# Analysis of Different Mechanisms of Photosynthetic Oxygen Reduction

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Three mechanisms of oxygen reduction by chloroplast lamellae in the presence of autoxidizable electron acceptors can be differentiated by product analyis in the presence or absence of either dibromothymoquinone (DBMIB) or superoxide dismutase (SOD) :

1) $H_2O_2$ is the product of two-electron oxygen reduction by 2,3-dimethyl-5,6-methylenedioxy-*p*-benzoquinone, involving only photosystem II. This reaction is not inhibited by either DBMIB or SOD.

2) Superoxide anion, and $H_2O_2$ as the product of its dismutation, are products of monovalent oxygen reduction following autoxidation of certain low potential electron acceptors (herbicides) of photosystem I. These reactions are not inhibited by SOD but are blocked by DBMIB.

3) $H_2O_2$ is the product of and "apparent" two-electron photoreduction of oxygen, mediated by certain *o*-diphenols (caffeic acid). These reactions are inhibited by both DBMIB and SOD indicating the involvement of photosystem I and of $O_2^-$ as an intermediate in the $H_2O_2$ producing reaction.

## Introduction

Hydrogen peroxide and the superoxide anion ($O_2^-$) are products of oxygen reduction by photosystem I of chloroplast lamellae [1, 2], stimulated by ferredoxin, certain quinones, or low potential dyes [1–4]. Oxygen reduction by photosystem II in the presence of dibromothymoquinone (DBMIB) has been reported [5, 6] yielding $H_2O_2$ apparently not derived via dismutation of $O_2^-$ [7].

Many of the above mentioned catalysts of oxygen reduction are herbicides [8] and/or inhibitors of photosynthetic electron transport [9, 10]. We wish to report on a testsystem, differentiating three mechanisms of oxygen reduction by different redox compounds.

## Materials and Methods

Chloroplast lamellae were isolated from spinach [11] by recentrifugation of intact chloroplasts in hypotonic buffer medium. Ferredoxin [12] and NADP-ferredoxin reductase [13] were isolated from spinach, superoxide dismutase from *Euglena gracilis* [14]. $O_2^-$ was determined as nitrite formation from hydroxylamine [15, 16] and $H_2O_2$ with the aid of NADH peroxidase (Boehringer, Mannheim). The oxygen reducing reactions were conducted as described in the tables and figures.

DIMEB and DBMIB were gifts from Prof. A. Trebst, Ruhr-Universität Bochum. Diquat, MPT and Bis-MPT were gifts from Drs. E. Ebert and H. P. Fischer, CIBA-Geigy AG Basel, Switzerland.

*Abbreviations:* DIMEB, 2,3-dimethyl, 5,6-methylenedioxy-*p*-benzoquinone; DBMIB, dibromothymoquinone; MPT, 2-(4-methyl-4-pyridinio), 1,3,5-triazinium bromide; Bis-MPT, 2,4-bis(4-methyl-4-pyridinio)-1,3,5-triazinium dibromide; AQ, anthraquinone-2-sulfonic acid; Diquit, 1,1′-dimethyline-2,2′-bipyridylium dibromide; Paraquat, MV, 1,1′-dimethyl-4,4′-bipyridylium dichloride.

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## Results

### *1) Effects of autoxidizable electron acceptors on photosynthetic, or on NADP-ferredoxin reductase-catalyzed oxygen reduction*

As recently communicated [7], DBMIB and DIMEB catalyze a two-electron transfer to oxygen forming $H_2O_2$ without $O_2^-$ as intermediate. Methylviologen (MV, paraquat) on the other hand seems to stimulate $H_2O_2$-formation via monovalent oxygen reduction, yielding $O_2^-$ and, $H_2O_2$ after dismutation of $O_2^-$. In Table I several compounds are compared for their activity in photosynthetic oxygen reduction. $NO_2^-$-formation from $NH_2OH$ is used as the test for the formation of $O_2^-$ [15, 16]. Two classes of compounds can be differentiated:

a) Compounds stimulating both $H_2O_2$- and $NO_2^-$-formation by illuminated chloroplast lamellae (AQ, diquat, paraquat, MPT, Bis-MPT) and,

Table I. Effects of DBMIB and DCMU on $H_2O_2$ production and hydroxylamine oxidation by chloroplast lamellae in the presence of autoxidizable electron acceptors.
The reaction mixture contained in 2 ml: phosphate buffer pH 7.8 (100 μmol); $NH_4Cl$ (2.5 μmol); $MgCl_2$ (2.5 μmol); KCN (1 μmol); $NH_2OH$ (1 μmol; only in the $NO_2^-$ vessels);
chloroplast lamellae with 100 μg Chl, indicated electron acceptors (0.04 μmol);
DCMU, DBMIB, ($10^{-5}$ M) where indicated.
The reactions were conducted for 10 min at 18 °C in white light (30,000 lx).

| Electron acceptor | Activity [μmol/mg Chl/h] | | | | | |
|---|---|---|---|---|---|---|
| | — | | +DCMU | | +DBMIB | |
| | $H_2O_2$ | $NO_2^-$ | $H_2O_2$ | $NO_2^-$ | $H_2O_2$ | $NO_2^-$ |
| none | 10.7 | 5.2 | 0 | 0 | 13 | 0.1 |
| AQ | 38.5 | 12 | 0 | 0 | 16.7 | 0 |
| diquat | 37.4 | 9.1 | 0 | 0 | 15 | 0 |
| paraquat (MV) | 36 | 11 | 0 | 0 | 17 | 0 |
| MPT | 74.8 | 14.3 | 0 | 0 | 17 | 0 |
| Bis-MPT | 66.8 | 12.5 | 0 | 0 | 16.6 | 0 |
| DIMEB | 30 | 2 | 0.6 | 0 | 25 | 0 |
| DBMIB | 13 | 0.1 | 1.5 | 0 | — | — |
| caffeic acid | 35 | 1.5 | 0 | 0 | 17 | 0.1 |

b) compounds stimulating $H_2O_2$-formation, but inhibiting $NO_2$-Formation (DIMEB, DBMIB, caffeic acid), as compared to oxygen reduction in the absence of an electron acceptor.

The inhibitors of photosynthetic electron transport (for a review see ref. [10]), DCMU and DBMIB, by ca. 100% inhibit $NO_2^-$-formation with all the compounds tested while $10^{-5}$ M DBMIB by roughly 50% inhibits $H_2O_2$ formation in the presence of all the tested compounds; DBMIB itself slightly stimulates $H_2O_2$ formation in the absence of other compounds (*c. f.* ref. [5]). DCMU inhibits $H_2O_2$ formation by ca. 100%, except a low rate remaining in the presence of either DBMIB or DIMEB (see also refs. [ 5 and 7]). A similar result as observed with illuminated chloroplasts is obtained if the above compounds are reduced by NADP-ferredoxin reductase with NADPH + $H^+$ as electron donor. As shown in Table II, AQ, diquat, paraquat, MPT and Bis-MPT to various extents produce both $H_2O_2$ and $NO_2^-$, AQ with an $E_0' = -0.2$ V being more active than the pyridylium salts with redox potentials ($E_0'$) of approx. $-0.35$ to $-0.45$ V [8] at physiological pH. DIMEB and DBMIB and, to some extent also caffeic acid stimulate $H_2O_2$-formation, but show no activity in hydroxylamine oxidation.

According to the results presented in Tables I and II, caffeic acid and DIMEB show similar behaviours as far as the product of oxygen reduction is concerned. This observation is emphasized by the results shown in Fig. 1: increasing concentrations of either DIMEB or of caffeic acid ($10^{-6}$ M up to $10^{-3}$ M) stimulate $H_2O_2$ formation (Fig. 1 b) but inhibit hydroxylamine oxidation (Fig. 1 a) by illuminated chloroplast lamellae, as compared to the catalysis by paraquat (MV).

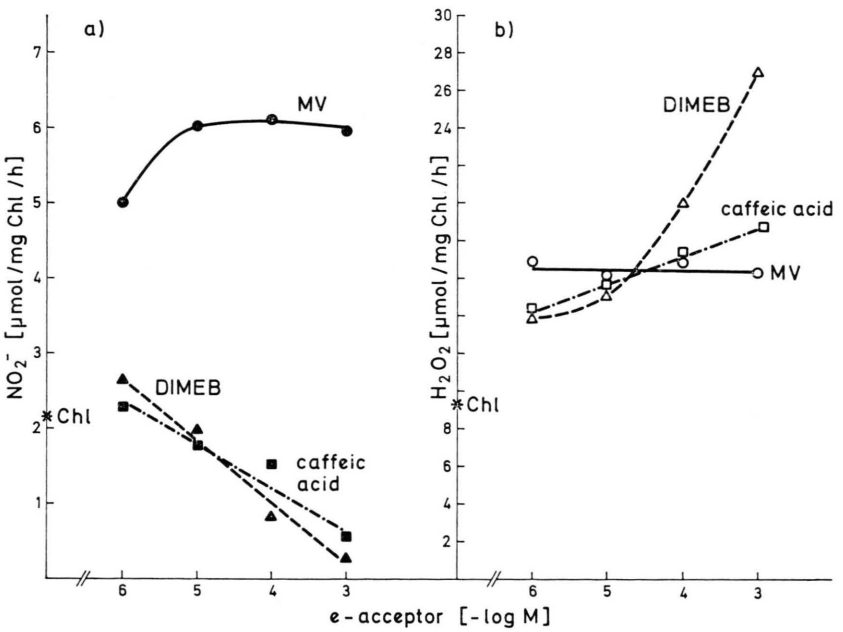


Fig. 1.
Effects of methylviologen (MV, Paraquat), 2,3-dimethyl-5,6-methylenedioxy-*p*-benzoquinone (DIMEB) and caffeic acid on $H_2O_2$ formation and hydroxylamine oxidation by illuminated chloroplast lamellae
a) hydroxylamine oxidation,
b) $H_2O_2$ formation.
The reaction mixtures and the reaction conditions were as outlined for Table I with the indicated additions.

Table II. Products of oxygen reduction by autoxidizable electron acceptors, reduced by NADP-ferredoxin reductase with NADPH+$H^+$ as electron donor.
The reaction mixture contained in 3 ml: Tris-HCl buffer pH 7.3 (150 μmol); $MgCl_2$ (2.5 μmol); NADP (1 μmol); glucose-6-phosphate (20 μmol); glucose-6-P-dehydrogenase (Boehringer, 25 μg);
KCN (1 μmol); $NH_2OH$ (3 μmol, only in the $NO_2^-$-vessels);
NADP-ferredoxin reductase (0.12 mg protein).
The reaction was conducted for 20 min at 18 °C in the dark.

| Electron acceptor | Products [nmol/h] | |
|---|---|---|
| | $H_2O_2$ | $NO_2^-$ |
| none | 40 | 0.1 |
| AQ | 3150 | 171 |
| Bis-MPT | 1190 | 54 |
| diquat | 650 | 33 |
| MPT | 340 | 16 |
| paraquat | 225 | 11 |
| DBMIB | 1460 | 0 |
| DIMEB | 790 | 0 |
| caffeic acid | 100 | 0 |

### 2) *Differentiation between two-electron (DIMEB) and two-times-one-electron (caffeic acid) oxygen photoreduction*

The apparently identical reactions in oxygen reduction (two-electron transfer) of DIMEB and caffeic acid can be differentiated by the addition of low concentrations of DBMIB ($10^{-6}$ M). As shown in Table III, similar to endogenous $H_2O_2$ formation, both paraquat- and caffeic acid-stimulated $H_2O_2$ formation are inhibited by $10^{-6}$ M DBMIB whereas the reaction in the presence of DIMEB is not influenced. Addition of SOD, on the other hand, inhibits endogenous as well as caffeic acid-catalyzed $H_2O_2$ formation, exhibiting no effect on paraquat- or DIMEB-catalyzed $H_2O_2$ production, however.

Table III. Effects of DBMIB or SOD on $H_2O_2$ formation by chloroplast lamellae in the presence of either caffeic acid, DIMEB or paraquat.
Reaction mixture and — conditions were as described for Table I.

A) Effect of $10^{-6}$ M DBMIB

| % Inhibition | Electron acceptor [$10^{-4}$ M] | $H_2O_2$ formed [μmol/mg Chl/h] | |
|---|---|---|---|
| | | −DBMIB | +DBMIB |
| 61 | none | 7 | 2.7 |
| 75 | caffeic acid | 28 | 7 |
| 67 | paraquat | 26 | 8.5 |
| 0 | DIMEB | 30.4 | 31 |

B) Effect of 60 * units SOD

| % Inhibition | Electron acceptor [$10^{-4}$ M] | $H_2O_2$ formed [μmol/mg Chl/h] | |
|---|---|---|---|
| | | −SOD | +SOD |
| 44 | none | 16 | 9 |
| 63 | caffeic acid | 35 | 13 |
| 0 | paraquat | 38 | 39 |
| 0 | DIMEB | 40 | 39 |

* *c. f.* ref. [16].

This result indicates that the apparently identical mechanisms of oxygen reduction in the presence of either DIMEB or caffeic acid (as indicated by product analysis) are different.

## Discussion

Photosynthetic formation of reduced oxygen species as the superoxide anion and $H_2O_2$ seems to play a role in plant metabolism (for reviews see refs. [17] and [18]). The stimulation of oxygen photoreduction, on the other hand, is apparently connected with the herbicidal activity of several commercially used low potential redox compounds [8, 19, 20]. The intent of the present paper is to differentiate between three mechanisms of oxygen reduction by illuminated chloroplasts in the presence of several cofactors of oxygen reduction. As outlined under "results", we differentiate:

### 1) *Catalysis of one-electron photoreduction by photosystem I*

This reaction, mediated by low potential redox compounds (AQ, diquat, paraquat, quaternary salts of pyridylium-triazines) forms $O_2^-$ and $H_2O_2$, inhibited by $10^{-6}$ M DBMIB, but not influenced by 60 units SOD [7, 14, 16]. The reaction involves reduction and autoxidation of the low potential compounds, forming $O_2^-$ and by dismutation of $O_2^-$, $H_2O_2$ [8, 21].

### 2) *Catalysis of $H_2O_2$ production, $O_2^-$ apparently not being the precursor of $H_2O_2$ nor an intermediary product or cosubstrate*

DBMIB and DIMEB have been shown to produce $H_2O_2$ not involving either photosystem I or $O_2^-$ [7]. In the presence of DIMEB, $H_2O_2$ is produced by a reaction which is not inhibited by DBMIB or by SOD. The mechanism of $H_2O_2$-formation may thus be an ionic one [22]; the electron donor is a component at the reducing site of photosystem II after the DCMU-block.

*3) Catalysis of an SOD-inhibitable $H_2O_2$ formation by photosystem I*

Stimulation of $H_2O_2$ production by *o*-diphenols has recently been investigated in connection with oxygen activation after mechanical wounding of sugar beet leaves [23]. In the case of caffeic acid, both DBMIB and SOD inhibit $H_2O_2$ formation indicating the involvement of photosystem I and $O_2^-$, where $O_2^-$ seems to be rather an intermediate or cosubstrate of the reaction than a product.

Since we have to assume that the primary acceptor of photosystem I of chloroplast lamellae (in the absence of ferredoxin and NADP) is a one-electron donor for oxygen ([1, 2], Table I), the following reactions may occur (see also refs. [24 and 25])

a) $O_2 + e^- \rightarrow O_2^-$ (initiation)

b) $O_2^- + o\text{-diphenol} + H^+ \rightarrow H_2O_2 + \text{semiquinone}$ (chainreaction)

c) $\text{semiquinone} + O_2 \rightarrow \text{quinone} + O_2^- + H^+$

d) $\text{quinone} + 2\,H^+ + 2\,e^- \rightarrow o\text{-diphenol}$

e) $2\,O_2^- + H^+ \xrightarrow{SOD} H_2O_2 + O_2$ (termination).

In the sequence a) to d), $e^-$ represents the reducing power of the primary electron acceptor of photosystem I. The sum of the chain-reaction (b – d) is:

f) $O_2 + 2\,H^+ + 2\,e \rightarrow H_2O_2$.

From this sequence b) to d) it is obvious that SOD blocks $H_2O_2$ formation since it terminates the cycle (reaction e) by avoiding reaction b and thus the formation of the autoxidizable semiquinone. In the presence of an *o*-diphenol that reacts with chloroplast-lamellar phenoloxidase (*e. g.* 3-hydroxytyramine) the above sequence (see ref. [23]) is less influenced by SOD (unpublished results) since not $O_2^-$ but phenoloxidase initiates the cycle.

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