobic decomposition of L-ascorbic acid in the presence of D-arabinoascorbic acid. *Yakugaku Zasshi*, **91**, 444–453. (*in Japanese*)
11) KURATA, T. and FUJIMAKI, M. (1976). Formation of 3-keto-4-deoxypentosone and 3-hydroxy-2-pyrone by the degradation of dehydro-L-ascorbic acid. *Agric. Biol. Chem.*, **40**, 1287–1291.
12) KURATA, T. and SAKURAI, Y. (1967). Degradation of L-ascorbic acid and mechanism of nonenzymic browning reaction. Part III. Oxidative degradation of L-ascorbic acid (degradation of dehydro-L-ascorbic acid). *Agric. Biol. Chem.*, **31**, 177–184.
13) KURATA, T. (1976). Mechanism of degradation and discoloration reaction of L-ascorbic acid. *Nippon Nogeikagaku Kaishi*, **50**, R209–216. (*in Japanese*)

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