*J. Biosci.,* Vol. 11, Numbers 1–4, March 1987, pp. 435–441. © Printed in India.

# Scavenging of superoxide radical by ascorbic acid

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**Abstract.** Using acetaldehyde and xanthine oxidase as the source of superoxide radical, the second order rate constant for the reaction between ascorbic acid and superoxide radical was estimated to be $8{\cdot}2 \times 10^7$ $M^{-1}$ $s^{-1}$. In rats, the average tissue concentration of ascorbic acid was of the order of $10^{-3}$ M and that of superoxide dismutase was of the order of $10^{-6}$ M. So, taking together both the rate constants and the tissue concentrations, the efficacy of ascorbic acid for scavenging superoxide radical in animal tissues appears to be better than that of superoxide dismutase. The significance of ascorbic acid as a scavenger of superoxide radical has been discussed from the point of view of the evolution of ascorbic acid synthesizing capacity of terrestrial vertebrates.

**Keywords.** Superoxide radical; ascorbic acid; superoxide dismutase; rate constant.

## Introduction

The potentiality of ascorbic acid as a scavenger of superoxide radical ($O_2^-$) has earlier been discussed by Nishikimi (1975). He has found the second order rate constant for the reaction between ascorbic acid and $O_2$, generated by xanthine-xanthine oxidase system, to be $2{\cdot}7 \times 10^5$ $M^{-1}$ $s^{-1}$. He has followed the rate of oxidation of ascorbic acid with $O_2^-$ at a wave length of 249·6 nm which is away from the absorption maximum of ascorbic acid at 265 nm. Moreover, when xanthine oxidase is added to a system containing xanthine and ascorbic acid about 23% of the decrease in absorbance at 265 nm (apparently taken as the oxidation of ascorbic acid) is contributed by the oxidation of xanthine to uric acid. Furthermore, uric acid is also a scavenger of $O_2^-$ (Ames *et al.,* 1981; Kellogg and Fridovich, 1977). Previously, we determined the rate constant ($K_{AH2}$) for the reaction between $O_2^-$ and ascorbic acid using a combination of titrimetric and photometric methods. The value was found to be $5{\cdot}4 \times 10^6$ $M^{-1}$ $s^{-1}$ (Som *et al.,* 1983). Considering that determination of the rate of oxidation of very small concentrations of ascorbic acid by titrimetric method might be subjected to significant error, we have reevaluated the rate constant for the reaction between $O_2^-$ and ascorbic acid using acetaldehyde instead of xanthine as a substrate for xanthine oxidase. The reaction between acetaldehyde and xanthine oxidase as a source of $O_2^-$ has been effectively used by Kellogg and Fridovich (1977). The results are presented in this communication.

## Materials and methods

Xanthine and xanthine oxidase (grade 1), cytochrome C, superoxide dismutase and triton X–100 were purchased from Sigma Chemical Co., St. Louis, Missouri, USA.

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Catalase was obtained from CSIR centre for Biochemicals, New Delhi, and this was found to be free from superoxide dismutase contamination. Acetaldehyde was purchased from British Drug House, Bombay and ascorbic acid was a product of Sarabhai M Chemicals.

The activity of superoxide dismutase was assayed by its ability to inhibit the reduction of cytochrome C by the xanthine-xanthine oxidase system (McCord and Fridovich, 1969). The molar concentration of superoxide dismutase was determined from its activity; one unit of superoxide dismutase was taken to be $3{\cdot}1 \times 10^{-9}$ M (McCord and Fridovich, 1969).

The assay mixture for the reaction between ascorbic acid and $O_2^-$ generated by acetaldehyde-xanthine oxidase system contained 50 mM air-equilibrated potassium phosphate bufer, pH 7·0, 0·1 mM EDTA, 25 mM acetaldehyde, 40$\mu$g catalase and graded concentrations of ascorbic acid in a final volume of 2 ml. The reaction was started by addition of 7 mU of xanthine oxidase and oxidation of ascorbic acid was followed by change of absorbance at 265 nm in a 10 cm cuvette using a Hitachi Model 200–20 spectrophotometer with recorder. After an equilibration period of 30 s, readings were taken for 2 min. Under the conditions, there was no spontaneous oxidation of ascorbic acid in the absence of $O_2^-$Also no change of absorbance at 265 nm was observed with acetaldehyde and ascorbic acid, acetaldehyde and xanthine oxidase or with ascorbic acid and xanthine oxidase. The optimum concentration of ascorbic acid at which the rate of its oxidation with $O_2^-$ generated by acetaldehyde and xanthine oxidase system showed saturation was determined. In a separate set of experiment, using this optimum concentration of ascorbic acid the rate of oxidation was determined in the presence of various concentrations of superoxide dismutase.

Superoxide dismutase activity in tissue homogenate was determined by xanthine-xanthine oxidase system (Crapo *et al*., 1978). Tissue homogenate (1:9 for lung and 1:4 for other tissues) was prepared in ice cold 0·25 M sucrose containing 0·5% Triton XX100. The crude homogenate was centrifuged at 17,000 *g* for 30 min and the supernatant was dialyzed against 25 mM potassium phosphate buffer, pH 7·8 and assayed for superoxide dismutase activity as described below. Erythrocyte superoxide dismutase activity was assayed after removing the haemoglobin by Tsuchihasi procedure (Crapo *et al.,* 1978). This was reported as the blood superoxide dismutase content in table 1. Plasma does not have any superoxide dismutase activity. The assay mixture contained $4 \times 10^{-5}$ M xanthine, 2 mM EDTA, $3 \times 10^{-5}$ M cytochrome C, 40 $\mu$g catalase, 10 $\mu$M KCN, required amount of tissue homogenate and 0·1 M air-equilibrated potassium phosphate buffer pH 7·8 in a final volume of 2 ml. The reaction was initiated by addition of 7 mU of xanthine oxidase. The increase of absorbance was measured at 550 nm for 2 min after an equilibration period of 30 s. Under the condition, the rate of change in absorbance in the absence of tissue homogenate was approximately 0·02 per min. One unit of superoxide dismutase is described as the amount of enzyme required to cause 50% inhibition of ferricytochrome C reduction per 3 ml of assay mixture. For different tissues, the amount of tissue homogenate added to the assay mixture was so adjusted that approximately 50% inhibition was observed in each case.

Ascorbic acid in different tissues was estimated as 2:4 dinitrophenylhydrazine derivative following the method of Chatterjee and Banerjee (1979).

## Results

*Oxidation of ascorbic acid by superoxide radical*

The rate of disappearance of $O_2^-$ ($V_{AH_2}$) in the presence of graded concentrations of ascorbic acid and xanthine oxidase is shown in figure 1. The saturating level of ascorbic acid was found to be $5{\cdot}75 \times 10^{-6}$ M.

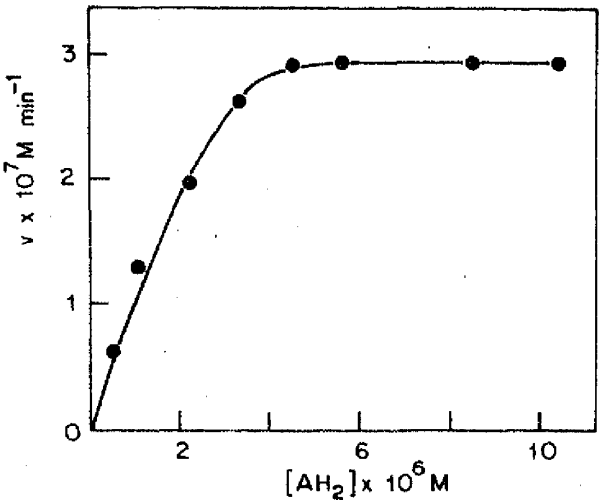


**Figure 1.** The rate of oxidation of ascorbic acid as a function of its concentration. Details of the method are given in the text.

*Effect of varied concentrations of superoxide dismutase on the oxidation of ascorbic acid by* $O_2^-$

Taking the saturating concentration of ascorbic acid *i.e.* $5{\cdot}75\times 10^{-6}$ M and keeping other conditions constant, the effect of varied concentrations of superoxide dismutase on the rate of oxidation of ascorbic acid was examined and data were plotted in figure 2.

*Rate constant* ($K_{AH_2}$) *of the reaction between ascorbic acid and* $O_2^-$

The reaction of $O_2^-$ with ascorbic acid ($AH_2$) can be represented in the following way:

$$O_2^- + AH_2 + H^+ \rightarrow AH + H_2O_2 \quad (1)$$

Where $AH$ is the ascorbate free radical. Therefore, the rate of disappearance of $O_2^-$ ($V_{AH_2}$) can be formulated as

$$V_{AH_2} = K_{AH_2}[AH_2]\,[O_2^-]. \quad (2)$$

$AH$ is a relatively non-reactive species (Bielski *et al.*, 1975) and it decays mainly by

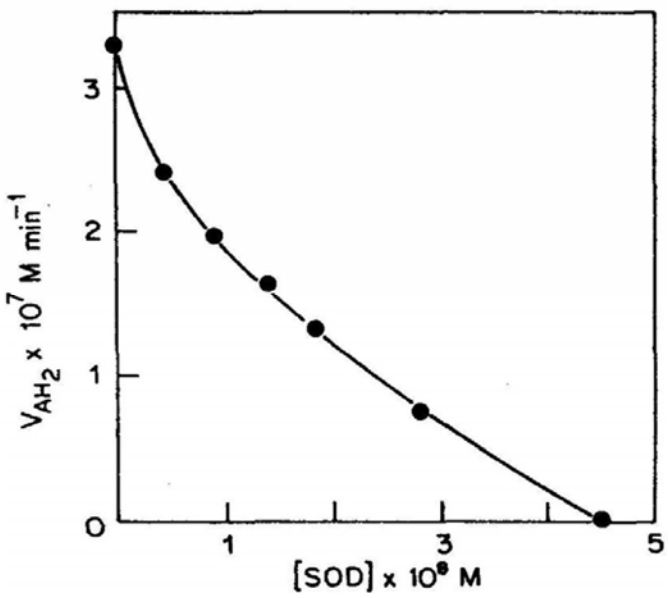


**Figure 2.** Inhibitory effect of superoxide dismutase on the rate of oxidation of ascorbic acid by $O_2^-$. The saturating concentration of ascorbic acid used was $5{\cdot}75 \times 10^{-6}$ M. Details of the method are given in the text.

disproportionation. A represents dehydroascorbic acid.

$$AH\cdot + AH\cdot \longrightarrow A + AH_2.$$

On the other hand, the reaction between $O_2^-$; and superoxide dismutase is as follows:

$$2O_2^{\cdot} + 2H^+ \xrightarrow{K_{SOD}} O_2^{\cdot} + H_2O_2. \tag{3}$$

This enzyme-catalyzed reaction is first order with respect to enzyme and to $O_2^-$ (Rotillo *et al*., 1972; Klug-Roth *et al.*, 1972) and the rate is given by

$$V_{SOD} = K_{SOD}[SOD][O_2^-]. \tag{4}$$

Under the experimental conditions used, at a saturating concentration of ascorbic acid and in the presence of superoxide dismutase, $O_2^-$ disappears through both reactions (1) and (3). Under such a condition at a steady state, the rate of generation of $O_2^-$ ($V$) by the reaction of acetaldehyde and xanthine oxidate is the sum of the rates of disappearance of $O_2^-$ through reactions (1) and (3).

$$i.e.\ V = V_{AH_2} + V_{SOD}. \tag{5}$$

Then the following equation can be derived from equations (2), (4) and (5):

$$\frac{V}{V_{AH_2}} = 1 + \frac{K_{SOD}[SOD]}{K_{AH_2}[AH_2]} \tag{6}$$

Using the saturating level of ascorbic acid, the effect of varied concentrations of superoxide dismutase on the rate of oxidation of ascorbic acid was examined and data were plotted according to equation (6). Figure 3 shows that a straight line intersecting the ordinate at a point $V/V_{AH_2} = 1$ is obtained. Assuming $K_{SOD} = 1{\cdot}9 \times$

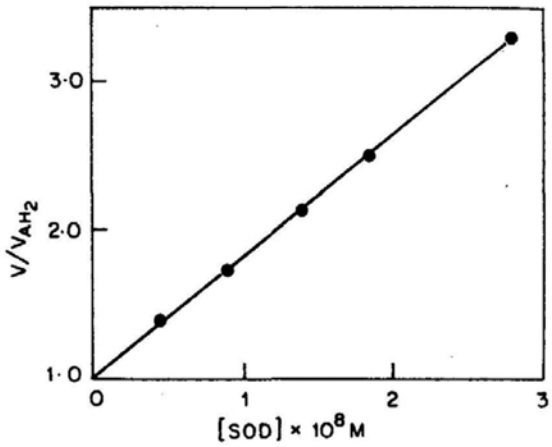


**Figure 3.** Relationship between $V/V_{AH_2}$ and concentration of superoxide dismutase. Details of the method are given in the text.

$10^9$ $M^{-1}$ $S^{-1}$ (Rotilio *et al.,* 1972), the rate constant $K_{AH_2}$ for the reaction between ascorbic acid and $O_2^-$ was calculated from the slope of the graph to be $8{\cdot}2 \times 10^7$ $M^{-1}$ $S^{-1}$ .

*Tissue concentrations of superoxide dismutase and ascorbic acid of rat*

The levels of ascorbic acid and total superoxide dismutase in different tissues of rat are given in table 1. The data indicate that the average concentrations of superoxide dismutase in rat tissues is about 1000 times less than that of ascorbic acid.

**Table 1.** Levels of total superoxide dismutase and ascorbic-acid in rat.

| Tissue | Superoxide dismutase M × $10^6$ | Ascorbic acid M × $10^3$ |
|---|---|---|
| Blood | 14 ± 0·20 | — |
| Plasma | — | 0·04 ± 0·006 |
| Liver | 2·60 ± 0·14 | 1·40 ± 0·110 |
| Adrenal glands | 2·60 ± 0·38 | 6·80 ± 0·700 |
| Heart | 1·40 ± 0·10 | 0·57 ± 0·060 |
| Brain | 0·80 ± 0·07 | 1·60 ± 0·140 |
| Kidney | 0·69 ± 0·08 | 0·79 ± 0·040 |
| Lung | 0·55 ± 0·08 | 1·20 ± 0·100 |
| Pancreas | 0·46 ± 0·06 | 0·89 ± 0·070 |

Each result represents an average (±SD) from 8 young male albino rats (Charles Foster strain) of body weight between 125–175 g. Details of methods are given in the text.

## Discussion

The biological function of ascorbic acid is not clear. The most discussed function is its involvement in collagen synthesis (Chatterjee, 1978). It should be mentioned that

the stimulatory effect of ascorbic acid in hydroxylation of proline in collagen synthesis does not appear to be specific and also this role does not explain the mechanism of capillary haemorrage in scurvy and dramatic cure of scurvy after ascorbic acid administration (Gallop *et al.,* 1972; Barnes, 1975). Purified preparations of collagen prolyl hydroxylase require molecular oxygen, $Fe^{2+}$ ions, and α-ketoglutarate for the activity. The requirement of ascorbic acid is not absolute (Barnes, 1975). Addition of ascorbic acid stimulates the hydroxylation, but ascorbic acid may be replaced by tetrahydropteridine, dithiothreitol, catalase or albumin (Ramachandran and Reddi, 1976). Thus, the role of ascorbic acid is difficult to explain, particularly because solutions of ascorbate, $Fe^{2+}$ ions, and oxygen are known to generate hydroxyl radicals non-enzymatically which can participate in hydroxylating reactions (Bade and Gould, 1969).

We consider that the biological function of ascorbic acid may well be explained from the view point of evolution of the ascorbic acid synthesizing capacity in terrestrial vertebrates. The capacity to synthesize ascorbic acid is absent in fishes and it in the amphibians (Chatterjee, 1973; Roy Chaudhuri and Chatterjee, 1969; 1975). The average oxygen concentrationofwateris0·3%, whereas that of air in the time of evolution of amphibians (about 350 million years ago) was about 10% (Cloud and Gibor, 1970). This means that during evolution from aquatic to the terrestrial mode of life the amphibians had to face an oxygen concentration 30 times that of fishes. One would conceive that such a tremendous increase in oxygen concentration would have been extremely toxic and fatal, unless the newly evolved tetrapods acquired a strong defence mechanism against oxygen toxicity. It is now known that the form of oxygen responsible for oxygen toxicity is $O_2^-$, which may act secondarily through hydroxyl radical. In microorganisms, superoxide dismutase increased markedly (15–20 times) with elevation of oxygen concentration, apparently to survive against oxygen toxicity (Fridovich, 1974). One would, therefore, expect that like that observed in the microorganisms, the superoxide dismutase should have adaptively increased many-fold in amphibians. However, we observed that superoxide dismutase activity of liver of amphibians did not significantly increase over that of fishes (Som *et al.*, 1983). Incidentally, the amphibians started synthesizing ascorbic acid, which is also a potential scavenger of $O_2^-$ (Nishikimi, 1975; Som *et al.*, 1983; Sawyer *et al.*, 1985). The second order rate constant $K_{AH_2}$ for the reaction between $O_2^-$ and ascorbic acid at pH 7·0 has been found to be $8{\cdot}2 \times 10^7\ M^{-1}\ s^{-1}$. Although the rate constant $K_{SOD}$ between $O_2^-$ and superoxide dismutase is $1{\cdot}9 \times 10^9\ M^{-1}\ s^{-1}$, which is approximately 23 times more than that of $K_{AH_2}$, the average concentration of superoxide dismutase in tissues of rat is in the order of $10^{-6}$ M which is about 1000 times less than that of the average tissue concentration of ascorbic acid. It would thus appear that taking together the rate constants and the tissue concentrations, the efficacy of ascorbic acid for scavenging $O_2^-$ in animal tissues is better than that of superoxide dismutase. There is a further advantage of ascorbic acid as a scavenger of $O_2^-$. The product of reaction between ascorbic acid and $O_2^-$ is dehydroascorbic acid. In animal tissues, dehydroascorbic acid is reduced back to ascorbic acid by glutathione resulting in recycling of ascorbic acid (Basu *et al.*, 1979; Som *et al.*, 1980). We have indicated before that the scavenging of $O_2^-$ by ascorbic acid in conjunction with glutathione is more economical (Som *et al.*, 1983). We consider that the evolution of vertebrates from aquatic medium to the terrestrial atmosphere was contingent on the evolution of a

new enzyme, namely L-gulonolactone oxidase, to provide the newly evolved tetrapods with adequate amount of ascorbic acid, a chemical defence against oxygen toxicity. Apparently, this might have happened to compensate the lack of adaptive increase of the enzymatic defence, namely superoxide dismutase, in the early tetrapods, so that evolution of terrestrial vertebrates could continue.

## Acknowledgements

This work was supported by grants-in-aid from the Indian Council of Medical Research and the University Grants Commission, New Delhi.

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