Biosci. Biotechnol. Biochem., **64** (3), 476–483, 2000

Interconversion between Dehydro-L-Ascorbic Acid and L-Ascorbic Acid

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Received August 9, 1999; Accepted November 30, 1999

L-Ascorbic acid (AA) plays an important role in biological systems as an electron donor. Erythorbic acid (EA) is the epimer of AA and has chemical characteristics very similar to those of AA. It is demonstrated in the present study by ^{1}H-NMR that dehydro-L-ascorbic acid (DAA) was reduced by EA under neutral conditions but not acidic, and that dehydroerythorbic acid (DEA) was also reduced by AA under the same conditions. These reactions also occurred at a low concentration close to the concentration of AA in such biological tissue as the liver. Furthermore, the interconversion of DAA and AA at neutral pH and low concentration was also confirmed by radioluminography. These results suggest the interconversion between DAA and AA *in vivo*.

Key words: dehydro-L-ascorbic acid; L-ascorbic acid; erythorbic acid; dehydroerythorbic acid; interconversion

L-Ascorbic acid (AA), also referred to as vitamin C, is an outstanding antioxidant in food and biological systems. In physiological terms, this means that AA provides electrons for various oxidants and for other electron acceptors. In these reactions, AA is reversibly oxidized with the loss of one electron to form the free radical, monodehydro-L-ascorbic acid (AFR),[1] which is further oxidized to dehydro-L-ascorbic acid (DAA) mainly *via* disproportionation (Fig. 1).[2] DAA can be reduced to AA or irreversibly hydrolyzed to 2,3-diketo-L-gulonic acid (DKG) which leads to a loss of the function of vitamin C. DAA is rapidly reduced to AA *in vivo* by both enzymatic and nonenzymatic reactions.[3–7] Therefore, the biological function of DAA is considered to be very similar to that of AA. However, DAA has been demonstrated to be unstable under neutral conditions and to be hydrolyzed to DKG by cleavage of the lactone ring, this degradation process having been reported to be dependent on both pH and temperature,[8] while the vitamin C activity of DAA does not equal that of AA.[9–11] In addition, AFR, which is thought to be an intermediate compound in the reaction from AA to DAA, is relatively unreactive as a common organic radical and to have a comparatively long life so that it can be easily detected by ESR.[12] These chemical characteristics of AA are thought to be the reason why AA serves as an outstanding electron donor in biological systems. It appears that all these oxidation processes of AA to DAA greatly affect the vitamin C activity of AA. However, the reaction mechanism between AA and DAA has not yet been fully clarified. For example,

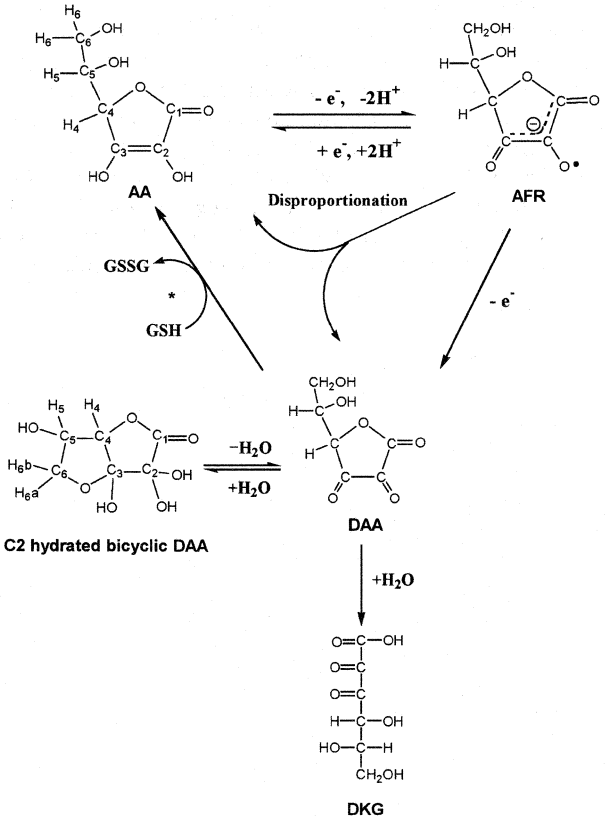

Fig. 1. AA and Its Oxidation Products.
DAA might exist in various forms, although only two are shown here for simplicity. *DAA reductase.

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Abbreviations: AA, L-ascorbic acid; EA, erythorbic acid; DAA, dehydro-L-ascorbic acid; DEA, dehydroerythorbic acid; AFR, monodehydro-L-ascorbic acid; DKG, 2,3-diketo-L-gulonic acid; TLC, thin-layer chromatography; RLG, radioluminography

there exists the conflicting finding of interconversion between DAA and AA. In several studies on AFR, ascorbate and DAA have been used to generate AFR[13,14] by presuming that AFR would be produced by the reaction between AA and DAA. However, Winkler *et al.* have reported that AFR could not be formed from a mixture of AA and DAA.[7]

On the other hand, erythorbic acid (EA) is a stereoisomer of AA, differing only in the spatial configuration of the hydroxyl group at carbon 5, and has almost the same acidic and reducing properties by its endiol group in the lactone ring as those of AA. EA is widely used as an antioxidant in various foods and beverages mainly due to its reducing properties. However, it is generally accepted that the vitamin C activity, the antiscorbutic potency, of EA is very low, being about one-twentieth that of AA,[15] and the side chains of AA and its isomer, including EA, are also thought to affect their vitamin C activity. Several reports on the nutritional interaction of AA and EA have been presented.[15-18] However, the interaction of EA and AA *in vivo* has not been fully elucidated, nor have the oxidation pathway of EA and the structure and chemical characteristics of the oxidized form of EA, dehydroerythorbic acid (DEA), been clarified.

As already mentioned, the reaction between AA and DAA is rather complicated and the reaction mechanism has not been clarified, despite elucidation of this reaction mechanism being very important to understand the biological function of AA. In this study, the oxidation reactions of AA and EA were compared in order to obtain useful information about the reaction mechanism for DAA and AA *via* AFR. The interconversion between DAA and AA at a physiological concentration under neutral pH conditions was also examined to determine the possible occurrence of the reaction *in vivo*.

Materials and Methods

Materials. The deuterium-labeled solvents and reagents used in ^{1}H-NMR and ^{13}C-NMR measurements were all guaranteed-grade reagents from Merck. AA and EA were purchased from Wako Pure Chemical Industries Co. (Tokyo). DAA was prepared in methanol by the method described in the literature[19] and obtained as pale syrup which was kept in a freezer at −20°C until its use. Identification was made by NMR and thin-layer chromatography (TLC), using a solvent system of acetonitrile:acetone:water:acetic acid = 80:5:15:1 (v/v) and an adsolvent of silicagel 60. After DAA had been reduced with dithioerythritol, the concentration of DAA was measured by a Shimadzu LC-9A high-performance liquid chromatograph under the following HPLC conditions: column, ODS-2 (150×4.6 mm i.d., GL Sciences); mobile phase, sodium phosphate buffer (50 mM at pH 2.3); flow rate, 0.7 ml/min; column temperature, room temperature; detection, electrochemical (BAS LC-4B 500 mV). The concentration of DAA in the syrup was estimated to be about 75% (w/w) or equivalent to 4.3 mmol/g. HPLC was used to confirm that the syrup of DAA was pure enough and did not contain any AA, the remaining 25% only being methanol. DEA was prepared by the same method as that used for the preparation of DAA. The potassium salt of DKG was prepared from AA according to the method of Kagawa,[20] its purity being confirmed by TLC. [1-^{14}C] AA was purchased from Amersham Pharmacia Biotech Co. (Tokyo). All other commercially obtained reagents (Wako Pure Chemical Industries Co. (Tokyo)) were of guaranteed grade.

Chemical interaction between DAA and EA and between DEA and AA, and the remaining amounts of DAA and AA in the buffer (pH 7.4). Evaluation of the chemical interaction between DAA and EA and between DEA and AA was performed by ^{1}H-NMR (JEOL GX 400 FT NMR spectrometer operated at 400 MHz). DAA or DEA syrup (40 mg = 0.17 mmol) was dissolved in 1.0 ml of a sodium phosphate buffer that had been prepared with deuterium oxide (0.5 M at pH 7.4), and to each solution was added 20 mg (0.11 mmol) of either AA or EA. The final concentration of the oxidized form and of the reduced form was *ca.* 170 mM and 110 mM, respectively. ^{1}H-NMR spectra were obtained after incubating for 5 min, 10 min, 30 min, 1 h, 2 h, 12 h and 24 h.

The remaining amounts of DAA and AA in the sodium phosphate buffer that had been prepared with deuterium oxide (0.5 M at pH 7.4) were calculated from each signal area by ^{1}H-NMR under the conditions already described.

Chemical interaction between DAA and EA and between DEA and AA at low concentrations. DAA and EA were mixed in a sodium phosphate buffer (0.05 M at pH 7.4) in equimolar concentrations (57 mM, 5.7 mM and 0.57 mM), and the mixture was incubated for 10 min at room temperature or at 37°C. A portion of the reaction solution was diluted with a 0.5% metaphosphoric acid solution when necessary and was subjected to HPLC to examine the reduction of DAA. The conditions for HPLC were as follows: column, LiChrosorb-NH_2 (150×4.6 mm i.d., GL Sciences); mobile phase, acetonitrile:water:acetic acid = 87:11:2 (v/v); flow rate, 2.0 ml/min; column oven, room temperature; detection, UV at 254 nm.

Confirmation of the interconversion between AA and DAA under physiological conditions. The inter-

conversion between AA and DAA at a low concentration close to the physiological level was confirmed by radioluminography (RLG), by which phosphor screen images are produced on an imaging plate (IP).[21,22] [1-^{14}C] AA and DAA were mixed and diluted with a phosphate buffer (pH 7.4 at 0.05 M) to obtain final concentrations of 60 μM and 90 μM, respectively. The mixture was analyzed by TLC, using an adsolvent of silicagel 60 (Merck) and a solvent system of acetonitrile:acetone:water:acetic acid = 80:5:15:1. Generally, under these development conditions, the R_f values of AA and DAA were around 0.5 and 0.7, respectively. After development, the IP was exposed to the silica gel plate. IP measurement was conducted with a BAS-2000 Bio-image Analyzer System (Fuji Photo Film Co. (Tokyo)) and the absorbed dose recorded on the IP was quantified by scanning with a fine laser beam of 633 nm, the resulting radioluminographic image being displayed on a computer screen (Macintosh 8500/180).

Results

^{1}H-NMR spectra of AA, EA, DAA and DEA in D_2O and the buffer (pH 7.4)

Several studies on the chemical structures of AA, EA and DAA by ^{1}H-NMR spectroscopy have been reported,[23-25] but not that of DEA. Figure 2 shows ^{1}H-NMR spectra of AA and EA in a sodium phosphate buffer (pH 7.4) (Figs. 2A and B) and of DAA and DEA in D_2O (Figs. 2C and D). In the typical ^{1}H-NMR spectrum of DEA in D_2O, the signal assignments were as follows: $\delta_{H4}=4.83$, $\delta_{H6a}=4.44$, $\delta_{H6b}=3.73$ ppm. The signal of H5 could not be correctly assigned because it overlapped with the signal of water, but it was deduced to be about $\delta_{H5}=4.87$ ppm. The spectrum of DEA clearly shows the characteristic signal pattern attributed to protons on the hemiketal ring like that of bicyclic DAA. Thus, the structure of DEA was also bicyclic in an aqueous solution, because the signals of two protons (H6a and H6b) bonded to C6 were not equivalent. However, the spectrum of DEA is quite different from that of DAA, suggesting different bicyclic structures for each (Figs. 2 C and D). On the other hand, the spectrum of EA is very similar to that of AA (Figs. 2A and B). Aqueous solutions of DAA and DEA showed acidity. When the concentration of DAA or DEA was 100 mM, the aqueous solution showed about pH 3.0. ^{1}H-NMR spectra of DAA and DEA taken in the buffer (pH 7.4) were confirmed to be almost the same as those in D_2O, although some minor signals due to the degradation products of DAA and DEA were observed (data not shown). These degradation products were mainly DKG and diketo-D-gluconic acid produced by hydrolysis of the

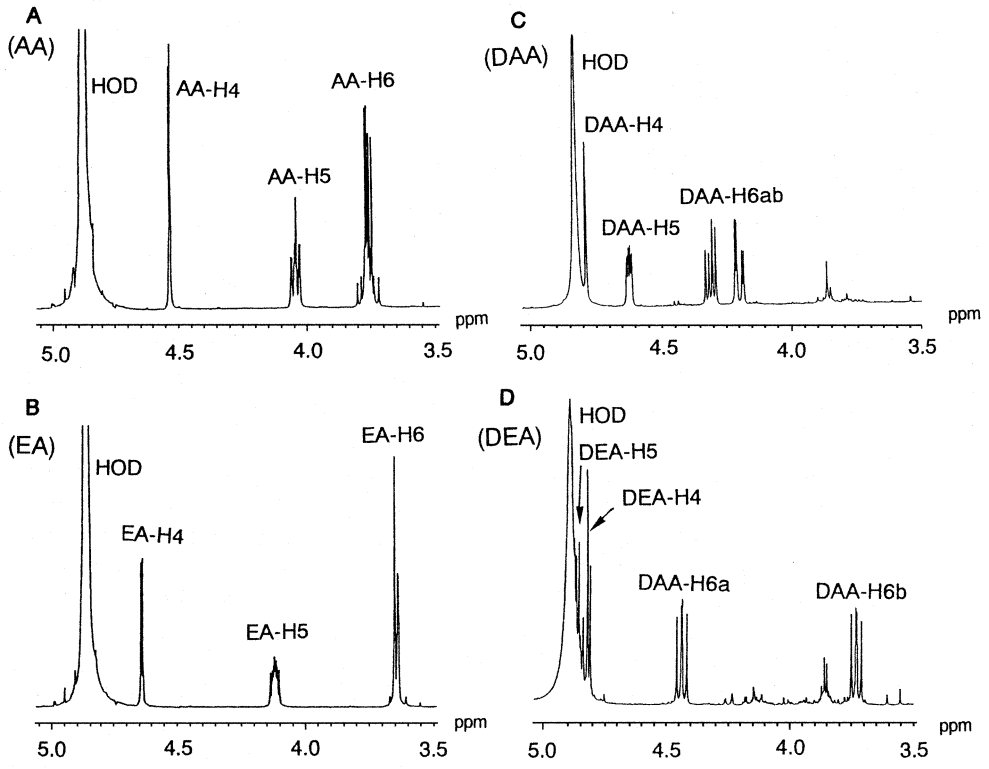


Fig. 2. ^{1}H-NMR Spectra of AA, EA, DAA and DEA.

(A) AA (20 mg/ml = 110 mM) dissolved in a sodium phosphate buffer prepared with D_2O (0.5 M at pH 7.4), **(B)** EA in the same preparation as **(A)**, **(C)** DAA (40 mg (syrup)/ml = 170 mM) in D_2O, **(D)** DEA in the same preparation as **(C)**. Small signals around 3.85 ppm in **(C)** and **(D)** are those of DKG. The numbering of each proton is given in Fig. 1.

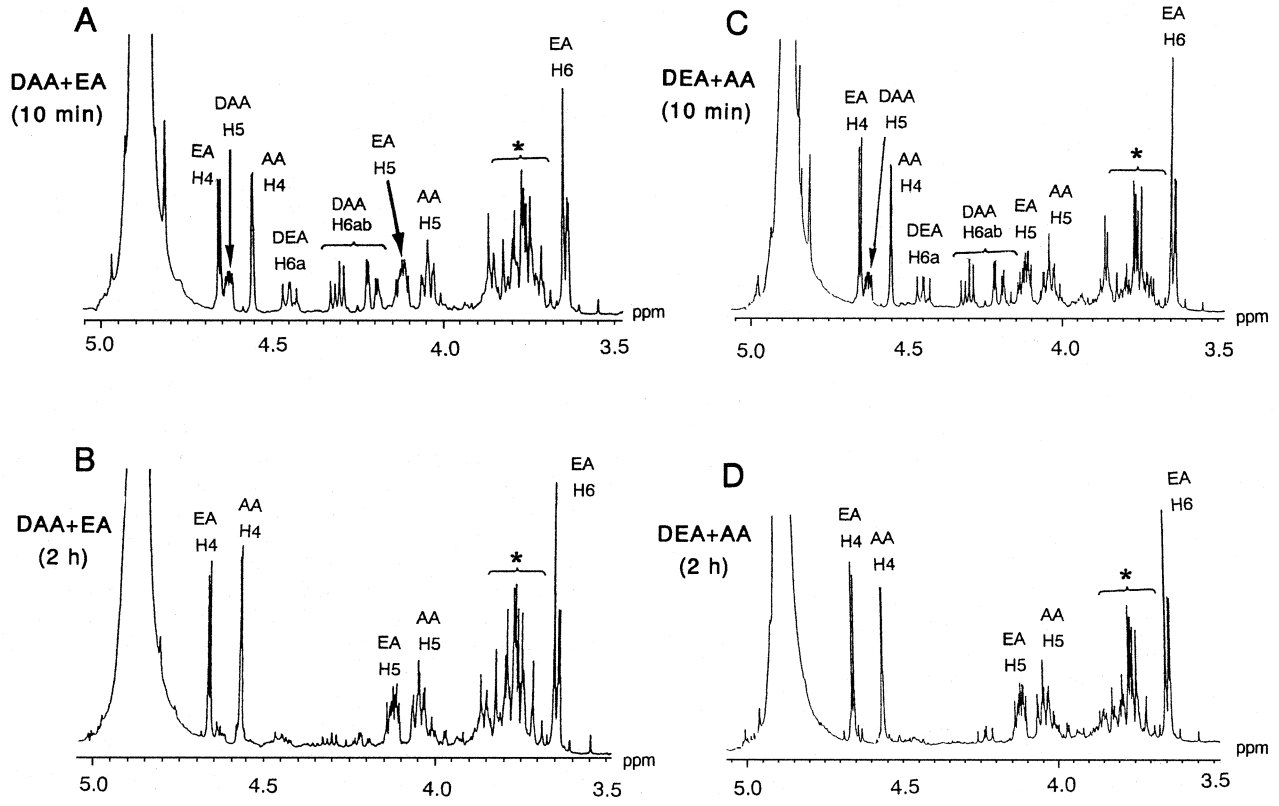


Fig. 3. ^{1}H-NMR Spectra of the Mixture of DAA and EA (**A** and **B**) and of the Mixture of DEA and AA (**C** and **D**) at pH 7.4.
DAA, EA, DEA and AA were mixed in a sodium phosphate buffer with D_2O (0.5 M at pH 7.4) to make final concentrations of 110 mM = 2 g/100 ml (AA and EA, reduced form) and 170 mM = 3 g/100 ml (DAA and DEA, oxidized form). The mixed sample was incubated for 10 min and 2 h at room temperature, and the solution was then applied to NMR. The signal of methanol from the solvent of the DAA or DEA syrup was used as the reference (3.37 ppm). * is the signal resulting from AA-H6 and DKG.

lactone rings of DAA and DEA. These results suggest that AA, DAA, EA and DEA could be separately identified by ^{1}H-NMR under neutral conditions (pH 7.4).

Chemical interaction between DAA and EA and between DEA and AA in the buffer (pH 7.4)

Figure 3 shows the NMR spectral changes due to the reduction of DAA by EA and of DEA by AA. When EA was added to DAA in the sodium phosphate buffer (0.5 M at pH 7.4) and left at room temperature (*ca.* 20°C) at final concentrations of 110 mM (2 g/100 ml) and 170 mM (3 g/100 ml), signals of AA and DEA, which were not apparent in the initial solution, were observed after 10 min of incubation, suggesting that chemical interaction between DAA and EA had occurred (Fig. 3A). As shown in Fig. 3B, the signals of DAA had almost disappeared, and signals of EA, AA and those due to degradation compounds, mainly DKG, remained after 2 h of incubation. After further incubation (24 h), AA and EA remained as the main compounds (data not shown). It is well known that some degradation products with an endiol group which have electron-donating properties are formed from DKG.[9,26] However, after 24 h of incubation of the DAA solution at pH 7.4 and room temperature, the formation of AA due to the reduction of DAA by the degradation products of DAA was hardly apparent by NMR (data not shown). Likewise, as shown in Fig. 3C, it was also confirmed that DEA was reduced by AA, yielding EA and DAA. In this mixture of DEA and AA, EA and AA remained after 2 h of incubation under the same conditions (Fig. 3D) as those for the reaction between DAA and EA (Fig. 3B).

Chemical interaction between AA and DEA and between EA and DAA at low concentrations

The chemical interaction between DAA and EA and between DEA and AA were confirmed at a very high concentration (2–3 g/100 ml) and at room temperature (*ca.* 20°C) by ^{1}H-NMR. The chemical interaction between DAA and EA at a low concentration (10 mg/100 ml) such as the physiological level, for example, a concentration of AA of 0.5–0.9 mg/100 ml in the blood, 30–50 mg/100 g in the adrenal glands, and 10–30 mg/100 g in the liver, was determined by HPLC (Fig. 4). The results show that AA, which was absent in the starting compounds of DAA and EA (Fig. 4A), was apparently produced in the mixture at high concentrations of DAA and EA

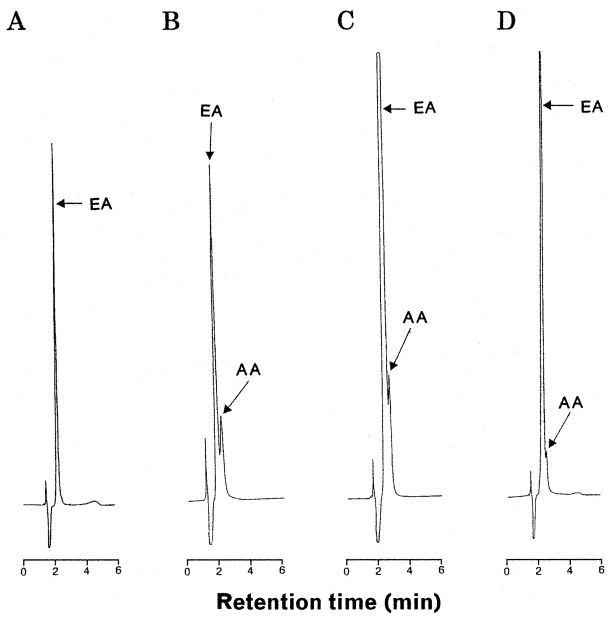


Fig. 4. Chromatograms of the Reaction Mixture of DAA and EA in a Sodium Phosphate Buffer (pH 7.4) at Various Concentrations.

DAA and EA were mixed in equimolar amounts; **A**, control, 57 mM = 1 g/100 ml (concentration of DAA and EA), 0 min (incubation time); **B**, 57 mM, 10 min at room temperature; **C**, 5.7 mM = 100 mg/100 ml, 10 min at room temperature; **D**, 0.57 mM = 10 mg/100 ml, 10 min at 37°C. The conditions for HPLC are described in the materials and methods section.

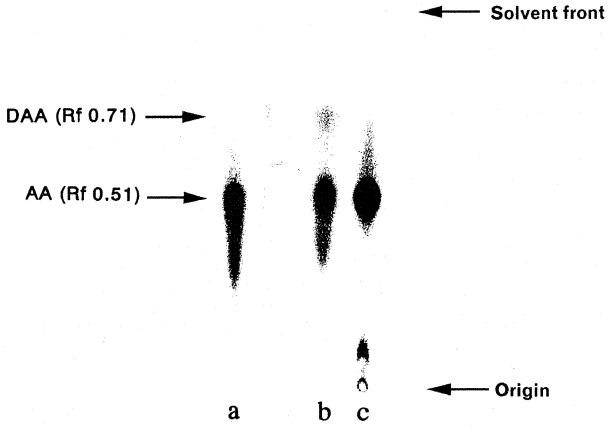


Fig. 5. Chromatogram of AA and the Mixture of AA and DAA Incubated at pH 7.4 at Room Temperature.

(a) [1-^{14}C] AA in the buffer (pH 7.4); **(b)** mixture of [1-^{14}C] AA and DAA in the buffer (pH 7.4), the concentrations of AA and DAA being 60 μM (1 mg/100 ml) and 90 μM (1.5 mg/100 ml), respectively; **(c)** [1-^{14}C] AA in 5% metaphosphoric acid as a reference. The condition for TLC are described in the materials and methods section.

(57 mM = 1 g/100 ml and 5.7 mM = 100 mg/100 ml, respectively) after 10 min of incubation in a sodium phosphate buffer (pH 7.4) at room temperature (*ca.* 20°C) (Figs. 4B and C). Furthermore, AA was also confirmed to have been produced at a lower concentration (0.57 mM = 10 mg/100 ml) after 10 min incubation at 37°C (Fig. 4D), although no formation of AA was apparent at room temperature. The chemical interaction between DEA and AA at low concentrations was also confirmed in the same way (data not shown).

Confirmation of the interconversion between AA and DAA under physiological conditions

The interconversion between DAA and AA could not be confirmed by ^{1}H-NMR, since no spectral changes were expected to be observable even when DAA was reduced to AA, or when AA was oxidized to DAA. We therefore tried to confirm the interconversion between DAA and AA by RLG with [1-^{14}C] AA. Almost equimolar DAA and [1-^{14}C] AA were mixed at low concentrations close to the physiological level, 90 μM = 1.5 mg/100 ml for DAA and 60 μM = 1 mg/100 ml for [1-^{14}C] AA, in a sodium phosphate buffer (0.05 M at pH 7.4). After incubating for a specified period of time, a portion of the reaction mixture was subjected to TLC, and AA and DAA were detected by RLG. Figure 5 shows that [1-^{14}C] AA alone was not oxidized to [1-^{14}C] DAA after incubating in the buffer solution (pH 7.4), but when AA was mixed with DAA, the formation of [1-^{14}C] DAA was positively detected under the same conditions. It was thus strongly suggested that DAA was reduced by AA, and that the interconversion between DAA and AA seemed to have occurred *in vivo*.

Stabilization of DAA in the presence of AA

Figure 6 shows the ^{1}H-NMR spectra of DAA and the mixture of DAA and AA after 50 min of incubation at pH 7.4 and room temperature (*ca.* 20°C). It seems that AA suppressed the degradation of DAA to DKG, because the signals of DAA remained after a longer period of incubation in the presence of AA than in the absence of AA, in spite of the same concentration of DAA being employed in both these cases. This result is also quantitatively supported by the NMR data (Fig. 7). Consequently, by estimating the change of each signal area in the ^{1}H-NMR spectrum, it was confirmed that the decrease in signal intensity of DAA in the absence of AA was more rapid than that in the presence of AA, although the decrease of AA was hardly apparent during the reduction of DAA. This result implies that the degradation of DAA to DKG would be suppressed in the presence of AA, and that the interconversion between DAA and AA might somehow be involved in this apparent stabilization of DAA by AA.

Discussion

This study has confirmed that DAA was reduced to AA by EA, and that DEA was also reduced to EA

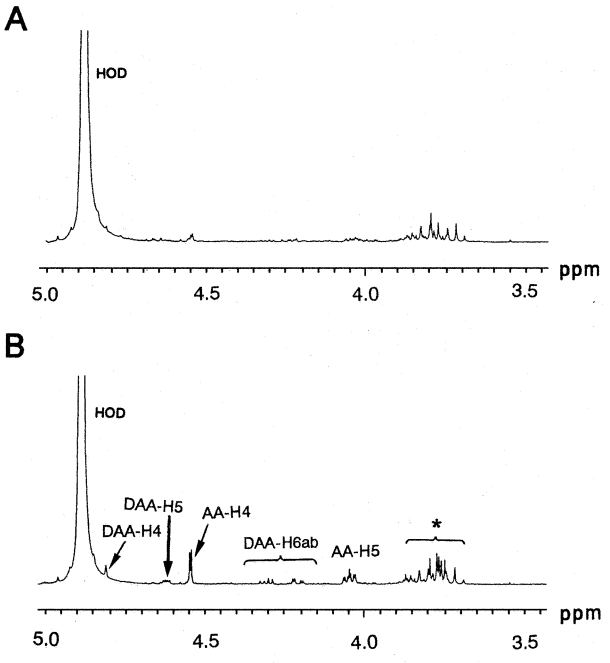


Fig. 6. ^{1}H-NMR Spectra of DAA (**A**) and the Mixture of DAA and AA (**B**) in a Buffer (pH 7.4) after 50 min of Incubation at Room Temperature.

The concentrations of DAA and AA were 25 mM and 12.5 mM, respectively. * is the signal resulting from AA-H6 and DKG.

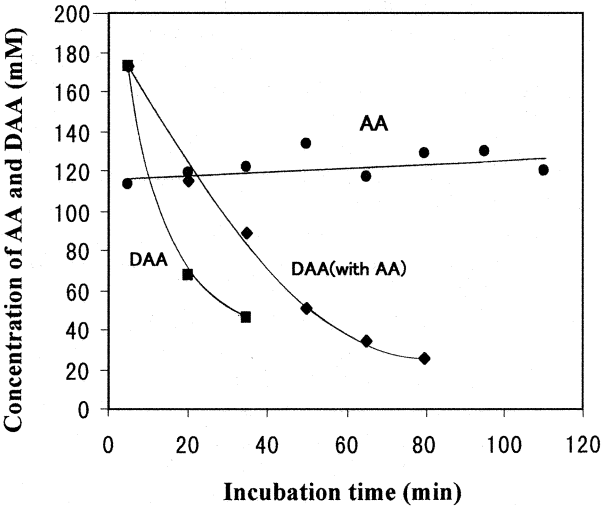
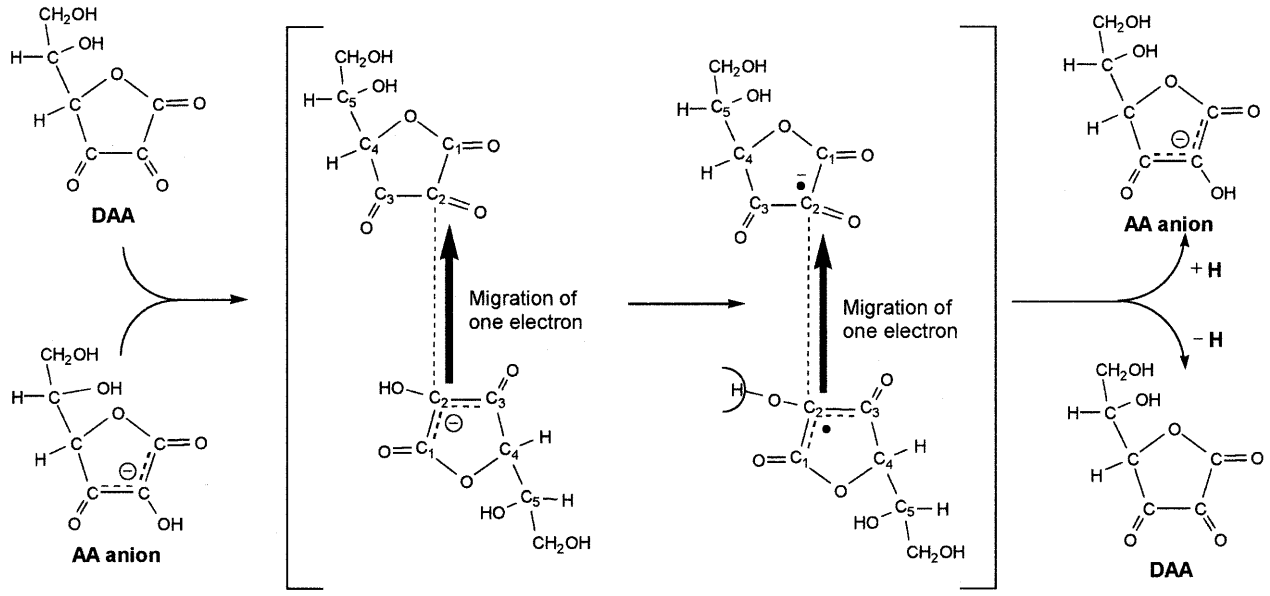


Fig. 7. Concentrations of DAA and AA in the Buffer (pH 7.4) Measured from Their Signal Area by ^{1}H-NMR.

Methanol was used as an internal standard for the chemical shift (3.37 ppm) and signal area. ● concentration of AA in the solution of AA alone with an initial concentration of 110 mM; ◆ concentration of DAA in the mixture of DAA and AA with initial concentrations of 170 mM and 110 mM respectively; ■ concentration of DAA in the solution of DAA with an initial concentration of 170 mM.

by AA, even at a comparatively low concentration (10 mg/100 ml) and at neutral pH (Figs. 3 and 4). This result is interesting because EA is the epimer of AA and is very similar to AA in its physicochemical characteristics. However, EA is a different compound from AA, its oxidation-reduction potential not being exactly equal to that of AA,[15] and it has also been reported that EA is able to prevent AA from being oxidized.[27] Nevertheless, both EA and AA reduced the oxidized form of the other as shown in this study. Furthermore, the interconversion between AA and DAA *in vivo* was strongly implicated from the result of the RLG analysis (Fig. 5) and from the stabilization of DAA by AA (Figs. 7 and 8). These results enable the interconversion mechanism between AA and DAA to be speculated as shown in scheme 1. The chemical interactions between DAA and EA, DEA and AA, and DAA and AA that were observed by NMR were found only to occur at neutral pH (pH 7.4) and not in the acidic range (pH. 3.0–4.0). In other words, it would seem that DAA and DEA could be reduced by the EA anion or AA anion and not by the non-dissociated form of AA and EA, which seems to be reasonable from the point of view of their chemical characteristics and structures. It has been clarified by molecular orbital methods that elements of the molecular structure of the AA anion, such as the inter-atomic distances of carbon atoms in the planar lactone ring, are very similar to those of the monocyclic tri-ketone form of DAA; that is, the structure including the conjugated system from C1 to C3 of the AA anion is very similar to the tri-ketone structure of DAA.[28] It has also been clarified that the π-electron system in the AA anion molecule is highly delocalized, and it can be expected that the AA anion would provide electrons very easily to the acceptors. Therefore, the AA anion is presumed to be able to interact with DAA more easily than AA is able. Furthermore, from the fact that DAA and EA, or DEA and AA can react with each other, it can be expected that the side chains of AA and EA are not very important for this interconversion reaction, and that monocyclic DAA reacts with the AA anion, although the most abundant element in the structure of DAA is the C2-hydrated bicyclic part in water.

There have been several reports suggesting interconversion between AA and DAA based on ESR studies that were focused on the generation of AFR,[13,14] but the notion of whether the interaction between DAA and AA might occur *in vivo* or not has not been completely resolved. Although an *in vivo* study on the interconversion between DAA and AA *in vivo* has not been carried out, the results from this present study seem to positively support this phenomenon *in vivo* (Fig. 5). This agrees with the recent report of Van Duijn *et al.*,[29] but contradicts that of Winkler *et al.*[7] In these reports, the interconversion between AA and DAA was discussed only by confirming the formation of AFR by using ESR, while the oxidation of AA by DAA, or the



Scheme 1. Interconversion Mechanism for AA and DAA *via* AFR.

reduction of DAA by AA was not directly addressed. Radical reactions are generally known to be affected by many factors, and the generation of AFR has actually been confirmed to be strongly affected by heavy metal ions.[29] This might be the reason why conflicting results about the generation of AFR have been reported hitherto, and why the reaction mechanism for the interconversion between AA and DAA seemed to be difficult to understand by only observing the formation of AFR. The reduction of DAA to AA by EA and the reduction of DEA to EA by AA (Figs. 3 and 4), as well as the oxidation of AA to DAA by DAA (Fig. 5) that was confirmed in this study are thought to imply interconversion between AA and DAA. Moreover, these results prompt the assumption that electron transfer from AA to DAA would occur *via* the transition state formed by an AA anion molecule and a DAA molecule of the triketone form as shown in scheme 1, and that the same transition state would also be involved in the disproportionation reaction of two AFR molecules to AA and DAA. A further study to elucidate the detailed reaction mechanism for the interconversion between DAA and EA, DEA and AA, and DAA and AA is underway and will be reported elsewhere.

Acknowledgment

We thank Dr. E. Furuta of the Radioisotope Laboratory in the Faculty of Science at Ochanomizu University for performing the RLG analysis.

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