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ON THE PROPERTIES OF ASCORBATE PHOTOOXIDATION IN ISOLATED CHLOROPLASTS
EVIDENCE FOR TWO ATP SITES IN NONCYCLIC PHOTOPHOSPHORYLATION

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SUMMARY

Ascorbate photooxidation was studied in isolated spinach chloroplasts. The stoichiometry of the reaction is 1 μmole ascorbate oxidized per 1 μmole O_2 taken up. The reaction is stimulated by the addition of an electron acceptor for light reaction I, like anthraquinone or methylviologen; it is inhibited by DCMU and other inhibitors of light reaction II and does not take place in light above 700 mμ. The rate of ascorbate photooxidation is controlled by the phosphorylating system, since it is stimulated by the addition of either the phosphorylating system or an uncoupler. Coupled ascorbate photooxidation is inhibited by DCCD, an inhibitor of ATP formation. Heated spinach chloroplasts, not able to evolve O_2, still oxidize ascorbate coupled to ATP formation in a 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea-sensitive reaction. It is concluded that ascorbate replaces water as electron donor for light reaction II and that both light reactions and the electron transport system between the two light reactions including one ATP site are participating in ascorbate photooxidation.

The measured stoichiometry of ATP formation to ascorbate oxidation and O_2 uptake is 0.5:1:1. By subtracting the basal rate of uncoupled electron flow in ascorbate photooxidation from the rate of coupled electron flow, the corrected stoichiometry of 1 mole ATP per 2 electrons transferred is obtained. By the same calculation 2 moles ATP are formed per 2 electrons transferred, if water is the electron donor in a Hill reaction. Therefore the results indicate that there may be an ATP site between water and pigment system II; this ATP site is not operating in ascorbate photooxidation.

INTRODUCTION

The photooxidation of ascorbate by isolated chloroplasts has been studied in several laboratories[1–5] (see also reviews in refs. 3 and 6). When ascorbate is oxidized, 1 mole O_2 is taken up and 1 mole H_2O_2 is formed[3]. It was shown that the reaction is stimulated by an electron acceptor for light reaction I and is coupled to ATP for-

Abbreviations: DCCD, dicyclohexylcarbodiimide; DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea.

mation[3]. Ascorbate photooxidation by chloroplasts is inhibited by 3-(3',4'-dichlorophenyl)-1,1-dimethylurea (DCMU)[3] and *o*-phenanthrolin[1], suggesting that ascorbate is an electron donor before light reaction II. A similar conclusion was reached by FORTI AND JAGENDORF[7] in order to explain ascorbate-stimulated cyclic photophosphorylation. The recent experiments of KATOH AND SAN PIETRO[8] with heated chloroplasts of Euglena and of YAMASHITA AND HORIO[9] and YAMASHITA AND BUTLER[10,11] with Tris-washed chloroplasts supported the view that ascorbate is an electron donor for light reaction II.

We wish to report here on further properties of ascorbate photooxidation in spinach chloroplasts, particularly on the coupled ATP formation. It is suggested that in ascorbate photooxidation light reactions I and II and the complete electron transport chain between the two light reactions, including one ATP site, are involved. The results furthermore suggest a second ATP site in open chain electron transport between water and light reaction II.

METHODS

The broken chloroplast particles were prepared from spinach leaves according to the method of ALLEN *et al.*[33].

Heated chloroplasts: broken chloroplasts were heated in small portions of 1 ml with a concentration of 1 mg chlorophyll/ml at 40° for 1.5 min in 0.02 M Tris buffer (pH 8.0). The suspension was centrifugated at high speed, and the pellet was resuspended in 0.02 M Tris buffer (pH 8.0).

The photosynthetic reaction was carried out in Warburg vessels with a volume of 14 ml at 15°. After equilibration in air, the vessels were illuminated with 35000 lux (Philips–Attralux).

O_2 evolution and uptake were measured manometrically.

NADPH was measured at 340 mμ in the supernatant of the samples after centrifugation at high speed.

After stopping the reaction with 0.2 ml 20% trichloroacetic acid solution ATP was measured in an aliquot either enzymatically[36] (with phosphoglyceric acid, NADH, triose phosphate dehydrogenase, phosphoglycerate kinase by the decrease of absorbance at 340 mμ), or by the incorporation of radioactive phosphate (90000 counts/min ^{32}P per vessel), in organic phosphate according to Y. SUGINO AND Y. MIYOSHI[34].

After stopping the reaction with 0.2 ml 20% trichloroacetic acid solution ascorbic acid was measured in an aliquot according to Mohr's method (ref. 35): ascorbic acid reacts with diazotized 2-nitraniline to yield oxalic acid–2-nitrophenylhydrazide. With an excess of NaOH the red-violet sodium salt of this compound is formed and measured at 540 mμ.

RESULTS

The stoichiometry of noncyclic photophosphorylation in air with an autooxidizable compound as electron acceptor and water as electron donor (pseudocyclic electron transport) is 0.5 mole O_2 taken up and 1 mole ATP formed per 2 electrons transferred, when the endogenous catalase of chloroplasts is inhibited by KCN (ref.

12). The O_2 taken up is then trapped as H_2O_2 (ref. 3) (*i.e.*, Mehler reaction). Under the same conditions the stoichiometry of ascorbate photooxidation is 1 mole O_2 taken up and 1 mole ascorbate oxidized per 2 electrons transferred. This expected stoichiometry of O_2 uptake and ascorbate consumption is, however, not obtained at high pH, since water and ascorbate photooxidation are superimposed: O_2 uptake is higher than the expected amount when compared with the ascorbate used up (Table I, pH 7.8). At lower pH the rate of pseudocyclic electron transport with or without ADP/P_i is significantly lower than at higher pH. Ascorbate photooxidation then approaches the expected stoichiometry of 1 μmole O_2 taken up per 1 μmole ascorbate oxidized (Table I, pH 7.4). In order to show the properties of ascorbate photooxidation all further experiments were run at pH 7.4. Table II shows that ascorbate photooxida-

TABLE I

RATES AND STOICHIOMETRY OF QUINONE REDUCTION WITH WATER OR ASCORBATE AS ELECTRON DONOR AT DIFFERENT pH'S

15 min light in air, 35000 lux at 15°. The reaction mixture contained in 3 ml: 150 μmoles Hepes buffer (*N*-2-hydroxyethylpiperazine-*N'*-2 ethane sulfonic acid + NaOH); 0.1 μmole anthraquinone-2-sulfonic acid; 1 mM KCN; broken chloroplasts (P_1S_1) from spinach with 0.2 mg chlorophyll; 10 μmoles ADP, 10 μmoles P_i, and 5 μmoles $MgCl_2$ were added as indicated.

Additions to anthraquinone-2-sulfonic acid	*pH*	*Electron donor*				
		Water		*Ascorbate (10 μmoles)*		
		O_2 taken up (μmoles)	*ATP formed (μmoles)*	*O_2 taken up (μmoles)*	*Ascorbate oxidized (μmoles)*	*ATP formed (μmoles)*
—	7.4	0.5	—	1.7	1.65	—
ADP, P_i, Mg^{2+}	7.4	1.0	1.9	3.7	3.6	1.7
—	7.6	0.75	—	2.3	2.3	—
ADP, P_i, Mg^{2+}	7.6	1.5	3.1	4.6	4.0	2.6
—	7.8	0.95	—	3.3	2.6	—
ADP, P_i, Mg^{2+}	7.8	2.1	4.2	6.0	4.7	3.75

TABLE II

PROPERTIES OF ASCORBATE PHOTOOXIDATION COMPARED WITH WATER AS ELECTRON DONOR

Experimental conditions as in Table I; Hepes buffer, pH 7.4. AQ, anthraquinone-2-sulfonic acid.

Additions	*Electron donor*				
	Water		*Ascorbate*		
	O_2 taken up (μmoles)	*ATP formed (μmoles)*	*O_2 taken up (μmoles)*	*Ascorbate oxidized (μmoles)*	*ATP formed (μmoles)*
Without AQ	0.0	—	0.0	0.3	—
AQ	0.3	—	1.8	2.3	—
AQ, ADP, P_i, Mg^{2+}	0.9	1.5	3.5	3.9	1.4
AQ, ADP, P_i, Mg^{2+}, $2 \cdot 10^{-3}$ M NH_4Cl	2.7	0.4	6.2	4.8	0.4
AQ, ADP, P_i, Mg^{2+}, $1 \cdot 10^{-4}$ M DCCD	0.2	0.1	1.7	2.6	0.1
AQ, ADP, P_i, Mg^{2+}, $1 \cdot 10^{-4}$ M DCCD, $2 \cdot 10^{-3}$ M NH_4Cl	3.0	0.1	6.7	5.0	0.1
AQ, $2 \cdot 10^{-3}$ M NH_4Cl	3.4	—	7.1	4.9	—

tion is coupled with ATP formation. The stoichiometry of the reaction averages 0.5 μmole ATP formed per 1 μmole ascorbate oxidized (see Table VIII). Ascorbate photooxidation (measured as O_2 uptake as well as ascorbate disappearance) is stimulated by the phosphorylating system and by the uncoupler NH_4Cl (Table II). The inhibitor of ATP formation, dicyclohexylcarbodiimide (DCCD)[13], inhibits coupled ascorbate photooxidation; this inhibition is overcome by an uncoupler. Ascorbate photooxidation therefore responds to stimulation and inhibition of the electron transport chain, indicating clearly that ascorbate photooxidation is mediated by the coupled electron transport chain and not, as would have been possible, by a chemical chlorophyll-sensitized photooxidation[14].

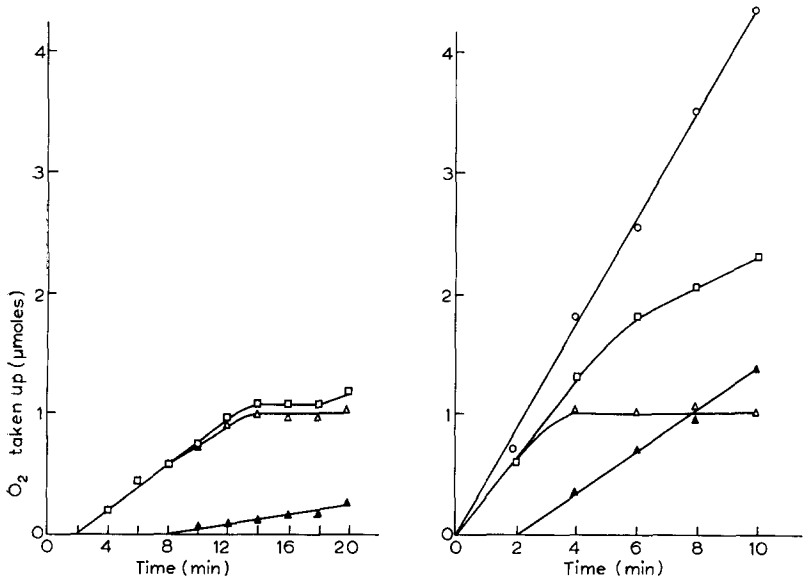


Fig. 1. Kinetics of ascorbate photooxidation at pH 7.4. Experimental conditions same as in Table I except that no ADP, P_i or Mg^{2+} was added. ▲—▲, without ascorbate; □—□, *plus* 1 μmole ascorbate; △—△, difference curve (□—□ *minus* ▲—▲).

Fig. 2. Kinetics of ascorbate photooxidation at pH 7.4 in the presence of an uncoupler. For experimental conditions, see Table I; $2 \cdot 10^{-3}$ M NH_4Cl in each vessel. ▲—▲, without ascorbate; □—□, *plus* 1 μmole ascorbate; ○—○, *plus* 10 μmoles ascorbate; △—△, difference curve (□—□ minus ▲—▲).

The kinetics of the photooxidation of a small amount of ascorbate in comparison with water as electron donor in the absence of the phosphorylating system or of an uncoupler is shown in Fig. 1. Under these conditions pseudocyclic electron transport with water as electron donor has a very low rate at pH 7.4, whereas O_2 is taken up after a lag phase when ascorbate is present. When the added amount of ascorbate is used up, O_2 uptake virtually comes to an end. By subtracting the two curves of O_2 uptake in the absence and presence of 1 μmole ascorbate, the difference curve approaches 1 μmole O_2 taken up, indicating again the stoichiometry of 1 μmole O_2 per 1 μmole ascorbate oxidized (Fig. 1). In the presence of the uncoupler, NH_4Cl, the rate of the pseudocyclic system with water as electron donor is 0.17 μmole O_2 taken up per min, whereas the rate in the ascorbate system (with 1 μmole ascorbate) is 0.32 μmole O_2 taken up per min, if the initial rates are compared (Fig. 2). Since in

the pseudocyclic system 0,5 mole O_2 and in ascorbate photooxidation 1 mole O_2 corresponds to 2 electrons transferred, the rates of the pseudocyclic and the ascorbate system are almost the same in the presence of an uncoupler. The difference curve in Fig. 2 indicates the stoichiometry of ascorbate oxidation and O_2 uptake as 1. The dependence of ascorbate photooxidation on the concentration of ascorbate is shown in Fig. 3. In the absence of the phosphorylating system the stimulation of O_2 uptake by ascorbate is about 4-fold, *i.e.*, the stimulation of electron flow is 2-fold (Fig. 3). In the presence of the phosphorylating system the rate of the pseudocyclic system is 0.07 μmole O_2/min and the rate of ascorbate photooxidation 0.23 μmole O_2/min, *i.e.*, the rates of electron flow are 0.07 to 0.115, respectively, the latter independent of

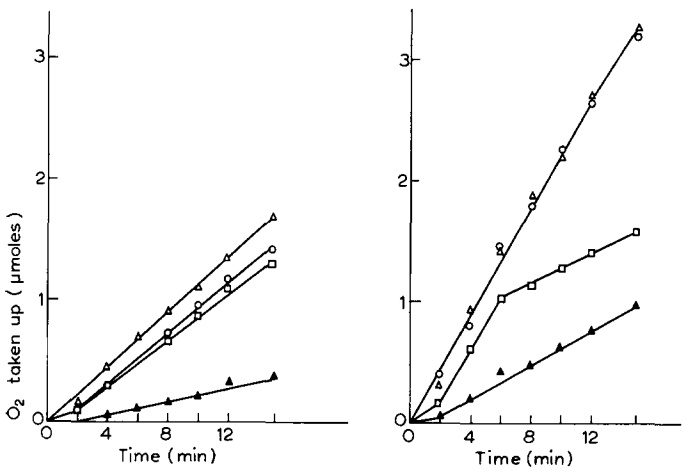


Fig. 3. Dependence of ascorbate photooxidation on ascorbate concentration at pH 7.4 in the absence of the phosphorylating system. For experimental conditions, see Table I; ▲—▲, without ascorbate; □—□, *plus* 1 μmole ascorbate; ○—○, *plus* 5 μmoles ascorbate; △—△, *plus* 20 μmoles ascorbate.

Fig. 4. Dependence of ascorbate photooxidation on ascorbate concentration at pH 7.4 in the presence of the phosphorylating system. For experimental conditions, see Table I; 10 μmoles ADP and P_i, 5 μmoles $MgCl_2$ in each vessel. ▲—▲, without ascorbate; □—□, *plus* 1 μmole ascorbate; ○—○, *plus* 5 μmoles ascorbate; △—△, *plus* 20 μmoles ascorbate.

ascorbate concentration, when initial rates are compared (Fig. 4). Therefore in the absence or presence of the phosphorylating system, but not in the presence of an uncoupler, ascorbate stimulates electron flow about 2-fold when compared with pseudocyclic electron transport.

Ascorbate photooxidation is stimulated by the addition of an electron acceptor for light reaction I; anthraquinone sulfonic acid and methyl viologen acting in the same way as shown in Table III. Both systems show the same response to an uncoupler and inhibitor of ATP formation, showing that, as expected, the nature of the electron acceptor at light reaction I does not matter.

Table IV shows the inhibition of ascorbate photooxidation by a number of inhibitors of light reaction II. All compounds inhibit at the same concentration, as they are known to inhibit O_2 evolution[15–17].

TABLE III

PROPERTIES OF ASCORBATE PHOTOOXIDATION MEDIATED BY TWO DIFFERENT ELECTRON ACCEPTORS

Experimental conditions as in Table I; Hepes buffer, pH 7.4.

Additions to 10 μmoles ascorbate	*Electron acceptor*					
	Anthraquinone-2-sulfonic acid (0.1 μmole)			*Methyl viologen (0.1 μmole)*		
	O_2 taken up (μmoles)	*Ascorbate oxidized (μmoles)*	*ATP formed (μmoles)*	*O_2 taken up (μmoles)*	*Ascorbate oxidized (μmoles)*	*ATP formed (μmoles)*
—	1.9	2.2	—	1.7	2.0	—
ADP, P_i, Mg^{2+}	3.3	3.3	1.0	3.0	3.0	1.1
ADP, P_i, Mg^{2+}, $2 \cdot 10^{-3}$ M NH_4Cl	6.7	5.4	0.4	5.8	4.6	0.4
ADP, P_i, Mg^{2+}, $1 \cdot 10^{-4}$ M DCCD	1.5	2.1	0.1	1.4	1.9	0.1
ADP, P_i, Mg^{2+}, $1 \cdot 10^{-4}$ M DCCD, $2 \cdot 10^{-3}$ M NH_4Cl	6.3	5.4	0.1	6.1	5.2	0.1
No electron acceptor	0.0	0.1	—	0.0	0.1	—

TABLE IV

INHIBITION OF ASCORBATE PHOTOOXIDATION BY A NUMBER OF INHIBITORS OF LIGHT REACTION II

Experimental conditions see Table I; Hepes buffer, pH 7.4; $2 \cdot 10^{-3}$ M NH_4Cl in each vessel.

Additions to 10 μmoles ascorbate	*O_2 taken up (μmoles)*	*Inhibition (%)*
—	5.7	
$2.0 \cdot 10^{-5}$ M DCMU	0.0	100
$2.0 \cdot 10^{-6}$ M DCMU	0.0	100
$2.0 \cdot 10^{-7}$ M DCMU	2.75	52
$1.6 \cdot 10^{-5}$ M trichloro-2-trifluoromethylbenzimidazole	0.0	100
$1.2 \cdot 10^{-5}$ M simazin	0.35	94
$0.8 \cdot 10^{-5}$ M 6-*n*-hexyl-3-methylmercapto-4-amino-1,2,4-triazine-5-on	0.1	99
$1.0 \cdot 10^{-4}$ M *o*-phenanthroline	1.55	73

TABLE V

ASCORBATE PHOTOOXIDATION AT DIFFERENT WAVELENGTHS

Experimental conditions, 5 min light ($6 \cdot 10^5$ erg/cm^2·sec) in air, $2 \cdot 10^{-3}$ M NH_4Cl and only 0.1 mg chlorophyll in each vessel.

Additions to 0.1 μmole anthraquinone-2-sulfonic acid	*Light >600 mμ (Schott RG 1)*	*Light >700 mμ (Schott RG 10)*
	Ascorbate oxidized (μmoles)	*Ascorbate oxidized (μmoles)*
10 μmoles ascorbate	1.0	0.0
10 μmoles ascorbate, 0.2 μmole diaminodurol, $2 \cdot 10^{-5}$ M DCMU	1.2	1.1

Table V shows that ascorbate photooxidation requires light reaction II, since ascorbate is oxidized by light above 600 mμ but not by light above 700 mμ. By adding an electron donor for light reaction I, for example diaminodurol, ascorbate is, however, oxidized by light above 700 mμ.

KATOH AND SAN PIETRO[8] have shown recently that in heated Euglena chloroplasts the photoreduction of $NADP^+$ at the expense of ascorbate is still possible. Ascorbate photooxidation is also possible in spinach chloroplasts heated for 1.5 min at 40°. In heated chloroplasts pseudocyclic electron transport with water as electron donor is no longer possible, since O_2 evolution has been destroyed. But quinone reduction with ascorbate as electron donor is still possible (Table VI). The stoichiometry of ATP formation to ascorbate disappearance and to O_2 uptake is exactly 0.5:1:1 (see also experiments in Table VIII). Since the pseudocyclic electron flow system cannot superimpose in these heated chloroplasts, the stoichiometry of the ascorbate photooxidation is clearly obtained. In heated chloroplasts ascorbate photooxidation is, as in untreated chloroplasts, stimulated by the phosphorylating system and by an uncoupler. Ascorbate photooxidation is still sensitive to DCMU in heated chloroplasts. The concentration necessary for 50% inhibition by DCMU ($2 \cdot 10^{-7}$ M) has not changed when heated and untreated chloroplasts are compared (Tables IV and VI).

TABLE VI

PROPERTIES OF ASCORBATE PHOTOOXIDATION IN HEATED CHLOROPLASTS

Experimental conditions see Table I; Hepes buffer, pH 8.0.

Additions to 0.1 μmole anthraquinone-2-sulfonic acid	*O_2 taken up (μmoles)*	*Ascorbate oxidized (μmoles)*	*ATP formed (μmoles)*
$2 \cdot 10^{-3}$ M NH_4Cl (H_2O as electron donor)	0.2	—	—
10 μmoles ascorbate	1.6	1.6	—
10 μmoles ascorbate, ADP, P_i, Mg^{2+}	3.1	3.2	1.6
10 μmoles ascorbate, $2 \cdot 10^{-3}$ M NH_4Cl	3.7	3.8	—
10 μmoles ascorbate, $2 \cdot 10^{-3}$ M NH_4Cl, $2 \cdot 10^{-6}$ M DCMU	0.5	0.5	—
10 μmoles ascorbate, $2 \cdot 10^{-3}$ M NH_4Cl, $2 \cdot 10^{-7}$ M DCMU	1.6	1.6	—

Though the experiments discussed so far clearly indicate that ascorbate is an electron donor for light reaction II, if a quinone is used as an electron acceptor, photosynthetic $NADP^+$ reduction does not seem to use ascorbate as electron donor in untreated broken chloroplasts. This is indicated by the known result that O_2 evolution is not depressed by addition of ascorbate when $NADP^+$ is used as electron acceptor, as would be expected when ascorbate replaces water as electron donor[18,19] (but see ref. 20). In heated chloroplasts, however, in which water can donate electrons to only a small extent[8], $NADP^+$ is reduced at the expense of ascorbate. The rates of $NADP^+$ reduction, however, are lower than the rate of the reduction of a quinone (Table VII).

The stoichiometry of ATP formation to O_2 uptake in coupled pseudocyclic electron transport with anthraquinone is, independently of pH, one, as it is in all Hill reactions, which is taken as evidence for the existence of only one ATP site in

TABLE VII

ASCORBATE AS ELECTRON DONOR FOR NADP REDUCTION IN HEATED CHLOROPLASTS

Experimental conditions as in Table I; Hepes buffer, pH 7.4; $2 \cdot 10^{-3}$ M NH_4Cl in each vessel; 6 μmoles $NADP^+$ were added together with 0.01 μmole ferredoxin. Chloroplasts (P_1S_1) were heated at 40° for 4 min.

Electron donor	*Electron acceptor*	*O_2 taken up or evolved (μmoles)*	*NADPH formed (μmoles)*	*Ascorbate oxidized (μmoles)*
Water	AQ	± 0.0	—	—
Ascorbate	AQ	− 2.6	—	2.6
Water	$NADP^+$	+ 0.2	0.4	—
Ascorbate	$NADP^+$	+ 0.15	1.3	0.9

open chain electron transport. However, KROGMANN *et al.*[21], GOOD[22] and IZAWA AND GOOD[23] have argued for many years that the stoichiometry of the Hill reaction becomes two when the basal rate of uncoupled electron transport (*i.e.*, in the absence of phosphorylating system) is subtracted from the rate of the coupled system. The ATP formation related to this difference of coupled and uncoupled electron transport is then two. If this type of calculation of the amount of ATP formed in noncyclic photophosphorylation could be applied to pseudocyclic electron transport of the Mehler-type reaction (Table I), it would follow that there are also two ATP sites in pseudocyclic electron transport. The stoichiometry of ATP formation to ascorbate photooxidation is 0.5 μmole ATP per 1 μmole ascorbate oxidized or 1 μmole O_2 taken up (see average of several experiments in Table VIII). If the calculation of GOOD is used for ascorbate photooxidation, it follows that in ascorbate photooxidation, only 1 μmole ATP is formed for 2 electrons transferred. The same is true for heated chloroplasts. In Table VIII the amount of ATP formed is calculated for the difference of the coupled *minus* the uncoupled basal rate of electron transport measured as O_2 uptake or as ascorbate oxidation. The average of the nine experiments shown there amounts to 0.96 μmole ATP formed per 2 electrons transferred.

DISCUSSION

Isolated chloroplasts oxidize ascorbate in the light, as shown by several laboratories[1–11] and reviews[3,6]. The stoichiometry of the reaction was determined to be 1 μmole of ascorbate per 1 μmole of O_2 taken up, if the endogenous catalase of the chloroplasts is inhibited by KCN and if the O_2 evolution is kept to a minimum by working at low pH or with heated chloroplasts. As already shown in a previous paper, 1 μmole H_2O_2 is formed per μmole O_2 taken up[3]. The reaction is strongly stimulated by a quinoid compound with a negative redox potential as final electron acceptor, like anthraquinone sulfonic acid or methylviologen. Ascorbate is therefore oxidized according to the following sequence.

$$\begin{array}{lll} Q + \text{ascorbate} \xrightarrow{h\nu} QH_2 & & + \text{dehydroascorbate} \\ QH_2 + O_2 \longrightarrow Q & & + H_2O_2 \\ \hline \text{Ascorbate} + O_2 \xrightarrow{h\nu} \text{dehydroascorbate} & & + H_2O_2 \end{array}$$

TABLE VIII

MEASURED STOICHIOMETRY OF ATP FORMATION TO ASCORBATE PHOTOOXIDATION AND CALCULATED STOICHIOMETRY FOR THE AMOUNT OF ATP FORMED TO THE DIFFERENCE OF COUPLED AND UNCOUPLED ELECTRON TRANSPORT

Experimental conditions as in Table I.

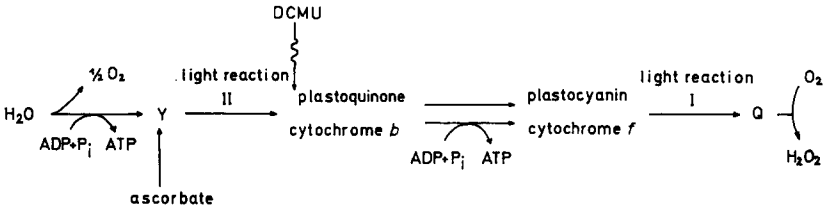
Expt. No.	*Additions to 10 μmoles ascorbate*	*O_2 taken up (μmoles)*	*Difference of coupled minus basal electron flow*	*Ascorbate oxidized (μmoles)*	*Difference of coupled minus basal electron flow*	*ATP formed (μmoles)*	*Measured stoichiometry* $\frac{ATP\ (\mu moles)}{Ascorbate\ (\mu moles)}$	*Calculated stoichiometry* $\frac{ATP\ (\mu moles)}{O_2\ (\mu moles)}$	*Calculated stoichiometry* $\frac{ATP\ (\mu moles)}{Ascorbate\ (\mu moles)}$
1	— ADP, P_i, Mg^{2+}	1.7 3.7	2.0	1.65 3.6	1.95	1.7	0.47	0.85	0.87
2	— ADP, P_i, Mg^{2+}	1.8 3.5	1.7	2.3 3.9	1.6	1.4	0.36	0.83	0.88
3	— ADP, P_i, Mg^{2+}	1.2 2.9	1.7	1.6 2.9	1.3	1.55	0.53	0.91	1.15
4	— ADP, P_i, Mg^{2+}	2.1 3.9	1.8	2.1 4.3	2.2	2.2	0.51	1.22	1.00
5	— ADP, P_i, Mg^{2+}	1.9 3.3	1.4	2.2 3.3	1.1	1.0	0.31	0.72	1.10
6	— ADP, P_i, Mg^{2+}	1.7 3.0	1.3	2.0 3.0	1.0	1.1	0.37	0.85	1.00
7	— ADP, P_i, Mg^{2+}	1.5 3.1	1.6	1.45 3.2	1.75	2.2	0.69	1.37	1.25
8 Heated chloroplasts	— ADP, P_i, Mg^{2+}	1.6 3.1	1.5	1.6 3.2	1.6	1.6	0.50	1.07	1.00
9 Heated chloroplasts	— ADP, P_i, Mg^{2+}	2.0 4.5	2.5	1.9 4.2	2.3	2.1	0.50	0.84	0.91
Average							0.47	0.96	1.02

A stoichiometric appearance of dehydroascorbic acid could not be measured, since dehydroascorbic acid is unstable under the conditions employed. There was no evidence, however, for the further photooxidation of dehydroascorbic acid or its degradation products by a similar reaction mechanism as proposed by HABERMANN AND HAYWARD[4]. This is concluded from the stoichiometry which is correct for the case where only 2 electrons are accepted from ascorbate. The disappearance of ascorbate and the concomitant O_2 uptake are sensitive to a number of inhibitors of light reaction II, like DCMU and other herbicides, as already shown earlier[1,3]. Ascorbate is photooxidized by light above 600 mμ, but not by light above 700 mμ. However, in the presence of diaminodurol as electron donor for pigment system I, ascorbate is also oxidized by the long-wavelength light. From these experiments it is concluded that ascorbate is an electron donor for light reaction II. This has also been recently proposed by KATOH AND SAN PIETRO[8], who showed that heated Euglena chloroplasts, unable to evolve O_2, are still able to reduce $NADP^+$ at the expense of ascorbate in a DCMU-sensitive and cytochrome$_{552}$-requiring reaction. YAMASHITA AND HORIO[9] and YAMASHITA AND BUTLER[10,11] showed that in Tris-washed chloroplasts (also unable to evolve O_2), $NADP^+$ reduction at the expense of ascorbate is still possible, and is DCMU sensitive, shows a red-light-drop phenomenon, and is stimulated by the addition of phenylene diamines. In addition to ascorbate several other compounds are now known to be electron donors for light reaction II: phenylene diamines[10,11,24], ketogulonic acid[5], hydroquinones[3,11], cysteine[8] and hydroxylamine[25].

According to the view that in quinone-stimulated ascorbate photooxidation, ascorbate is an electron donor for light reaction II and the quinone electron acceptor after light reaction I, the electron transport system between the two light reactions participates. This is strongly supported by the result that ascorbate photooxidation is coupled with ATP formation[3]. Furthermore ascorbate photooxidation is stimulated by the addition of ADP and P_i or by an uncoupler. An inhibitor of ATP formation, DCCD (ref. 13), inhibits coupled ascorbate photooxidation, and this inhibition is overcome by an uncoupler. Therefore ascorbate photooxidation shows a photosynthetic control as expected if a coupled electron transport chain is participating. The amount of ATP formed is now found to be 1 mole ATP per 2 moles ascorbate or O_2 consumed, under conditions where pseudocyclic photophosphorylation is suppressed (low pH or heated chloroplasts). Therefore the ratio of ATP formation per 2 electrons transferred in ascorbate photooxidation is 0.5:1; in comparison, the known stoichiometry of a Hill reaction or of pseudocyclic electron transport is 1 mole ATP per 2 electrons transferred. It seems difficult to understand how 0.5 mole ATP can be formed in an electron transport chain.

However, KROGMANN *et al.*[21], GOOD[22] and IZAWA AND GOOD[23] have put forward the hypothesis that 2 moles ATP are formed per 2 electrons transferred in the Hill reaction. According to the hypothesis of IZAWA AND GOOD[23], an uncoupled basal electron-transport chain runs parallel to a coupled chain. By subtracting the rate of the basal electron transport from that of the coupled electron transport, the corrected stoichiometry of 2 moles ATP per 2 electrons transferred is obtained. By using the same kind of calculation for the ascorbate photooxidation system, 1 mole ATP is formed per 2 electrons transferred (the average of several experiments is 0.96) when ascorbate is used as electron donor instead of water. This suggests that one ATP site is common to electron transport whether water or ascorbate is used as electron donor,

whereas the second ATP site is located in that electron transport chain operating only when water is oxidized. The validity of GOOD's argument has been questioned[26,27], but by accepting GOOD's argument, it follows that ascorbate photooxidation by-passes one of the two ATP sites operating according to GOOD in a Hill reaction. Scheme 1 shows this argument in a scheme, tentatively putting one ATP site between water and Y, and the ATP site which is responsible for the photosynthetic control in ascorbate photooxidation between the two light reactions. An ATP site between water and the second light reaction has also been proposed by SCHWARTZ[28,38] and KOK *et al.*[37]. Recent schemes of ARNON *et al.*[29] and ARNON[30] also put the ATP site in noncyclic phosphorylation between water and the light reaction. The results of YAMASHITA AND HORIO[9] and YAMASHITA AND BUTLER[10,11] suggest two ATP sites in open chain electron transport.



Scheme 1. Scheme for photosynthetic electron transport in coupled ascorbate photooxidation showing a second ATP site participating in water oxidation but not in ascorbate photooxidation.

As mentioned above the stoichiometry of 1 μmole O_2 taken up per μmole ascorbate photooxidized is obtained only at low pH or in heated chloroplasts, where pseudocyclic electron transport with the photooxidation of water is not superimposed. As has been known for some time[31], open chain electron transport from water has a low rate at low pH, whether the phosphorylating system is present or not. Since the uncoupler NH_4Cl strongly stimulates noncyclic electron transport, it follows that it is the pH profile of the phosphorylating system which is limiting electron transport[31]. At this low pH ascorbate stimulates O_2 uptake about 4-fold, which corresponds to a 2-fold stimulation of electron flow, whether the phosphorylating system is present or not. When the uncoupler NH_4Cl is present, addition of ascorbate stimulates O_2 uptake about 2-fold, which means that there is no additional stimulation of electron flow by ascorbate. Since ascorbate is able to stimulate electron transport in the presence of the phosphorylating system which controls the rate of electron flow from water, ascorbate must by-pass the limiting ATP site. This could also be taken as support for the participation of two ATP sites in water oxidation, but only one ATP site in ascorbate photooxidation. The ATP site before light reaction II has an optimum at a higher pH than the one between the two light reactions. Therefore at low pH, the phosphorylating site before light reaction II limits the rate of the electron transport system when water is the electron donor. When ascorbate is the electron donor, the phosphorylating site between the two light reactions becomes limiting. In the presence of an uncoupler the electron flow from ascorbate or water has the same rate, independent of pH, since all the limiting ATP sites are uncoupled. This interpretation also explains the older result that $NADP^+$ reduction at the expense of an electron donor for pigment system I at low pH is less limited by the ATP system than $NADP^+$ reduction with water as the electron donor[32].

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