**Oxygen Dependency of One-Electron Reactions Generating Ascorbate Radicals
and Hydrogen Peroxide from Ascorbic Acid**

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Short Title: Oxygen Dependency of One-Electron Reactions...

Key words: ascorbic acid, hydrogen peroxide, ascorbate radical, ascorbyl radical and
dehydroascorbate

33

34 **Abstract**

35 The effect of oxygen on the two separate one-electron reactions involved in the
36 oxidation of ascorbic acid was investigated. The rate of ascorbate radical ($\text{Asc}^{\bullet-}$)
37 formation (and stability) was strongly dependent on the presence of oxygen. A product
38 of ascorbic acid oxidation was measurable levels of hydrogen peroxide, as high as 32.5
39 μM from 100 μM ascorbic acid. Evidence for a feedback mechanism where hydrogen
40 peroxide generated during the oxidation of ascorbic acid accelerates further oxidation of
41 ascorbic acid is also presented.

42 The second one-electron oxidation reaction of ascorbic acid leading to the
43 disappearance of $\text{Asc}^{\bullet-}$ was also strongly inhibited in samples flushed with argon. In the
44 range of 0.05 to 1.2 mM ascorbic acid, maximum levels of measurable hydrogen
45 peroxide were achieved with an initial concentration of 0.2 mM ascorbic acid. Hydrogen
46 peroxide generation was greatly diminished at ascorbic acid levels of 0.8 mM or above.

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1. Introduction

The oxidation of ascorbic acid has great importance in biological and food sciences, yet many details of this two-electron reaction sequence are not well understood. Barron and others (Barron, 1936) investigated a variety of buffers at pH 1–10 containing 0.02 mM ascorbic acid. They proposed that ascorbic acid was oxidised to dehydroascorbate via the transfer of two electrons that reduced two atoms of Cu^{2+} . In the second step of their proposed reaction, four cuprous ions reduced 2 molecules of oxygen to regenerate cupric copper and produce 2 molecules of hydrogen peroxide. Neither ferrous nor ferric iron was found to act as a catalyst for the oxidation of ascorbic acid under the parameters examined. Silverblatt and King (Silverblatt, Robinson, & King, 1943) elaborated on the generation of hydrogen peroxide during the copper catalysed oxidation of ascorbic acid, and reported that the H_2O_2 was relatively stable under the conditions of their experiments. Udenfriend and others (Udenfriend, Clark, Axelrod, & Brodie, 1954) reported that at neutral pH, aromatic compounds were hydroxylated by molecular oxygen in the presence of ferrous iron, ascorbic acid and EDTA. This mixture (often referred to as the “Udenfriend system”) is proposed to involve a 2-electron transfer via a complex of oxygen, Fe^{2+} and ascorbic acid that transfers triplet oxygen to a substrate. Khan and Martell (Khan & Martell, 1967a) (Khan & Martell, 1967b) proposed that, in the pH range between 2 and 5.5, the first electron transferred from the ascorbate monoanion occurs via an ascorbate-metal-oxygen complex results in the formation of hydroperoxy radicals ($\text{HO}_2^\bullet$). In their proposed mechanism, the second electron is transferred directly from the ascorbate radical ($\text{Asc}^\bullet$) to reduce a transition metal and to form dehydroascorbate (DHA). Within the literature

the terms ascorbyl radical and ascorbate radical are often used interchangeably to refer to the radical resulting from the loss of one electron from the ascorbate monoanion. In this paper we refer to the ascorbyl radical as $\text{AscH}^\bullet$, which has a pK_a of -0.86 and in biological media will lose a proton to form the ascorbate radical ($\text{Asc}^\bullet$) (Bors & Buettner, 1997).

Jameson and Blackburn (Jameson & Blackburn, 1976) proposed a chain-reaction mechanism in the pH range from 1.8 to 5. Similar to the Khan and Martell mechanism (Khan & Martell, 1967a) (Khan & Martell, 1967b), their mechanism also involved the formation of an ascorbate-copper-oxygen complex prior to the formation of the ascorbate radical. The mechanism favoured involved the formation of the peroxide ion (O_2^{2-}) intermediate resulting from the transfer of two electrons to molecular oxygen via a copper(II)-ascorbate dimer. A copper-copper binuclear species containing ascorbate and chloride ligands were considered components of the reactive species. Bielski and others (Bielski, Allen, & Schwarz, 1981) generated ascorbic acid radicals via pulse radiolysis of ascorbic acid in N_2O saturated solutions (i.e, in the absence of oxygen), and examined parameters affecting the rate of DHA generation. In their proposed mechanism ascorbate radicals undergo disproportionation via an ascorbate dimer that combine with a proton to form a molecule of DHA and the ascorbate monoanion. Because molecular oxygen was excluded from their experiments, a proposed mechanism based on the observations of Bielski and others (Bielski, Allen, & Schwarz, 1981) should not be viewed as conclusive when considering the reaction mechanism occurring in systems containing oxygen. Several review articles and books published on the subject appear to have used the work of Bielski and others (Bielski,

Allen, & Schwarz, 1981) to conclude that degradation of ascorbic acid in oxygenated mediums occurs via an independent dismutation reaction and that oxygen is not involved in the reaction.

Scarpa, Stevanato, Viglino and Rigo (Scarpa, Stevanato, Viglino, & Rigo, 1983) reported that, in the presence of catalase and preformed-copper-histidine or preformed-iron-EDTA complexes, the oxidation of ascorbic acid was dependent on the formation of superoxide radical anions. From the data reported, the rate of superoxide generation was about 10-times higher from the Fe^{2+} -EDTA complex than the Cu^{2+} -(His)₂ complex. Copper is about 80 times more efficient than iron as a catalyst of ascorbic acid oxidation (Buettner, 1988). Because of the use of preformed-metal complexes with molecules other than ascorbic acid, it is difficult to draw any comparisons with other studies of ascorbic acid oxidation, other than that externally manufactured superoxide radical anions can contribute to the oxidation of ascorbic acid.

Strizhak and others (Strizhak, Basylichuk, Demjanchyk, Fecher, Schneider, & Munster, 2000) monitored the oxidation of ascorbic acid in a continuous flow stirred tank reactor at pH 4.8, using an $\text{H}_2\text{SO}_4/\text{Na}_2\text{SO}_4$ buffer. They discussed a reaction sequence where the first one-electron oxidation to generate the ascorbate radical is catalysed by Cu^{2+} . Subsequent reaction(s) between oxygen and ascorbate radicals would then form the hydroperoxyl radical ($\text{HO}_2^\bullet$) radical and DHA. The hydroperoxyl radical could then react with ascorbic acid to further generate ascorbate radicals and H_2O_2 . Unlike most other investigations, Strizhak and others concluded that hydroxyl radicals produced via degradation of the hydrogen peroxide produced during the oxidation of ascorbic acid could also further oxidise ascorbic acid. Bradshaw and others (2002) examined the

formation of H₂O₂ in a model wine system using square-wave voltammetry. These experiments were conducted at pH 3.2, 45 °C, 1.14 mM ascorbic acid, and assays were conducted on an hourly basis over the course of 12 hours. Evidence that H₂O₂ generated under these conditions reacted with residual ascorbic acid was presented. The vastly different reaction conditions used in these various studies are likely to have contributed to disagreements in the proposed mechanisms for ascorbic acid oxidation.

There have also been numerous publications addressing the ability of reaction products from ascorbic acid oxidation to cause damage to biological materials. Chen, Espey, Sun, Lee, Krishna, Shacter, et al. (Chen, Espey, Sun, Lee, Krishna, Shacter, et al., 2007) demonstrated that hydrogen peroxide generation in the extracellular fluid of rats was dependent on ascorbate concentration and occurred only when the ascorbate radical exceeded 100 nM. The mechanism of H₂O₂ production was not addressed further. Oikawa and Kawanishi (Oikawa & Kawanishi, 1998) reported that ascorbate in the presence of copper promoted DNA damage, but there appears to be no follow-up investigation into the mechanism. Other investigations that reported the generation of reactive oxygen species (ROS) and damage to biological material as a result of ascorbic acid redox cycling include Norkus, Kuenzig and Conney (Norkus, Kuenzig, & Conney, 1983), Chiou (Chiou, 1983), Samuni and others (Samuni, Aronovitch, Godinger, Chevion, & Czapski, 1983), Marx and Chevion (Marx & Chevion, 1986), and Fisher and Naughton (Fisher & Naughton, 2003).

When commercially-available powdered protein drink mixes were hydrated, elevated levels of luminol-enhanced luminescence were produced (after an initial lag phase) that lasted for an hour and beyond (Boatright, 2013). The addition of catalase

strongly inhibited the ROS-generated luminescence, implicating the role of hydrogen peroxide formed from the interaction of the drink mix ingredients after the powder was hydrated. The amount of ascorbic acid found in these products ranged from 0.3 mM to none over the course of 60 minutes after being hydrated. Copper contents of these hydrated products ranged from 3.0 to 40.6 μ M, and the pH was 6.5. The level of ascorbate radicals in the drink mix that produced the highest level of luminol-enhanced luminescence was estimated to be 379 nM. To date, this is the only published example where ascorbate radicals were quantified in a “non-manipulated” food product, and evidence is presented for the generation of H_2O_2 via ascorbic acid oxidation.

In the current investigation data obtained in the pH range from 6.5 to 9.0 strongly suggests that the oxidation of the ascorbate monoanion to the ascorbate radical is a one-electron step that is strongly dependent on oxygen. The subsequent transfer of the second electron to form DHA is also strongly oxygen dependent, either directly or indirectly. Additional data demonstrate the optimum level of ascorbic acid for hydrogen peroxide generation, as well as the relative rate and amount of hydrogen peroxide resulting from the oxidation of ascorbic acid.

2. Materials and Methods

2.1 Chemicals

Sodium phosphate dibasic, diethylenetriaminepentaacetic acid (DTPA), leucocrystal violet (LCV) (product No. 219215), horseradish peroxidase (HRP) (product No. P8250) (EC1.11.1.7, oxidoreductase), catalase (from bovine liver) (product No. C9322) (EC 1.11.1.6, oxidoreductases), bovine serum albumin (BSA), and 3-carboxy-PROXYL were obtained from the Sigma-Aldrich (St. Louis, MO). Ascorbic acid, carbonate buffers and sodium phosphate monobasic were from Thermo Fisher (Pittsburgh, PA).

2.2 Oxygen concentration measurement

Oxygen concentrations were measured using an Extech 407510 dissolved oxygen meter (Extech Instruments Corp., Nashua, NH). Three different levels of oxygen concentrations were achieved by 1) using buffers in equilibrium with ambient conditions (8.3 ± 0.1 mg/L), 2) “oxygenated” buffers and 3) buffers flushed with argon to minimise available oxygen. Where noted, “oxygenated” conditions were achieved by bubbling 99.99% oxygen at a rate of 1.25 L/min through a 2-mm I.D. Teflon tube into buffer solutions for 15 s. Initial values prior to adding reactants were 34.0 ± 0.64 mg/L (0.991 mM). At the end of reactions with ascorbic acid (in beakers open to the atmosphere), oxygen contents were 21.0 mg/L ± 0.10 (0.66 mM). Because the amount of oxygen available in the “oxygenated” buffers was always at least 6.5 orders of magnitude greater than the amount of ascorbic acid present in the reaction mixtures, reaction rates were not considered to be limited due to the availability of oxygen. This technique is similar to that used by Khan and Martell (1967a). When flushed with argon for 5 min there was no detectable oxygen.

2.3 *Hydrogen Peroxide quantification*

Hydrogen Peroxide was quantified using the method of Mottola, Simpson and Gorin (Mottola, Simpson, & Gorin, 1970). Leucocrystal violet (LCV) (50 mg) was dissolved in 100 mL of 0.5 % HCl. Horseradish peroxidase (HRP) (10 mg) was dissolved in 10 mL nanopure water. Assays were conducted by adding 1 mL of the LCV solution, 0.5 mL of the HRP mixture and the designated amount of reaction mixture (either 0.5 or 1.0 mL) into a 10-mL volumetric flask. Then 0.2 M phosphate buffer (pH 6.5) was used to bring the volume to 10 mL. Spectrophotometric readings at 590 nm were obtained between 1–2 min of combining reagents. Values reported are $\mu\text{M H}_2\text{O}_2$ in the reaction mixture containing ascorbic acid. Because the LCV assay can be affected by changes in pH, an H_2O_2 standard curve at pH 6.5 was prepared in the range of 0.85 to 5.1 $\mu\text{g H}_2\text{O}_2$. A molar absorptivity of 70,950 was calculated from the standard curve when using a pH 6.5 phosphate buffer. Molar absorptivity of 75,000 was reported by Mottola, Simpson and Gorin (Mottola, Simpson, & Gorin, 1970) when using a pH 4.5 buffer. Control samples measured after 12 min of reaction, without added peroxidase, were not significantly different to the corresponding blank (without peroxidase or ascorbic acid), indicating that only H_2O_2 was measured by the LCV reaction. All experiments were conducted at 22 °C.

2.4 *Spectrophotometric measurement of ascorbic acid degradation*

Spectrophotometric measurement of ascorbic acid degradation was followed by the decrease in absorbance at 265 nm, according to the methods of Puget and Michelson (Puget & Michelson, 1974), and Halliwell and Foyer (Halliwell & Foyer, 1976). Ascorbic acid was prepared in a premix and then portions were added to the reaction mixtures

(after the addition of other reactants) to achieve the noted ascorbic acid concentrations. After the reaction conditions were achieved, a quartz cuvette was filled and placed in an Agilent 8453 diode array spectrophotometer. The degradation of ascorbic acid to either the ascorbate radical or DHA results in the loss of absorbance at 265 nm (Bielski, Comstock, & Bowen, 1971) (Oberbacher & Vines, 1963). The appropriate amounts of catalase or DTPA were weighed using a Mettler Toledo MT5 microbalance (Columbus, OH). The addition of catalase or DTPA occurred just prior to the addition of ascorbic acid, and an absorbance blank was subtracted where appropriate. Catalase was denatured by preparing a premix (50 mg per 10 mL) in the appropriate buffer. The premix was then rapidly brought to 95 °C, held above 90 °C for 5 min, and then cooled on ice to 25 °C. Then the appropriate amount of premix was added to the reaction mixture. Some samples (where noted) were flushed with 99.998% argon for 5 min in a Teflon-capped vial (10 mL sample with 30 mL headspace). Once ascorbic acid was added through the septum, the sample was removed through the septum with a syringe and transferred to an argon-flushed capped quartz cuvette. If H₂O₂ was added, the cuvette and cap was flushed with argon during the brief and minimal removal of the cuvette cap. All experiments were conducted at 22 °C.

2.5 *Electron paramagnetic resonance (EPR) spectroscopy*

Electron paramagnetic resonance (EPR) spectroscopy was performed at 20 °C on a Bruker EMX EPR spectrometer (Billerica, MA). Parameters for measuring the ascorbate radical were 10 mW microwave power scans; microwave frequency of 9.86 GHz; receiver gain 2×10^5 , modulation amplitude 0.5 G; modulation frequency 100 kHz; time constant 40.96 ms; conversion time 163.84 ms, resolution 1024 and field sweep of

140 Gauss. The ascorbate radical peak area was quantified using 3-carboxy-PROXYL as a spin standard according to the method of Buettner (Buettner, 1990). Aqueous samples were introduced into the EPR in a Bruker AquaX sample holder. Because of the need to introduce the samples into the AquaX holder and conduct the described field sweep, the earliest possible reading obtained was 20 s after mixing reagents. Analyses of EPR peaks were determined using the Bruker WINEPR System software. The g -value axis was calibrated relative to a crystalline 2,2-diphenyl-1-picrylhydrazine (DPPH) standard at 2.0036 using Bruker WINEPR System software. Tuning the EPR with the AquaX holder and performing a scan of the reagent blank (no added ascorbic acid) produced no ascorbate radical signal.

2.6 Atomic absorption spectroscopy.

Powdered samples were ashed according to AOAC Official Method 985.35. Iron and copper contents of the samples were then determined with a PerkinElmer AAnalyst 200 atomic absorption spectrometer (PerkinElmer, Waltham, MA).

2.7 Luminescence measurements

Luminescence was measured using a Promega GloMax 20/20 Luminometer (Madison, WI). Ascorbic acid was prepared in a premix and then the appropriate amount was mixed in a 35-mL glass beaker with the appropriate buffer, which was covered and inserted into the luminometer. No oxygen, copper or luminescence enhancer was added. All experiments were conducted at 22 °C.

2.8 Statistical evaluations

248 All data requiring statistical analysis were evaluated by analysis of variance using the
249 Statistical Analysis System (SAS Institute Inc., Cary, NC) software package. Significant
250 differences between means were identified by the least significant differences (LSD)
251 procedures at a significant level of 0.05, and comparison of means was done using the
252 Tukey-Kramer HSD test. Sample replicates are indicated by $n = 3$.

253

3. Results and Discussion

3.1 *Generated H₂O₂ accelerates ascorbic acid oxidation*

A comparison of the rate of ascorbic acid degradation (by the decrease in absorbance at 265 nm) in oxygenated 0.1 M phosphate buffer at pH 6.5 demonstrates the well-documented acceleratory effect of copper (**Figure 1**). The 0.1 M phosphate buffer used in this investigation contributed 0.29 μM copper and 1.77 μM iron to the reaction mixtures. At 5.0 μM added copper, the initial rate of ascorbic acid degradation (ca. 0–2 min) in the pH 6.5 phosphate buffer was approximately $3.5 \times 10^{-7} \text{ M} \cdot \text{s}^{-1}$. The change in rate of ascorbic acid degradation when the amount of added copper was increased from 5.0 to 8.0 μM under the same conditions was not discernible, and the curves in **Figure 1** from these two concentrations closely overlap.

The measureable hydrogen peroxide in the oxygenated 0.1 M phosphate mixture at pH 6.5 containing 2.5 μM added copper exhibited an increase over the first 15 minutes from non-detectable to $22.94 \pm 0.49 \mu\text{M}$ (**Figure 2**). The initial rate of ascorbic acid degradation ($2.37 \times 10^{-7} \text{ M} \cdot \text{s}^{-1}$) corresponds to a maximum rate of measureable H₂O₂ formation of $7.67 \times 10^{-8} \text{ M} \cdot \text{s}^{-1}$. H₂O₂ detected by the leucocrystal violet method is referred to as “measurable H₂O₂” in the presence of ascorbic acid because ROS emanating from H₂O₂ are known to contribute to the oxidation of ascorbic acid (Martinello & da Silva, 2006), (Deutsch, 1998) and not all the hydrogen peroxide generated was detected due to these reactions. The slowing generation of hydrogen peroxide at ca. 15 min corresponds to the nearly complete disappearance of ascorbic acid at about the same time. The maximum level of hydrogen peroxide in the pH 6.5 phosphate buffers occurred with 0.2 mM ascorbic acid, reaching $32.46 \pm 3.7 \mu\text{M}$ by 15

min (**Table 1**). At levels above 0.2 mM ascorbic acid there was an inhibitory effect on the reaction that generates hydrogen peroxide.

The association between ascorbic acid oxidation and hydrogen peroxide generation has been acknowledged since the 1930's. However, the correlation of H₂O₂ production with the rate of ascorbic acid degradation, in the pH range and concentrations of ascorbic acid, oxygen and copper examined here, have not been previously reported. Kimoto, Tanaka, Gytoku, Morishige and Pauling (Kimoto, Tanaka, Gytoku, Morishige, & Pauling, 1983) reported a single observation that 10 µM H₂O₂ was produced from the oxidation of ascorbic acid after 10 min in 0.25 mM ascorbate and 2.5 µM added copper at 37 °C in Krebs-Ringer phosphate buffer at pH 7.4. The EPR signal from their mixture when combined with the spin trap *N-tert-butyl-α*-phenylnitrone indicated the presence of oxygen-centred radicals.

The contribution of hydrogen peroxide and daughter radicals to the further degradation of ascorbic acid is further indicated with the addition of catalase at 150 U/mL, which inhibited the rate of ascorbic acid degradation (**Figure 3**). As a control, heat-denatured catalase exhibited a much lower inhibitory effect than the non-denatured catalase. Similar results were reported by Samuni and others (Samuni, Aronovitch, Godinger, Chevion, & Czapski, 1983). Flushing the sample with argon for 5 min prior to adding ascorbic acid had a very strong inhibitory effect on ascorbic acid oxidation (**Figure 3**). This strong inhibitory effect was still observed when the amount of added copper was increased to 10 µM (**Figure 4**). After 10 minutes there was no significant difference in absorbance values between samples with 2.5 and 10 µM added copper under argon ($p > 0.05$). In the reduced oxygen environment, the effect of added H₂O₂

after 12 minutes on the degradation of ascorbic acid can be seen (**Figure 4**). Addition of H_2O_2 to bring the content in the reaction mixture to 100 μM (while flushing the headspace with argon) strongly accelerated the degradation of ascorbic acid in the absence of oxygen. The 100 μM level is about four times higher than the “measurable H_2O_2 ” generated during ascorbic acid oxidation shown in **Figure 2**. If H_2O_2 and daughter radical generation occur via a transition metal-oxygen-ascorbate complex, then the rate of interaction with molecules in the near neighbourhood (“active site chemistry”) would be greatly accelerated. The addition of H_2O_2 under argon demonstrates that H_2O_2 generated by the oxidation of ascorbic acid can, in turn, contribute to the acceleration of the overall oxidative reaction rate.

3.2 Effect of excluding oxygen on formation of $\text{Asc}^{\cdot-}$ and DHA

The oxidation of ascorbic acid to dehydroascorbic acid is a two-electron process. At pH 6.5, loss of the first electron from the ascorbate monoanion results in the formation of the ascorbate radical ($\text{Asc}^{\cdot-}$). Subsequent loss of a second electron results in the disappearance of $\text{Asc}^{\cdot-}$ and formation of DHA. By comparing the absorbance data in **Figures 3 & 4** with the EPR data in **Figure 5**, factors affecting the disappearance of the ascorbate monoanion and the formation of the ascorbate radical, and the disappearance of the ascorbate radical, can be observed. When samples were flushed with argon (to minimise available oxygen) at pH 6.5, initiation of the first one-electron oxidation reaction that produces the ascorbate radical as well as the second one-electron reaction that produces DHA is strongly inhibited (**Figures 3, 4 & 5**). However, the first one-electron reaction forming the ascorbate radical is not completely inhibited.

This very slow formation and degradation of ascorbate radicals under argon corresponds to the stabilisation of the ascorbate monoanion under the same conditions as measured by A_{265} (**Figure 3**). Such an observation is in agreement with the mechanisms discussed by Jameson and Blackburn (Jameson & Blackburn, 1976). They addressed the possibility of a limited degree of charge transfer within a copper-chloride-ascorbate complex in the absence of oxygen that would encourage the binding of oxygen. All else being the same, increasing the level of added copper from 2.5 to 10 μM had no significant effect on the levels of ascorbate radicals formed or their stability under argon (data not shown).

Because oxygen has such a strong effect on these two one-electron ascorbic acid oxidation reactions, it is necessary to consider oxygen as an intricate component when considering the overall series of mechanisms. A series of reaction steps that included the formation of the hydroperoxyl radical and hydrogen peroxide, and exhibits an autocatalytic feedback, was proposed by Strizhak and others (Strizhak, Basylichuk, Demjanchyk, Fecher, Schneider, & Munster, 2000) where:

If this series of reactions are representative of the oxidation of ascorbic acid,

reaction (2) would be the rate-limiting step in the absence of oxygen. Such a characterisation of the two one-electron oxidation steps for ascorbic acid into an oxygen-independent electron transfer producing the ascorbate radical, and subsequent oxygen-dependent reactions (that also generate H₂O₂) are in disagreement with some of the previously proposed reaction mechanisms discussed in the “Introduction” section of this paper and the current findings which indicate that the first electron transfer leading to the formation of the ascorbate radical is strongly dependent on oxygen.

Two more recent review articles (Du, Cullen, & Buettner, 2012) (Frei & Lawson, 2008) have expressed this complex sequence of reactions in terms of:

This sequence of reactions disagrees with most of the references cited in the introduction of this paper, as well as the current findings, except reaction (7) where the work of Bielski and others (Bielski, 1982) is cited. Because the ascorbate radicals in the works by Bielski and others (Bielski, Allen, & Schwarz, 1981) (Bielski, 1982) were not created in the presence of oxygen (pulse radiolysis in N₂O saturated solutions was used), nor did they disproportionate in the presence of oxygen, no reactions involving O₂ would be expected to occur. The Fe²⁺ (or Cu⁺) formed from reaction (6) is postulated to then reduce molecular oxygen to the superoxide radical anion, followed by dismutation of the superoxide radical anion to form hydrogen peroxide. It appears that

there were no references provided in these reviews to support this mechanism. With this model, no ascorbate-oxygen complex is formed, and no interaction of oxygen with ascorbate radicals was addressed. The redox potential of the $\text{Cu}^{++}/\text{Cu}^+$ complex is about -180 mV, and that of the $\text{O}_2/\text{O}_2^{\bullet-}$ couple is -330 mV. It is therefore very unlikely for molecular oxygen to be reduced by Cu^+ alone (as presented in equation 8) at a rate sufficient to account for the observations in the current and previous investigations. However, transition metal complexes, similar to the copper-chloride-ascorbate complex described by Jameson and Blackburn (Jameson & Blackburn, 1976) are capable of achieving this reduction. Details of the chemistry of oxygen and transition metal interactions have been covered extensively by Hamilton (Hamilton, 1974) and Martel and Sawyer (Martell & Sawyer, 1988). Superoxide radical anions are generated from electron transport chains within mitochondria (Boveris and Cadenas, 1982) and during respiratory bursts of phagocytic cells (e.g., neutrophils, monocytes and macrophages), as a side product of prostaglandin synthesis and in the oxidation of hypoxanthine or xanthine by xanthine oxidase (Bostek, 1989; Halliwell & Gutteridge, 1989). Potassium superoxide has also been used to induce the formation of $\text{O}_2^{\bullet-}$ (Bielski & Allen, 1977). Simply mixing cuprous copper, or ferrous iron, with oxygenated water is not found among the published methods used to generate $\text{O}_2^{\bullet-}$ in vitro. In oxygenated 0.2 M phosphate buffer at pH 6.5 with 100 μM added ferrous sulfate, no H_2O_2 was detected at 0.5 or 15 min after mixing using the LCV assay ($n = 3$ for each time).

3.3 Ascorbic acid degradation at alkaline pH

Because food products containing ascorbic acid are, on occasion, exposed to alkaline processing conditions (Favell, 1998; Sumner, Nielsen, & Youngs, 1981), the

effect of alkaline pH on the degradation of ascorbic acid was also examined. The amount of hydrogen peroxide generated at pH 8.0 is presented in Table 1. In **Figure S1** the rates of ascorbic acid degradation in non-oxygenated nanopure water, and in 0.2 M carbonate buffers at pH 8.0, 9.0 and 10.2 are presented. The acceleratory effect of alkaline conditions on the degradation of ascorbic acid is well known (Buettner & Jurkiewicz, 1996) and the relative stability of ascorbic acid in nanopure water at pH 4 is also evident. The lower initial absorbance is due to the difference in the absorption spectrum of ascorbic acid in an acidic pH (Haase & Dunkley, 1969). The contribution of ROS generated from hydrogen peroxide, which is produced from the degradation of ascorbic acid, is evident from the stability of the samples flushed with argon at pH 9.0. The addition of 1 mM of the chelating agent diethylenetriaminepentaacetic acid (DTPA) (which is particularly effective at binding copper) also strongly inhibited the degradation of ascorbic acid. The initial rate of ascorbic acid degradation in carbonate buffer without added oxygen at pH 10.2 occurred at approximately $1.45 \times 10^{-7} \text{ M} \cdot \text{s}^{-1}$. Changing the carbonate buffers pH to 8.0 reduced the rate of ascorbic acid degradation to approximately $4.02 \times 10^{-8} \text{ M} \cdot \text{s}^{-1}$.

3.4 *Carbonate luminescence from ascorbic acid degradation*

Another potential consequence of the production of hydrogen peroxide, and subsequent formation of the hydroxyl radical, is the generation of carbonate radicals. Chemical luminescence (CL) was produced in the carbonate buffers in the presence of ascorbic acid without the addition of any CL enhancers (e.g., luminol) (**Figure S2**). Under these conditions peak luminescence occurred at about 22 min. This indicates that the hydrogen peroxide and subsequent daughter radicals produced from the

reaction of ascorbic acid, oxygen and copper produce sufficient levels of ROS to convert the carbonate or bicarbonate ions into carbonate radicals, which can themselves produce chemical luminescence without added luminescence enhancers (Hodgson & Fridovich, 1976). Several investigations have demonstrated the ability of powerful radicals (e.g., hydroxyl and peroxynitrite-mediated processes) to produce carbonate radicals from carbonate ions (Bonini, Radi, Ferrer-Sueta, Ferreira, & Augusto, 1999) (Queliconi, Marazzi, Vaz, Brookes, Nehrke, Augusto, et al., 2013).. The carbonate radical ($E^\circ=1.78$ V, pH 7.0), while not as strong an oxidant as the hydroxyl radical, has a longer lifetime and the ability to diffuse and cause significant biological damage when reacting with tyrosine and tryptophan (Augusto, Bonini, Amanso, Linares, Santos, & De Menezes, 2002), and DNA damage (Shafirovich, Dourandin, Huang, & Geacintov, 2001) and oxidative damage occurring after ischaemia-reperfusion (Queliconi, et al., 2013).

4. Conclusions

The findings reported in this paper demonstrate that both the first and second one-electron oxidative reactions of ascorbic acid (forming $\text{Asc}^{\cdot-}$ and then DHA) are strongly dependent on the presence of oxygen. The paper is also the first to document the rate of hydrogen peroxide generation in relationship to the rate of ascorbic acid degradation in simple buffered solutions at concentrations of ascorbic acid, oxygen and copper relevant to food products. Furthermore, the occurrence of a feedback mechanism is demonstrated where the hydrogen peroxide and daughter radicals generated during ascorbic acid oxidation in turn contribute to the oxidation of the

residual ascorbic acid molecules. The findings of this paper most closely support the proposed mechanisms of Jameson and Blackburn (Jameson & Blackburn, 1976); however, the existence of a hydrogen peroxide/hydroxyl radical feedback mechanism greatly complicates these reactions. The findings of this current study also demonstrate the optimum levels of ascorbic acid affecting the generation of hydrogen peroxide under the conditions examined. When the level of ascorbic acid is decreased or increased from this optimum range (0.1 to 0.2 mM), the amount of hydrogen peroxide generated decreased. Knowing the optimum level of ascorbic acid in these ROS-generating reactions can be used to control the generation of hydrogen peroxide and daughter radicals, and minimise oxidative damage in food products.

Research Support: The information reported in this paper (No. 15-07-017) is part of a project of the Kentucky Agricultural Experiment Station and is published with the approval of the Director.

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Figures for: Oxygen Dependency of One-Electron Reactions Generating Ascorbate Radicals and Hydrogen Peroxide from Ascorbic Acid

Figure Captions

Figure 1. Degradation of ascorbic acid (0.1 mM) with different levels of added copper(II) sulfate at pH 6.5 in 0.1 M phosphate buffer as measured by the decrease in absorbance at 265 nm. All buffers were oxygenated except where indicated otherwise. $n = 3$

Figure 2. Hydrogen peroxide concentrations generated over time in mixtures of “oxygenated” 0.1 M phosphate buffer pH 6.5, added copper(II) sulfate (2.5 μ M) and ascorbic acid (0.1 mM) using the leuco crystal violet assay. The corresponding degradation of ascorbic acid was measured by the decrease in absorbance at 265 nm under the same conditions. $n = 3$

Figure 3. Effect of removing oxygen via an argon flush and catalase (150 U/mL) on the degradation of 0.1 mM ascorbic acid with 2.5 μ M added copper(II) sulfate at pH 6.5 in “oxygenated” 0.1 M phosphate buffer as measured by the decrease in absorbance at 265 nm. Non-enzymatic effect from added protein is shown using heat-denatured catalase and the same amount of BSA. $n = 3$

Figure 4. Degradation of ascorbic acid (0.1 mM) as measured by the decrease in absorbance at 265 nm in 0.2 M phosphate buffer pH 6.5 flushed with argon to remove oxygen, and with different levels of copper. Where noted H_2O_2 was added to achieve a concentration of 100 μ M after 12 min ($n = 3$).

Figure 5. Comparison of ascorbate radical levels in buffered solutions flushed with argon vs oxygen in samples which contained 0.1 mM ascorbic acid and 2.5 μ M added copper in 0.2 M phosphate buffer at pH 6.5 ($n = 3$)

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Tables for: Oxygen Dependency of One-Electron Reactions Generating Ascorbate Radicals and Hydrogen Peroxide from Ascorbic Acid

Table 1. Hydrogen peroxide (μM) generated from buffered solutions of ascorbic acid¹

Buffer	Initial Ascorbic Acid Concentration					
	0.05 mM	0.1 mM	0.2 mM	0.4 mM	0.8 mM	1.2 mM
0.2 M Phosphate Buffer (pH 6.5) ²	15.90 (1.61)a	22.94 (0.49)ab	32.46 (3.70)c	25.85 (4.60)bc	1.42 (0.79)d	None Detected
0.2 M Carbonate Buffer (pH 8.0) ³	–	22.97 (2.33)	–	–	–	–

Values in parentheses are standard deviations

¹ Mean of values taken at 15 min (n=3)

² Oxygenated buffer with 2.5 μM copper(II) sulfate added

³ Without added oxygen or copper

Values within rows with different letters differ ($P < 0.05$)