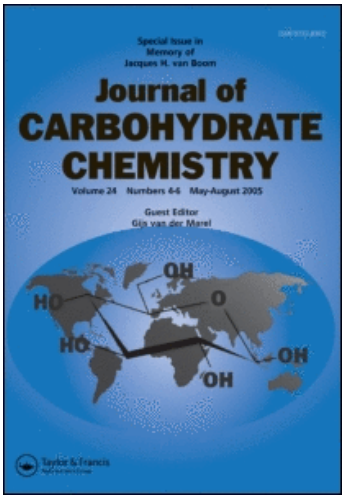

Journal of Carbohydrate Chemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713617200

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To cite this Article Shin, Dong Bum and Feather, Milton S.(1990) 'The Degradation of L-Ascorbic Acid in Neutral Solutions Containing Oxygen', Journal of Carbohydrate Chemistry, 9: 4, 461 — 469
To link to this Article: DOI: 10.1080/07328309008543846
URL: http://dx.doi.org/10.1080/07328309008543846

J. CARBOHYDRATE CHEMISTRY, 9(4), 461-469 (1990)

THE DEGRADATION OF L-ASCORBIC ACID IN NEUTRAL SOLUTIONS CONTAINING OXYGEN[1]

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Received October 12, 1989 - Final Form March 2, 1990

ABSTRACT

The stability of L-ascorbic acid was examined by incubating it alone and in the presence of α-*N*-formyl-L-Lysine (NFL) at pH 7.0 (0.1 M phosphate buffer) in the presence of oxygen, a metal ion chelator, and in both the presence and absence of sodium cyanoborohydride. Among the large number of degradation products produced, threonic, oxalic, glyceric and glyoxylic acids, as well as threose were all identified by GLC-MS. Decarboxylation is also a feature of the degradation, with approximately 30% of the ascorbic acid undergoing degradation via this pathway. In the presence of cyanoborohydride and NFL, ε-*N*-carboxymethyl-L-lysine (CML) was detected as a reaction product as well. Using GLC-MS as the detection method and ascorbic acid-1-^{13}C as the reactant, only glyoxylic acid and CML were found to contain a ^{13}C label, indicating that, of the compounds identified above, only these latter two contain C-1 of ascorbic acid.

INTRODUCTION

Ascorbic acid is known to be unstable in solution, and numerous studies have been reported on the products of the degradation reaction under a variety of reaction conditions.[2-4] We have recently reported that ascorbic acid, in the presence of oxygen and at pH 7.0, causes the formation of cross links in lens protein, as evidenced by PAGE SDS gel electrophoresis.[5] In the presence of oxygen, ascorbic acid undergoes a

free radical-mediated oxidation to dehydro-L-ascorbic acid,[6] and it has been shown that the latter compound undergoes hydrolysis to 2,3-diketo-L-gulonate as well.[7] Both of these compounds are unstable in solution and undergo a variety of further degradation reactions involving fragmentation to lower molecular weight compounds and further dehydration to other reactive intermediates. Decarboxylation is also a feature of the degradation reaction. In acidic media L-ascorbic acid dehydrates to give 2-furaldehyde and carbon dioxide, the latter being formed in nearly quantitative yields.[8] In an earlier report[5] we discussed the cross-linking reaction of lens protein in terms of a possible Maillard reaction, since, by visual observation, the reaction resembled a Maillard reaction involving a reducing sugar and an amino group. The solutions were brown in color, and carbonyl-containing fragmentation products were observed, as evidenced by thin layer chromatograms. In this paper we wish to report some studies aimed at identifying some of the reaction products that are produced when L-ascorbic acid undergoes degradation at the conditions where protein cross linking is observed, i.e., at pH 7.0 and 37 °C.

RESULTS AND DISCUSSION

The solution stability of L-ascorbate was studied by measurement of the loss of absorbance at 265 NM. L-ascorbate slowly disappeared from solution over a period of 300 hours, at which time the absorbance decreased by about 80% in intensity. The presence of NFL (at equimolar concentration) and protein (lens protein and lysozyme) had little effect on the rate of the disappearance. During the early stages of the reaction (the first 48 hours), dehydro-L-ascorbate, the product of direct oxidation of L-ascorbate, as well as 2,3-diketo-L-gulonate, the hydrolysis product derived from the latter, appear, as evidenced by TLC (irrigant A). The following Rf values were observed for these compounds: L-ascorbic acid, 0.45; dehydro-L-ascorbic acid, 0.70; 2,3 diketo-L-gulonic acid, 0.15. None of the latter show any absorption at 265 nm. At later stages, a number of carbonyl-containing compounds were visible as well, one of which co-chromatographed with L-threose.

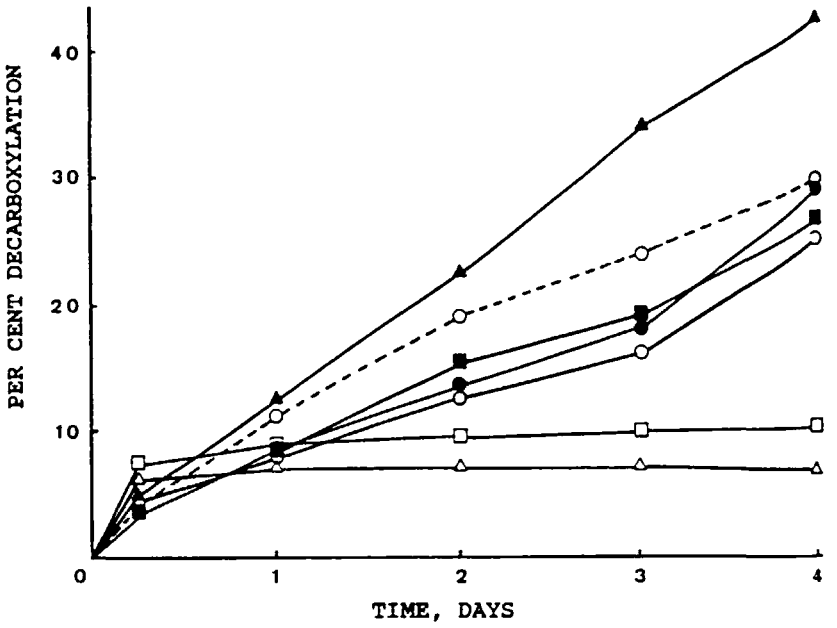


Fig. 1. Rates of decarboxylation of L-ascorbic acid in the presence of possible reactants. Measurement involved the collection of $^{14}CO_2$ from L-ascorbic acid-1-^{14}C. Each data point used a solution that was 20 mM in L-ascorbic acid (1.0 Ci) in 1.0 ml. of phosphate buffer (pH=7.0). (▲—▲), + α-*N*-acetyl lysine (20 mM); (○—○), control (no reactants added); (●—●), + lens protein (25 mg.); (■—■), +lens protein (50 mg.); (○—○), + cyanoborohydride (40 mM); (□—□), + glutathione (40 mM); (△—△), + dithiothreitol (40 mM).

Decarboxylation is known to be a feature of the degradation of L-ascorbic acid in strongly acidic solution.[15,16] Under these conditions, this represents a major degradation reaction, with carbon dioxide and 2-furaldehyde (the major decarboxylation product) being produced in near quantitative yields. In order to examine the extent of decarboxylation under physiological conditions, a sample of L-ascorbic acid-1-^{14}C was incubated and the extent of decarboxylation estimated by determining the total amount of $^{14}CO_2$ produced in the reaction (Figure 1).

The results suggest that approximately 30% of total carboxyl carbon can be accounted for as CO_2 during the first 48 hours of the

degradation reaction, and, as with the rate of disappearance, the presesence of NFL and protein appear to have little effect on the overall rate of the reaction. It is noteworthy that von Abt and co-workers[17] reported that when animals (including human subjects) are fed L-ascorbate-1-^{14}C, about 25 % of the isotope is expired as $^{14}CO_2$.

The fact that a number of other compounds including L-threose are produced in the reaction suggests that, under these conditions, chain fragmentation of the 6 carbon compounds is a feature of the degradation reaction as well. Aliquots of incubation solutions were concentrated to dryness and converted to the trimethylsilyl ether derivatives. GLC indicated a complex mixture of components. In order to examine the nature of these fragmenation products further, incubation mixtures were fractionated into neutral and acidic fractions by ion exchange chromatography, converted to appropriate derivatives and further examined by GLC-MS. Although not all components were identified, a number of major products could be positively identified and are listed in Table 1, along with relevant GLC and MS data.

Incubations performed with all reactants (L-ascorbic, dehydro-L-ascorbic or 2,3 diketo-L-gulonic acids as the starting materials) gave rise to similar mixtures of compounds, as evidenced by TLC and GLC. The only exception was CML, which was produced in major amounts only in the presence of cyanoborohydride.

Fragmentation reactions in conjunction with the degradation of ascorbic acid are well known, especially the formation of oxalate, which is generally assumed to originate from carbons 1 and 2. The finding of more than one type of two carbon acid prompted us to examine the origin of these more carefully, as well as the other compounds identified. Incubation reactions were repeated using L-ascorbic acid-1-^{13}C as the reactant. That the ascorbic acid was labeled at C-1 was clearly evident from ^{13}C NMR spectra collected for the compound. Spectra were run at a concentration of 10 mg per mL of deuterium oxide and showed only one signal for the enriched carbon at 178 PPM. Two experiments were performed, one in the presence of NFL and cyanoborohydride, the other in the absence of cyanoborohydride. The reaction mixtures were then processed and examined by GLC and GLC-MS. For all the acidic compounds

TABLE 1. GLC[1] and MS Parameters of some fragmentation products produced by the degradation of L-ascorbic acid at pH 7.0 and 37 °C after 48 hours of incubation.

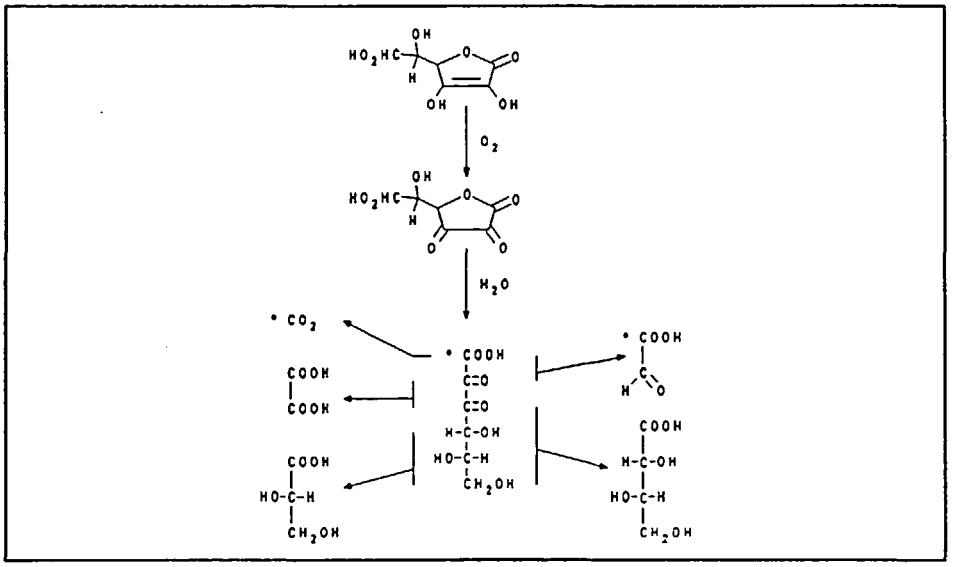
COMPOUND	GLC RETENTION TIME	MAJOR MASS SPECTRAL PEAKS
L-ascorbate[2]	39.8	575
Oxalate[2]	9.6	261
Glyoxalate[2]	6.1	261
Threonate[2]	29.8	535
Glycerate[2]	17.4	391, 433
Threose[3]	11.5	320, 408, 423 (M^+)
CML[4]	15.5	317, 284, 273, 257

1. See **EXPERIMENTAL** for GLC conditions and parameters. 2. As the *tert*-butyldimethylsilyl (TBDMS) ether derivative. 3. Determined as the oxime-TMS derivative. 4. ϵ-*N*-carboxymethyl-L-lysine.

listed in Table 1, a strong mass spectral peak corresponding to M-57 (representing the parent ion minus the tertiary butyl group) was observed, as has been reported by Mawhinney et al.[9] for a number of carboxylic acids. With the exception of CML and glyoxylic acid derived from L-ascorbic acid-1-^{13}C, all compounds gave mass spectra superimposable with the original spectra collected on the unlabeled compounds and listed in Table 1. For the case of CML, peaks were observed at M/Z=318, 285, and 274, indicating that the compound contained the original C-1 of ascorbic acid. For the case of glyoxylic acid, peaks were observed at 220, and 233, also one mass unit higher than is observed for the ordinary compound.

Baynes and his group have detected CML in human lens protein hydrolyzates and in model systems composed of NFL and D-glucose. It is also found in model systems composed of ascorbate and protein models, but at very low concentrations (Baynes, et. al, personal communication). In view of the relatively large amounts found herein, coupled with the fact that it is produced only in the presence of cyanoborohydride, it seems likely that the CML is produced by reaction of NFL with glyoxylate in the presence of cyanoborohydride, and may not neccessarily represent a direct reaction product between ascorbate and NFL.

It is interesting to note that, while the glyoxylate contains C-1 of ascorbate, the oxalate is unlabeled. Based on the large number of compounds produced, the degradation reaction probably involves a number of different pathways with carbon chain fragmentation a prominent feature of the degradation. The reaction scheme shown below is consistent with the known facts about the degradation with respect to the carbon pathway. The fact that all three of the 6 carbon compounds give the same pattern of degradation products suggests that 2,3-diketo-L-gulonate, the final oxidation product, serves as the starting material for the degradation. The reaction scheme shown below is consistent with the findings.



The combination of CO_2 (which arises from C-1), oxalate and glycerate are consistent with fragmentations between C-1 and C-2, as well as between C-3 and C-4 of DKG, while labeled glyoxylate and unlabeled threonate suggests cleavage between C-2 and C-3 of DKG. Clearly, the reaction is more complex than shown, since threose is also produced in the reaction, as well as a number of unidentified minor components. Decarboxylation may account for up to 30% of the reaction pathway, with carbon chain fragmentation, possibly by hydrolytic cleavage reactions or reverse aldol reactions accounting for at least some of the remainder of the products.

EXPERIMENTAL

Materials and Methods. TLC was performed on silica gel plates using the following irrigants and sprays: acetonitrile:water:acetone: acetic acid (80:15:5:1, v/v), irrigant A, and butanol:acetic acid:water (4:1:1, v/v), irrigant B. For the detection of carbonyl-containing compounds, the chromatograms were sprayed with 0.5 % dinitrophenyl-hydrazine in 2 N hydrochloric acid, and for amino-containing compounds, with 0.5% ninhydrin in ethanol. Mass spectra were collected using a double focussing Kratos MS-25 instrument equipped with a DS-55 data system, interfaced with a Hewlett-Packard GC equipped with bonded SE-30 capillary column. For routine detection of compounds a Shimadzu model number GC-9A GLC was equipped with an FID detector, a 30 meter SPB-1 capillary column having a film thickness of 0.25 μm (Supelco) and helium as the carrier gas. Parameters for GLC separations were as follows: initial oven temperature, 60 °C for 4 min and then an increase of 10 °C per min to 270 °C. Reactions involving α-*N*-formyl lysine (NFL) were hydrolyzed in 2 N HCl for 30 min at 65 °C prior to derivatization. For derivatization for GLC and MS studies, acidic compounds were converted to the *tert*-butyldimethylsilyl ether derivative by reaction with *N*-(*tert*-butyldimethylsilyl)-*N*-methyltrifluoroacetamide (MTBSTFA, Aldrich Chemical) and *tert*-butyldimethylsilyl chloride (Aldrich Chemical) in *N,N*-dimethyl formamide (DMF), as described by Mawhinney et al.[9] Oxime derivatives were prepared as described by Mawhinney et al.[10], and these were converted to the trimethylsilyl derivatives by reaction with *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA, Pierce Chemical) containing 1% trimethylchlorosilane (TMCS, Pierce Chemical).

Preparation of Reactants. Dehydro-L-ascorbic acid (DHA) was prepared by oxidation of L-ascorbic acid (ASA) with oxygen in the presence of charcoal as described by Ohmori, et al.[11] The product was obtained as a chromatographically pure syrup and could be stored at -10 °C for several months in that state of purity. Sodium 2,3-diketo-L-gulonate (DKG) was prepared by hydrolysis of DHA as described by Otsuka, et al.[12] The compound was obtained as a chromatographically pure solid and was stored under nitrogen at -10 °C until used. ϵ-*N*-Carboxymethyl-L-lysine

(CML) was prepared from iodoacetic acid and NFL, as described by Ahmed et al.[13] This material, when converted to the acetylated methyl ester gave a single peak by GLC, and a mass spectrum identical to that reported by Baynes et al.[14]

Incubation Conditions. A typical incubation experiment involved sterile filtering 2.0 mL of a solution that was 0.1 M in either ASA, DHA or DKG (in 0.2 M phosphate buffer at pH 7.0) into a screw capped vial, which was placed in a 37 °C incubator. Vials were opened at approximately 24 h intervals, examined by TLC, and/or processed for conversion to the appropriate derivative for GLC or GLC-MS.

Measurement of the Rates of Decarboxylation of ASA. A solution of ASA-1-^{14}C (3.96 mg, containing 1.0 μCi) in 0.2 M phosphate buffer (pH 7.0) was placed in the bottom of a septum stoppered 1 X 12 cm test tube that was equipped with a hanging center well which contained a 2.5 cm square of Whatman 1 mm filter paper saturated with 5 M ethanolamine, to trap the evolved CO_2. A series of tubes so prepared were placed in a 37 °C incubator. For sampling, 0.5 mL of 4 M HCl was injected through the septum into the incubation solution, and, after 30 min, the paper in the center well was removed, placed in a scintillation vial and counted useing conventional methodology.

Fractionation of the Degradation Products. Aliquots of the incubation reactions were desalted by passage through a column of Dowex 50 (hydrogen form) ion exchange resin and the effluent passed through a column of Dowex 1 (acetate form). The effluent and washings (which contained the neutral components) were concentrated to dryness at 30 °C, derivatized and examined by GLC. The acidic components were eluted from the column with 2 void volumes of 2 N trifluoroacetic acid, the effluent evaporated to dryness as described above, derivatized and examined by GLC. For examination of the solutions for CML, an aliquot was processed using ion exchange techniques and then converted to the acetylated methyl ester as described by Baynes.[14]

ACKNOWLEDGEMENT

This work was supported in part by NIH grant EY 07070 and in part by Research to Prevent Blindness, Inc.

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