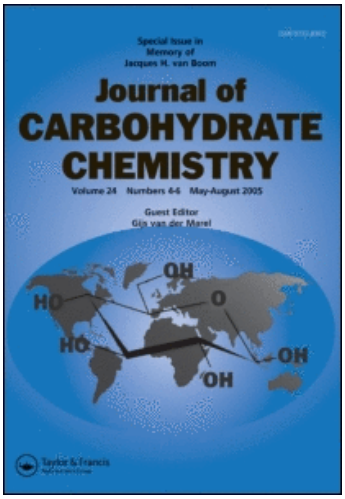

Journal of Carbohydrate Chemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713617200

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To cite this Article López, Mercedes G. and Feather, Milton S.(1992) 'The Production of Threose as a Degradation Product from L-Ascorbic Acid', Journal of Carbohydrate Chemistry, 11: 6, 799 — 806
To link to this Article: DOI: 10.1080/07328309208020093
URL: http://dx.doi.org/10.1080/07328309208020093

J. CARBOHYDRATE CHEMISTRY, 11(6), 799-806 (1992)

THE PRODUCTION OF THREOSE AS A DEGRADATION PRODUCT FROM L-ASCORBIC ACID[1]

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Received November 18, 1991 - Final form May 20, 1992

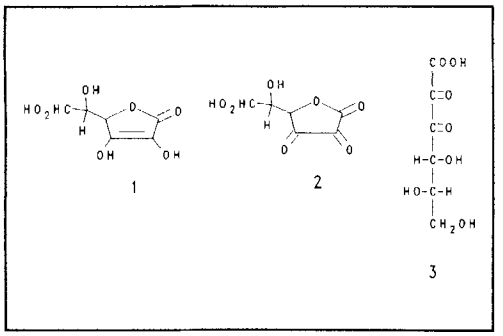
ABSTRACT

In order to examine the nature of *in vivo* Maillard reactions that involve L-ascorbate and proteins, rates of formation of threose from L-ascorbic acid (**1**), dehydro-L-ascorbic acid (**2**), and 2,3-diketo-L-gulonic acid (**3**) were measured in the presence and absence of oxygen at pH 7.0 (phosphate buffer) and at 37 °C. Threose is produced in measurable quantities from L-ascorbate only in the presence of oxygen. Compounds **2** and **3** both give rise to threose in both the presence and absence of oxygen. Compound **3** gives threose at a faster rate and in higher amounts than **1** or **2**, suggesting that it is the primary source of threose in this reaction. Incubations of dehydro-L-ascorbate with N^{α}-acetyl-L-lysine in the presence of cyanoborohydride gave N^{α}-acetyl-N^{ϵ}-(1-deoxy-L-threitol-1-yl)-L-lysine which was chemically synthesized (from threose and N^{α}-acetyl-L-lysine) and unequivocally characterized. The data suggest that the Maillard reaction observed when L-ascorbic acid is incubated with protein may well arise as a result of interactions of L-threose with amino groups, and that the function of oxygen in the reaction is to convert **1** into the more reactive **2**, and **3**. Oxygen does not appear to be necessary for the further degradation of **2** or **3**.

INTRODUCTION

The existence of the Maillard reaction as an *in vivo*, nonenzymatic process is now a well accepted fact.[2,3] The reaction, which is degradative in nature, involves the initial interaction of a reducing sugar with a protein to give 1-amino-1-deoxy-2-ketose derivatives (Amadori compounds), followed by their further degradation. During this process, proteins become cross-linked, flourescent compounds are formed, and other protein modifications take place. Ascorbic acid, or a degradation product derived from it is known to be a participant in this type of reaction in the sense that protein modification is observed when proteins and L-ascorbic acid are incubated together.[4] One of the major degradation products produced when ascorbic acid is incubated at pH 7.0 and 37 °C is the reducing sugar threose, which represents a candidate for the Maillard reaction. Threose has been identified as a degradation product derived from ascorbic acid in solution in the presence of oxygen,[5] and Baynes and his collaborators[6] have identified N^{ϵ}-(carboxymethyl)lysine (CML) in an incubation reaction that contained N^{α}-formyl-L-lysine and threose. They also identified 1-deoxy-1-N^{ϵ}-(N^{α}-acetyl-L-lysino)-L-threulose, the Amadori product derived from threose and the amino acid. They hypothesized that CML is formed by a sequence of reactions involving the production of threose from ascorbic acid, its reaction with the ϵ-lysyl amino group to form an Amadori compound and the subsquent oxidative cleavage of this compound between C-2 and C-3 of the sugar residue to give CML.

At pH 7.0 and 37 °C, ascorbic acid (**1**) is relatively unstable and gives rise to dehydroascorbic acid (**2**), a free radical-mediated oxidation product, which then undergoes hydrolysis to 2,3-diketo-L-gulonic acid (**3**), the open chain form of dehydroascorbate (structures, next page). These compounds all appear to undergo complex degradation reactions as well.[5,7-9] The purpose of the present study was to examine this system in an attempt to determine the species in solution responsible for the generation of threose and to examine the nature



of the interactions of threose and dehydroascorbic acid with protein amino groups. The latter was accomplished by incubating threose with N^{α}-acetyl-L-lysine in the presence of cyanoborohydride, a reagent that is reported to reduce Schiff bases, which, presumably, represent the first formed reaction product of a carbonyl group with an amino group.

RESULTS AND DISCUSSION

Figure 1 provides information on the stability of threose at the conditions studied.

As expected, threose is significantly less stable in solution than aldopentoses and -hexoses, with a half life of approximately 3.5 days (84 h) at conditions of this study.

Since **1** shows a strong absorption band at 265 nm (A=16,500), its level in incubation solutions was monitored by spectral measurement as well as by TLC. In the absence of oxygen, after 96 hours of reaction time, 80% of **1** remained (UV measurement), and traces of **2** (Rf=0.70) and **3** (Rf=0.27) were visible (by TLC). In the presence of oxygen at the same conditions, most of **1** had disappeared. These observations and the data on the rate of formation of threose (Fig. 2) suggest that **1** is not a major source of threose in this reaction.

Oxygen has a negligible effect on the rate of threose production from both **2** and **3**. Solutions of **2** and **3** are relatively unstable, and threose can be observed by TLC after 12 h of incubation. Clearly, **1** (and probably **2** and **3**) undergo degradation by a multiplicity of pathways, given the large number of compounds that have been identified as degradation products. The data are, however, consistent with **3** serving as the source of threose in these reactions. It is noteworthy that relatively high yields (maximum yield = 19.7%) of threose are obtained from **3** early in the reaction.

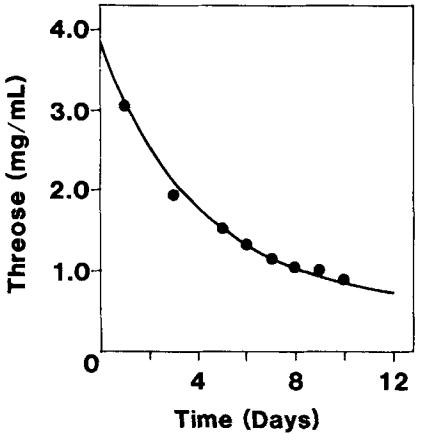


Fig. 1. Rate of disappearance of threose from solution at pH 7.0, 37 °C in the presence of oxygen.

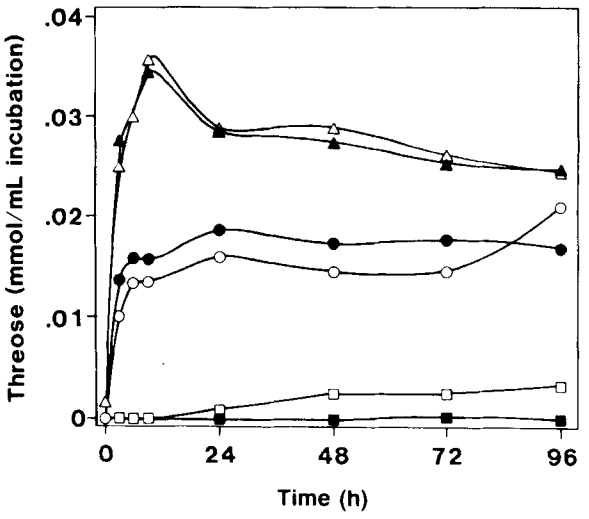


Fig 2. Rates of appearance of threose from reactants at pH 7.0 and 37 °C in the presence (open symbols) and absence of oxygen (closed symbols). L-Ascorbic acid (□,■), Dehydro-L-ascorbic acid (○,●) and 2,3-diketo-L-gulonate (△,▲).

With respect to incubation of **1** with N^{ϵ}-acetyl-L-lysine in the presence of cyanoborohydride; an examination by TLC (irrigant B, spray A) of an aliquot of the incubation solution after 96 h time showed only unreacted N^{α}-acetyl-L-lysine and CML to be present. This was confirmed by GLC. For the case of **2**, TLC (irrigant B, spray A) showed the presence of only unreacted N^{α}-acetyl-L-lysine. GLC, however, showed the presence of N^{ϵ}-(1-deoxy-L-threitol-1-yl)-L-lysine, which was identified as the methyl ester-trifluoroacetate derivative. This material had an MS identical to the authentic compound described below.

EXPERIMENTAL

General Procedures. TLC was performed using silica gel plates using the following irrigants: Pyridine-water-acetic acid (90:10:2, v/v, irrigant A) and acetonitrile-water-acetone-acetic acid (80:15:5:6, v/v, irrigant B). Plates were visualized using ultraviolet light (for UV absorbing compounds) and sprayed with the following sprays: ninhydrin (spray A), permanganate (spray B) or 2,6-diclorophenol in 95% ethanol (spray C). GLC was performed using a Varian 3400 instrument in the split mode. Separations were done using a 25 meter Quadrex 001-OV-17 capillary column having I. D. 0.025 mm. For sugar derivatives, the parameters were as follows: 150 - 250 °C at 10 °C per min with a hold time of 2 min. NMR spectra were collected using a Bruker AMX-500 instrument. GLC/MS measurements were carried out using a double focusing Kratos MS-25 instrument equipped with a DS-55 data system and interfaced with a Hewlett-Packard GLC instrument equipped with a column similar to the one used in this study.

Preparation of Starting Compounds. Dehydro-L-ascorbate (**2**) was prepared from L-ascorbate using the slightly modified procedure that was originally described by Tolbert and Ward[10] using ethanol as the solvent and activated charcoal (Mallinckrodt) and bubbling air through the solution for 16 h rather than the recommended 1 h. The product was obtained as a clear syrup which was chromatographically pure (Rf = 0.70, irrigant B, spray C). The ^{13}C NMR spectrum showed signals at δ 173.1 (C-1), 90.9 (C-2), 105.2 (C-3),

87.2 (C-4), 72.5 (C-5) and 76.7 p.p.m. (C-6), consistent with previous reports.[10] 2,3-Diketo-L-gulonic acid (**3**) was prepared as described by Otsuka and co-workers[11] and was obtained (after overnight desiccation *in vacuo*) as a dry solid. This material had Rf=0.27 (irrigant B, spray C). ^{13}C NMR showed signals at δ 174.2 (C-1), 94.9 (C-2), 93.3 (C-3), 74.1 (C-4), 68.2 (C-5), and 62.1 (C-6), consistent with literature reports.[11] L-Threose was prepared by periodate oxidation of 1,3-*O*-benzylidene-L-arabinitol, followed by hydrolysis in acetic acid as described by Perlin.[12] This preparation was also chromatographically homogeneous and the ^{13}C NMR spectrum was identical to that reported in the literature.[13] N^{ϵ}-(1-Deoxy-L-threitol-1-yl)-L-lysine was prepared by reacting L-threose (0.86 g) with N^{α}-acetyl-L-lysine (0.43 g) in the presence of cyanoborohydride (0.43 g) in 50 mL of buffer (pH=7.0) at room temperature. The reaction was stopped after three weeks, concentrated to 2.0 mL and N^{α}-acetyl-N^{ϵ}-(1-deoxy-L-threitol-1-yl)-L-lysine (Rf=0.51, irrigant A, spray B) isolated in pure form by preparative TLC using irrigant A. ^{13}C NMR showed the following signals: δ 169.8 (amino acid carboxyl), 166.8 (acetyl carbonyl), 74.6 (C-2, threitol), 69.2 (C-3, threitol), 64.4 (C-4, threitol), 56.0 (C-2, amino acid), 52.3 (C-1, threitol), 49.5 (C-6, amino acid), 33.2 (C-3, amino acid), 27.3 (C-5, amino acid), 24.6 (acetyl methyl carbon), and 24.2 (C-4, amino acid). This compound was further characterized by hydrolysis in 3N trifluoroacetic acid at 100 °C for 12 h. The ^{13}C NMR spectrum of this material showed signals at δ 166.7 (amino acid carboxyl) 74.3 (C-2, threitol), 69.1 (C-3, threitol), 64.7 (C-4, threitol), 53.8 (C-2, amino acid), 52.1 (C-1, threitol), 49.7 (C-6, amino acid), 32.5 (C-3, amino acid), 27.2 (C-5, amino acid) and 24.1 (C-4, amino acid). Conversion of the compound to the methyl ester-trifluoroacetate derivative, followed by GLC/MS gave the following peaks: 712 (M - CH_3OH), 685 (M - $COOCH_3$), 571 (M - CF_3COOCH_3 - H), 458 (571 - $NHCOCF_3$), 180 (305 - CH_2NCOCF_3) and 69 (180 - $NCOCF_3$).

Measurement of threose formation. Threose was measured by GLC as the alditol acetate. All incubation solutions were 0.12 M in mannitol (internal standard) and 1.0 mM in diethylenetriaminepentaacetic acid (chelator). In a typical experiment, a 0.12 M solution of **2** in the above solution

was incubated in a water bath at 37 °C. At intervals, 0.5 mL aliquots were removed, treated with sodium borohydride for 0.5 h, evaporated to dryness, and acetylated by treatment with acetic anhydride (1.0 mL) and pyridine (1.0 mL) at 75 °C for 1 h. This solution was concentrated to dryness under a stream of air and the residue dissolved in 0.5 mL of methylene chloride. One microliter used for a GLC run. For studies of the rate of disappearance of L-threose from solution, a solution of L-threose (50 mg) and mannitol (25 mg, internal standard) in 12 mL of buffer, was incubated at 37 °C in the presence of oxygen. An aliquot was immediately withdrawn to determine the threose concentration at 0 time, and at timed intervals, 0.5 mL aliquots were withdrawn, reduced with 10 mg of sodium borohydride (30 min) evaporated to dryness and converted to the acetate derivative as described above. The dried residue was dissolved in 0.5 mL of methylene chloride and 1.0 μL was injected for the analysis. Threitol tetraacetate is readily identified by its mass spectrum, which shows the following peaks: 289 (M-H), 217 (M-CH_2OCOCH_3), 145 (217-$CHOCOCH_3$) and 115 (145-CH_2O).

Incubation of 1 and 2 with N^{α}-acetyl-L-lysine in the presence of cyanoborohydride. Solutions of **1** and **2** (0.12 M) were incubated with an equimolar amount of N^{α}-acetyl-L-lysine in the presence of 0.24 M sodium cyanoborohydride and oxygen. For GLC analysis, the samples were removed after 96 h reaction time and treated as described above for threose analyses. Aliquots were also removed and examined by TLC using irrigant B and sprayed with spray A.

ACKNOWLEDGEMENT

This work was supported, in part, by NIH grant EY 07070. The 500 MHz NMR Spectrometer was partially funded by the National Science Foundation (CHE 89-08304).

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