

# ASCORBATE OXIDATION: UV ABSORBANCE OF ASCORBATE AND ESR SPECTROSCOPY OF THE ASCORBYL RADICAL AS ASSAYS FOR IRON

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Catalytic transition metals are an absolute requirement for the aerobic oxidation of ascorbate monoanion. Thus, for example, the concentration of iron can be determined by the metal-dependent rate of ascorbate oxidation in near-neutral solutions. The lower limit of detection of iron, as Fe(III)EDTA, by monitoring the decrease in absorbance at 265 nm of ascorbate is about 200 nM. However, by measuring the concentration of the ascorbyl radical by ESR spectroscopy the lower limit is about 10 nM.

Using these assays, I have shown that the typical microliter laboratory syringe can introduce significant iron into solutions. Thus, for studies involving iron, these two tests can be used to determine the amount of contaminating iron in reagents as well as iron from other sources such as laboratory equipment.

**KEY WORDS:** Ascorbate, ascorbyl radical, ESR, iron, iron-EDTA, syringe.

**ABBREVIATIONS:** A<sup>-</sup>, ascorbate radical; AH<sup>-</sup>, ascorbate monoanion; EHPG = N,N'-ethylenebis [2-(2-hydroxyphenyl) glycine], CAS Reg # 1170-02-1; 3-CP, 3 carboxy-proxyl, CAS Reg # 2154-68-9.

## INTRODUCTION

Adventitious iron, because of its efficacy as a redox catalyst, can pose problems for researchers in numerous fields, particularly in the study of oxygen radicals<sup>1</sup> and ascorbate chemistry.<sup>2,3</sup> I have recently demonstrated that with diligent efforts it is possible to remove adventitious metals from buffers and in these buffers ascorbate is quite stable,<sup>2</sup> even at pH 7. Because the oxidation of ascorbate is strictly metal-dependent, the rate of ascorbate oxidation or the concentration of ascorbyl radical can be used to determine the concentration of iron in buffered solutions. With these two assays I also demonstrate that typical  $\mu$ l laboratory syringes can introduce substantial iron into solutions.

## MATERIALS AND METHODS

Ascorbic acid and chelating resin (sodium form, dry mesh 50-100) were from Sigma; EHPG from Aldrich; and EDTA from Fisher. Adventitious metals were removed from the 50 mM pH 7.40  $\pm$  0.04 phosphate buffer solution by batch treatment with chelating resin.<sup>2</sup> After treatment with chelating resin, the decrease in absorbance of ascorbate in the standard 15 minute test<sup>2</sup> was reduced from 11% to 0.3% or less. Stock FeCl<sub>3</sub> in 0.01 M HCl was standardized with EHPG ( $\epsilon_{470}$  of Fe(III)EHPG = 4800 M<sup>-1</sup> cm<sup>-1</sup>, pH 7.4). The EDTA (tetra acid) contains 40 ppm iron, Fisher lot analysis. Thus, a 250  $\mu$ M EDTA solution contains  $\approx$  50 nM

Fe(III)EDTA, which causes a significant interference in the ESR-ascorbyl radical assay. Four recrystallizations of the EDTA using high purity water yielded an EDTA that produced no significant interference from adventitious Fe(III)EDTA in the assay.

The standard curve of ascorbate oxidation was determined by the decrease of its absorbance at 265 nm ( $\epsilon_{265} = 14,500 \text{ M}^{-1} \text{ cm}^{-1}$ )<sup>2</sup> in 50 mM pH 7.4 phosphate buffer solutions containing 125  $\mu\text{M}$  ascorbate (initial absorbance 265 nm = 1.8, 1 cm), 50  $\mu\text{M}$  EDTA, and varying amounts of Fe(III). The order of addition was EDTA, iron, buffer, and ascorbate, respectively.

The ascorbyl radical concentration was determined by using 3-carboxy-proxyl nitroxide radical as a standard. Because many factors affect ESR signal area measurements,<sup>4</sup> the 3-CP standard and  $\text{A}^\cdot$  experimental spectra were obtained using identical physical arrangements of the samples and identical instrument settings, except for receiver gain. ESR spectra were recorded with a Varian E-4 system equipped with a  $\text{TM}_{110}$  cavity; samples were placed in a TM flat cell. All ESR spectra were collected using a modulation amplitude of 0.63 G. The (time constant)/(scan rate) ratio was kept constant at  $1\text{s}/2.5 \text{ G min}^{-1}$  for all spectra. Double integration of the spectra was accomplished with aid of the free radical simulation program of Oehler and Janzen.<sup>5</sup> The 3-CP lineshape could be simulated with  $\Delta H_{\text{pp}} = 1.275 \text{ G}$  and 70% Gaussian-30% Lorentzian shape function, while the  $\text{A}^\cdot$  radical lineshape was simulated using  $\Delta H_{\text{pp}} = 0.60 \text{ G}$ ,  $a^{\text{H}} = 1.83 \text{ G}$ , and a 100% Gaussian shape function. These parameters showed that for  $\text{A}^\cdot$  and 3-CP lines of equal height, the relative area for the two species is: (area 3-CP)/(area  $\text{A}^\cdot$ ) = 8.62, for the instrument settings used. This information allowed the calculation of the  $\text{A}^\cdot$  concentration from simple signal height measurements using 3-CP as a standard. The ascorbate concentration used in the ESR studies was 250  $\mu\text{M}$ .

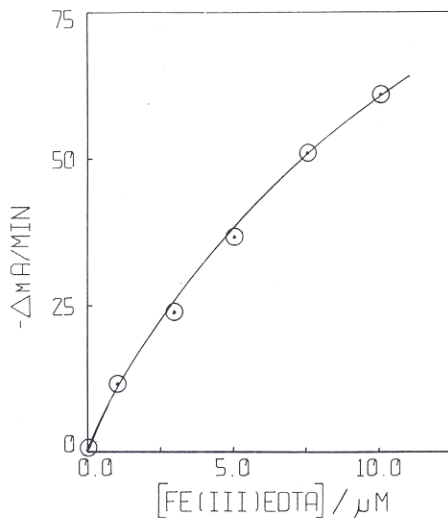


FIGURE 1 This curve was obtained in 50 mM demetallated phosphate buffer pH 7.40 containing 50  $\mu\text{M}$  EDTA and 125  $\mu\text{M}$  ascorbate (265 nm absorbance, 1 cm, at time zero = 1.8). mA = milliabsorbance units

The syringes were from the Hamilton Company, Reno, Nevada, Models 705-N, 1705-TEF LL (gas tight), and 1725 TEF LL (gas tight). EDTA ( $50\ \mu\text{M}$ )-was added to the buffer to convert the iron introduced by the syringe to EDTA chelated iron. Subsequently,  $125\ \mu\text{M}$  ascorbate was added and the rate of ascorbate oxidation was determined. The iron content was calculated from a standard curve.

## RESULTS

### *Loss of Ascorbate, 265 nm*

As seen in Figure 1, the rate of ascorbate oxidation in the air-saturated  $50\ \text{mM}$  pH 7.4 phosphate buffer is a function of the amount of Fe(III)EDTA present. Converting iron in the buffered solution to Fe(III)EDTA allows one to determine the concentration of iron by comparison to a standard curve of ascorbate oxidation versus Fe(III)EDTA concentration. This allows the determination of the iron in the buffer, provided the iron is present as Fe(III)EDTA. The lower limit of detection appears to be  $\approx 0.2\ \mu\text{M}$  iron.

### *Ascorbyl Radical*

The ascorbyl radical concentration is also a function of the amount of Fe(III)EDTA present in the buffer solution (Figure 2). Thus, it is possible to determine the iron concentration of a buffer solution provided the iron is present in a defined catalytic state such as Fe(III)EDTA. However, with the ESR-ascorbyl radical method, the lower limit of detection is on the order of  $10\ \text{nM}$  Fe(III)EDTA. It must be emphasized that is necessary to remove adventitious iron from the EDTA to achieve this level of

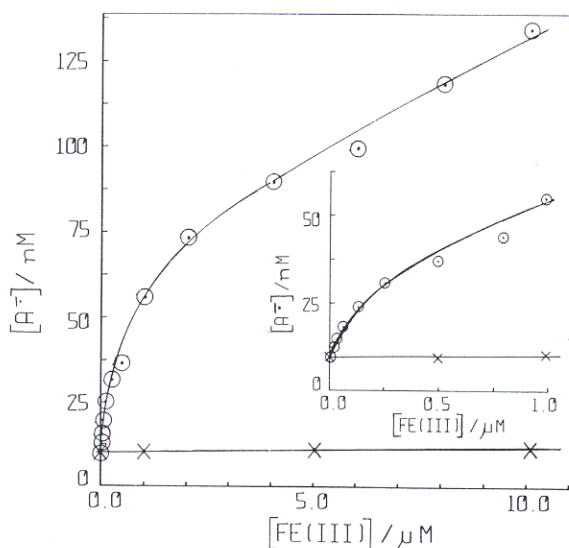


FIGURE 2 These curves were obtained in  $50\ \text{mM}$  demetalled phosphate buffer pH 7.40 containing  $250\ \mu\text{M}$  ascorbate and  $250\ \mu\text{M}$  EDTA (O) or  $50\ \mu\text{M}$  Desferal (X).

TABLE 1

Syringe	[iron]/ $\mu\text{M}$	
	Method: $\text{AH}^-$ oxidation rate	ESR- $[\text{A}\cdot^-]$
none; demetalled buffer only	$< 0.2^a$ (4)	$< 0.010$ (8)
Hamilton, 705-N <sup>b</sup>	$5.8 \pm 3.9$ (5)	$5.0 \pm 2.9$ (5)
Gas-tight, Hamilton 1705-TEF LL <sup>c</sup> (stainless steel needle <sup>d</sup> )	$< 0.2$ (4)	$0.18 \pm 0.12$ (5)
1705-TEF (with Teflon <sup>®</sup> needle)	$< 0.2$ (4)	$0.14 \pm 0.03$ (5)
1725-TEF LL <sup>c</sup> (stainless steel needle <sup>d</sup> )	$< 0.2$ (4)	$0.061 \pm 0.008$ (5)
1725-TEF LL (Teflon <sup>®</sup> needle)	$< 0.2$ (4)	$0.015 \pm 0.007$ (5)

<sup>a</sup>The value quoted is the median value (or upper limit) along with the 95% confidence interval, when appropriate; (n) = number of trials.

<sup>b</sup>Ground glass barrel, stainless steel plunger.

<sup>c</sup>This gas-tight syringe has a smooth barrel, stainless steel plunger and Teflon<sup>®</sup> plug.

<sup>d</sup>2 inch 22S stainless steel needle.

<sup>e</sup>This gas-tight syringe has a smooth barrel and Teflon<sup>®</sup> plunger and plug.

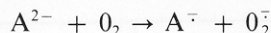
sensitivity. Desferal<sup>®</sup> is able to remove completely the catalytic activity of iron in the oxidation of ascorbate. See Figure 2. However, to achieve these results, especially at the higher concentration of iron, it was necessary to incubate the Desferal<sup>®</sup> with the iron supplemented buffer for approximately one to two hours before the chelation of iron by Desferal<sup>®</sup> was complete.

### Syringes

Both methods for Fe(III)EDTA determination were applied to buffer solutions that had been in typical laboratory syringes (Table 1). There was great variability in the observed iron levels and as expected the longer the buffer solution was in the syringe the higher the iron level observed (not shown). All syringes added iron to the buffer, and not surprisingly, the less contact made with stainless steel syringe components, the lower the level of iron added by the syringe to the buffer.

## DISCUSSION

Two analytical approaches using ascorbate were compared for their sensitivity to detect iron in buffer solutions. Monitoring the decrease in the 265 nm absorbance of ascorbate is a quick and easy method that uses readily available equipment. However, it is less sensitive than the ESR-ascorbyl radical method. To achieve high sensitivity in the ESR-ascorbyl radical approach it is imperative that adventitious iron be removed from the EDTA. It should be noted that the concentration of  $\text{A}^-$  in the absence of catalytic transition metals or the presence of Desferal<sup>®</sup> is not zero. I believe this nonzero  $[\text{A}^-]$  is due to the true autoxidation\*\* of ascorbate; it is most likely the ascorbate dianion that is reacting with oxygen



The chemistry and kinetics of this process are now under investigation.

\*\*True autoxidation is defined as the spontaneous oxidation in air of a substance not requiring catalysts.



Copper is also a common contaminant in buffer solutions and because of its high catalytic efficiency in ascorbate oxidation it could produce significant interference in these assays.<sup>2,3</sup> However, EDTA significantly slows the catalysis of ascorbate oxidation by copper and therefore low levels of Cu(II)EDTA ( $\leq 1 \mu\text{M}$ ) will not be an interference in the UV absorbance method. However, in the ESR-A<sup>-</sup> assay for iron, as Fe(III)EDTA,  $1 \mu\text{M}$  Cu(II)EDTA will be equivalent to  $\approx 20 \text{ nM}$  Fe(III)EDTA. This possible low level interference should be kept in mind.

As seen in Table 1 the use of common laboratory  $\mu\text{l}$  syringes can introduce substantial iron. However, the use of the gas tight syringes substantially reduces this possible iron contamination, because it is the Teflon<sup>®</sup> plug that provides the plunger seal in the smooth barrel, rather than a stainless steel plunger in a ground glass barrel. With the ESR-A<sup>-</sup> approach it was even possible to determine that the 2 inch 22S gauge stainless steel needle used in these experiments introduced  $\approx 50 \text{ nM}$  iron into the buffer solution. See Table 1. Naturally if syringes are required the best results will be obtained with a syringe that has both a Teflon<sup>®</sup> plunger and needle.

I have previously described a method to test for the presence of adventitious catalytic metals in simple near-neutral buffer solutions.<sup>2</sup> This method monitors the loss of the 265 nm absorption of ascorbate. However, the ESR-ascorbate radical approach could also be used as a test. The ESR approach would provide a more sensitive test for low levels of contamination.

## CONCLUSION

If iron is diligently removed from buffer solutions, the rate of ascorbate oxidation can provide an analytical approach to the determination of micromolar levels of iron in near-neutral simple buffers. However, the ESR-ascorbyl radical method is able to estimate nanomolar levels of iron, as low as  $10 \text{ nM}$  iron as Fe(III)EDTA.

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