Preparation and Characterization of a 345 nm Absorbing Reductant Derived from Dehydro-L-Ascorbic Acid

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The addition of sodium hydroxide to an ethanolic dehydro-L-ascorbic acid solution produced a yellowish precipitate which was enriched by a reductant possessing absorption maxima at 345 and 225 nm. This reductant was purified by DEAE-Sepharose column chromatography and Cellulofine-gel chromatography; on the basis of physicochemical analyses it was deduced to be the 2,3-enediol form of 2,3-diketo-L-gulono-δ-lactone (2,3,6-trihydroxy-4-oxo-2-hexen-5-olide). The process, derived from dehydro-L-ascorbic acid, involved a hydrolytic cleavage of a 5-membered γ-lactone ring, conversion to the 2,3-enediol form and rearrangement to 6-membered δ-lactone. The marked bathochromic shift of the absorption maximum of this reductant from that of ascorbic acid (265 nm) could be due to an extended conjugation among the enediol group and two carbonyl groups on both sides.

Dehydro-L-ascorbic acid (DHA), the oxidized form of L-ascorbic acid (AsA), is very unstable in a neutral aqueous solution and is easily delactonized to 2,3-diketo-L-gulonic acid (DKG) and further receives degradation, oxidation, and decarboxylation leading to uncontrolled variables.[1,2]

Polarographic studies of neutral aqueous solutions of DHA[3–5] have revealed complicated oxidation waves, suggesting the occurrence of several reductants which have an enediol group or some other functional groups that are readily oxidizable as an enediol. It seems that the autoxidation of AsA constitutes a coordinated chain of redox reactions in which it is oxidized, and subsequently converts, at least in part, to reducing compounds.

A reductant possessing an absorption maximum at 265 nm, the 3,4-enediol form of 2,3-diketogulono-δ-lactone, was isolated by Otsuka et al.[6,7] from an aqueous solution of DKG potassium salt. This compound was reported to be very unstable and to easily develop an intense brown color, even in the cold; it was therefore proposed to be an important intermediate of the browning reaction of AsA under oxidative conditions.

The present study deals with the preparation and characterization of a reductant possessing an absorption maximum at 345 nm (R-345) derived from ethanolic DHA solution.

Experimental

Preparation of Oxidation Products of AsA. An ethanol solution of DHA was prepared by the method of Doner and Hicks.[8] AsA (10 g) was suspended in 500 ml ethanol containing 12 g of activated charcoal, Norit "SX Plus" (WAKO). Oxygen gas was continuously bubbled through the solution at room temperature for 15—20 hours until the disappearance of the 245 nm absorption peak of AsA and the appearance of the 230 nm peak of DHA. Upon removal of charcoal by filtration, an ice-cold ethanol solution was mixed with 150—180 ml of an ethanolic NaOH (2%) solution until no more precipitation occurred. The precipitate, collected by centrifugation, was washed twice with cold ethanol and dried under vacuum. The yield of a yellowish powder (NaOxA, Na salts of oxidation products of AsA) was about 9 g. NaOxA was highly soluble in distilled water.

For the preparation of a neutral aqueous solution of DHA, the ethanolic DHA solution was evaporated thoroughly under vacuum, and then dissolved in a dilute NaOH solution and adjusted to pH 7.0.

HPLC in the Reversed-Phase, Ion-Pairing Mode. Analyses were performed, according to the method of Finley and Duang,[9] with a JASCO 880-PU liquid chromatograph. Separation was achieved on a Radial-PAK cartridge Resolve C_{18} column (0.8×10 cm), Waters Assoc. The mobile phase was 50 mM potassium phosphate buffer, pH 6.0, containing 1 mM EDTA, 2.5 mM tetrabutylammonium hydrogensulfate and 3% methanol. The eluent was pumped at a flow rate of 1 ml min^{-1}. Three-dimensional (retention time: wavelength: absorbance) chromatographic data were obtained with a JASCO MULTI 330 multi channel detector. The reducing compounds in the eluate were monitored according to a method of Lankelma and Poppe[10] with the aid of electrochemical detector (Model ICA-3060, Toa Electronics LTD) set at 600 mV vs. Ag/AgCl.

Purification of R-345. DEAE-Sepharose Fast Flow (Pharmacia) was first washed with 0.2 M HCl (1 M=1 mol dm^{-3}) and then 0.2 M NaOH and finally thoroughly with distilled water. It was then equilibrated with a 10 mM sodium phosphate buffer, pH 7.0, and packed into a column (2.5×33 cm). NaOxA (0.5 g) in 5 ml phosphate buffer was applied to a column and eluted with phosphate buffer. The eluates were examined for absorbance at 345 and 265 nm. The fraction possessing an absorption maximum at 345 nm was passed through a Cellulofine GCL-25-m column (Seikagaku Kogyo Co) (2.0×33 cm) for desalting as well as for removing impurities.

Physicochemical Measurements. To obtain information regarding the molecular weight, FAB-MS was carried out with a JEOL JMS-AX505W spectrometer. The sample was dissolved in dimethyl sulfoxide-glycerol and then deposited on the tip of a probe. A negative ion $[M]^-$ spectrum was obtained. The Na content was determined with a Nippon Jarrell Ash FLA-100 Flameless Atomizer. Polarographic

analyses were carried out in a 10 mM sodium phosphate buffer, pH 7.0, with a YANACO P-1100 voltammeter (Yanagimoto), using a dropping mercury electrode and SCE as a reference electrode. The half-wave potential, $E_{1/2}$, was obtained as the peak potential on the derivative polarogram. ESR spectra of free radicals were measured with a JEOL JES FE-1X spectrometer at 9.43 GHz with 100 kHz modulation and at 25 mW microwave power in a glass capillary tube at room temperature. The ^{13}C and ^{1}H NMR spectra were taken with a JEOL JNM-GSX400 spectrometer. The IR spectra in a KBr disk were recorded on a Hitachi 345 IR spectrophotometer.

Results and Discussion

Profiles of NaOxA. In a neutral aqueous solution NaOxA possessed high absorption bands at 345 and 225 nm and lower band at 265 nm while DHA had only a low band at 265 nm (Fig. 1). The absorbance of NaOxA was about ten times higher than that of DHA.

Three-dimensional HPLC of NaOxA (Fig. 2a) revealed an abundance of a component possessing absorption maxima at 345 and 225 nm at 8 min retention time and smaller amounts of two 265 nm absorbing components at a retention time between 5.6 and 6.0 min. The ECD response of NaOxA on the ECD-HPLC (Fig. 2b) was the highest at 8 min retention time, corresponding to the 345, 225 nm absorbing component.

Purification of R-345. The elution curve of NaOxA on the DEAE-Sepharose column chromatography (Fig. 3) represents three major fractions: two 265-nm absorbing components in 400—550 ml and 345-nm absorbing component in 550—700 ml. The latter fraction was passed through a Cellulofine column for desalting and for removing 265-nm absorbing impurities. The purity of R-345 was ascertained by means of HPLC and then lyophilized. The yield of a faintly yellowish powder was about 3 g from 10 g of NaOxA. The absorption spectrum of purified R-345 in a neutral aqueous solution (Fig. 4) represented two absorption maxima at 345 and 225 nm, but not around 265 nm. The ε_{max} at 345 nm was ca. 14,000. On

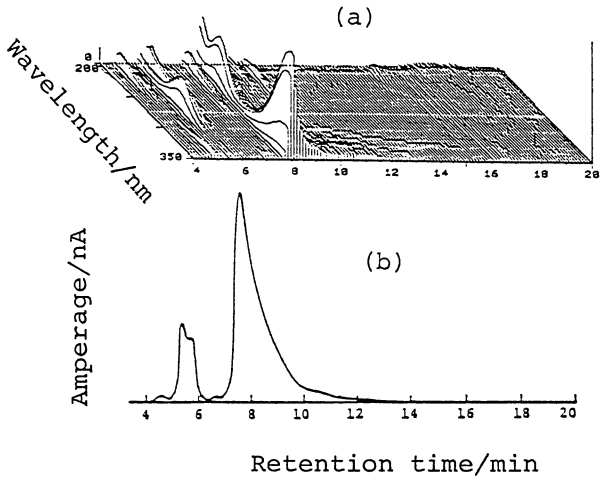

Fig. 2. HPLC profiles of NaOxA. (a) Three dimensional (retention time, wavelength and absorbance) chromatogram. (b) ECD chromatogram of reducing compounds.

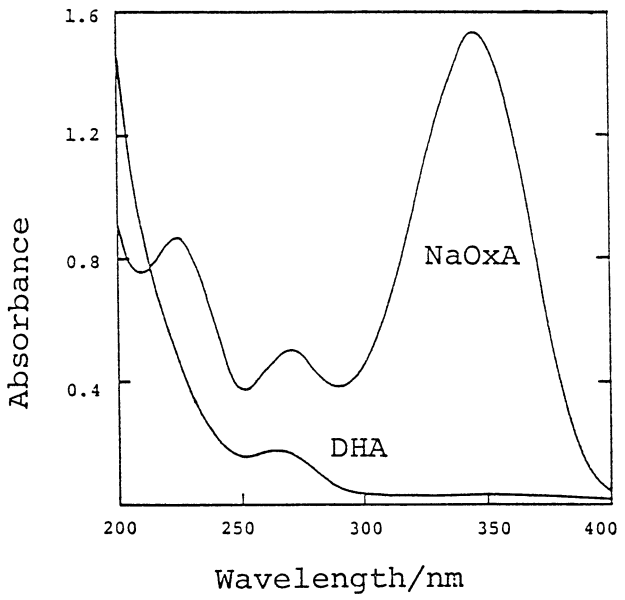

Fig. 1. Absorption spectra of NaOxA and DHA in neutral aqueous solution. From 1 ml each of ethanolic DHA solution (oxidation product of 1 g AsA in 50 ml ethanol), 300 ml NaOxA and 30 ml DHA aqueous solutions of pH 7.0 were prepared and their absorption spectra were measured.

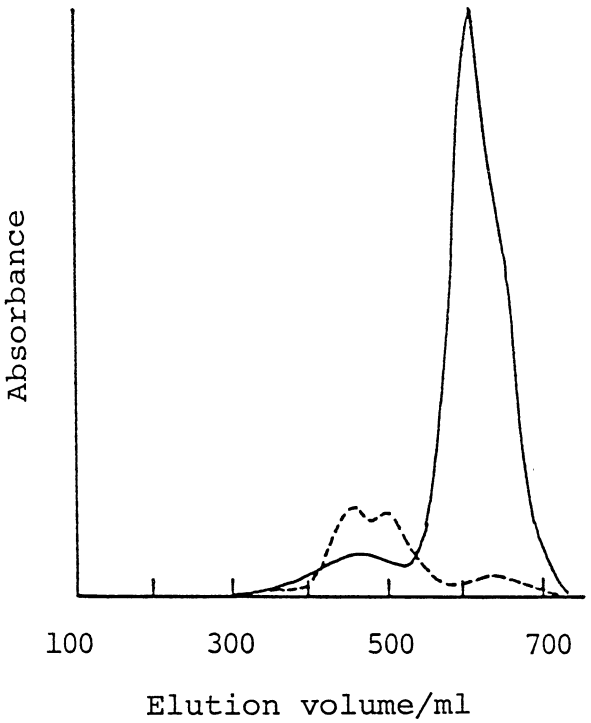

Fig. 3. DEAE-Sepharose column chromatography of NaOxA. (—) 345 nm; (---) 265 nm.

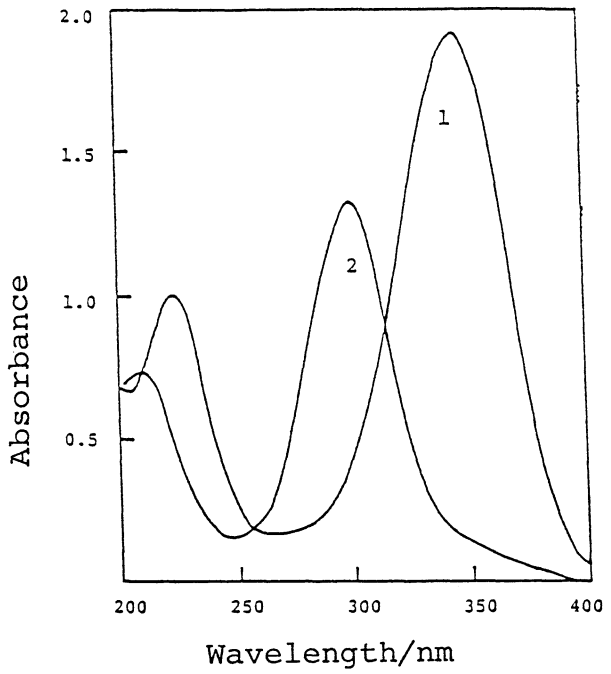

Fig. 4. Absorption spectra of R-345 in aqueous solution at pH 7.0 and 2.0. 1, pH 7.0; 2, pH 2.0.

acidification below pH 3.0, the absorption maximum at 345 nm underwent a marked hypsochromic shift to 300 nm.

Long ago, Herbert et al.[11] reported that a slightly alkaline solution of oxidative product of AsA displayed relatively intense absorption bands at 265 and 340 nm which, on acidification, shifted to 245 and 300 nm, respectively. From our results, these absorption bands could be ascribed to those of a mixture of AsA and R-345.

Physicochemical Characterization. The FAB-MS of the $[R\text{-}345]^-$ ion exhibited an m/z of 173, which was smaller by two hydrogen units than $[AsA]^-$. The Na content of R-345, dried at 105 °C overnight, was 12.0%. Thus, R-345 may exist as a monosodium salt. The half-wave potential, $E_{1/2}$, of R-345 in a phosphate buffer of pH 7.0 was −0.060 V vs. SCE which was a slightly lower negative value than that of AsA (−0.075 V vs. SCE). Though AsA produced a stable ascorbyl free radical, as shown by Lagercrantz,[12] R-345 did not exhibit any ESR signal in the free-radical region.

Six signal of the ^{13}C NMR spectrum of R-345 (Fig. 5) were assigned reference to the data Berger[13] and Kang et al.[2]; one triplet at 63.0 ppm for C-6, one doublet at 82.7 ppm for C-5 and four singlets at 138.7, 155.8, 166.3 and 182.5 ppm for other four carbons. Although both AsA and DKG possessed two doublets for C-4 and C-5, R-345 showed only one doublet for C-5. The ^1H NMR spectrum of R-345 in D_2O (Fig. 6) represented two signals: one signal of the characteristic ABX type splitting pattern around 4.040 ppm for H_2-6 and one triplet at 5.004 ppm for H-5, though AsA had three

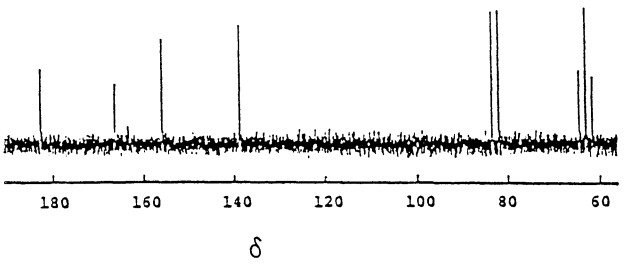

Fig. 5. Off-resonance spin-decoupled ^{13}C NMR spectrum of R-345.

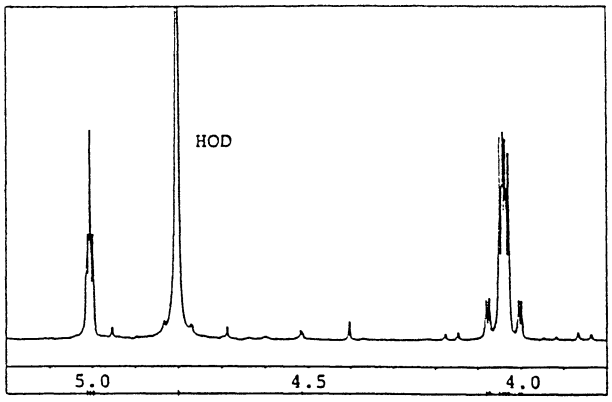

Fig. 6. ^1H NMR spectrum of R-345 in D_2O.

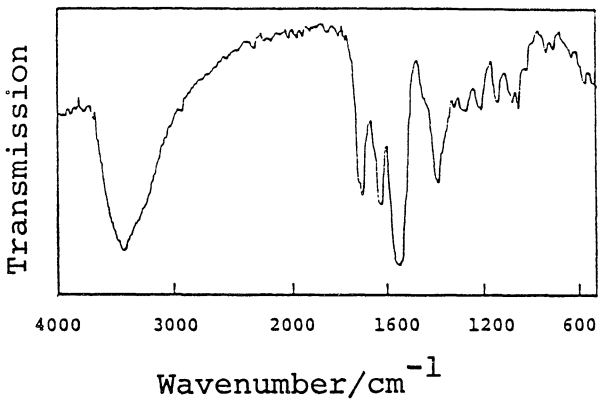

Fig. 7. IR spectrum of R-345 in KBr disk.

signals for H_2-6, H-5 and H-4, as reported by Sapper et al.[14] and Lohmann et al.[15] These NMR spectra indicated that no hydrogen atom was bound directly to the C-4 carbon atom of R-345. Both the C-5 and H-5 signals of R-345 were located downfield as low as those of C-4 and H-4 of 5-membered (γ-lactone) AsA. These NMR data as well as molecular weight information suggest that R-345 has a 6-membered (δ-lactone) ring in which C-1 and C-5 are linked through the oxygen atom, and that C-2 and C-3 (138.7, 155.8 ppm) constitute the enediol group and C-1 and C-4 (182.5, 166.3 ppm) are carbonyl carbon. Upon acidification, a singlet signal at 155.8 ppm received a pronounced upfield shift to 140.0 ppm, as did the C-3

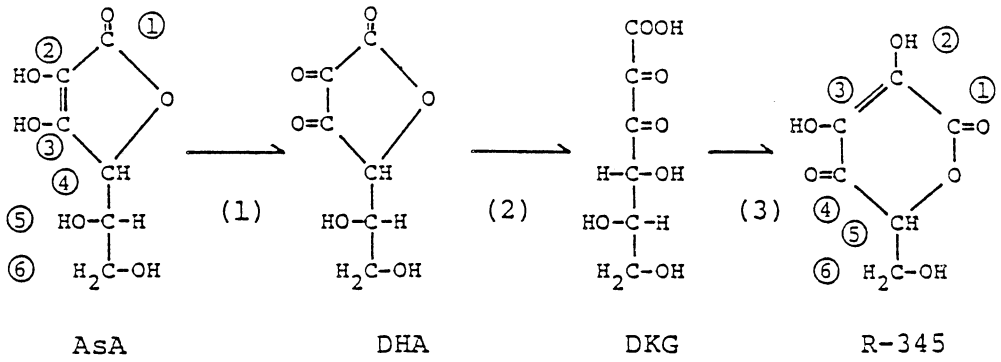

Fig. 8. Proposed structure of R-345 and reaction pathway from AsA to R-345. (1) removal of two hydrogen atoms by oxidation, (2) hydrolytic cleavage of 5-membered γ-lactone, (3) conversion to the enediol form and rearrangement to 6-membered δ-lactone. Encircled Arabic numerals indicate the carbon position in the molecule.

of AsA. The C-3 carbon atom of R-345 may receive the first deprotonation in a neutral aqueous solution.

The absorption maximum at 265 nm and ε_{max}= 14,700 of ionized AsA was assigned, by Ogata and Kosugi,[16] to be the π–π^* excitation of an enediol C=C bond conjugated with lactone carbonyl C=O. The marked bathochromic shift of the absorption maximum of R-345 from that of AsA may be due to an extended conjugation among the enediol group and two C=O groups on both sides. IR spectrum of R-345 (Fig. 7) represented the presence of OH (3400 cm^{-1}), C=O (1720 cm^{-1}, 1630 cm^{-1}) and C=C (1560 cm^{-1}) bands, similarly to those of AsA.[15] The band at 1720 cm^{-1} was identical with that (1721 cm^{-1}) of the lactone carbonyl of dehydroacetic acid.[17] The band at 1630 cm^{-1}, which was not demonstrable in the Na salt of AsA, could be assigned to the C-4 carbonyl group. The absorption frequency of the C=C band was lowered by 40 cm^{-1} due to an extended conjugation with two adjacent carbonyl groups than that (1600 cm^{-1}) of the Na salt of AsA. The band at 1390 cm^{-1} could be assigned to the ring vibration of δ-lactone.

From the above-described analyses, R-345 could be deduced to be the 2,3-enediol form of DKG (2,3,6-trihydroxy-4-oxo-2-hexen-5-olide); the possible reaction pathway from AsA to R-345 is shown in Fig. 8.

An addition of NaOH to an ethanolic DHA solution facilitated the cleavage of a 5-membered γ-lactone ring of DHA and the high rate of enolic isomerization and rearrangement to a 6-membered δ-lactone, thus producing a large amount of R-345. On the other hand, when DHA was dissolved in a neutral aqueous solution, it predominantly underwent hydrolytic degradation, as well as further oxidation and decarboxylation, reverting only inconsiderably to the lactonized reductants of the enediol type.

During 1 hour incubation at 37 °C, the loss of 345 nm absorbance of R-345 (0.1 mM) and 265 nm absorbance of AsA (0.1 mM) was about 20 and 30 per cent, respectively. The stabilities of both reductants were nearly equal.

The neutral aqueous solution of R-345 did not develop a brown color on standing for several days at room temperature, differently from the 265 nm absorbing reductant isolated by Otsuka *et al.*[6,7] from an aqueous DKG solution. R-345 may be stabilized in favor of extended conjugation among the enediol group and two adjacent carbonyl groups.

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References

1) W. H. Kalus, W. G. Filby, and R. Munzner, *Z. Naturforsch.*, **37c**, 40 (1982).
2) S-O. Kang, H. Sapper, and W. Lohmann, *Z. Naturforsch.*, **37c**, 1064 (1982).
3) F. Petuely and U. Kunssberg, *Monatsch*, **83**, 80 (1952).
4) S. Ono, M. Takagi, and T. Wasa, *Bull. Chem. Soc. Jpn.*, **31**, 356 (1958).
5) S. Ono, M. Takagi, and T. Wasa, *J. Am. Chem. Soc.*, **75**, 4369 (1958).
6) M. Otsuka, T. Kurata, and N. Arakawa, *Agric. Biol. Chem.*, **50**, 531 (1986).
7) M. Otsuka, T. Kurata, and N. Arakawa, *Dev. Food Sci.*, **13**, 77 (1986).
8) L. W. Doner and K. B. Hicks, *Anal. Biochem.*, **115**, 225 (1981).
9) J. W. Finley and E. Duang, *J. Chromatogr.*, **207**, 449 (1981).
10) J. Lankelma and H. Poppe, *J. Chromatogr.*, **125**, 375 (1976).
11) R. W. Herbert, E. L. Hirst, E. G. V. Percival,R. J. W. Reynolds, and F. Smith, *J. Chem. Soc.*, **1933**, 1270.
12) C. Lagercrantz, *Acta Chem. Scand.*, **18**, 562 (1964).
13) S. Berger, *Tetrahedron*, **33**, 1587 (1977).
14) H. Sapper, S-O. Kang, H-H. Paul, and W. Lohmann, *Z. Naturforsch.*, **37c**, 942 (1982).
15) W. Lohmann, E. Pagel, and V. Penka, *Eur. J. Biochem.*, **138**, 479 (1984).
16) Y. Ogata and Y. Kosugi, *Tetrahedron*, **26**, 4711 (1970).
17) L. J. Bellamy, "The Infra-Red Spectra of Complex Molecules," Methuen & CO., Ltd., London (1958), pp. 185—186.