The non-oxidative degradation of ascorbic acid at physiological conditions

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Abstract

The degradation of L-ascorbate (AsA) and its primary oxidation products, L-dehydroascorbate (DHA) and 2,3-L-diketogulonate (2,3-DKG) were studied under physiological conditions. Analysis determined that L-erythrulose (ERU) and oxalate were the primary degradation products of ASA regardless of which compound was used as the starting material. The identification of ERU was determined by proton decoupled 13C-nuclear magnetic resonance spectroscopy, and was quantified by high performance liquid chromatography, and enzymatic analysis. The molar yield of ERU from 2,3-DKG at pH 7.0 37°C and limiting O2, was >97%. This novel ketose product of AsA degradation, was additionally qualitatively identified by gas-liquid chromatography, and by thin layer chromatography. ERU is an extremely reactive ketose, which rapidly glycates and crosslinks proteins, and therefore may mediate the AsA-dependent modification of protein (ascorbylation) seen in vitro, and also proposed to occur in vivo in human lens during diabetic and age-onset cataract formation. ß 2000 Published by Elsevier Science B.V. All rights reserved.

Keywords: Ascorbic acid; Dehydroascorbate; Diketogulonate; Erythrulose; Glycation; Cataract

1. Introduction

L-Ascorbate (AsA) serves as the primary water soluble antioxidant in cells [1]. AsA preferentially interacts with reactive oxygen species (ROS) generated during oxidative stress, thus protecting cell constituents from oxidative damage [2]. Recent interest in the potential toxicity of AsA [3–5] has focused attention on the primary oxidation product produced when AsA interacts with ROS, L-dehydroascorbate (DHA). Unlike AsA, DHA is both unstable in the absence of oxygen [6], and is a reactive electrophile, which generates further reactive degradation products over time in solution [5]. These electrophilic products react with nucleophiles on proteins, specifically lysinyl and arginyl residues, resulting in structurally deleterious, non-enzymatic modifications of proteins [7]. Tissues maintain a low concentration of DHA due to its reduction back to ASA by glutathione, both enzymatically and non-enzymatically [8]. However, there is an equilibrium level of DHA equivalent to ~5% the AsA pool, which may rise...
after oxidative stress [9–11]. Increases in DHA degradation have been hypothesized to be involved in the etiology of a variety of diseases, including senile cataract [12], aging [13], diabetes [14] and Alzheimer’s disease [15]. These diseases have all been associated with increases in ROS production and protein glycation. Hypothetically, increased ROS production in disease could lead to increased AsA oxidation, and therefore increased DHA accumulation and degradation to glycation-active products. It is therefore important to determine the products of AsA non-enzymatic degradation, as they may mediate the deleterious physiological effects of DHA. Determination of the major non-enzymatic degradation product(s) of AsA at physiological conditions will also enhance the understanding of the catabolism of AsA in general.

DHA rapidly hydrolyzes in solution at pH 7.0 to L-diketogulonate (2,3-DKG) [6], which is very unstable and degrades further. Forty years after the first investigation in this area, it has been variously reported, that the non-enzymatic/catabolic degradation pathway of AsA leads through 2,3-DKG to CO₂ and L-xylose [16], CO₂ and L-xylosone [17], CO₂, and L-lyxonate plus L-lyxonate [18–20], oxalate and L-threonoate [17,19], or oxalate and L-threose [21]. These divergent reports have prevented consensus on, and new investigations into, which degradation products of 2,3-DKG are produced under physiological conditions. Thus both the non-enzymatic and catabolic fates of vitamin C in vivo still remain in dispute. Recently 2,3-DKG concentrations have been shown to rise in oxidatively stressed human erythrocytes in vitro [22], and in the lens of rats placed under diabetic stress [23]. High lenticular rates of 2,3-DKG degradation in rats were correlated with increased cataract formation in vivo [24]. Importantly, the half life of DHA in vivo may be as short as 2 min [25] with rapid production of 2,3-DKG. However the fate of 2,3-DKG was not determined in these studies. Our interest in the ultimate degradation product(s) responsible for the ascorbylation of lens proteins led us to reinvestigate the AsA degradation pathway. The in vitro fates of AsA and its oxidation products DHA and 2,3-DKG, were followed in a pH 7.0 buffer at 23 or 37°C, and in human lens extracts, using nuclear magnetic resonance (NMR) spectroscopy.

2. Materials and methods

2.1. Materials

L-Ascorbate, L-dehydroascorbate, and L-erythrulose (97%) were purchased from Aldrich, Milwaukee, WI. These were assayed for purity by ¹³C-NMR, and were found to contain no detectable impurities. Additionally commercial ERU was assayed by proton NMR, further confirming its purity. L-[2,3-¹³C]AsA and L-[3,13C]AsA were purchased from Omicron Biochemicals, South Bend, IN and the white crystalline acids (99% ¹³C) contained no impurities as determined by NMR, and melting point. NADH and sorbitol dehydrogenase (EC 1.1.1.14, from sheep liver) were purchased from Sigma, St. Louis, MO, as were all other reagents used in the analytical studies. Potassium L-2,3-DKG was synthesized from AsA exactly as described by [26], and L-xylosone was prepared as reported in [27]. 3-Deoxyglucosone was synthesized by the method as described in [28]. Thin layer plates (Silica Gel 60 F 254, 5×20 mm) were obtained from Alltech, Deerfield, IL.

2.2. Thin-layer chromatography

TLC was conducted on silica gel plates using the following irrigants: butanol–acetic acid–water (3:1:1, v/v, irrigant A) and acetonitrile–water–acetone–acetic acid (80:15:5:6, v/v, irrigant B). Plates were stained using 2% vanillin (in 5% H₂SO₄, 95% ethanol; spray 1), or 0.93% aniline (free base)–1.66% O-phthalic acid in wet butanol (90% n-butanol, 10%, water; spray 2), and heated at 120°C for 10–15 min for development.

2.3. High performance liquid chromatography

HPLC was carried out using a Phenomenex Rezex-RMN-carbohydrate column (in the Na⁺ form), with dimensions of 300×7.8 mm. The run flow rate was 0.6 ml/min, with dH₂O as the mobile phase. Aliquots were taken from the reaction mixtures containing 0.1–0.2 M DHA or 2,3-DKG in 0.2–0.4 M phosphate buffer and diluted 1:4 with dH₂O. To remove UV absorbing chromophores, 50 mg of activated carbon was added per ml of the mixture. Samples were then sterile filtered through a 0.2-μm sterile
acrodisc (Gelman Sciences, Ann Arbor MI), yielding a clear sample eluant. The injection volume for each sample run was 250 µl. The chromatogram was monitored at 278 nm, using a Kratos spectrflow 773 UV detector.

2.4. Gas-liquid chromatography

GLC was performed using a Varian 3400 instrument in the split mode. Separations were done using a 25-m Quadex 001-Ov-17 capillary column with an i.d. 0.025 mm. Analysis of sugar derivatives was done with a hold time of 2 min after injection, followed by a 150–250°C temperature gradient at 10°C/min. Reaction mixtures containing 2,3-DKG or sugar standards were prepared for chromatography by preparing the alditol acetate derivatives according to the method described in [21].

2.5. Nuclear magnetic resonance spectroscopy

Proton decoupled 13C-NMR spectra were acquired at 23 or 37°C on 1 ml samples of DHA, or 2,3-DKG, in potassium phosphate buffer, pH 7.0 prepared in 9:1, H2O:D2O. A Bruker DRX500 (13C-unlabeled studies) or DRX300 (13C-labeled studies) spectrometer were used to collect spectra. The acquisition parameters used were; sweep frequency 125.757 MHz; pulse duration 8.24 µs (90°); interpulse delay 4.5 µs; and 1.0 Hz line broadening. The number of scans and other data specific to a particular experiment are reported in the figure legends. All samples were run with 3-(trimethylsilyl)propionic-2,2,3,3-d4 acid, sodium salt as the internal standard, with its signal set at 0.00 ppm.

2.6. Ultraviolet spectroscopy

A solution of commercial ERU (97%) was prepared quantitatively in dH2O and purified by HPLC. The fraction containing the sugar was dried at 4°C. This was dissolved in phosphate buffer, pH 7.0, and the absorbency constants were determined (λ = 278 nm, εM = 30 L/M·cm−1) representing the n to π* transition characteristic for ketones. Quantification of ERU in the HPLC chromatograms was done by relating the observed ERU peak area to a standard curve of area vs. concentration, prepared by chromatography of known amounts of commercial ERU.

2.7. Sorbitol dehydrogenase assay

A commercial enzyme solution was prepared by dissolving 2 mg of sorbitol dehydrogenase (6.3 U/mg) in 2 ml of 0.2 M KPO4 buffer, pH 7.0, and diluting with 8 ml of 1.25% BSA, prepared in the same buffer. The enzyme prepared in this manner was found to be stable for at least 1 week at 4°C. ERU was assayed within the range from 0.025–0.2 M in 0.1 M phosphate buffer, pH 7.0, containing 13–15 µl of the BSA/enzyme solution (or the amount of enzyme needed to reduce 0.1 M D-fructose at a rate of ~0.6 ΔA340/min). NADH was added at a level of 375 µM, and ΔA340 followed for 1 min. Each assay was carried out in duplicate. The Km for commercial ERU reduction was 0.260 M (± 17.6 M), while Vmax was 707 nmol ml−1 min−1 (± 3.2 nmol ml−1 min−1). Kinetic parameters were determined according to [29]. The kinetics of the HPLC purified product of 2,3-DKG, matched those determined with commercial ERU to within experimental error.

2.8. Human lens homogenates

Human lenses were obtained from Missouri Lions Eye Tissue Bank, and Human lens homogenates (HLH) were prepared as previously described [30], with 1 ml of buffer per lens. Donors were aged 30–40 years, and the protein concentration of the homogenate was determined to be 25 mg/ml by the method of Bradford [31] for kinetic studies. Homogenates were prepared to 50 mg/ml for 13C-AsA studies, were 500 µl homogenate is 1 human lens. All lens protein extracts were prepared in chelex-treated phosphate buffer, pH 7.0, and sterile filtered before use. No homogenates were dialyzed.

2.9. Degradation incubations

Incubations of DHA and 2,3-DKG were routinely carried out in phosphate buffers pH 7.0 at 23 or 37°C, and sterile filtered before incubation or before analysis by NMR, HPLC, GLC, or TLC. 5 mM 13C-AsA (1 ml) was oxidized with ascorbate oxidase 20 U in 0.2 M phosphate buffer, pH 7.0, for 15 min. The
enzyme was the rapidly removed by ultracentrifugation, and 500 µl of the labeled DHA diluted to 2.5 mM for NMR with 400 µl dH₂O and 100 µl D₂O. Initial spectra showed only signals of DHA and 2,3-DKG.

2.10. SDS-PAGE

Gel electrophoresis of proteins cross-linked by sugars was conducted under reducing conditions as described by Laemmli [32]. All sugars were at a concentration of 10 mM and were incubated with 6 mg/ml calf lens proteins for 5 days at 37°C, then diluted and assayed for crosslinking by SDS-PAGE.

2.11. Kinetics and 13C-NMR studies in human lens homogenates

The NADH-dependent reduction of ERU by HLH (prepared as in Section 2.8 except in 0.05 M potassium phosphate buffer, pH 7.0) was studied with ERU and l-[3-13C]-DHA/DKG (prepared as in Section 2.9) as starting materials. Assays of ERU were done within the range from 2.5 to 50 mM. NADH (0.425 mM) was preincubated for 2–3 min with 50 µl HLH, which had been diluted with 300 µl of buffer. All assays of ERU were done at a total volume of 1 ml and the decrease at A₃₄₀ was followed after the addition of ERU. Each data point was done in duplicate and kinetic parameters determined as in Section 2.7, over the first 2 min. The Kₘ for reduction of ERU was 21.3 mM (± 2.5 mM), while Vₘₐₓ was 66.2 nmol ml⁻¹ min⁻¹ (± 3.2 nmol ml⁻¹ min⁻¹). Studies to determine the effect of varying the NAD⁺/NADH ratio on ERU reduction were done similarly as in determination of kinetic constants. However, at the start [NADH] was 0.37 mM, while [NAD⁺] was varied from 0 to 1.5 mM. ERU was added to start the reduction at 0.020 M for all assays. In the determination of the effects of d-sorbitol on ERU reduction both [NADH] 0.37 mM and [ERU] 0.15 mM were the same in all assays, while [d-sorbitol] was varied from 0 to 0.45 M.

NMR studies on the reduction of ERU by HLH, were done using 0.5 ml of oxidized l-[3-13C]AsA prepared as in Section 2.9. This was added to 0.5 ml of HLH prepared as in Section 2.8. Therefore, in these studies, 0.5 ml HLE is equivalent to ~1 human lens.

3. Results

3.1. Oxalate and ERU are the products of 2,3-DKG degradation

2,3-DKG degradation was followed continuously by 13C-NMR over 40 h at 23°C under essentially anaerobic conditions (Fig. 1). The initial NMR spectrum of 2,3-DKG with C1–C6 resonances at 176.98, 97.87, 97.28, 77.05, 71.07 and 64.92 (Fig. 1a) corresponded to the reported proton decoupled spectra [33]. After 24 h, the 2,3-DKG signals had decreased substantially and five new resonances appeared at 215.23, 176.01, 78.74, 68.74 and 65.80 ppm. (Fig. 1b). After 40 h of incubation, the degradation was nearly complete (Fig. 1c) with the production an apparent five carbon product in nearly quantitative

Fig. 1. 13C-NMR proton decoupled spectra showing the degradation over time of 0.1 M 2,3-DKG in 0.2 M phosphate buffer, pH 7.0, 23°C. Each spectra was acquired with a minimum of 2000 scans at 0 h (a), after 24 h (b), after 40 h (c), and final spectra of degradation showing products (d) at 48 h, and a spike of D with an ~ equal molar amount of ERU (e). Note the increase in only the four signals corresponding to ERU.
yields. The identification of two products, ERU with four carbons at 215.23, 78.74, 68.74, and 65.80 ppm, and oxalate with two carbons (one resonance), was confirmed by spiking the NMR tube at the end of the degradation (Fig. 1d) with ERU (Fig. 1e). A similar spike with authentic oxalate confirmed the peak at 176.01 ppm as oxalate (data not shown).

The production of ERU was also detected by TLC (Table 1). This analysis agreed with the NMR results, showing that the 2,3-DKG degradation product consistently migrated with the ERU standard in two solvent systems. In irrigant A, ERU was clearly separated from L-threose, a previously proposed product. GLC analysis showed that both erythritol and threitol were formed following NaBH₄ reduction of the 2,3-DKG degradation product (Table 2). Since both alditols were produced by the reduction of the product, the 2,3-DKG degradation product could again be identified as ERU.

### Table 1

<table>
<thead>
<tr>
<th>Carbohydrate</th>
<th>( R_f ) Irrigant A</th>
<th>( R_f ) Irrigant B</th>
</tr>
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<tbody>
<tr>
<td>L-Xylose</td>
<td>0.48</td>
<td>0.33</td>
</tr>
<tr>
<td>L-Xylosone</td>
<td>0.43</td>
<td>0.43</td>
</tr>
<tr>
<td>D-Erythrose</td>
<td>0.53</td>
<td>0.52</td>
</tr>
<tr>
<td>L-Threose</td>
<td>0.62</td>
<td>0.54</td>
</tr>
<tr>
<td>L-Erythrulose</td>
<td>0.52</td>
<td>0.52</td>
</tr>
<tr>
<td>L-2,3-DKG degradation product</td>
<td>0.52</td>
<td>0.52</td>
</tr>
</tbody>
</table>

Results of chromatograms with reported 2,3-DKG degradation products, L-erythrulose standard, and the actual product from a degradation of 0.2 M 2,3-DKG in 0.4 M phosphate buffer, pH 7.0, and 23°C.

### Table 2

<table>
<thead>
<tr>
<th>Alditol acetates of:</th>
<th>Retention time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythritol</td>
<td>11.93</td>
</tr>
<tr>
<td>Threitol</td>
<td>12.13</td>
</tr>
<tr>
<td>Xylitol</td>
<td>15.83</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>16.85</td>
</tr>
<tr>
<td>L-2,3-DKG degradation product</td>
<td>11.93, 12.14</td>
</tr>
</tbody>
</table>

Samples from alditol standards were modified to the alditol acetates as described in [20]. Aliquots from a degradation of 0.2 M L-2,3-DKG in 0.4 M phosphate buffer, pH 7.0, 23°C, were reduced with NaBH₄ to alditol acetates before GLC analysis as in [20].

### 3.2. ERU is produced quantitatively from both DHA and 2,3-DKG

The degradation of DHA was similarly followed by \(^{13}\)C-NMR. As shown in Fig. 2, the rate profile for production of 2,3-DKG from DHA and that for the sequential degradation of 2,3-DKG to ERU, were obtained at pH 7.0 and 37°C. ERU was still the final product with DHA as the starting material, and was detected after 1.5 h of the DHA incubation. To quantify the yield of ERU from 2,3-DKG, we developed an HPLC method for its separation and detection. The chromatogram (Fig. 3) shows that the 2,3-DKG degradation product eluted with authentic ERU as confirmed by spiking the reaction with the commercial compound. The product was quantified by HPLC and UV-VIS spectroscopy (Fig. 4). The ketose could be detected after only 1 h by this method. At the end of the 2,3-DKG incubation, the ketose accounted for 63.5% of the initial molar amount of 2,3-DKG, and the half-life \((t_{1/2})\) of ERU under these conditions (see inset) was determined to be \(\sim 32\) h. Considering both the production of ketose from 2,3-DKG and the \(t_{1/2}\) of the ketose, the molar...
Fig. 3. HPLC chromatogram of the degradation products of 0.2 M DHA in 0.4 M phosphate buffer, pH 7.0, 37°C after 24 h. Top panel: the initial separation. Bottom panel: the same sample with excess ERU added as a spike.

Fig. 4. Production of ERU from 0.2 M DKG in 0.4 M phosphate buffer, pH 7.0, 37°C over time as determined by HPLC-UV. Inset shows the degradation of 0.1 M ERU over time in 0.2 M phosphate buffer under the same conditions. Quantification was determined by HPLC-UV followed at 278 nm.

Fig. 5. $^{13}$C-NMR spectrum showing the products of 0.2 M 2,3-DKG reacted with 0.2 M H$_2$O$_2$ in 0.4 M phosphate buffer for 2 h. Spectrum obtained using a Bruker DRX500 spectrometer. The numbers indicate the four most intense resonances of the major product of diketogulonate oxidation, which correspond exactly with those of an L-threonate standard previously run under the same conditions. 1, 181.69 ppm; 2, 75.70 ppm; 3, 75.70 ppm; 4, 65.79 ppm.
percentage of 2,3-DKG which was generated as 
ERU was estimated to be >97%. An enzymatic as-
say using sorbitol dehydrogenase was performed on 
the HPLC fraction containing the product. Since this 
enzyme recognizes only the L-enantiomer of ERU 
[34,35], the NADH-dependent reduction of the 
HPLC eluant confirmed that it had retained the L-
stereochemical configuration of the progenitor mole-
cule L-2,3-DKG. Additionally, the kinetics of the en-
zymatic reduction of the 2,3-DKG product matched 
those obtained with commercial ERU (see Section 
2.7). The single predicted product L-threitol (data

Fig. 6. $^{13}$C-NMR spectra following the degradation of 2.5 mM $^{13}$C-labeled AsA in 50 mM chelx-treated 0.1 M phosphate buffer, pH 
7.4, and 23°C over 12 h. Spectra were acquired using a Brunker DRX300 spectrometer, with 2880 scans for each 2-h spectrum. Upper 
panel shows near complete conversion of C-2 signal of 2,3-DKG to oxalate. Lower panel shows near complete conversion of C-3 sig-
nal of 2,3-DKG to ERU.
3. ERU is the product of AsA degradation under non-oxidative conditions; D-threonic acid is produced under oxidative conditions

We next tested whether ERU could be produced from AsA. The degradation of L-[2-13C]AsA and L-[3-13C]AsA were followed after oxidation with ascorbate oxidase, which produces the products DHA and H2O from ascorbate and O2, in a chelex-treated phosphate buffer, at 23°C. The use of labeled AsA allowed the analysis of the degradation pathway by NMR at more physiological concentrations of AsA oxidation products. The results seen in Fig. 5, clearly show that the major products after 12 h, are oxalate (Fig. 5, upper panel) from L-[2-13C]AsA. Concomitantly ERU production (Fig. 5, lower panel) from L-[3-13C]AsA is seen. In both cases the major signal after 2 h is that of 2,3-DKG (13C-2, at 97.87 ppm, 13C-3 at 97.28 ppm), which rapidly and quantitatively became only the signal for oxalate at 176.01 ppm (upper panel), and the C-1 signal of ERU at 68.74 ppm (lower panel). In both spectra, unidentified intermediates can readily be detected. These results suggest that the mechanism by which oxalate and ERU are produced is via a carbon-carbon bond scission between C2, and C3 of 2,3-DKG, or an intermediate produced from 2,3-DKG.

Having established 2,3-DKG as the progenitor of ERU, we questioned why this ketose had not been found previously in AsA degradation studies, since it was the only product seen in our studies. H2O2 (the product of AsA oxidation) was incubated with 2,3-DKG for 2 h (Fig. 6) in a phosphate buffer which had not been treated with chelex. The resulting NMR spectrum of the reaction clearly shows D-threonic as the major product (numbers are shown above the four signals of threonic), and a strong \( \text{HCO}_3^- \) signal is also seen along with several unidentified acid signals. In this experiment, no ERU was detected. This suggest that \( \text{H}_2\text{O}_2 \) produced by AsA oxidation, can oxidize 2,3-DKG preventing the formation of ERU.

3.4. ERU crosslinks lens protein

We tested the protein crosslinking ability of ERU to determine if it could account for the reported AsA-dependent modification of protein [5]. As seen in Fig. 7, ERU crosslinked bovine lens proteins to a greater extent than the previously proposed glycation-active sugars glucose, fructose, or 3-deoxyglucosone after 4 days of incubation. This was primarily indicated by the large amount of high molecular weight aggregates that did not enter the resolving gel.

3.5. Human lens effectively reduces ERU to D-threitol

Since ERU production during AsA catabolism would be potentially deleterious, we studied the capacity of HLH to reduce ERU to the tetritol. As determined (see Section 2.11) lens contains \( \approx 1.33 \) U of NADH-dependent activity towards ERU as a substrate. The kinetic parameters were very similar to those found for the reduction of ERU by purified...
human sorbitol dehydrogenase [36]. We next evaluated the activity profile over physiological ratios of NAD$^+$/NADH known to exist in the lens [37]. As can be seen (Fig. 8, upper panel) even at the most inhibitory ratio of NAD$^+$/NADH of 4:1 the reductive activity was still ~75% of that in the absence of NAD$^+$. The effects of varying the ERU/D-sorbitol ratio were more significant. At a ratio of 1:12 the activity was inhibited ~50%, (Fig. 8, lower panel).

Finally, we used $^{13}$C-NMR to study the reduction of ERU by HLH. The homogenate slowly reduced ERU to l-threitol (65.60 ppm) using only the NADH present in the HLH (Fig. 9, upper panel). As calculated from relative signal intensities of C1 of ERU and C1 of l-threitol, the breakdown of the entire 2.5 μmol of 2,3-DKG over 6 h (~7 nmol min$^{-1}$), is met by a corresponding reduction rate of ~1.1 nmol min$^{-1}$ and a total of ~400 nmol of L-threitol. At 14 h, ~600 nmol of L-threitol were produced. Since human lens contains only 51–210 nmol of NADH [37], the reduction rate is clearly limited by the availability of endogenous NADH, and the recycling of NAD$^+$ to NADH. However, in the presence of excess of 5.0 μmol added NADH, the tremendous capacity of the lens to reduce the 2.5 μmol of ERU produced from 2,3-DKG is apparent (Fig. 9, lower panel). Even at a flux rate equivalent to the turnover of the entire physiological level of 2.5 μmol of AsA in only 4 h, little ERU can be detected and the C3 resonance of 2,3-DKG is converted to the C1 of L-threitol rapidly.

4. Discussion

ERU is the major product of the non-oxidative degradation of DHA, and 2,3-DKG. To our knowledge, this ketose product, which is formed in ≥97% yield from 2,3-DKG, has not been previously reported. The in vitro degradation of AsA has been studied extensively under a variety of conditions to determine the fate of AsA oxidation products, both in solution, and in tissue homogenates. From these previous studies, an astounding variety of degradation/catabolic products of vitamin C have been reported. The conditions of these studies were primarily oxidative, owing to the need to initially oxidize AsA with O$_2$ to initiate the subsequent degradation. The reaction of H$_2$O$_2$, (the ignored product of AsA oxidation to DHA), with DHA and 2,3-DKG during their degradative fates has not been generally considered. Since many of the studies on the degradation and catabolism of AsA have been carried out in buffers containing Fe$^{3+}$ and Cu$^{2+}$ impurities [38], hydroxyl radical would be generated in significant amounts from the H$_2$O$_2$ product by the Fenton reaction [39]. It has been determined that H$_2$O$_2$ and DHA react directly at pH 7 to produce l-threonate and oxalate [40] quantitatively. Importantly, these products are among the reported catabolic metabolites of AsA [17,19]. It is essential to emphasize that no l-threonate can be produced from DHA or 2,3-DKG without their oxidation. In addition, it is well established that H$_2$O$_2$ reacts with 2-keto aldonic acids such as 2,3-DKG with sequential decarboxyla-
tion of C1 and C2 [41]. This oxidation could account for the numerous reports of CO₂, and the five carbon or four carbon sugar acid products seen in ascorbate degradations. The known effect of hydroxyl radical production on sugars is to cause oxidative fragmentation [41], with production of a variety of products, which may also have been erroneously reported as AsA catabolic products.

Our results support the hypothesis that two major AsA degradation pathways exist at physiological pH,
one oxidative, and the second non-oxidative (see Scheme 1). Central to the determination of which pathway predominates is the oxidative status of the milieu during 2,3-DKG degradation. In the presence of high concentrations of H₂O₂, 2,3-DKG produces L-threonate, oxalate, and CO₂. In the absence of H₂O₂, 97% ERU and oxalate are produced. The autoxidation of AsA in the absence of metal ions to L-threonolactone and oxalate has been recently reported by Miyake et al. This new pathway proceeds independent of DHA, but concomitant with the DHA-dependent pathway. Though potentially important, DHA was still the major product (> 85%) even during AsA autoxidation to L-threonolactone and oxalate [42].

Only a few studies have been done on the non-oxidative degradation of AsA at physiological conditions. In two such studies, ERU has been previously detected, but misidentified. Kang et al. [17] assessed the ¹³C-NMR spectrum of the major DHA degradation product as L-xyllosone (a 5-carbon product), when it clearly shows both ERU and oxalate. Also their spectrum does not correspond to the published spectra of L-xyllosone [27]. Lopez and Feather [21] detected a tetrose produced in high yield in AsA, DHA and 2,3-DKG degradations. However, they misidentified it as l-threose, using a TLC solvent system, which did not adequately separate erythrose and threose (see Table 1, irrigant B).

Although none of our incubations were under strictly anaerobic conditions, our determination of the non-oxidative degradation pathway of AsA was dependent on several factors. First, the use of DHA and 2,3-DKG eliminated production of H₂O₂ during the obligatory oxidation of AsA. Additionally, in each case, dissolved O₂ was insignificant (~0.2 mM), because the concentration of DHA, or 2,3-DKG (0.1–0.2 M) were three orders of magnitude of greater than O₂. We used ascorbate oxidase when confirming ERU production from AsA, which prevented the production of H₂O₂ during the oxidation of ascorbate (the products of ascorbate oxidase are H₂O and DHA). The use of chelex-treated buffers reduced participation of the Fenton reaction in the studied degradation pathway. These conditions more closely reflect those in vivo under which DHA and 2,3-DKG would degrade, namely in an environment with no free metals, and in low levels of H₂O₂ due to its rapid endogenous detoxification by the antioxidant enzymes catalase and glutathione peroxidase. However although we do not know which pathway operates in vivo, the discovery of the non-oxidative pathway and its novel product ERU is significant, and may be important to the final determination of AsA catabolism.

We have shown that the major non-oxidative degradation product of AsA under physiological conditions is ERU. The ketose should be very reactive towards protein amino groups, since (by NMR) ERU appears in solution to exist nearly 100% in the free carbonyl form. Sugar reactivity towards protein amino groups has been strongly correlated with the percentage of free carbonyl of a particular monosaccharide in solution [43,44]. Therefore, ERU is implicated as the species responsible for the faster and greater extent of protein crosslinking seen in incubations of AsA and protein, compared to similar incubations to those with using glucose [5]. The probability that ERU mediates AsA-dependent crosslinking is also argued by the relative half-lives of the AsA degradation products under conditions similar to those used in protein crosslinking studies (see Figs. 2 and 3). With a half-life of ~32 h in phos-
phate ERU based on structural considerations, should also be much more reactive than the short-lived DHA or 2,3-DKG with protein nucleophiles. This is especially true since DHA and 2,3-DKG are predominantly in unreactive hydrated and cyclic forms in solution. In addition to protein crosslinking, ERU produces more $O_2^\bullet^-$ upon glycation than other proposed glycators [45], which would increase its potential toxicity.

ERU may be a previously unidentiﬁed catabolite of AsA in vivo. We have determined that human lens has substantial NADH-dependent sorbitol dehydrogenase reductive activity for this ketose producing, NAD$^+$ and L-threitol. The kinetics of ERU reduction, production of the product L-threitol, and the inhibition of reduction by D-sorbitol support both the identiﬁcation of the reductive activity as that of lens sorbitol dehydrogenase, and further verify the product of 2,3-DKG degradation as ERU. We have shown that given a supply of NADH human lens has the capacity to prevent the accumulation of ERU, even at rapid rates of 2,3-DKG degradation.

Therefore ERU may be the progenitor of lens L-threitol, a metabolite whose origin is still unknown [46], and may originate from AsA catabolism. Additionally, a role for sorbitol dehydrogenase in reduction and detoxiﬁcation of ERU could associate increased AsA oxidation and catabolism with the complications resulting from altered sorbitol pathway function. The fate of lenticular threitol is unknown; however, increased polyol production has been strongly implicated in cataract formation and diabetic complications.

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References
