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"Antioxidant" (reducing) efficiency of ascorbate in plasma is not affected by concentration

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Ascorbate (vitamin C), an important dietary derived antioxidant, reportedly shows decreasing "antioxidant efficiency" with increasing concentrations in indirect radical trapping methods of antioxidant capacity. This study investigated the effect of concentration on antioxidant efficiency of ascorbate using a direct test of antioxidant capacity, the ferric reducing/antioxidant power test (FRAP assay). Results showed that the antioxidant efficiency factor of ascorbate was 2 and was constant over a wide concentration range in both plasma and pure aqueous solution. However, the absolute amount of ascorbate lost per unit of time increased with concentration. Furthermore, ascorbate was less stable in plasma than in aqueous solutions of similar pH and less stable in ethylenediamine tetraacetic acid (EDTA) than in heparinized plasma. Results indicate that previously reported concentration-dependent changes in antioxidant efficiency of ascorbate may have been caused by loss of ascorbate prior to and during testing, and by methodologic characteristics of indirect peroxyl radical trapping tests of antioxidant capacity. Therefore, it is suggested that the premise that the antioxidant efficiency of ascorbate is concentration-dependent is largely methodologically derived and does not reflect the antioxidant behavior of ascorbate per se. (J. Nutr. Biochem. 10:146–150, 1999)

Keywords: ascorbate; vitamin C; antioxidant; reductant; FRAP assay

Introduction

Results of various studies are supportive of the health benefits of diets rich in vitamin C (ascorbic acid; ascorbate).[1–8] Ascorbate's benefits are thought to be conferred largely by its powerful antioxidant properties.[9] In addition to its role as the major water soluble scavenging antioxidant,[9,10] ascorbate may recycle alpha tocopherol (vitamin E),[11] the major lipid soluble antioxidant,[12] from its tocopheroxyl radical form.[13] This channeling of reducing power from aqueous to lipid compartments of the body may be central to the maintenance of a favorable pro-oxidant: antioxidant balance within lipid rich structures such as membranes and lipoproteins. Increased ascorbate intake, therefore, may augment the antioxidant capacity of both aqueous and lipid systems in vivo.[14]

A biological antioxidant has been defined as "any substance that, when present at low concentrations compared to those of an oxidisable substrate, significantly delays or prevents oxidation of that substrate."[15] This definition is clear and covers the many diverse antioxidants found in vivo. However, unless an antioxidant prevents the generation of an oxidizing species (e.g., by metal chelation or enzyme catalyzed removal of a potential oxidant), the antioxidant reacts with the oxidizing species instead of the substrate (i.e., the antioxidant reduces the oxidant). In simple terms, nonenzymic antioxidants such as ascorbate can be described as reductants. Therefore, in this context antioxidant capacity is equivalent to reducing capacity.

In peroxyl radical trapping tests of total antioxidant capacity[14,16–22] the stoichiometric factor, or antioxidant efficiency, of vitamin E is reportedly constant and equal to 2 (i.e., each molecule of vitamin E reduces two peroxyl

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radicals). The stoichiometry of ascorbate in these tests, however, varies with concentration.[9,23,24] Radical trapping tests of antioxidant capacity measure the ability of the sample to withstand the oxidizing effects of reactive species purposefully generated in the test reaction mixture.[14] The action of antioxidants capable of scavenging these reactive species induces a period of inhibition or delay in an oxidation-induced signal, such as oxygen utilization,[16] chemiluminescence,[17–20] fluorescence,[21,22] or absorbance.[18] If radicals are generated at a constant rate, inhibition time is taken to be proportional to the combination of the molar concentration and the efficiency of the reacting antioxidant(s). However, radical trapping tests are often lengthy procedures, and there is a significant and inevitable delay between sample preparation and the end result. This may pose problems when samples contain an unstable antioxidant such as ascorbate, because autoxidation may lead to artefactually lower than expected results.

Ascorbate's activity factor in peroxyl radical trapping tests of total antioxidant capacity is, in theory, 2. Published values, however, are surprisingly low and variable,[16–23] ranging from approximately 1.7 at 1 μM to approaching zero at 1 mM.[24] In terms of contributing to the total antioxidant capacity of biological fluids, this apparent concentration effect cancels out any putative benefit of increasing the ascorbate concentration. Indeed, this has been proposed[25] as a buffering mechanism by which ascorbate may be regulated in cerebrospinal fluid (CSF). Ascorbate concentrations are three to four times higher in CSF than in plasma, and the antioxidant efficiency of ascorbate in CSF is reportedly 30% less than that of ascorbate in plasma.[25] However, CSF is low in urate and protein and contains negligible amounts of lipid soluble antioxidants,[25] suggesting that ascorbate is the first and last line of scavenging antioxidant defense in CSF. Therefore, a concentration-dependent mechanism to downregulate antioxidant efficiency is a puzzling biochemical phenomenon of questionable physiologic use.

It is also puzzling that most figures reported for the antioxidant efficiency of ascorbate are not integers.[16–25] Electrons must be transferred in their entirety; therefore, the reported figures, if accurate, could be taken to indicate that some ascorbate molecules in the test mixture are nonreactive or react only very slowly, and/or that some molecules are undergoing one-electron and some two-electron transfer reactions. However, if reaction conditions are such that there is a redox potential difference between two interacting species present in a reaction mixture, the reaction limiting factor is likely to be the relative amounts of the oxidized and reduced forms of each species. If electron transfer is less than expected, the cause may be lack of electron donor species—the reduced form of the antioxidant (or reducing species)—and/or lack of electron acceptor species—the oxidized form of the oxidizing species. It is unlikely to be caused by individual antioxidant molecules performing less efficiently—or not at all—if, under the reaction conditions, there is a redox potential difference favoring a kinetically possible electron transfer between the oxidizing and reducing species present. What is likely is that lower than expected efficiency results are caused by a combination of pre-analytical changes in the sample and methodologic characteristics of radical trapping tests.

This study aimed to re-examine the relationship between concentration and the antioxidant efficiency, defined here as the reaction stoichiometric factor, of ascorbate. A rapid, automated, direct test of antioxidant capacity, the ferric reducing/antioxidant power (FRAP[1]) assay,[26] was used. The speed and ease of the FRAP assay permits batch analysis of antioxidant capacity to be performed on fresh samples, and exactly timed repeat analyses of samples can be performed. Antioxidant capacity is defined here as the product of the molar concentration and the antioxidant efficiency. Using the FRAP assay, experimental work was performed to answer the following questions:

1. Is the antioxidant efficiency of ascorbate concentration dependent? If so, a nonlinear dose-response relationship with ascorbate in the FRAP assay would be obtained in both pure and complex solution.
2. Does ascorbate autoxidize faster at high concentration? If so, time-related changes in the concentration of ascorbate would be expected to be greater in solutions of high ascorbate concentration than in more dilute ascorbate solutions.

A related point of interest in this study was the effect of commonly used anticoagulants on the stability of ascorbate in plasma. Most studies employing radical trapping methods have used ethylenediamine tetraacetic acid (EDTA) plasma; however, antioxidants appear to be less stable in EDTA plasma than in heparinized plasma.[27] It is possible that pre-analytical loss of ascorbate in studies using radical trapping methods could be significant. This would help account for the low efficiency values previously reported.

Methods and materials

The FRAP assay was performed, as described in detail elsewhere,[26] using a Cobas Fara centrifugal analyzer (Roche Diagnostics, Basel, Switzerland). In brief, the FRAP assay measures the change in absorbance at 593 nm owing to the formation of a blue colored Fe^{II}-tripyridyltriazine compound from the colorless oxidized Fe^{III} form by the action of electron donating antioxidants. In- and between-run coefficients of variation (CVs) in the FRAP assay are, respectively, less than 1% and less than 3% at 50 to 1,000 μM total antioxidant capacity.

Ascorbate solutions between 10 and 1,000 μM were prepared as required from extra pure ascorbic acid crystals (E. Merck, Darmstadt, Germany) in distilled, deionized water, in phosphate buffered saline [PBS; 10 mM phosphate buffer pH 8.0 containing 2.7 mM potassium chloride and 137 mM sodium chloride (Sigma, St. Louis, MO USA)], and in aged plasma. Aged plasma refers to pooled nonhemolyzed plasma stored for at least 1 week at −70°C and thawed at 4°C for at least 24 hours before use; this plasma contains no detectable ascorbate (Benzie, unpublished results) and was used as diluent in preparing plasma-based ascorbate solutions. The FRAP values of the ascorbate solutions were measured immediately after preparation. Standard solutions of Fe^{II} in the range of 100 to 1,000 μM were prepared from ferrous sulphate ($FeSO_4 7H_2O$, Riedel de Haen, Seelze, Germany) in distilled, deionized water. In the FRAP assay, the antioxidant efficiency of

[1]U.S. patent pending.

the antioxidant under test, in this case ascorbate, is calculated with reference to the reaction signal given by an Fe^{II} solution of known concentration, this representing a one-electron exchange reaction. Ascorbate concentrations in water, PBS, and aged plasma-based solutions (using citrated, EDTA, and heparinized plasma) were measured immediately after preparation and at timed intervals after storage at room temperature and at 4°C using an automated spectrophotometric method[28]; this method has a lower limit of detection of 2 μM, and in- and between-run CVs of less than 5%. The concentrations of citrate, EDTA, and heparin in plasma were as recommended for standard anticoagulant purposes in blood collection, typically 3.0 mg, 1.5 mg, and 15 IU/mL of blood, respectively, and was achieved by collecting blood in commercially available blood collection tubes. The measured pH values of citrated, EDTA, and heparinized plasma were 8.0, 7.6, and 7.7, respectively.

All procedures involving human subjects had prior approval from the Ethics Committee of the Hong Kong Polytechnic University and complied with the Declaration of Helsinki, as revised in 1983.

Results

Results showed that:

1. The antioxidant efficiency of ascorbate was constant, at 2, over the concentration range tested; that is, efficiency was independent of concentration in the FRAP assay system.
2. Ascorbate was less stable in plasma than in pure aqueous solution and pH-matched PBS.
3. Stability of ascorbate in plasma was different depending on the anticoagulant used.
4. The amount of ascorbate lost per unit of time correlated directly with the ascorbate concentration in absolute, but not relative, terms.

The FRAP dose-response relationship of ascorbate in both pure aqueous solution and in plasma was linear up to at least 1,000 μM ascorbate, the highest concentration tested. *Figure 1* shows the ascorbate dose-response line over the concentration range of 50 to 1,000 μM in water, in aged EDTA plasma, and in aged heparinized plasma. It can be seen that the slope of each line (the dose-response relationship) was the same for freshly prepared water-based and for freshly prepared plasma-based ascorbate solutions, indicating no matrix effects. Slopes of the dose-response lines of the ascorbate-containing solutions were twice that of the aqueous Fe^{II} dose-response line (also shown in *Figure 1*). Therefore, results show that the antioxidant efficiency of ascorbate in this test system was constant at 2.0 and remained so over a wide concentration range, indicating that the antioxidant efficiency of ascorbate is not concentration dependent.

Figure 2 shows the effect of concentration on loss of ascorbate in aqueous and citrated plasma-based solutions kept at room temperature and re-measured at timed intervals. There was a significant direct relationship ($r > 0.977$; $P < 0.001$ in each case) between the initial concentration and the absolute decrease in ascorbate concentration at various times after preparation of the ascorbate solutions. This relationship also was seen in aqueous ascorbate solutions kept at room temperature and at 4°C for up to 24 hours; although destruction of ascorbate was slower at 4°C, higher concentrations showed greater absolute decreases at both temperatures (results not shown). *Figure 3* shows the

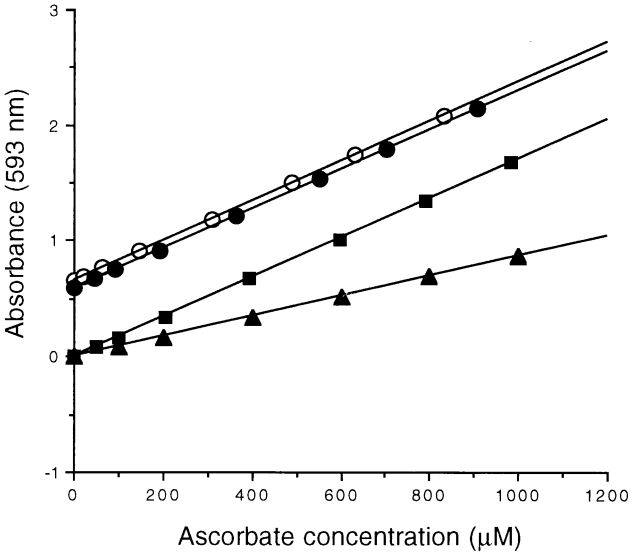

Figure 1 Dose-response of ascorbate in the ferric reducing/antioxidant power test (FRAP) assay for reducing (antioxidant) activity. In pure aqueous solution (squares), aged ethylenediamine tetraacetic acid (EDTA) plasma (open circles), and aged heparinized plasma (filled circles), ascorbate gave a linear concentration-related change in signal (absorbance), showing no change in relative antioxidant activity with concentration. The relative antioxidant efficiency of ascorbate was 2.0; that is, on a mole for mole basis ascorbate gave twice the signal induced by Fe^{II} (triangles), Fe^{II} representing a one-electron exchange reaction in the FRAP assay. Each point represents the mean of three (for each ascorbate solution) or two (Fe^{II} solutions) readings; precision is good with the FRAP assay, and therefore, although ±1 SD error bars are plotted, they do not show.

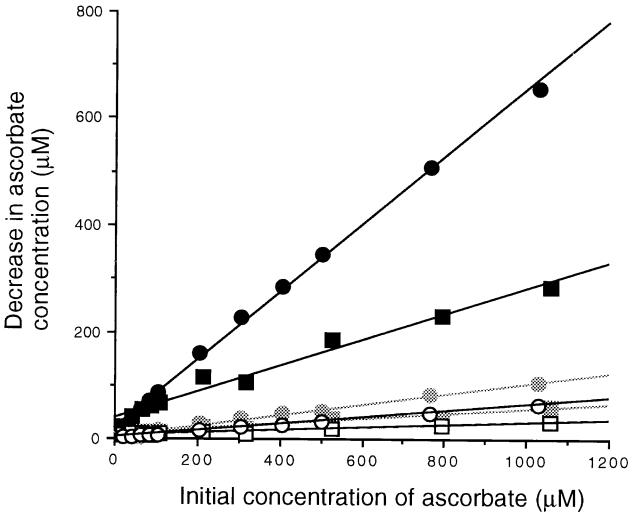

Figure 2 Decrease in ascorbate after 3.5, 5.5, and 23.5 hours (open, shaded, and solid shapes, respectively) at room temperature in citrated plasma (circles) and in pure aqueous solution (squares). Results showed significant correlation between the decrease in ascorbate (rate of destruction or autoxidation) and its initial concentration ($r > 0.977$; $P < 0.001$ in each case). Each point represents the mean of duplicate readings.

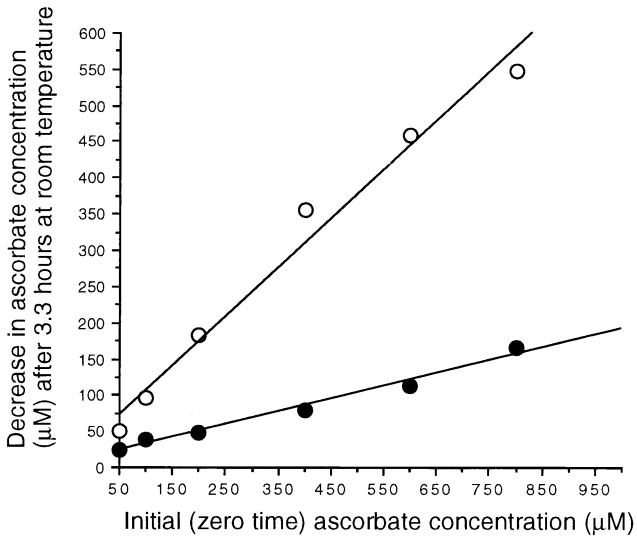

Figure 3 Decrease in ascorbate in ethylenediamine tetraacetic acid (EDTA) plasma (open circles) and heparinized plasma (filled circles) after 3.3 hours at room temperature; each point represents the mean of three readings. Results showed a significant direct correlation ($r \geq 0.988$; $P < 0.001$) between the decrease in ascorbate concentration and its initial concentration in both types of plasma. The rate of destruction of ascorbate in EDTA plasma was almost four times faster than that in heparinized plasma.

decrease in ascorbate in EDTA and in heparinized plasma after 3.3 hours at room temperature; a significant direct correlation ($r \geq 0.988$; $P < 0.001$) was seen between the absolute decrease in ascorbate and its initial concentration in both types of plasma. However, the rate of destruction of ascorbate in plasma was faster than in pure aqueous solution and faster in EDTA than in heparinized plasma. Differences could not be accounted for simply by the difference in pH between plasma-based (pH 8.0) and pure aqueous (pH 4.0) solutions, because ascorbate in PBS (pH 8.0) was destroyed at an intermediate rate (results not shown). The difference in the rate of loss of ascorbate between aqueous and plasma-based solutions is likely to reflect the availability of a large number of suitable redox partners in a heterogeneous mixture such as plasma and perhaps available redox active metal ions in EDTA plasma.

Discussion

The results of this study showed that the ability of ascorbate to act as an electron donating antioxidant is not affected by its concentration per se, as is generally believed. Results also showed that ascorbate is less stable in plasma than in pure aqueous solution and is less stable in EDTA than in heparinized plasma, indicating that heparin may be the anticoagulant of choice when collecting blood for measuring plasma ascorbate and/or antioxidant power. The rate of ascorbate oxidation is reportedly dependent on the concentration and form of transition metal ions present[29]; however, protein binding of iron and copper in native plasma effectively removes these ions from the oxidation equation, and the extremely low levels of free iron and copper in plasma, estimated to be 10^{-23} and 10^{-18} mol/L, respectively,[30] make endogenous transition metal-mediated oxidation of ascorbate in plasma unlikely. Iron bound to EDTA, however, is redox active.[29] Traces of iron in EDTA blood collection tubes could explain the faster loss of ascorbate in EDTA plasma seen in this study.

EDTA plasma has been commonly used in radical trapping tests of antioxidant capacity. Most of these tests are lengthy, manual procedures, and samples tested were unlikely to be fresh. Instability-related loss of ascorbate prior to and during the test would result in a lower than expected antioxidant capacity result. This could be misinterpreted as being caused by a decrease in antioxidant efficiency. Furthermore, when oxygen uptake is used as the signal,[24] autoxidation involving reaction of ascorbate with oxygen would indicate, erroneously, antioxidant depletion, further lowering the apparent antioxidant efficiency in peroxyl radical tests.

In peroxyl radical trapping tests the antioxidant efficiency of ascorbate has been reported[24,25] to approach zero as the molar concentration of the vitamin increases. Peroxyl radical trapping methods define antioxidant efficiency as the number of peroxyl radicals reduced by each molecule of the antioxidant; interaction with oxidant species other than the target peroxyl radicals does not count, and so leads to an underestimation of antioxidant efficiency. The explanation given for the low antioxidant efficiency of ascorbate was a concentration-dependent increase in the reaction of ascorbate with oxygen in the sample.[24] Autoxidation would lead to a decrease in the number of target peroxyl radicals scavenged per mole of ascorbate, and hence to a lower measured antioxidant capacity in the sample. However, if the nominal ascorbate concentration is used to calculate antioxidant efficiency, the net result is an apparently concentration-related decrease in the efficiency. This concentration-related decrease, however, is a methodologic feature, not a characteristic of the general antioxidant behavior of ascorbate, as is widely believed.

Conclusion

Results of this current study indicate that increasing the concentration does not change ascorbate's ability to act as an electron donating antioxidant in either pure solution or in complex mixtures such as plasma. However, the type of anticoagulant used affects stability of ascorbate in plasma, and there may be significant pre-analytical loss of ascorbate unless rapid measurement of fresh plasma samples is performed. It is likely that methodologic characteristics of indirect peroxyl radical trapping tests of antioxidant capacity, combined with the instability of ascorbate, are responsible for the low and apparently concentration-related antioxidant efficiency values previously reported. Therefore, the generally held view that the antioxidant efficiency of ascorbate is concentration-dependent may be based on a misunderstanding of results obtained by and characteristics of indirect radical trapping test methods of antioxidant capacity, rather than on the antioxidant behavior of ascorbate per se, and should be re-examined.

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