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ROLE OF THE SUPEROXIDE FREE RADICAL ION IN PHOTOSYNTHETIC ASCORBATE OXIDATION AND ASCORBATE-MEDIATED PHOTOPHOSPHORYLATION

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SUMMARY

The mechanism of ascorbate photooxidation in isolated chloroplasts has been studied. The enzyme superoxide dismutase has been used as a tool to show that ascorbate is oxidized by the superoxide free radical ion, which is formed during the autooxidation of a low-potential electron acceptor.

In the absence of an artificial, low-potential electron acceptor, addition of ascorbate stimulates photophosphorylation in isolated chloroplasts. This effect of ascorbate is abolished by superoxide dismutase, indicating that both the superoxide free radical ion and ascorbate are responsible for the stimulation of photophosphorylation. In this case, the superoxide free radical ion seems to be formed during the autooxidation of an endogenous electron acceptor.

In the presence of ferredoxin and $NADP^+$, photophosphorylation in isolated chloroplasts stops as soon as the available $NADP^+$ is fully reduced. If ascorbate is present in this system, however, a linear rate of photophosphorylation is maintained in spite of the fact, that $NADP^+$ is fully reduced. This ascorbate-mediated photophosphorylation again is abolished by superoxide dismutase.

During the catalysis of this oxygen-dependent photophosphorylation, ascorbate consumption is not observed. These findings support the idea, that in chloroplasts ascorbate together with the superoxide free radical ion may function in providing additional ATP by an oxygen-dependent photophosphorylation.

INTRODUCTION

The photooxidation of ascorbate by isolated chloroplasts has been investigated by many authors (*cf.* ref. 1), and the effects of ascorbate on numerous photosynthetic chloroplast reactions have been described. These observations might be ordered in three groups: (1) Stimulation of endogenous photophosphorylation[2–4]. (2) Stimulation of coupled ATP formation in the $NADP^+$ reduction[5,6]. (3) Quinone-stimulated photooxidation[7].

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; AQ, anthraquinone-2-sulfonic acid.

The following reasons for the observed effects have been offered: (a) Poising of the redox state of an endogenous system and/or acting as a protective agent. (b) Cycling of the three redox states of ascorbate. (c) Stoichiometric consumption of ascorbate in photooxidations. It is difficult to be absolutely certain which of the above three explanations (a, b or c) best account for stimulations of the individual photosynthetic reactions. For example, (a) or (b) could be responsible for the stimulation of endogenous photophosphorylation or of coupled noncyclic ATP formation in photosynthetic $NADP^+$ reduction. (b) or (c) could be the cause of the stimulation of oxygen uptake in pseudocyclic electron transport (Mehler reaction). Furthermore two possible mechanisms for ascorbate photooxidation are conceivable. Ascorbate could be the electron donor for the electron transport chain before Photosystem II[1,7] or ascorbate is oxidized by a peroxide formed by Photosystem I[8,9].

The present paper tries to find a common cause for the effects of ascorbate. Our approach has been to pinpoint the conditions under which ascorbate is an electron donor for the photosynthetic electron transport chain and those in which it is oxidized by a peroxide radical, formed by Photosystem I. For this purpose, the effect of superoxide dismutase on photosynthetic reactions has been studied. This enzyme catalyzes the dismutation of the superoxide free radical ion into O_2 and H_2O_2 and, therefore, inhibits those reactions, in which a superoxide free radical anion participates[10,11].

MATERIALS AND METHODS

Superoxide dismutase was isolated from dry peas. The purification of the enzyme from a crude extract by $(NH_4)_2\,SO_4$ and acetone precipitation was carried out using a procedure modified from Sawada *et al.*[12]. The protein fraction of the second $(NH_4)_2SO_4$ precipitation was taken up in 2.5 mM potassium phosphate buffer, pH 7.8, and heated in portions of 2 ml for 2 min at 60 °C, since superoxide dismutase proved to be rather heat stable. After centrifugation, the supernatant was chromatographed on a Whatman DE 52 column (13 cm × 5 cm). The enzyme, visible as one blue-green zone, was eluted with a linear gradient from 0 to 0.1 M NaCl in 2.5 mM potassium phosphate buffer, pH 7.8. All 10-ml fractions, in which 0.1 ml at a dilution of 1:400 showed an inhibition of the cytochrome *c* reduction by the xanthine oxidase reaction[10] of more than 50%, were concentrated by $(NH_4)_2SO_4$ precipitation, adsorbed on another column of Whatman DE 52 (1 cm × 8 cm) and eluted with 0.2 M NaCl in 2.5 mM potassium phosphate buffer, pH 7.8. The number of activity units was determined by means of the inhibition of either the cytochrome *c* reduction or epinephrine oxidation[10,13], with xanthine oxidase as the generator of the superoxide radical ion. The overall purification was 150-fold, compared with a crude extract from dried, green peas. The average preparation had about 1000 enzyme units per mg protein. The purified preparation contained 1.14 μg Cu/mg protein (0.18 gatom Cu/10 mg protein). The molecular weight of green pea superoxide dismutase was estimated to be 31000, containing 2 gatoms Cu and 2 gatoms Zn per mole enzyme protein[12]. From the protein and copper determinations we calculated (assuming that no other copper-containing protein was present) that our preparation was about 30% pure. About 50 enzyme units ($6.2 \cdot 10^{-5}$ μmole) of superoxide dismutase were used in the experiments, where indicated. This is 5-fold the amount, which gave a

90% inhibition in the cytochrome *c* reduction test. In addition to superoxide dismutase, about 8000 units of catalase were present in most of the experiments as indicated in the tables and figures.

The photosynthetic assays were carried out at 15 °C in Warburg vessels. Oxygen uptake or evolution was measured manometrically.

Broken chloroplasts were prepared from spinach leaves as described by Nelson *et al.*[14]. Heated chloroplasts were prepared and ascorbate was determined as described by Böhme and Trebst[1]. The NADPH present in the supernatant was determined at 340 or 366 nm after centrifugation of the samples at 15000 × *g*. Absorbances were measured and adsorption spectra were recorded in either a Zeiss PMQ II or a Cary 15 spectrophotometer. Protein was determined according to the method of Murphy and Kies[15]. Copper was determined with a Zeiss PMQ spectrophotometer fitted to a FA 2 atomic absorption unit with a hollow cathode lamp (λ = 324.7 nm). Ferredoxin and a ferredoxin–$NADP^+$ reductase-containing fraction were isolated from spinach leaves as described by Tagawa and Arnon[16] and Shin *et al.*[17], respectively. ATP was determined as [^{32}P]ATP[18]. Cytochrome *c*, catalase, xanthine oxidase, xanthine, glucose 6-phosphate and glucose-6-phosphate dehydrogenase were obtained from Boehringer, Mannheim. Epinephrine was obtained from Merck, Darmstadt.

RESULTS

(*1*) *The photooxidation of ascorbate*

The stoichiometry of the photooxidation of ascorbate during the pseudocyclic photophosphorylation was studied in detail by Trebst *et al.*[7] and by Böhme and Trebst[1]. These authors could show, that in isolated chloroplasts with an autooxidizable electron acceptor, the photooxidation of ascorbate was stoichiometric to photophosphorylation, O_2 uptake and H_2O_2 formation. From the inhibition of these reactions by 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) it was concluded, that ascorbate might act as an electron donor for Photosystem II. Studying the oxidation of hydroxylamine and ascorbate in digitonin-fragmented chloroplasts and with artificial

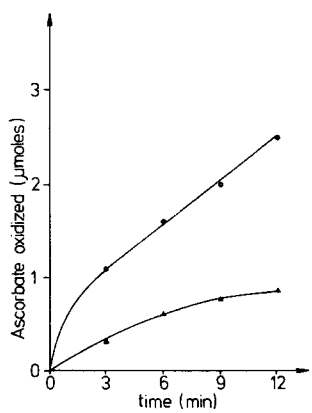


Fig. 1. Kinetics of ascorbate photooxidation. For experimental conditions see Table I. ●—●, without superoxide dismutase; ▲—▲, *plus* superoxide dismutase.

enzymatic systems, Elstner *et al.*[8] proposed an alternative mechanism for the oxidation of ascorbate, in which a peroxide other than H_2O_2 (formed during the autooxidation of the reduced electron acceptor) is the oxidant.

Fig. 1 shows the kinetics of the ascorbate photooxidation by isolated chloroplasts with a low-potential electron acceptor. Superoxide dismutase inhibits the reaction to about 65%. After the light reaction proceeds for 10–12 min, the oxidation in the vessel which contains superoxide dismutase comes to a virtual halt, while the one without superoxide dismutase still proceeds linearly. An almost complete inhibition of ascorbate photooxidation can be achieved by the addition of both superoxide dismutase and catalase. Catalase alone has no or very little effect on the ascorbate photooxidation (Table I).

TABLE I

INHIBITION OF ASCORBATE PHOTOOXIDATION IN ISOLATED CHLOROPLASTS WITH METHYL VIOLOGEN AS ELECTRON ACCEPTOR BY SUPEROXIDE DISMUTASE AND CATALASE

The reaction medium contained in 3 ml: broken chloroplasts with 0.1 mg chlorophyll, 80 μmoles Tris–HCl, pH 8.0, 0.2 μmoles methyl viologen, 10 μmoles ascorbate, 5 μmoles NH_4Cl, 5 μmoles $MgCl_2$; the reaction was allowed to proceed for 15 min at 25000 lux and 15 °C in air.

Additions	*μmoles ascorbate oxidized/mg chlorophyll per h*	*μmoles O_2 taken up/mg chlorophyll per h*
None	112	88
plus catalase	100	52
plus superoxide dismutase	28	28
plus superoxide dismutase *plus* catalase	4	12

The kinetics of oxygen uptake during the ascorbate oxidation with anthraquinone as electron acceptor are shown in Fig. 2. No or little oxygen uptake is observed when only anthraquinone is present; after a lag phase (probably due to the reduction of AQ, *cf.* ref. 1), an oxygen uptake is measured, if in addition to AQ, 10^{-3} M KCN is present. Addition of ascorbate causes a rapid oxygen uptake, which can be inhibited by superoxide dismutase. The vessel with both AQ and ascorbate continues taking up oxygen in the dark after switching off the light for at least 5 min. Addition of 10^{-3} M KCN stimulates the rate of oxygen uptake in the dark, due to the inhibition of an endogenous catalase activity, which simultanously splits the H_2O_2 accumulated during the light reaction. A similar observation was made by Good and Hill[19] with ascorbate in quinone-treated chloroplasts.

Oxygen uptake varied with different chloroplast preparations in the absence of KCN, probably because of different endogenous catalase activities (*cf.* refs 7 and 20). The values for oxygen uptake are omitted in the following tables since KCN cannot be added to our test system, because it also inhibits the superoxide dismutase[21] (*cf.* Table II).

Ascorbate oxidation by an enzymatic system which mimics the "pseudocyclic" conditions was reported by Elstner *et al.*[8] and was confirmed by Epel and Neumann[9].

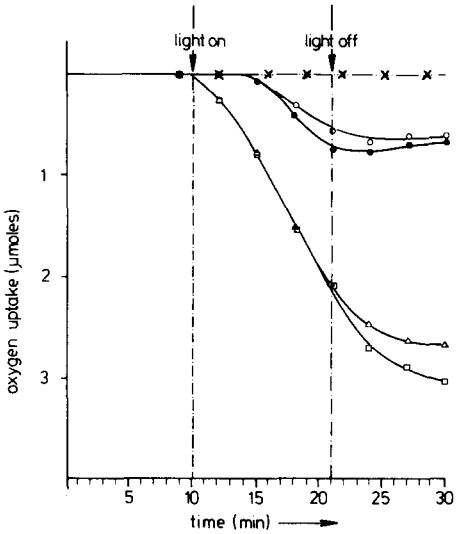


Fig. 2. Kinetics of oxygen uptake by isolated chloroplasts with 0.6 μmole anthraquinone-2-sulfonic acid (AQ) as electron acceptor. For experimental conditions see Table I. × · · —· · × , *plus* AQ; ○—○, *plus* AQ *plus* 10^{-3} M KCN; ●—●, *plus* AQ *plus* 10 μmoles ascorbate *plus* superoxide dismutase *plus* catalase; △—△, *plus* AQ *plus* 10 μmoles ascorbate; □—□, *plus* AQ *plus* 10 μmoles ascorbate *plus* 10^{-3} M KCN.

TABLE II

THE CO-OXIDATION OF ASCORBATE DURING THE DIAPHORASE-CATALYZED OXIDATION OF NADPH WITH ANTHRAQUINONE AND ITS INHIBITION BY SUPEROXIDE DISMUTASE AND CATALASE

The reaction mixture contained in 3 ml: 10 μmoles glucose 6-phosphate, 10 μg glucose-6-phosphate dehydrogenase, 1 μmole $NADP^+$, an $NADP^+$–ferredoxin reductase fraction containing 2 mg protein, 0.2 μmole AQ; 15-min dark reaction in air at 15 °C.

Additions	*μmoles ascorbate oxidized/15 min*
Complete	1.9
plus 10^{-3} M KCN	2.7
plus superoxide dismutase *plus* catalase	0.3
plus superoxide dismutase *plus* catalase *plus* 10^{-3} M KCN	2.5
minus glucose 6-phosphate	0
minus AQ	0.1
minus $NADP^+$–ferredoxin reductase	0

This system with AQ ($E_0 = -200$ mV), reduced by NADPH in a diaphorase-catalyzed reaction, in the dark oxidizes ascorbate apparently *via* superoxide free radical ion. KCN stimulates the oxidation of ascorbate in this system as previously reported[8]. In the presence of 10^{-3} M KCN the ascorbate oxidation is no longer inhibited by superoxide dismutase and catalase (Table II).

As shown in Table III, the addition of both superoxide dismutase and catalase has no influence on the ATP formation in pseudocyclic photophosphorylation regardless whether ascorbate is present or not, while the photooxidation of ascorbate is inhibited to about 90%.

(2) The stimulation of the endogenous photophosphorylation by ascorbate

The photophosphorylation in isolated chloroplasts without an artificial electron acceptor ("endogenous" photophosphorylation, *cf.* ref. 4) is stimulated by ascorbate as already reported by other groups[2–4]. This stimulatory effect of ascorbate can be inhibited by the addition of both superoxide dismutase and catalase or by DCMU (Table IV).

As shown in Table IV, ascorbate brings about a 4-fold stimulation of the "endo-

TABLE III

THE LACK OF INFLUENCE OF SUPEROXIDE DISMUTASE ON THE PSEUDOCYCLIC PHOTOPHOSPHORYLATION IN THE PRESENCE AND ABSENCE OF ASCORBATE

Experimental conditions, see Table I. Changes: *minus* NH_4Cl, *plus* 10 μmoles phosphate, *plus* 10 μmoles ADP.

Additions	*μmoles ATP formed /mg chlorophyll per h*	*μmoles ascorbate oxidized /mg chlorophyll per h*
None	50	—
plus 10 μmoles ascorbate	52	88
plus superoxide dismutase *plus* catalase	50	—
plus 10 μmoles ascorbate *plus* xsuperoxide dismutase *plus* catalase	54	10

TABLE IV

THE EFFECT OF ASCORBATE ON THE "ENDOGENOUS" PHOTOPHOSPHORYLATION IN ISOLATED CHLOROPLASTS AND ITS INHIBITION BY SUPEROXIDE DISMUTASE AND CATALASE

Experimental conditions, see Table III. Change: *minus* methyl viologen.

Additions	*μmoles ATP formed /mg chlorophyll per h*	*μmoles ascorbate oxidized/mg chlorophyll per h*
None	6.0	—
plus $2 \cdot 10^{-5}$ M DCMU	1.0	—
plus 10 μmoles ascorbate	25	0
plus 10 μmoles ascorbate *plus* $2 \cdot 10^{-5}$ M DCMU	1.5	0
plus 10 μmoles ascorbate *plus* superoxide dismutase *plus* catalase	7.5	0

genous" photophosphorylation, but no consumption of ascorbate is observed. In the presence of superoxide dismutase and catalase, the stimulatory effect of ascorbate disappears, and the rate of photophosphorylation is reduced to the endogenous rate. $2 \cdot 10^{-5}$ M DCMU inhibits both the endogenous and the ascorbate-mediated photophosphorylation.

Similar results are obtained with heat-treated chloroplasts (Table V). The important difference in this case, compared to the experiment described in Table IV, is the neglegible rate of endogenous phosphorylation, which is not further inhibited by DCMU. However, as in the experiment shown in Table IV no oxidation of ascorbate can be observed. This result is in agreement with the findings of Forti and Jagendorf[4]. The stimulatory effect of ascorbate can be inhibited again by the addition of both superoxide dismutase and catalase and by DCMU.

TABLE V

THE EFFECT OF ASCORBATE ON THE PHOTOPHOSPHORYLATION IN HEAT-TREATED CHLOROPLASTS AND ITS INHIBITION BY SUPEROXIDE DISMUTASE AND CATALASE

Experimental conditions, see Table III. Changes: heat-treated chloroplasts were used, in the absence of methyl viologen.

Additions	*μmoles ATP formed/mg chlorophyll per h*
None	1.6
plus $2 \cdot 10^{-5}$ M DCMU	1.0
plus 10 μmoles ascorbate	10
plus 10 μmoles ascorbate *plus* $2 \cdot 10^{-5}$ M DCMU	1.2
plus 10 μmoles ascorbate *plus* superoxide dismutase *plus* catalase	3.5

(3) Stimulation of the ATP formation in a $NADP^+$-reducing system

Jacobi[6] showed that, with $NADP^+$ as the acceptor in the presence of ascorbate and air, photophosphorylation can still proceed in spite of the fact that all of the added $NADP^+$ was reduced. The rate of this photophosphorylation seemed to be independent of anaerobic photophosphorylation, which is coupled to the $NADP^+$ reduction and was thus interpreted as an extra ATP site.

By following the kinetics of the $NADP^+$ reduction and ATP formation with limiting amounts of added $NADP^+$ (in the presence or absence of ascorbate) (Fig. 3), we found a stoichiometry of about 1:1 between ATP formation and $NADP^+$ reduction after 8–10 min of light. At all shorter times more NADPH than ATP is formed. After 10 min, some NADPH is reoxidized and reaches a steady state at about 15 min. Around 75% of the added $NADP^+$ is found reduced at all times after 15 min. At about 15 min, the amount of ATP formed in experiments without added ascorbate reaches a plateau which is equivalent to the amount of $NADP^+$ added (2 μmoles). In the presence of ascorbate, however, photophosphorylation proceeds even after NADPH formation has reached a steady state. The rate of this ATP formation is

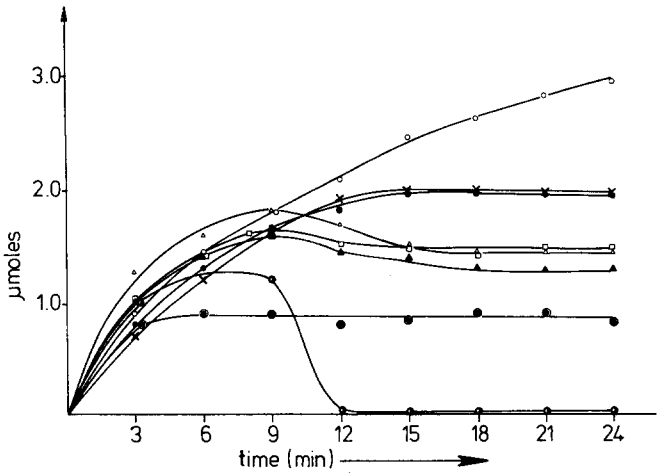


Fig. 3. Kinetics of photophosphorylation and $NADP^+$ reduction in the presence or absence of ascorbate and with or without superoxide dismutase *plus* catalase. Experimental conditions: chloroplast fragments with 0.2 mg chlorophyll, 10 nmoles ferredoxin, 2 μmoles $NADP^+$, 10 μmoles phosphate, 10 μmoles ADP, light reaction in air, 25000 lux, 15 °C. ○—○, ATP in the presence of 10 μmoles ascorbate; ×—×, ATP in the presence of 10 μmoles ascorbate *plus* superoxide dismutase *plus* catalase; ●—●, ATP; △—△, NADPH; ▲—▲, NADPH in the presence of 10 μmoles ascorbate; □—□, NADPH in the presence of 10 μmoles ascorbate *plus* superoxide dismutase *plus* catalase; ⊙—⊙, ascorbate oxidized; ◑—◑, ascorbate oxidized in the presence of superoxide dismutase *plus* catalase.

about half of the initial rate. Another unexpected result is that the oxidation of ascorbate reaches a plateau at about 8% oxidized after only 3–5 min of light.

The effect of superoxide dismutase and catalase on photophosphorylation under conditions of limiting amounts of $NADP^+$in the presence of ascorbate is the following. The amounts of reduced $NADP^+$ and formed ATP at all times are identical with those in the absence of ascorbate. The amount of initially oxidized ascorbate disappears quantitatively. After 15 min of light (the time at which the systems without ascorbate reach their plateau or steady state) we observe the stoichiometries shown in Table VI.

TABLE VI

STOICHIOMETRY OF PHOTOSYNTHETIC PARAMETERS OBSERVED AT THE BEGINNING OF THE NADPH STEADY STATE

For the experimental conditions, see Fig. 3.

Additions	*μmoles $NADP^+$ formed*	*Δ*	*μmoles ATP formed*	*Δ*	*μmoles ascorbate oxidized*	*Δ*
None	1.5		1.9		—	
10 μmoles ascorbate	1.4	0.1	2.3	0.4	0.8	
10 μmoles ascorbate *plus* superoxide dismutase *plus* catalase	1.5	0.1	1.9	0.4	0	0.8

The same stoichiometric relationships were found in several independent experiments. As shown in Table VI, at the endpoint of photophosphorylation in the absence of ascorbate, the difference between photophosphorylation *plus* ascorbate and photophosphorylation *minus* ascorbate is about 0.4 μmole; exactly the same difference can be obtained if we subtract the ATP values, which are found with ascorbate in the presence of superoxide dismutase and catalase from those in the absence of superoxide dismutase and catalase. This difference we should like to refer to as "ascorbate-mediated photophosphorylation". In the presence of superoxide dismutase and catalase no ascorbate oxidation can be observed, starting from the point of the apparent end of the $NADP^+$-coupled photophosphorylation. At this point we find a stoichiometry of 0.4 μmole ATP formed by the ascorbate-mediated photophosphorylation for 0.8 μmole of ascorbate oxidized. One might conclude from the stoichiometry at this point, that the oxidation of 2 equiv of ascorbate can mediate the formation of 1 equiv of ATP. The question arises, however, why this stoichiometry is only observed at a certain point and why the ratio of ascorbate reduced to ascorbate oxidized is constant during the $NADP^+$/NADPH steady state. After the light reaction has proceeded for 21 min, for example, 0.8 μmole of ATP are formed by the ascorbate-mediated photophosphorylation. According to the above mentioned stoichiometry, we should observe the oxidation of 1.6 μmoles of ascorbate. As a possible explanation for this discrepancy, the possibility of the rereduction of the oxidized ascorbate by NADPH as pointed out by Marrè and co-workers[22,23] was tested. By measuring the oxidation of NADPH in the presence of ascorbate, an enzyme fraction from spinach which contains $NADP^+$–ferredoxin reductase, and in the presence of xanthine *plus* xanthine oxidase as the generator couple for superoxide free radical ion (which can oxidize ascorbate), we made the observation, that the fastest oxidation of NADPH occurs in the presence of all components: xanthine oxidase, xanthine, and the enzyme fraction from spinach and ascorbate. The observed rates under the described conditions (0.3 μmole NADPH oxidized/h), however, are too small, to count as the sole mechanism for the reduction of oxidized ascorbate. The finding, that in isolated chloroplasts without an added electron acceptor, ascorbate can stimulate the photophosphorylation without being oxidized[4] (Table IV) suggests, that another or additional mechanisms than the one described by Marrè and co-workers[22,23] are responsible. We, therefore, tested the influence of dehydroascorbate on photophosphorylation in isolated chloroplasts without any other electron acceptor and found a marked stimulation of the endogenous rate of ATP synthesis (Table VII).

As shown in Table VII, the stimulation of the photophosphorylation by dehydroascorbate is inhibited by DCMU and proceeds independently from (essentially superimposed on) the photophosphorylation, which is mediated by ascorbate. This result can be explained by the assumption, that dehydroascorbate is acting as a Hill reagent.

Another observation supports this assumption. In heat-treated chloroplasts, ascorbate can mediate a photophosphorylation as already shown in Table V, whereas dehydroascorbate has no effect on the photophosphorylation in this system. Dehydroascorbate is apparently only a weak electron acceptor in systems where an electron donor is present. A reduction of dehydroascorbate by isolated chloroplasts in the light has been observed by other workers[24–26].

TABLE VII

EFFECT OF DEHYDROASCORBATE ON PHOTOPHOSPHORYLATION IN ISOLATED CHLOROPLASTS

Experimental conditions, see Table IV.

Additions	*μmoles ATP formed/mg chlorophyll per h*
None	5.5
10 μmoles dehydroascorbate	11.0
10 μmoles ascorbate	21
10 μmoles ascorbate *plus* 10 μmoles dehydroascorbate	30
10 μmoles dehydroascorbate *plus* $2 \cdot 10^{-5}$ M DCMU	1.2
10 μmoles dehydroascorbate *plus* 10 μmoles ascorbate *plus* $2 \cdot 10^{-5}$ M DCMU	1.6

DISCUSSION

The stimulation of oxygen uptake by isolated chloroplasts with a low-potential electron acceptor and the concomitant oxidation of ascorbate[7] on one hand and the stimulation of photophosphorylation under certain conditions[2–6] on the other hand, were the focus of investigations, concerning the function of ascorbate in photosynthetic reactions. We have attempted to find a common mechanism for the effects of ascorbate on the photosynthetic reactions in isolated chloroplasts. Our results can be summarized as follows:

(a) Ascorbate photooxidation, mediated by an autooxidizable acceptor of Photosystem I, like methyl viologen, is inhibited by superoxide dismutase. This inhibition is independent of whether the acceptor is reduced by light or by NADPH.

(b) ATP formation coupled to pseudocyclic electron transport is not influenced by superoxide dismutase, regardless of whether ascorbate is oxidized concomitantly.

(c) The stimulation of endogenous photophosphorylation by ascorbate (*i.e.* without any other addition) is abolished by superoxide dismutase.

(d) The endogenous photophosphorylation is also stimulated by dehydroascorbate, but ascorbate and dehydroascorbate together are even more effective.

(e) The stimulation of ATP formation coupled to photosynthetic $NADP^+$ reduction by ascorbate, when followed kinetically, becomes apparent only when 75% of the added $NADP^+$ has been reduced. ATP formation proceeds in the presence of ascorbate, after $NADP^+$ reduction has levelled off. This additional ATP formation is abolished by superoxide dismutase.

(f) NADPH oxidation by the superoxide radical ion (regenerated by xanthine oxidase) is stimulated by the addition of ascorbate.

These results suggest that a superoxide radical ion participates in the reactions described and is oxidizing ascorbate to monodehydroascorbate. The superoxide radical ion is formed by the reaction of oxygen with the reduced form of an electron acceptor like methyl viologen or AQ, which might either be reduced by light or by NADPH *via* the $NADP^+$-ferredoxin reductase.

The autooxidation of a reduced low-potential acceptor results in the formation of superoxide radical ion. According to Musso and Döpp[27], the events of the reoxidation of a reduced quinone-type electron acceptor can be written as follows (Reactions a and b; Acc, electron acceptor).

$$\begin{array}{l} |O| \\ \| \\ Acc + 2e \rightarrow \\ \| \\ |O| \end{array} \qquad \begin{array}{l} |\overline{O}|^{(-)} \\ | \\ Acc \\ | \\ |\underline{O}|_{(-)} \end{array} \tag{a}$$

$$\begin{array}{l} |\overline{O}|^{(-)} \\ | \\ Acc + O_2 \rightarrow \\ | \\ |\underline{O}|_{(-)} \end{array} \qquad \begin{array}{l} |\dot{O}| \\ | \\ Acc + O_2^{\cdot -} \\ | \\ |\underline{O}|_{(-)} \end{array} \tag{b}$$

The superoxide radical ion thus formed can be further reduced to hydrogen superoxide by ascorbate ($AscH_2$), forming the monodehydroascorbate radical AscH·, (Reaction c).

$$O_2^{\cdot -} + AscH_2 \rightarrow AscH\cdot + HOO^- \tag{c}$$

The fact that the oxidation of ascorbate in this reaction is accompanied by an increased oxygen uptake might be explained by the following assumption, though nothing is known about the thermodynamic equilibrium. Monodehydroascorbate radical can reduce the acceptor anion radical $\begin{pmatrix} O\cdot \\ Acc \\ O^- \end{pmatrix}$ to the dianion (Reaction d), which in turn can take up a second molecule of oxygen during its reoxidation (Reactions b and e). Reaction b is probably the rate-limiting step.

$$\begin{array}{l} \quad |O| \\ \quad | \\ AscH\cdot + Acc \rightarrow \\ \quad | \\ \quad |\underline{O}|_{(-)} \end{array} \qquad \begin{array}{l} |\overline{O}|^{(-)} \\ | \\ + Acc + H^+ + Asc_{ox} \\ | \\ |\underline{O}|_{(-)} \end{array} \tag{d}$$

$$\begin{array}{l} |\overline{O}|^{(-)} \\ | \\ Acc + O_2 \rightarrow \\ | \\ |\underline{O}|_{(-)} \end{array} \qquad \begin{array}{l} |O| \\ | \\ Acc + O_2^{\cdot -} \\ | \\ |\underline{O}|_{(-)} \end{array} \tag{e}$$

The acceptor anion radical $\begin{pmatrix} O\cdot \\ Acc \\ O^- \end{pmatrix}$ on the other hand can dismutate into the oxidized and reduced form. The reduced acceptor can autooxidize again, so that per mole of ascorbate oxidized the number of moles oxygen taken up may become more than 1, depending on the pH of the reaction medium and on the autooxidability of the acceptor[27]. A 2–4-fold stimulation of oxygen uptake by ascorbate, which has been observed by many groups[1,4,7,9] can be explained by the sequence of the Reactions b–e as the induction of a chain reaction (c, d, e,→c, d, e,...), which is independent of light as soon as a certain amount of the reduced low-potential acceptor is formed. A light-dependent production of a catalyst for oxidation of ascorbate in quinone-treated chard chloroplasts has been shown by Good and Hill[19]. These authors observed, that the oxidation of ascorbate and the oxygen uptake by isolated chloroplasts could carry on in the dark after an illumination period in the presence of a quinone. The above mechanism is in good agreement with these results (Fig. 2). The simultaneous dismutation of the acceptor anion radical, however, leads to a slow termination of this chain reaction. If superoxide dismutase is present in the reaction mixture, the concentration of $O_2^{\cdot -}$ is decreased drastically, since the rate constant of the superoxide dismutase reaction has been found to be about $1 \cdot 10^9$ to $2 \cdot 10^9$ at 25 °C[21,28]. The reaction sequence a to e, may be a feasible explanation for our results, especially as far as the oxygen uptake in the dark after an illumination period is concerned, if quinone-type electron acceptors like AQ are used. In the case of methyl viologen as autooxidizable electron acceptor, however, it seems rather unlikely, that the increased oxygen uptake in the presence of ascorbate can be explained by a reduction of the autooxidized dye by monodehydroascorbate radical, because of the very negative redox potential of methyl viologen. The increased oxygen uptake in this case might simply be explained by the fact, that ascorbate can reduce the superoxide radical ion to H_2O_2, avoiding the dismutation[9]. Since methyl viologen or AQ are the better electron acceptors for Photosystem I compared with monodehydroascorbate radical or dehydroascorbate, a net oxidation of ascorbate is observed. The observed stimulation of photophosphorylation in the absence of a low-potential electron acceptor by ascorbate is abolished by superoxide dismutase. Since ascorbate is not consumed in these reactions, we would like to propose a mechanism for these photophosphorylations, which includes the participation of the three redox states of ascorbate in a cyclic process as well as the superoxide radical ion.

We assume, that a compound (y), which acts as electron donor for Photosystem II, is oxidized; an electron acceptor (Acc–Fe(III)) is reduced by Photosystem I:

$$2y + ADP + P_i \xrightarrow{\text{Photosystems}} 2y^+ + 2e + ATP \qquad (I)$$

$$2e + 2\ \text{Acc–Fe(III)} \rightarrow 2\ \text{Acc–Fe(II)} \qquad (II)$$

The reduced acceptor is autooxidizable and can reduce oxygen:

$$2\ \text{Acc–Fe(II)} + 2\ O_2 \rightarrow 2\ \text{Acc–Fe(III)} + 2\ O_2^{\cdot -} \qquad (III)$$

The formed superoxide radical ion is reduced by ascorbate, forming a stoichiometric amount of peroxide:

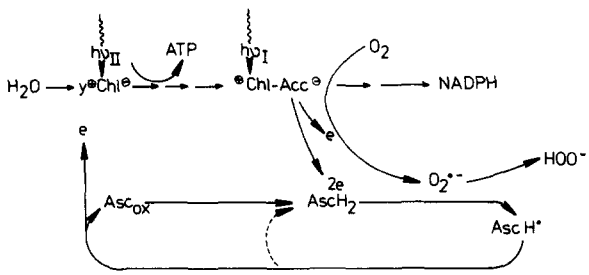
$$2\,AscH_2 + 2\,O_2^{\cdot -} \rightarrow 2\,AscH\cdot + 2\,HOO^- \qquad (IV)$$

The monodehydroascorbate radical, in turn can donate one electron to the photooxidized y^+:

$$2\,AscH\cdot + 2y^+ \rightarrow 2y + 2\,Asc_{ox} + 2\,H^+ \qquad (V)$$

This sequence of reactions can be inhibited by $2 \cdot 10^{-5}$ M DCMU at Reaction I and by superoxide dismutase at Reaction IV. In the system with $NADP^+$ as electron acceptor, Acc–Fe(II, III) is probably ferredoxin, which was added to the reaction mixture and which has been shown to reduce oxygen during its autooxidation[29].

According to this mechanism, the superoxide radical anion formed by the reaction of an unidentified acceptor for Photosystem I with oxygen (possibly bound ferredoxin[30–33]) oxidizes ascorbate and leads to the formation of monodehydroascorbate. The latter is an electron donor for Photosystem, II (at least in heat-treated chloroplasts, Table V, *cf.* ref. 1), or eventually (in the absence of a low-potential electron acceptor) an electron acceptor for Photosystem I in isolated chloroplasts with an intact water-splitting system. In both cases, a stimulation of electron transport is achieved, and it is measured as an increase in photophosphorylation. The above statements are summarized in Scheme 1.



Our results are not consistent with the proposal, that ascorbate acts only as a protective agent[34,35] in respect to photophosphorylation. We wish to support the idea, that ascorbate together with oxygen is able to maintain photophosphorylation under conditions, where the $NADP^+$-coupled photophosphorylation is bound to stagnate. As demonstrated by Champigny and Gibbs[36] the CO_2 fixation in isolated chloroplasts is stimulated several-fold by the addition of ascorbate. It has been shown, that about 40% of the total content of ascorbate in plant cells is located in the chloroplasts[37]. If the assumption is correct, that the rate of CO_2 fixation is governed by the availability of ATP, ascorbate may play a physiological role in the energy balance of the chloroplasts.

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