

SUPEROXIDE REDUCTION AS A MECHANISM OF ASCORBATE-  
STIMULATED OXYGEN UPTAKE BY ISOLATED CHLOROPLASTS

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Received April 16, 1973

**SUMMARY:** Addition of 1mM ascorbate to isolated chloroplasts with methyl viologen (MV) as electron acceptor trebled the rate of oxygen uptake and decreased the ADP/O ratio to a third of that with no ascorbate present. These effects of ascorbate were reversed by superoxide dismutase (SOD), which in the absence of ascorbate had little effect on O<sub>2</sub> uptake or ADP/O ratio. A chloroplast-associated SOD activity equivalent to 500 units/mg chlorophyll was detected. The effects of ascorbate and SOD on O<sub>2</sub> uptake were similar in both coupled and uncoupled chloroplasts. The results are consistent with the hypothesis that ascorbate stimulates O<sub>2</sub> uptake by reduction of superoxide, which is formed by autoxidation of the added electron acceptor (MV), and which dismutates in the absence of ascorbate. Ascorbate does not seem to stimulate O<sub>2</sub> uptake by replacing water as the photosystem II donor.

Ascorbate has been reported to act as a photosystem (PS) II donor in chloroplasts with water-oxidation inactivated by heating<sup>1,2</sup> or tris-treatment<sup>3</sup>. Bohme and Trebst<sup>2</sup> have suggested that ascorbate-stimulated O<sub>2</sub> uptake by isolated chloroplasts - which are still able to perform the Hill reaction in the absence of ascorbate - may be explained by ascorbate replacing water as the PSII electron donor. They suggest that electron transport in coupled chloroplasts is accelerated by ascorbate, since electron donation by ascorbate bypasses a normally rate-limiting PSII phosphorylation site.

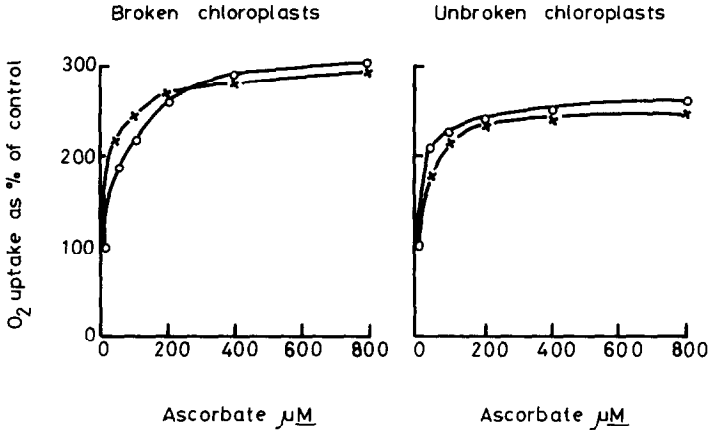
Epel and Neumann<sup>4</sup> have, however, proposed an alternative mechanism of ascorbate-stimulated O<sub>2</sub> uptake in the presence of a low potential PSI acceptor. In this scheme superoxide, produced by autoxidation of the acceptor, is reduced by ascorbate to peroxide (as has also been proposed by Elstner *et al*<sup>5</sup>), rather than the superoxide dismutating to peroxide and oxygen, which occurs in the absence of ascorbate. Aerobic oxidation of the semiquinone of low potential dyes, such as methyl viologen, has been

reported to involve superoxide formation<sup>6,7</sup>.

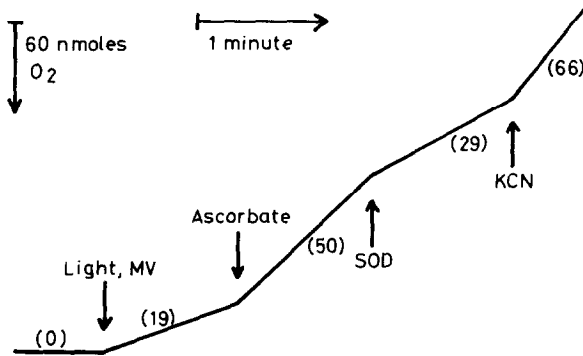
The results reported here support Epel and Neumann's scheme in three ways: 1) the observed ascorbate stimulation of  $O_2$  uptake and depression of the ADP/O ratio require only that ascorbate trebles the net  $O_2$  uptake per pair of electrons transferred from water, by reducing the superoxide formed to  $H_2O_2$  rather than the superoxide dismutating to  $H_2O_2$  and oxygen; 2) the addition of superoxide dismutase reverses the effects of ascorbate; 3) the effects of ascorbate and SOD on  $O_2$  uptake are similar for both coupled and  $NH_4Cl$ -uncoupled chloroplasts.

**METHODS:** Chloroplasts (unbroken; type B of Hall's classification<sup>8</sup>) were isolated from greenhouse spinach by a method based on that of Hall *et al*<sup>9</sup>, using a Polytron homogenizer, with ascorbate omitted from the grinding medium. Broken, washed chloroplasts (type C) were prepared by resuspending the chloroplasts in 50 mls of tenfold diluted resuspending medium; centrifuging for 4 minutes at 4000g and resuspending in undiluted medium. Chlorophyll estimation was performed as described by Arnon<sup>10</sup>.  $O_2$  uptake was measured in a Rank  $O_2$  electrode. Illumination by two 300W slide projectors gave an intensity of  $8.8 \times 10^4 \text{ erg cm}^{-2} \text{ sec}^{-1}$ , with Cinemoid 5A filters transmitting light of wavelengths between 540 nm and 740 nm. ADP/O ratios were calculated by the method of Hall *et al*<sup>9</sup>. SOD, isolated as bovine erythrocyte superoxide dismutase by the method of Weser *et al*<sup>11</sup>, was kindly supplied by U. Weser and was added to the reaction vessel at 8000 units/ml.

**RESULTS AND DISCUSSION:** Addition of ascorbate to both coupled and  $NH_4Cl$ -uncoupled broken, washed (type C) chloroplasts gave a three-fold stimulation of  $O_2$  uptake at concentrations greater than 0.5mM (Figure 1). With unbroken type B chloroplasts (which were swollen in the hypotonic reaction medium) maximal ascorbate stimulation was only 250% of the rate without ascorbate, but there was still no significant difference between ascorbate stimulation of the coupled and uncoupled rates.



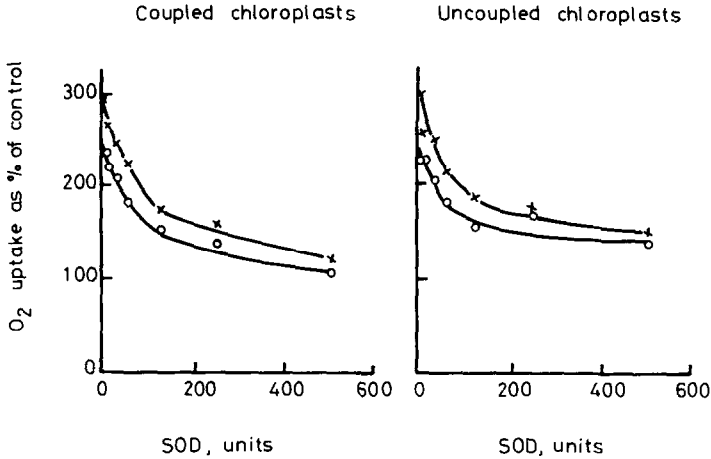
**Figure 1** O<sub>2</sub> uptake as % of the minus-ascorbate rate versus ascorbate concentration in the reaction medium, which also contained 0.1M sorbitol, 5mM MgCl<sub>2</sub>, 20mM NaCl, 2mM EDTA, 50mM HEPES pH 7.5, 3mM NaN<sub>3</sub>, 50μM MV, and chloroplasts equivalent to 100 μg of chlorophyll, in a final volume of 2 ml. ○—○; coupled rate. ×—×; uncoupled rate (+5mM NH<sub>4</sub>Cl).



**Figure 2** The effects of ascorbate (1mM), SOD (400 units), and KCN (10mM) on O<sub>2</sub> uptake by broken chloroplasts in the electrode. Other conditions as in Figure 1. Bracketed figures are the rates of O<sub>2</sub> uptake in μmoles/mg chl/hr.

The inhibitory effect of SOD was completely reversed by 10mM KCN, which caused up to a trebling of the SOD-inhibited rate, and which caused only a 20% stimulation of the rate of O<sub>2</sub> uptake in the absence of SOD (Table 1). The effects on chloroplast O<sub>2</sub> uptake of addition of ascorbate, SOD and KCN, are shown in the trace reproduced in Figure 2.

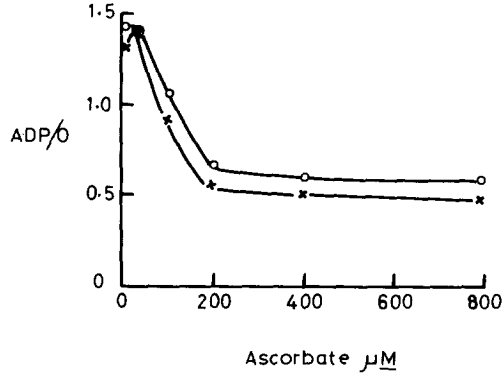
The effect of SOD on O<sub>2</sub> uptake stimulated by 1mM ascorbate is shown for coupled and uncoupled chloroplasts in Figure 3. In both cases the



**Figure 3** O<sub>2</sub> uptake with 1mM ascorbate, as % of the rate without ascorbate, versus SOD in the 2 ml reaction medium. Other conditions as in Figure 1. o—o ; unbroken (type B) chloroplasts; x—x ; broken (type C) chloroplasts.

difference between unbroken (type B) and broken (type C) chloroplasts corresponds to a chloroplast-associated SOD activity of about 50 units in chloroplasts containing 100  $\mu$ g of chlorophyll. From this one can calculate that there are 5-10 SOD molecules per electron transport chain.

Ascorbate and SOD had effects on the ADP/O ratios of both unbroken and broken, washed chloroplasts (Table 1), analogous to their effects on O<sub>2</sub> uptake. Figure 4 shows the ADP/O ratio of the chloroplasts plotted against the concentration of ascorbate in the reaction medium. For broken chloroplasts, the ADP/O ratio is depressed by ascorbate to about a third of its original value, and to slightly more than a third for unbroken chloroplasts. If the oxygen taken up per electron pair is trebled by the addition of ascorbate, as suggested by Figure 1, the decrease in the observed ADP/O ratio (Figure 4) does not require the assumption that the ATP/2e<sup>-</sup> has decreased. Hence these results do not require that electrons from ascorbate pass through only one of the two phosphorylation sites that are now thought to be involved in non-cyclic photophosphorylation<sup>9,12-16</sup>.

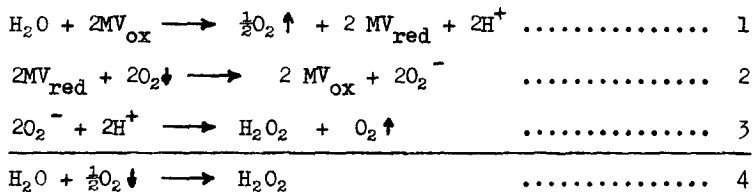


**Figure 4** ADP/O versus ascorbate concentration in reaction medium. Conditions as for Figure 1, with 10mM  $\text{K}_2\text{HPO}_4$  and 0.125 mM ADP added.  $\circ$ — $\circ$ ; unbroken (type B) chloroplasts.  $\times$ — $\times$ ; broken, (type C) chloroplasts.

**Table 1** The effects of ascorbate (1mM), SOD (400 units), and KCN (10mM) on coupled  $\text{O}_2$  uptake and the ADP/O ratio for broken (type C) chloroplasts. Reaction conditions as in Figure 4, i.e. all rates are with methyl viologen as electron acceptor.

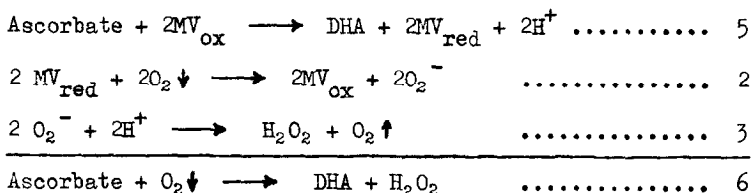
<u>Additions</u>	<u><math>\text{O}_2</math> uptake</u> <u><math>\mu\text{moles/mg chl/hr}</math></u>	<u>ADP/O</u>
None	33	1.44
KCN alone	38	1.49
SOD alone	29	1.67
Ascorbate alone	86	0.63
Ascorbate + KCN	84	0.67
Ascorbate + SOD	20	1.44
Ascorbate + SOD + KCN	72	0.77

Photosynthetic transport of a pair of electrons from water to a low potential dye such as methyl viologen is summarized in equation 1. Aerobic autoxidation of the dye may produce superoxide ions, (equation 2), and spontaneous superoxide dismutation may then occur (equation 3). If catalase is inhibited by azide so that no breakdown of  $\text{H}_2\text{O}_2$  occurs, the net  $\text{O}_2$  uptake per pair of electrons ( $\text{O}_2\downarrow/2e^-$ ) may be deduced from the summation of equations 1-3 to give equation 4, the Mehler reaction:



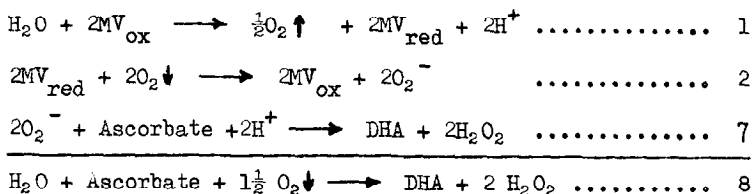
Hence  $O_2\downarrow / 2e^- = \frac{1}{2}$  in this system; i.e.  $O_2$  uptake proceeds at the same rate as  $O_2$  evolution in a Hill reaction with ferricyanide.

If ascorbate replaces water as the PSII donor, producing dehydro-ascorbate (DHA), equation 5 replaces equation 1, and the net reaction becomes that shown in equation 6, the same as that proposed by Elstner *et al*<sup>5</sup>



In this case  $O_2\downarrow / 2e^- = 1$ . Hence the effect of ascorbate addition would be to double the rate of  $O_2$  uptake for any given rate of electron transport, as proposed by Bohme and Trebst<sup>2</sup>.

If, however, ascorbate exerts its effect by reducing to  $H_2O_2$  the superoxide formed in reaction 2 (as suggested by Epel and Neumann<sup>4</sup>), equation 7 replaces equation 3, and the overall reaction becomes that expressed in equation 8.



Here  $O_2\downarrow / 2e^- = 1\frac{1}{2}$ ; thus the ratio is trebled (from  $\frac{1}{2}$  to  $1\frac{1}{2}$ ) by the addition of ascorbate. SOD catalyzes reaction 3 and may interact with  $O_2^-$  before ascorbate can, so the observed reversal of ascorbate's effect is predicted by the scheme summarized in equation 8.

The data presented here also show the predicted trebling of the rate of  $O_2$  uptake on addition of ascorbate (Figure 1), which occurs in both coupled and uncoupled chloroplasts. Since the trebling occurs in uncoupled chloroplasts it cannot be a result of electrons from ascorbate by-passing a rate-limiting PSII phosphorylation site<sup>2</sup>. Bohme and Trebst<sup>2</sup> report a constant rate of ATP formation in chloroplasts with and without ascorbate, which in

our view may be adequately explained by an unchanged rate of electron transport through both phosphorylation sites. This conclusion is supported by the reversal by added SOD of ascorbate-depression of the ADP/O ratio (Table 1) and of ascorbate-stimulation of O<sub>2</sub> uptake (Figure 3).

The mechanism of ascorbate-stimulated O<sub>2</sub> uptake, proposed by Epel and Neumann<sup>4</sup>, has the added advantage that it predicts an absence of stimulation by ascorbate of NADP<sup>+</sup> reduction in untreated chloroplasts<sup>2</sup>, since it does not require the ad hoc assumption that the effectiveness of a PSII electron donor depends directly on the chemical nature of the terminal acceptor.

Thanks are due to Dr. S.G. Reeves, Dr. M.C.W. Evans and Mr. J. Lumsden for helpful discussion. JFA is in receipt of a Science Research Council Research Studentship.

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