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In the absence of catalytic metals ascorbate does not autoxidize at pH 7: ascorbate as a test for catalytic metals

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Summary

Trace amounts of adventitious transition metals in buffer solutions can serve as catalysts for many oxidative processes. To fully understand what role these metals may play it is necessary that buffer solutions be 'catalytic metal free'. We demonstrate here that ascorbate can be used in a quick and easy test to determine if near-neutral buffer solutions are indeed 'catalytic metal free'. In buffers which have been rendered free of catalytic metals we have found that ascorbate is quite stable, even at pH 7. The first-order rate constant for the loss of ascorbate in an air-saturated catalytic metal free solution is less than 6×10^{-7} s^{-1} at pH 7.0. This upper limit appears to be set by the inability to completely eliminate catalytic metal contamination of solutions and glassware. We conclude that in the absence of catalytic metals, ascorbate is stable at pH 7.

Key words: Ascorbate; Iron; Copper; Autoxidation

Introduction

Trace amounts of adventitious transition metals, which are naturally present in buffer solutions, have been recognized as presenting many problems for researchers [1–17]. These trace levels of transition metals are able to catalyze reactions such as ascorbate oxidation [1,4,16] and the metal-catalyzed Haber-Weiss reaction [11]. Thus, for experiments in which low concentrations of catalytic metals are an important determinant of the result, these metals must be sequestered in an inactive form or removed from the reaction mixture. While ascorbate readily autoxidizes,

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Abbreviations: Åbs, d(Absorbance)/dt; AH_2, ascorbic acid, $pK_1 = 4.2$, $pK_2 = 11.6$ [21]; AH^-, ascorbate monoanion; A^{2-}, ascorbate dianion; A^-, ascorbate radical; mAU, milliabsorbance units, 1 cm path.

catalytic transition metals such as copper or iron are required for this oxidation to proceed with an appreciable rate at acid or neutral pH [1,16,18–20]. Removal of these metals from buffer salt solutions significantly slows this process [16]. We demonstrate that in the absence of catalytic metals, ascorbate does not autoxidize at pH 7. Thus, ascorbate can be used as a test for the presence of adventitious catalytic metals in buffer salt solutions. We present here a quick and easy method by which near-neutral buffer salt solutions can be checked for their catalytic metal activity. In addition, we offer a reinterpretation of some of the ascorbic acid literature in which contaminating catalytic metals were present.

Materials and Methods

Ascorbic acid (lot analysis indicates 0.0002% Fe), sodium monohydrogen phosphate (lot analysis indicates 0.0003% Fe), sodium dihydrogen phosphate (lot analysis indicates 0.001% Fe), and Tris (tris(hydroxymethyl)aminomethane) were from J.T. Baker. Iminodiacetic acid, Tris-HCl, and conalbumin (Type IV) were from Sigma. Chelex 100 (200-400 mesh sodium form) was from Bio-Rad Laboratories. Water used in solution preparations was purified with a Milli-Q water system (resistivity = 18 $M\Omega \cdot cm^{-1}$). All reagents were used as received, except where otherwise noted.

Absorbance measurements were done with a Perkin-Elmer Lambda 5 UV/VIS spectrophotometer. Each ascorbate sample (in a standard 1 cm quartz cuvette) was continuously monitored at 265 nm.

For the standard test, ascorbic acid is prepared as a 0.100 M stock solution (10 ml) using high purity water such as is produced by the Milli-Q water system. This solution is colorless, having a pH of ≈ 2. It is stored in a volumetric flask with a tight-fitting plastic stopper, thus oxygen is kept from the solution during long-term storage. As the solubility of oxygen in air-saturated water is ≈ 0.25 mM, the solution will become anaerobic with loss of $< 1\%$ of the original ascorbate. We have found that the solution can be kept for several weeks without significant loss of ascorbate due to the low pH and lack of oxygen. The appearance of a yellow color is an indication of ascorbate deterioration. We avoid the use of sodium ascorbate as it invariably contains a significant quantity of oxidation products as evidenced by the yellow color of the solution.

In a typical test, ≈ 3.5 µl of the ascorbate stock is added to 3 ml of the buffer salt solution to be tested. This results in an initial ascorbate absorbance of 1.5–2.0 at 265 nm. The molar extinction coefficient of ascorbate used here is 14500 $M^{-1} \cdot cm^{-1}$ at 265 nm. However, values ranging from 7500 to 20400 $M^{-1} \cdot cm^{-1}$ have been reported [21]. The ascorbate absorbance at 265 nm is followed for 15–30 min.

When possible, buffer solutions are prepared by weighing out the appropriate amounts of buffer salts for a 1.0 M stock solution that will produce the desired pH upon dilution to the working concentration. This avoids the use of acids or bases for pH adjustment which may be a source of additional adventitious catalytic metals.

Chelex 100 treatment for the removal of contaminating metals can be accomplished using the batch method, the dialysis method or the traditional column method. In the batch method, the resin is washed with portions of the buffer solution to be treated, then rinsed with high purity water. The washed resin is then added to the solution, using 5–10 ml of resin per liter of solution, and stirred overnight (shorter times can be used if more resin is used). After any necessary pH adjustments and additional stirring, the resin is usually filtered from the buffer. However, it may be left in the solution and allowed to settle to the bottom before withdrawing portions for use, being sure to check for suspended resin.

In the dialysis method, ≈ 7 g of washed Chelex 100 resin is suspended in ≈ 9 ml of the buffer. The end of a disposable 5 ml plastic pipette tip is enlarged and used to transfer the Chelex 100 suspension to a suitable length of boiled Visking^{R} (or similar) dialysis tubing. The cleaned sack is placed in 250–500 ml of buffer and stirred overnight. The dialysis sack may be left in the solution to help overcome problems with catalytic metal enrichment from the glassware and atmosphere. The dialysis method provides a simple way to easily remove the Chelex 100 from the solution.

For experiments in which the catalytic activity of < 1 μM Cu(II) was examined, stock solutions of 0.060 mM (pH 1.5) and 0.60 mM (pH 1.5) $CuCl_2$ were prepared. The order of reagent addition to the cuvette containing the chelexed buffer solution was: Cu(II) stock (followed by shaking), then enough ascorbate to produce a solution with an absorbance of 1.8 ± 0.1 at 265 nm, 1 cm, corresponding to a concentration of 125 ± 7 μM. The loss of ascorbate (absorbance at 265 nm) was followed for ≈ 5 min. As the loss was nearly linear in this time, the slope was determined for the time of approximately 1–4 min after the introduction of the ascorbic acid.

For the iron experiments, a stock solution of 0.60 mM $FeCl_3$ was prepared in dilute HCl, pH = 2.0. Standardization of the iron concentration was accomplished using EHPG (ethylenediamine *N,N'*-bis[2-(2-hydroxyphenyl) acetic acid]) [22,23]. Experiments were performed in the same manner as with the copper, except the loss of ascorbate was followed for 15 min.

Adsorption of catalytic metals on the cuvette surfaces proved to be a significant limiting factor for achieving a near 0% loss (pH 7.0 phosphate) of ascorbate in the typical 15-min test period. This problem was especially frustrating after experiments in which micromolar Fe(III) or Cu(II) was added to the reaction mixture. We found the most effective way to reduce this effect was simply to fill the cuvettes with dilute HCl (0.02 M) and let them stand overnight. After running only a few samples in an acid-soaked cuvette, significant loss of ascorbate (greater than 1% in chelexed 50 mM phosphate, pH 7.0) was often observed even if the cuvette was copiously washed with dilute acid and purified water.

Dialysis against conalbumin was done as outlined by Gutteridge [17]. 0.5 g conalbumin and 40 mg $NaHCO_3$ were dissolved in 10 ml of the buffer and transferred to the boiled Visking^{R} dialysis tubing. After thorough washing to remove any protein that might have been spilled on the outside surface, the sack was placed in 300 ml of buffer and stirred overnight ($\approx 4\,^{\circ}$C).

Results

Ascorbate test

As seen in Table 1, ascorbate autoxidation provides a simple test to determine if buffer solutions are 'catalytic metal free'. The per cent ascorbate lost ranged from 5–11% in untreated phosphate buffer to 0.6% or less after Chelex 100 treatment. The changes observed in Tris or Krebs-Ringer phosphate buffer were less dramatic, but at pH 7.4 ascorbate is a good indicator for catalytic metal content, provided clean cuvettes are used. However, at pH 9.0 or above, where A^{2-} will be a significant species, ascorbate is of little value as an indicator.

TABLE 1

ASCORBATE LOSS IN BUFFER SOLUTIONS

Solution	Treatment & additions	% Ascorbate lost in 15 min [a]
50 mM acetate buffer, pH 5.0	none	1.1 (0.1) [b]
50 mM acetate buffer, pH 5.0	Chelex 100	0.1 (0.09)
50 mM phosphate [c], pH 6.0	none	4.8 (0.8)
50 mM phosphate, pH 6.0	Chelex 100	0.3 (0.2)
50 mM phosphate, pH 7.0	none	10.7 [d] (2.7)
50 mM phosphate, pH 7.0	none + 50 μM EDTA	1.1 (0.4)
50 mM phosphate, pH 7.0	Chelex 100	0.5 (0.05)
50 mM phosphate, pH 7.0	Chelex 100	< 0.05 [e] (0.04)
50 mM phosphate, pH 7.0	dialysis-conalbumin	1.1 (0.6)
50 mM phosphate, pH 7.0	dialysis-conalbumin + 50 μM EDTA	0.4 (0.04)
50 mM phosphate, pH 7.0	dialysis-Chelex 100	0.5 (0.5)
50 mM phosphate, pH 7.0	Chelex 100 + 1 μM Cu(II)	27 (2.5) [f]
50 mM phosphate, pH 7.0	Chelex + 1 μM Cu(II) + 50 μM EDTA	0.4 (0.05)
50 mM phosphate, pH 7.8	none	8.4 (2.5)
50 mM phosphate, pH 7.8	Chelex 100	0.6 (0.2)
Krebs-Ringer phosphate, pH 7.4 [g]	none	1.1 (0.1)
Krebs-Ringer phosphate, pH 7.4	Chelex 100	0.5 (0.1)
50 mM Tris, pH 7.4	none	1.8 (0.2)
50 mM Tris, pH 7.4	Chelex 100	0.5 (0.3)
50 mM Tris, pH 9.0	none	3.6 (0.5)
50 mM Tris, pH 9.0	Chelex 100	3.1 (0.7)

[a] The results quoted are the median of at least four trials.

[b] Errors quoted are standard deviations.

[c] Phosphate implies a NaH_2PO_4-Na_2HPO_4 buffer prepared as in Materials and Methods.

[d] This may appear to be at odds with the results reported in [16], but different sources and grades of phosphate were employed.

[e] Results obtained from the very first test using cuvettes that had been soaked in HCl (0.02 M) overnight.

[f] This is the loss in 5 min rather than 15 min of the standard test.

[g] Ca-free Krebs-Ringer solution contained 120 mM NaCl, 5 mM KCl, 1.3 mM $MgCl_2$ and 16 mM sodium phosphate buffer, pH 7.4.

Iron and copper

Iron and copper are common trace level contaminants in reagents. From the lot analyses provided by Baker Chemical, the Fe(III) concentration in the 50 mM, pH 7.0, phosphate buffer is estimated to be 0.7 μM. In Fig. 1, we see that the rate of ascorbate loss is directly proportional to the iron concentration. EDTA increased the catalytic activity of Fe(III), as is well known [11,18–20]. However, Cu(II) is a much more effective catalyst than Fe(III) or Fe(III)-EDTA. In comparing Fig. 1 and 2, we see that Cu(II) is $\approx$ 80 times more effective than Fe(III) and $\approx$ 20 times more effective than Fe(III)-EDTA (50 mM phosphate buffer, pH 7.0). But in contrast to Fe(III), the addition of EDTA to the copper-ascorbate system stops the oxidation of ascorbate. See Table 1.

The inclusion of EDTA in the untreated phosphate buffer (50 mM, pH 7.0) -ascorbate system slowed the oxidation in the standard test from 10.7% loss ($\dot{A}bs$ = 12.8 mAU/min) to 1.1% ($\dot{A}bs$ = 1.3 mAU/min). From this observation we conclude that the majority of the catalytic activity observed is not due to iron. Using the Fe(III)-EDTA data presented in Fig. 1, the 1.1% loss of ascorbate indicates an Fe(III) concentration of $\approx$ 0.3 μM, which is in remarkable agreement with that estimated from the Baker analyses.

If we subtract the catalytic activity due to Fe(III) in the untreated buffer from the total activity ($\dot{A}bs$ = 12.8 − 0.3 = 12.5 mAU/min), we have the catalytic activity

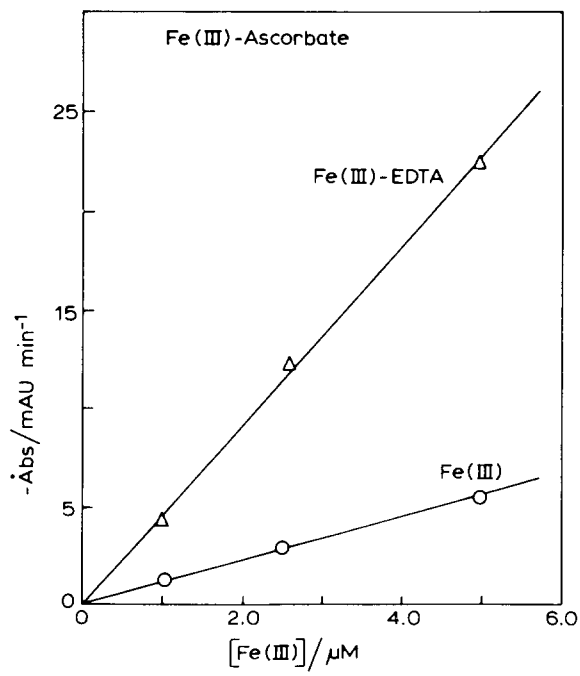

Fig. 1. Fe(III) and Fe(III)-EDTA catalyzed oxidation of ascorbate (125 ± 7 μM) in Chelex 100-treated 50 mM phosphate buffer, pH 7.0. ○ = Fe(III); △ = Fe(III)-EDTA. The ordinate represents the rate of loss in the 265 nm absorption of ascorbate in units of milliabsorbance units/min (1 cm path).

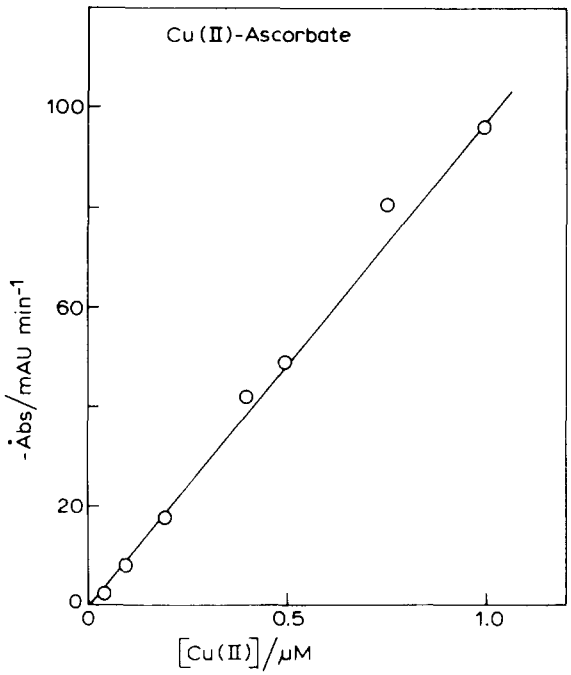

Fig. 2. Cu(II) catalyzed oxidation of ascorbate (125 ± 7 μM) in Chelex 100-treated 50 mM phosphate buffer, pH 7.0. The ordinate represents the rate of loss in the 265 nm absorption of ascorbate in units of milliabsorbance units/min (1 cm path). The addition of 50 μM EDTA to a 1 μM Cu(II) solution reduced the rate of ascorbate loss from 96 to 0.4 mAU/min^{-1}.

due to copper. Then from the data of Fig. 3 we can estimate that the concentration of copper in the untreated phosphate buffer is ≈ 0.13 μM. Unfortunately, the Baker lot analyses did not include copper to allow a comparison.

After Chelex 100 treatment of the pH 7.0 phosphate buffer, the loss of ascorbate in the standard test is reduced to 0.4% ($\dot{A}bs = 0.5$ mAU/min). However, in trials in which the standard test was performed as the first sample in a cuvette which had been soaked overnight in dilute HCl, this value was 0.1% ($\dot{A}bs = 0.12$ mAU/min) or less, the median value being 0.05%

From the values for the rate of ascorbate loss presented in Fig. 1 and 2, we can set an upper limit of 0.1 μM, using 0.1% loss, for the concentration of Fe(III) in Chelex 100-treated 50 mM phosphate buffer, pH 7.0. For copper this limit is on the order of 0.001 μM. These concentrations must be considered as estimates as we are at the limit of detection by this technique. We are near (or in) the noise level of the experiment due to additional phantom sources of catalysis such as Fe or Cu in the ascorbate, metals on the glassware and pipette surfaces, dust in the air and the like.

Dialysis of the 50 mM, pH 7.0, phosphate buffer against conalbumin or Chelex 100 reduced the catalytic metal content of the buffer as demonstrated by the standard ascorbate test (see Table 1). However, residual catalytic activity was observed in the buffer dialyzed against conalbumin. The addition of 50 μM EDTA

to this buffer reduced this 1.1% loss to 0.4%. Thus, iron appears to be removed from the solution, but some residual copper remains, 10–15 nM. Dialysis against Chelex 100 reduced both the copper and iron catalytic activity, yielding the same results in the standard test as the Chelex 100 batch method.

Kinetics

The oxidation of ascorbate (excess oxygen) has been found to be first-order with respect to the concentration of ascorbate anion in the pH range 2–6 [19]. At pH 7.0, the dominant species for ascorbate will be AH^- (99.9%) with low concentrations of AH_2 (0.1%) and A^{2-} (0.005%). Under these conditions the pseudo-first-order rate equation can be written as

$$-dT_A/dt = k_2[AH^-].$$

where T_A is the total concentration of all ascorbate species.

In the experiments with acid-soaked cuvettes and chelexed pH 7.0 phosphate buffer, the lowest consistent loss (median of eight samples) of ascorbate in the standard 15 min test was 0.05%. This yields an upper limit for k_2 of 6×10^{-7} s^{-1}.

For the iron- and copper-catalyzed oxidation of ascorbate (excess oxygen) the rate of loss of ascorbate in pH 7.0 phosphate buffer may be written as

$$-dT_A/dt = k_{cat}[AH^-][\text{Cu(II) or Fe(III)}].$$

From the data of Figs. 1 and 2, k_{cat} for Fe(III) = 10, k_{cat} for Fe(III) – EDTA = 42, and k_{cat} for Cu(II) = 880 ($M^{-1} \cdot s^{-1}$, 20 °C). It should be kept in mind that ferric and cupric ions will undergo hydrolysis at pH 7. Thus, these rate constants do not represent rate constants for $Fe(III)_{aq}$ or $Cu(II)_{aq}$, but rather are rate constants for the analytical concentration of Fe(III) and Cu(II) without regard to the concentration of the various hydrolysis species present.

Discussion

Tables 2 and 3 are a compilation of the levels of contaminating iron and copper found in commonly used reagents. Unless care is taken to reduce these naturally occurring concentrations, or the metals are complexed in a catalytically inactive form, these catalytic metals can present serious problems, in the interpretation of experimental results [1–17]. These problems can in principle be overcome by use of ion-exchange resins such as Chelex 100 or by dialysis such as used by McDermott et al. [4] and modified by Gutteridge [17]. However, each technique has its problems.

Chelex 100

The use of Chelex 100 to de-metal acids or bases will result in an increase in the catalytic metal concentration [13] (see Table 2). This is to be expected as strong acid

TABLE 2

TRACE IRON CONCENTRATIONS IN SOLUTIONS

Reagent	Treatment	[Fe]/μM [a]	Ref.
200 mM acetate, pH 5.5 & 6.5	none	10.1 - 27.8	4
200 mM acetate, pH 5.5 & 6.5	Chelex-100	~ 0.014 [b]	4
200 mM acetate/ceruloplasmin, pH 6.5	Chelex-100	~ 0.4 [c]	4
200 mM acetate/ceruloplasmin, pH 6.5	Chelex-100 & dialysis against transferrin	0.007–0.021	4
50 mM phosphate, pH 7.0	none	0.7	Here [d]
50 mM phosphate, pH 7.0	none	0.3	Here [e]
50 mM phosphate, pH 7.0	Chelex-100	< 0.1	Here
100 mM phosphate, pH 7.4	none	1.1	6
150 mM phosphate, pH 7.4	none	2.2	7
150 mM phosphate, pH 7.4	Chelex-100	0.055	7
20 mM phosphate, pH 7.4	none	0.2	14
67.5 mM phosphate, pH 7.4/4.0 mM KCl	none	9.7–19.4	12
100 mM Tris, pH 7.4	none	1.7	17
100 mM Tris, pH 7.4	dialysis against conalbumin	0 [f]	17
100 mM KCl	none	2.5	13
500 mM sodium formate	none	9.3	12
500 mM urea	none	6.2	12
500 mM thiourea	none	3.1	12
100 mM ascorbic acid	none	2.2	13
20 mM ascorbic acid	none	4.3	12
30% H_2O_2	none	1.8–9.1	13
10 mM EDTA	none	0.7	13
10 mm EDTA	Chelex-100	> 75	13
50 mM EDTA	none	7.7–8.9	12
10 mM DETAPAC [g]	none	1.8	13
10 mM DETAPAC	Chelex-100	8.4–24.7	13
4% NaOH	none	7.3	13
4% NaOH	Chelex-100	19.3	13
5% HCl	none	3.6	13
5% HCl	Chelex-100	11.4	13
0.5 mM xanthine	none	1.5	13
500 mM mannitol	none	0 [f]	12
400 mM sucrose	none	1–2	2
21 U/ml xanthine oxidase	none	12.5 [h]	13
8.5 mg/ml hyaluronic acid	none	10.3–14.5	12
Water	double-distilled	0 [f]	12
Water	Chelex-100	0 [f]	12

[a] Most of the values summarized here fall within the range expected when making a solution from reagent grade stock. See, for example, the analyses provided by suppliers such as J.T. Baker, Fluka A.G. or Merck.

[b] We have made this estimate from the data presented in [4], assuming an initial iron concentration of $\approx$ 20 μM. ^{59}Fe was used for the determination. Its concentration decreased to 0.075% of the concentration before Chelex-100 treatment.

[c] We have made this estimate from the data presented in [4], assuming an initial iron concentration of $\approx$ 20 μM. ^{59}Fe was used for the determination. Its concentration decreased to 2% of the concentration before Chelex-100 treatment.

TABLE 2 (continued)

[d] Calculated using the lot analysis supplied by Baker, assuming no contribution from the water.

[e] Determined from the Fe(III)-EDTA data of Fig. 1.

[f] To be interpreted as below the limit of detection, which was not given. A statistical analysis of the limited data presented in [30] suggests that 0.5–0.75 μM Fe is the approximate lower limit of detection by the bleomycin assay.

[g] DETAPAC = DTPA or diethylenetriaminepentaacetic acid.

[h] This is a shockingly large value, however, it must be kept in mind that 21 units/ml is on the order of 1000 times more than would be used in a typical experiment in which xanthine oxidase is used as a means to generate superoxide. In addition, xanthine oxidase contains four irons as Fe_2S_2 groups as part of its catalytic site. This iron, rather than adventitious iron, could easily account for the bulk of the iron observed in this atomic absorption analysis. The authors gave no indication as to whether any correction was made for this intrinsic iron.

TABLE 3

TRACE COPPER CONCENTRATIONS IN SOLUTIONS

Reagent	Treatment	[Cu]/μM [a]	Ref.
1 M KNO_3, pH 6.0	none	0.035	10
1 M KNO_3, pH 6.0	Chelex 100	ND [b]	10
1 M KNO_3, pH 6.0	APDTC [c]	ND	10
1 M NaCl, pH 6.0	none	0.05	10
1 M NaCl, pH 6.0	Chelex 100	ND	10
1 M NaCl, pH 6.0	APDTC	ND	10
1 M KH_2PO_4, pH 6.0	none	0.3	10
1 M KH_2PO_4, pH 6.0	Chelex 100	0.04	10
1 M KH_2PO_4, pH 6.0	APDTC	< 0.03	10
50 mM phosphate buffer, pH 7.0	none	≈ 0.13	Here [d]
50 mM phosphate buffer, pH 7.0	Chelex 100	≈ 0.001	Here [d]
400 mM sucrose	none	0.08–0.3	2
100 mM acetate, pH 5.4 or 6.0	Chelex 100	< 0.16	3
50 mM phosphate, pH 6.0 or 7.4	Chelex 100	< 0.16	3
1 M glycine buffer, pH 8.9	Chelex 100	ND	9

[a] Most of the values summarized here fall within the range expected when making a solution from reagent grade stock. See, for example, the analyses provided by suppliers such as J.T. Baker, Fluka A.G. or Merck.

[b] ND, not detected using atomic absorption.

[c] APDTC, complexing with ammonium pyrrolidinedithiocarbamate followed by extraction with chloroform.

[d] The data of Figs. 1 and 2 were used to make this estimate.

and base are used to regenerate Chelex 100 *. In addition, strong complexing agents such as EDTA are also able to extract metals from Chelex 100 resin, thus it is futile to attempt to de-metal such reagents with Chelex 100 [13]. It follows logically that chelating agents should not be present in buffer solutions when they are to be de-metalled with Chelex 100.

* See Bio-Rad Laboratories product information bulletin 2020.

Chelex 100 provides a means to de-metal simple salt and buffer solutions. However, attempts to remove adventitious metals from protein solutions can present problems. For example, Binder et al. [5] found that Chelex 100 formed a complex with copper-collagen. The collagen could only be eluted from the Chelex 100 column by acidifying the resin. In addition, Buettner [24] observed that Chelex 100 formed a complex with cytochrome *c* at neutral pH, removing it from solution. Thus, protein-metal-Chelex 100 complex formation must be considered when attempts are made to remove metals from protein solutions.

Biological effects are also possible when using Chelex 100-treated solutions. Rayment and Andrew [10] observed retardation of plant growth when Chelex 100-treated solutions were used in their studies. They attributed this to free iminodiacetic acid which can form when Chelex 100 stands for some time. Removal of free iminodiacetic acid from the resin prior to its use eliminated this effect.

Dialysis

Dialysis is an effective method of removing metals from buffer and protein solutions. Transferrin or conalbumin will be effective in removing iron, but some copper may remain and it is possible that protein contaminants may be introduced into the solution. Dialysis against Chelex 100 will remove both copper and iron, providing a means of circumventing the possible introduction of protein contaminants as well as avoiding formation of protein-metal-Chelex 100 complexes. In our experiments Chelex 100 was placed in the dialysis sack to de-metal the buffer. However, to de-metal a protein solution, it may be more appropriate to place the protein in the dialysis sack and dialyze it against a suspension of Chelex 100. This method was used effectively by Harris et al. [8] in their hyaluronic acid depolymerization studies.

Glassware

Buffer solutions can also undergo catalytic metal enrichment during storage ([8,12], personal observations and frustrations). We have found, as have Harris et al. [8], that keeping Chelex 100 in contact with buffer solutions during storage overcomes this problem. However, the ascorbate test provides a quick and easy method to monitor for this potential problem.

Limits of the ascorbate test

The ascorbate test for catalytic metals is most effective in the near-neutral or slightly acid pH range where AH^- is the dominant species. At higher pH values, $> \approx 8$, A^{2-} becomes a significant species. Because it rapidly autoxidizes, the sensitivity of the test is lost. Likewise, at low pH values, AH_2 will dominate and it autoxidizes too slowly [20] to provide a sensitive test. In addition, high concentrations of Cl^- in the media will also reduce sensitivity because high chloride concentrations inhibit (low concentrations can accelerate) the copper-catalyzed oxidation of ascorbate [25,26].

Large variations in the loss of ascorbate in untreated buffers should be anticipated depending on the source and grade of the chemicals and history of the

glassware. Compare, for example, results reported in [16,33,35] and here. Thus, removal of these catalytic metals is often essential for a comparison of experimental results from different laboratories or even within the same laboratory.

Reinterpretation of ascorbic acid literature

The spontaneous oxidation of ascorbate anion at pH 7.0, i.e. in the absence of catalytic metals, is quite slow. We have set an upper limit of $6 \times 10^{-7}\ s^{-1}$. This limit may be the result of catalytic metals which were not removed from the solution by Chelex 100 treatment as well as possible catalytic metals on the cuvette surfaces. Khan and Martell [19] have reported a value of $5.87 \times 10^{-4}\ s^{-1}$ for the spontaneous oxidation of AH^- (in 0.1 M KNO_3). As no efforts were made to remove contaminating catalytic metals, there was undoubtedly substantial Cu(II) and Fe(III) present (see Tables 2 and 3). Thus, we believe this rate constant is grossly overestimated. Using data from other studies, the observed first-order rate constant for the spontaneous oxidation of AH^- was found to be $2 \times 10^{-6} s^{-1}$ (chelexed pH 7.0 phosphate buffer, oxygen-saturated) [31], which is comparable to the value reported here.

In a study of copper and ascorbate oxidation in aqueous humor, Fong et al. [29] have demonstrated that aqueous humor can accelerate or inhibit copper-catalyzed oxidation of ascorbate. Those aqueous humor samples with high copper content (25 μM) contained little or no ascorbate (< 0.1 mg%), while those with low copper (0.5 μM) contained substantial ascorbate (2.2–30 mg%). However, in the light of the data presented here, it is clear they had substantial adventitious copper present in their buffer, ≈ 0.1 μM. In most experiments they added 0.4 μM copper to the incubations. Thus, the actual copper concentration is 0.5 μM. Although the overall conclusions are not changed by the high backround level of copper, the exact interpretation of the data is altered and is consistent with ≈ 0.1 μM adventitious copper.

Mishra and Kovachich have examined the inhibition of ascorbate oxidation by extracts from mammalian nervous tissue and serum [32–34], plant tissues [35] and microorganisms [36]. Their assay medium was typically Krebs-Ringer phosphate buffer, pH 7.4. Thus, the autoxidation of ascorbate they observed is due to adventitious catalytic metals, which are an uncertain element in their control experiments. Without additional control experiments using de-metalled medium, the mechanism for the inhibition of ascorbate oxidation by their extracts cannot be deduced.

Another possible problem which can result from adventitious metals in buffer solutions is synergistic catalytic activity. For example, Matsumura and Pigman [37] found that copper and iron acted synergistically in the metal-catalyzed depolymerization of hyaluronic acid by ascorbate. In a somewhat similar case, Yamazaki [38] found that A^- generated by ascorbate oxidase will reduce cytochrome *c*. However, Weis ([39] and references therein) was unable to reproduce this result unless Cu(II) was present. He suggested that contaminating copper was present in the ascorbate oxidase used by Yamazaki. Thus, adventitious metals can alter reaction endpoints, consequently altering conclusions.

Superoxide and metal catalysis

The generation of the very reactive hydroxyl radical in superoxide-generating systems was first proposed to result from the reaction of O_2^- with H_2O_2 [40]. However, it was soon recognized that iron played a role in this reaction [41,42]. Only trace amounts of iron, the levels present in typical buffer solutions, were required to alter the results observed in superoxide-generating systems [11]. Thus, just as in ascorbate oxidation, adventitious metals can alter the results observed in superoxide-generating systems and must be removed to insure that baseline or control experiments are reliable standards for interpretation of experimental results.

Simplified description of the method and its applications

An easy and rapid method, using ascorbic acid, is presented to determine if simple near-neutral buffer salt solutions are free of catalytic metals. In the standard test, approximately 3.5 μl of 0.100 M ascorbic solution are added to 3 ml of the buffer solution being tested. The loss of ascorbate is monitored for 15 min using its 265 nm absorbance. A loss of more than 0.5% of the ascorbate in this time indicates significant metal contamination. The advantages of this method are: (1) very low cost, (2) short time of determination, (3) it requires no specialized instrumentation, and (4) it provides an easy and repeatable method to determine and report experimental conditions.

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