Toxicity of Copper and Ascorbic Acid to *Serratia marcescens*

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ABSTRACT

ZIMMERMAN, LEONARD (Fort Detrick, Frederick, Md.). Toxicity of copper and ascorbic acid to *Serratia marcescens*. J. Bacteriol. 91:1537-1542. 1966.—Neutral solutions of ascorbic acid were antibacterial to *Serratia marcescens* at low but not at high population densities. The toxicity of ascorbate was eliminated by metal-sequestering treatments, and was restored only by the addition of trace amounts of copper salts. Copper-ascorbate was equally toxic under aerobic or anaerobic conditions; its toxicity was abolished by (i) chelating agents that sequestered the copper, (ii) metal-complexing agents that bound to the cells but did not sequester copper, and (iii) iron salts in the presence of air. On the basis of these observations, the toxic effects of copper-ascorbate were attributed to its reaction with vital Fe-containing cellular components.

Neutralized 1% ascorbic acid is one of the solutes that enhances the survival of *Serratia marcescens* during freeze-drying (12) of suspensions containing 10^{10} to 10^{11} cells per milliliter. When these suspensions were diluted with neutralized 1% ascorbate to 10^3 cells per milliliter for plate count population assay, however, no colonies were obtained on plates. The parent suspensions yielded the expected numbers of colonies when they were diluted with water instead of ascorbate. Exploratory testing showed that ascorbate was antibacterial in suspensions containing fewer than 10^5 cells per milliliter, but was nontoxic in more densely populated suspensions. Galacturonic acid, glucuronic acid, and other hexuronic acids, were nontoxic to *S. marcescens* at any population density.

A review (2) of the antibacterial effects of ascorbic acid has stated that the findings of different workers are often contradictory. For example, Lwoff and Morel (8) reported that the toxicity of ascorbate was modified by the cell concentration of test suspensions, but this effect was not confirmed by Ericsson and Lundbeck (3, 4). Experiments to resolve this point, and to obtain additional insight into the putative toxic reaction between ascorbate and *S. marcescens*, have been conducted in our laboratory. Results of those investigations are reported here, together with speculations upon their significance.

MATERIALS AND METHODS

Resting cells of *S. marcescens* strain 8UK, as routinely produced at Fort Detrick, were used in all these studies.

Ascorbic acid USP was used throughout this work. All other chemicals were of reagent grade or of highest purity commercially available, and were used without further purification. All test solutions were prepared fresh each day, with the use of sterile glassware and sterile distilled water but unsterilized chemicals. Although these solutions were not subsequently sterilized, their level of bacterial contamination was undetectably low on plate count assay.

Standard test procedure. An 0.1-ml amount of cell suspension was added to 9.9 ml of ascorbate solution brought to pH 7 with NaOH to make a suspension containing 1% ascorbic acid and 1,000 cells per milliliter. The viable cell population of this suspension was determined 30 to 45 sec after mixing (zero-time) and again after 15 min, by spreading 0.1 ml of the undiluted suspension on the surface of each of three agar plates. In a typical toxicity assay, 0.1 ml of suspension yielded 100% recovery or 100 colonies (range of 85 to 115) per plate at zero-time, and 0% recovery or zero colonies (range of 0 to 5) when plated after 15 min. Variations of this procedure will be described in conjunction with the results obtained by their use.

Oxygen-uptake rates were determined by use of a Warburg apparatus and standard techniques.

RESULTS

A 1% solution of ascorbic acid neutralized with NaOH was toxic to *S. marcescens* only in the sparsely populated (or dilute) cell suspensions,

TABLE 1. Correlation of antibacterial and of auto-oxidative activity of ascorbic acid

<i>Serratia marcescens</i> per ml of sodium ascorbate solution*	Rate of ascorbate oxidation† (μliters of O ₂ /min)	Cell survival after 15 min‡
0	4.3	—
10 × 10 ²	4.3	0
20 × 10 ⁹	0.6	100

* A solution of 1% ascorbic acid adjusted to pH 7 with NaOH.

† Difference between oxygen uptake rates of suspensions containing or lacking ascorbate.

‡ Viable-cell survival in all suspensions was 100% at zero-time.

where it auto-oxidized freely (Table 1). Ascorbate auto-oxidation is catalyzed by copper ions (10), as shown in Fig. 1, and copper is a common contaminant of laboratory-quality distilled water (5). Thus, it seemed plausible that the death of the cells was caused either by peroxides or some other toxic products of Cu-catalyzed ascorbate auto-oxidation. Since bacteria are known to adsorb multivalent cations (9), the densely populated (or dense) cell suspensions probably sequestered the Cu catalyst, and so simultaneously suppressed both the auto-oxidation and the toxicity of ascorbate. These speculations were tested by preparing dilute suspensions in ascorbate solutions whose auto-oxidation rate was lowered by the addition of chelating agents or by prior metal-extractive processing. As shown in Table 2, viable-cell survival in these suspensions was unimpaired. Toxicity was restored to these metal-depleted ascorbate solutions by the addition of 0.00001% CuSO₄·5H₂O, although that concentration of the salt was nontoxic in the absence of ascorbate. (This mixture of CuSO₄ and Na ascorbate

TABLE 2. Suppression of the auto-oxidation and the toxicity of sodium ascorbate^a by metal-sequestering treatments

Metal-sequestering process	Rate of ascorbate oxidation (μliters of O ₂ /min)	Cell survival after 15 min ^b
		%
<i>Addition of solutes^c</i>		
None.....	4.3	0
0.5% Na ethylenediamine tetraacetate (pH to 7.0 with NaOH).....	0.4	100
0.4% Na arsenite (pH to 7.0 with H ₂ SO ₄).....	0.5	100
0.1% Na diethyldithiocarbamate.....	0.2	100
0.001% NaCN.....	0.6	100
<0.1% (saturated) 8-hydroxyquinoline.....	0.5	100
<0.1% (saturated) dithizone.....	0.2	100
0.1% thiosemicarbazide.....	0.6	100
0.1% thiourea.....	0.3	100
<i>Extraction^d</i>		
With dithizone-CCl ₄	0.5	100
With Dowex 50 resin, Na form.....	2.1	100
With Duolite C-3 resin, Na form.....	3.6	100

^a In suspensions containing 1,000 *Serratia marcescens* cells per milliliter and 1% ascorbic acid brought to pH 7 with NaOH; these were prepared with laboratory-quality distilled water.

^b 100% viable-cell survival in all suspensions was after "zero-time."

^c Added to ascorbate during preparation of cell suspensions.

^d Ascorbate solutions were extracted before preparation of cell suspensions.

FIG. 1. Auto-oxidation of ascorbic acid.

will be termed Cu-ascorbate.) The salts of 14 other metals (0.001% concentrations of AgNO₃, NiSO₄·7H₂O, CoCl₂·6H₂O, MnSO₄·7H₂O, Cr(NO₃)₃·9H₂O, ZnSO₄·7H₂O, Ba(C₂H₃O₂)·H₂O, Ca(NO₃)₂·4H₂O, MgSO₄·7H₂O, 3CdSO₄·8H₂O, Pb(C₂H₃O₂)₂·3H₂O, SnCl₂·6H₂O, LiSO₄·H₂O, and Al₂Cl₆·12H₂O) did not restore the toxicity of metal-depleted ascorbate solutions.

The observations above all fitted the conventional explanation of ascorbate toxicity, but the additional observations below did not.

(i) Ascorbate solutions made with laboratory-quality distilled water auto-oxidized freely in the presence of aminoguanidine bicarbonate, dipyridyl, or ammonium salts, but these solutions were nontoxic to *S. marcescens* (Table 3). Since

TABLE 3. Noncorrelation of antibacterial activity and auto-oxidation of ascorbic acid

Solute content of cell suspension ^a	Rate of ascorbate oxidation (μ liters of O ₂ /min)	Cell survival after 15 min ^b
		%
None (control).....	0	100
1% Na ascorbate ^c	4.3	0
1% NH ₄ ascorbate + 0.01% dipyridyl.....	11.6	100
1% Na ascorbate + 0.5% aminoguanidine H ₂ CO ₃ ...	4.1	100
1% NH ₄ ascorbate ^d	15.0	100
0.5% NH ₄ ascorbate + 0.5% Na ascorbate.....	12.0	100

^a All suspensions yielded 100% recovery when assayed at zero time.

^b All suspensions contained 10×10^2 cells per milliliter.

^c Ascorbic acid, 1%, made to pH 7 with NaOH.

^d Ascorbic acid, 1%, made to pH 7 with NH₄OH.

the auto-oxidation of ascorbate and its toxicity were thus not invariably associated, further measurements of auto-oxidation rates were discontinued.

(ii) As shown in Table 4, many other solutes known to form complexes with multivalent metal cations also detoxified Cu-ascorbate, including amino acids and other primary amines, and sodium salts of some halogen, phosphate, and sulfoxy acids. Solute that failed to detoxify Cu-ascorbate are also listed in Table 4. With two exceptions, these compounds were not known to form complexes with transition metal cations; fluoride and thiocyanate form stable complexes only with Fe⁺⁺⁺ (1).

(iii) Additional observations were made by use of specially fabricated three-compartment Thunberg tubes. These were prepared for use by placing 0.1 ml of cell suspension in one side arm, 0.5 ml of test-solute solution in a second side arm, and 9.4 ml of neutralized ascorbate solution in the main compartment. The Thunberg tubes were evacuated with a vacuum pump (protected with a Dry Ice trap) to a dry-gas pressure below 5μ of Hg. The solutions in the tubes became chilled during this degassing. After they returned to room temperature, the solution of test solutes was mixed with the ascorbate solution, and then the cells were tipped in, making a suspension containing 10×10^2 cells per ml and 1% ascorbate. Two tubes were processed in parallel for each test-solute treatment, one being opened and assayed for viable-cell population at zero-time, and the other after 15 min under vacuum.

TABLE 4. Suppression of the toxicity* of ascorbic acid† plus copper‡ by added solutes‡

Toxicity not suppressed by	Toxicity suppressed by
Urea	All natural amino acids
Acetamide	Creatine
Tetramethylammonium sulfate	Glutathione
Tetramethylammonium sulfate	Guanidine carbonate
Trimethylamine oxide	Glycocyamine
Dimethylamine sulfate	Hydrazine sulfate
Sodium nitrate	β -Alanine
Sodium fluoride	Ammonium sulfate
Sodium thiocyanate	Sodium chloride, 0.3%
Sodium sulfate, 1.2%	Sodium bromide
Na glucuronate, 1%	Na iodide
Sucrose, 1%	Na citrate
Sucrose, 10%	Na picolinate
	Na phosphate(s)
	Na thioglycolate
	Na sulfite
	Na thiosulfate
	Na hydrosulfide
	Na hydrosulfite

* Killing of >95% of *Serratia marcescens* in suspensions containing 1,000 cells per milliliter after 15 min. All suspensions yielded 100% recovery at zero-time.

† Ascorbic acid, 1%, brought to pH 7 with NaOH, and 0.0001% CuSO₄·5H₂O.

‡ Except as noted, at 0.5% concentration.

The results obtained with this technique are summarized in Table 5. Both aerobically and anaerobically, Cu-depleted ascorbate was non-toxic; Cu-ascorbate was toxic, and this toxicity was suppressed by 0.5% (NH₄)₂SO₄ or 0.5% NaCl. The effects of 0.001% FeSO₄·7H₂O were unforeseen, however; this salt detoxified Cu-ascorbate in the presence but not in the absence of air. The salts of 14 other metals, listed above, did not suppress the antibacterial activity of Cu-ascorbate either aerobically or anaerobically.

Like copper salts, iron salts catalyze the auto-oxidation of neutral solutions of ascorbate. The colorless Fe⁺⁺-ascorbate complex formed by the addition of iron salts to ascorbate is stable in the absence of oxygen, but is converted to the violet-purple Fe⁺⁺⁺-ascorbate by shaking in air. This Fe⁺⁺-Fe⁺⁺⁺ transformation is analogous to the behavior of the iron complexes of cysteine and glutathione (15). The violet Fe⁺⁺⁺-complexes of the latter compounds spontaneously return to the colorless Fe⁺⁺-complexes on standing, whereas the Fe⁺⁺⁺-ascorbate complex does not, thus indicating that Fe⁺⁺⁺-ascorbate is not less stable

TABLE 5. *Suppression of the toxicity* of copper-ascorbate by solutes in the presence and absence of air*

Solute content of cell suspension in addition to 1% ascorbic acid, Na salt†	Viable cell survival‡ after 15-min exposure	
	Aero-bically	Anaero-bically
No added solutes (control)	100	100
0.00001% $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0	0
0.00001% $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ + 0.001% $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	100	0
0.00001% $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ + 0.5% NaCl	100	100
0.00001% $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ + 0.5% $(\text{NH}_4)_2\text{SO}_4$	100	100

* In suspensions containing 1,000 organisms per milliliter.

† Prepared with distilled water passed through a Barnstead demineralizer cartridge.

‡ Survival in all suspensions was 100% at zero-time.

than Fe^{++} -ascorbate. Treatment of Fe^{+++} -ascorbate with sodium hydrosulfite, however, regenerates the colorless Fe^{++} -ascorbate.

It seemed paradoxical that iron salts and iron-complexing solutes like dipyriddy and ammonium salts should both abolish the antimicrobial activity of Cu-ascorbate. However, *S. marcescens* was known to contain iron, and this fact was confirmed as follows. Cells grown at 37 C (to prevent pigment formation) were suspended in a solution of 0.05% dipyriddy and held at 4 C overnight. Centrifugation of this suspension created a pink pellet of packed cells and a colorless supernatant fluid, whereas a control sample not treated with dipyriddy gave a white cell pellet. The addition of dipyriddy to solutions containing ferrous salts produces a soluble red complex that is a classic analytical test for Fe^{++} . The formation of an insoluble red color by dipyriddy-treated *S. marcescens* was interpreted as evidence of the presence of Fe^{++} ions firmly bound to the cells.

DISCUSSION

It is widely believed that ascorbate solutions are antimicrobial only when they are auto-oxidizing: the mechanism of toxicity may involve direct oxidation of cell components by ascorbate (3, 4), or production of H_2O_2 (8), oxidized enediols (11), or hydroxyl free-radicals (14). Reversal of ascorbate toxicity by inorganic or organic iron compounds has been ascribed to their ability to catalyze the breakdown of the postulated toxicants through nonbactericidal mechanisms (8, 14).

Turner, however, reported that vaccinia virus was inactivated by ascorbate whose auto-oxidation was catalyzed by Cu; ascorbate auto-oxidation catalyzed by enzymes or by high pH in the absence of copper was nontoxic, but Cu-ascorbate was virucidal anaerobically (14). These data and ours conflict with the concept that a product of ascorbate auto-oxidation must be responsible for its toxicity. Furthermore, if iron-containing solutes detoxified solutions of Cu-ascorbate by catalytically decomposing a toxic product of its aerobic auto-oxidation, then the stabilizing effects of iron salts should have been enhanced under our conditions of anaerobiosis or (at worst) reduced oxygen tension. The observation that the anaerobic toxicity of Cu-ascorbate was unmodified by the presence of iron salts was taken as critical evidence that the auto-oxidation of ascorbate was not the basis for its toxicity.

The following hypotheses, summarized in Fig. 2, might account for the observed interactions between Cu-ascorbate and other solutes on the viability of *S. marcescens*.

(i) The active toxicant in suspensions containing Cu-ascorbate may be Cu^+ , which is cyclically oxidized to Cu^{++} during its lethal reaction with the cells and then regenerated by reaction with ascorbate. Because of this cyclic regeneration, the effective Cu^+ concentration in such a reaction mixture may far exceed its nominal concentration. Also, nontoxic concentrations of Cu^{++} salts might become antibacterial in the presence of ascorbate because of the conversion of less-toxic Cu^{++} to more-toxic Cu^+ . The concept that the toxicity of a cation may vary with its valence is consistent with the results of Grumbach (6, 7), who observed that certain concentra-

FIG. 2. Possible reactions between *Serratia marcescens*, ascorbic acid, oxygen, copper salts, iron salts, and other solutes.

tions of Cu salts were antibacterial to facultative anaerobes only in the anaerobic zones of shake-agar cultures, whereas Hg salts in the same system were bactericidal only in the aerobic zones. These data may reflect the fact that Cu^+ is more toxic than Cu^{++} but Hg^{++} is more toxic than Hg^+ .

(ii) The addition of metal-binding agents to cell suspensions containing Cu-ascorbate would sequester most of the Cu, suppressing both the toxicity of Cu-ascorbate and its auto-oxidation. Probably Cu-ascorbate was nontoxic to densely populated *S. marcescens* because enough cells were present to adsorb and sequester most of the Cu.

(iii) Iron salts and iron-complexing solutes both interfered with the toxicity of Cu-ascorbate. If Cu-ascorbate killed *S. marcescens* by reacting with vital Fe^{++} -containing cellular compounds, then the protective effects of the compounds above might occur as follows. (a) Dipyriddy and ammonium salts might have combined with the cellular Fe^{++} -containing compounds to form complexes that are unreactive with and insensitive to Cu^+ or Cu-ascorbate, in the presence or absence of oxygen. The fact that Cu-ascorbate still auto-oxidized freely in the presence of these complex-forming agents indicated that their viability-preserving effects could not be due to their Cu-sequestering activity. (b) A mixture of Cu-ascorbate and Fe-ascorbate complexes would be formed in suspensions containing Cu salts, Fe salts, and ascorbate. In the presence of oxygen, Cu^+ in this solution could be oxidized to Cu^{++} either by O_2 or by Fe^{+++} -ascorbate, and the Fe^{++} -ascorbate generated in the latter reaction which would then auto-oxidize back to Fe^{+++} -ascorbate. Thus an artificial Fe-containing electron-transport system might be set up, catalyzing the auto-oxidation of Cu-ascorbate and also diverting its reducing activity away from the Fe^{++} of the cells. The effects of Fe-ascorbate were apparently comparable to those of catalase, which also interfered with the toxicity of Cu-ascorbate but did not suppress its auto-oxidation (14). According to this analysis, the protective effects of Fe-ascorbate depend upon the continuing reoxidation of Fe^{++} -ascorbate to Fe^{+++} -ascorbate by atmospheric oxygen. In the absence of oxygen, this reoxidation would stop, and the protective activity of Fe salts would be expected to disappear, as it did.

No details of the mechanism through which Cu^+ is toxic to *S. marcescens* can be inferred from the data and hypotheses presented here. Possibly Cu^+ denatures the Fe^{++} -containing molecules directly, by breaking one or more of

the nonvalence bonds that hold the metal atom firmly to a large organic molecule. It is also possible that the Fe^{++} -containing structure is a terminal electron-transport enzyme which, in the presence of Cu^+ , allows electrons to flow into the cells, opposite to the normal direction of electron transport, and so disorganizes bacterial metabolism. Other unknown mechanisms, however, may be responsible for the toxicity of Cu^+ and Cu^+ -ascorbate to *S. marcescens*.

It may be wondered how cells can survive if Fe^{++} groups in their vital components are firmly complexed with chelating agents such as dipyriddy. The most probable explanation is that the integrity of these cell components is indispensable for growth and ultimate colony formation, but not for maintenance of viability in resting cells. None of the chelating agents listed in Table 2 is toxic to resting *S. marcescens*, whereas they all prevent cell growth when present in liquid or solid media at the indicated concentrations. Probably the chelating agent molecules attached to the cells are competitively removed by cations in the media when cells in suspensions containing chelators are diluted and plated with solutions and media free of metal-binding agents.

Finally, the major source of copper contamination in our solutions proved to originate from the copper pipette cans in which our unwrapped pipettes were sterilized. Replacing the copper cans with aluminum or stainless-steel ones has diminished the troublesome effects that led to this investigation.

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