Univalent Reduction of Molecular Oxygen by Spinach Chloroplasts on Illumination*

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SUMMARY

Spinach chloroplasts induced the photoreduction of cytochrome c and photooxidation of epinephrine, both of which depended on oxygen and were inhibited by superoxide dismutase. This confirmed the univalent reduction of molecular oxygen in illuminated chloroplasts.

In the presence of cytochrome c, hydrogen peroxide formation by chloroplasts was inhibited completely. Rates for the photoreduction of cytochrome c and the formation of hydrogen peroxide were equivalent, indicating that the univalent reduction of oxygen is a part of the Mehler reaction and the divalent reduction of oxygen is absent in chloroplasts.

Spinach ferredoxin, cytochrome c-reducing substance, and phenazine methosulfate did not stimulate or decrease the superoxide dismutase-inhibited rates of the photoreduction of cytochrome c and photo-oxidation of epinephrine. Chloroplasts treated with histone, which blocked Photosystem I, could not photooxidize epinephrine. Thus, we propose that the univalent reducing site of oxygen is a primary electron acceptor in Photosystem I.

The production of hydrogen peroxide in illuminated chloroplasts was demonstrated by Mehler et al. from the formation of acetaldehyde in a system of ethanol and catalase (1-3). This divalent photoreduction of molecular oxygen has been confirmed in chloroplasts of several organisms (4-8) and a relationship between the Mehler reaction and photophosphorylation suggested (8-10). Recently, in vivo formation of hydrogen peroxide by Anacystis nidulans was reported by Patterson and Myers (11), indicating that the Mehler reaction is not an artifact of isolated chloroplasts.

Previously, we showed that aerobic oxidation of sulfite is induced in chloroplasts on illumination. This oxidation was supposed to be initiated by superoxide radicals produced through the univalent reduction of oxygen by illuminated chloroplasts (12). We here report the photoreduction of cytochrome c and photooxidation of epinephrine by spinach chloroplasts under aerobic conditions and the inhibition of both reactions by superoxide dismutase, thus confirming the production of superoxide radicals in chloroplasts. On trapping superoxide radicals with cytochrome c no formation of hydrogen peroxide was found, showing that the univalent reduction of molecular oxygen is part of the Mehler reaction. Some evidence for the univalent reducing site of oxygen is also presented. Part of these experiments has appeared in preliminary form (13, 14).

MATERIALS AND METHODS

Once washed spinach chloroplasts were prepared in a grinding medium containing 0.4 M sucrose, 50 mM Tricine-KOH, pH 7.7 at 25° and 10 mM NaCl by the procedure described previously (15). To assay cytochrome c reduction and the formation of hydrogen peroxide, chloroplasts were purified by sucrose density centrifugation which removed contaminating ferrocytochrome c oxidase in the mitochondria and catalase in the peroxisomes. The chloroplasts isolated from 80 g of leaves were suspended in 6 ml of the grinding medium, then 2-ml aliquots were layered on a discontinuous sucrose density gradient consisting of 3 of 2.0 M, 3 ml of 1.8 M, 10 ml of 1.5 M, and 10 ml of 1.3 M sucrose. All sucrose fractions contained 10 mM Tricine-KOH, pH 7.7, and 10 mM NaCl. Centrifugation was carried out at 24,000 rpm (37,000 to 90,000 X g) for 3 hours in a Hitachi 55PA ultracentrifuge with an RPS-25A rotor. Mitochondria were found in the lower green band, based on the occurrence of ferrocytochrome c oxidase. The upper green band which sedimented in 1.3 M sucrose was collected and washed by centrifugation with the grinding medium.

The chloroplasts isolated were suspended in 50% glycerol, then stored at -20° as described previously (15). Under these conditions chloroplast retained their ability to reduce cytochrome c and to oxidize epinephrine for at least a month. The concentration of chlorophyll was determined spectrophotometrically (16).

The photoreduction of ferrocytochrome c was determined by the absorbance change at 550 nm. The difference between the absorption coefficients of ferro- and ferricytochrome c at 550 nm was assumed to be 19,000 M⁻¹cm⁻¹ (17). Light-induced oxidation of epinephrine was determined by the increase in absorbance at 480 nm due to the formation of adrenochrome, assuming an absorption coefficient of 4020 M⁻¹cm⁻¹ (18). Reactions were carried out at 25° using a Shimadzu UV-200 spectrophotometer modified so that the absorbance change could be monitored continuously with the appropriate interference filter while the cuvette was being irradiated from the side. Actinic light, provided by a 500-watt tungsten projector (50,000 lux), was passed through a red filter which cut off rays below 650 (cytochrome c) or 620 nm (epinephrine). The standard reaction mixture contained, in a final volume of 2 ml: 50 mM potassium phosphate, pH 7.8, 10 mM NaCl, chloroplasts containing 10 μg (cytochrome c) or 7.5 μg (epinephrine) of chlorophyll and 20 μM ferrocytochrome c or 500 μM DL-epinephrine. Reaction rates were determined from the initial absorbance change 20 s after illumination. The small dark rate was subtracted.

Hydrogen peroxide was measured fluorometrically according to

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1 The abbreviation used is: Tricine, 2-(hydroxymethyl)-2-(morpholinyl)-ethane sulfoacid

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the method of Guibault et al. (19) with a modification. Just after the photoreaction, 0.16 n mole of spinach peroxidase B (20) and 5 μmoles of homovanillic acid were added and the fluorescence intensity at 425 nm was determined while the sample was being excited at 315 nm with a Hitachi MPF-2 spectrofluorophotometer. After the photoreaction approximately 30 s were needed before the measurement could be taken. The fluorescence yield was not affected even when the two reagents were added 60 s after the onset of the photoreaction. This shows the absence of catalase in these sucrose density centrifugation-purified chloroplasts. The fluorescence yield due to hydrogen peroxide was determined in each sample from the increase in fluorescence intensity by adding an aliquot of hydrogen peroxide to the reaction mixture after each measurement. Hydrogen peroxide was standardized from its absorbance at 230 nm using an absorption coefficient of 71 M⁻¹ cm⁻¹ (21).

Superoxide dismutase and ferredoxin from spinach leaves purified to crystals were used (20, 22). The peroxidase used was a homogeneous preparation from spinach leaves (20). dl-Epinephrine, phenazine methosulfate, horse heart cytochrome c (type I), crystalline catalase from beef liver, and histone from calf thymus (type II) were obtained from Sigma. Other chemicals were reagent grade. Glass-distilled water was used throughout.

RESULTS

**Photo-reduction of Cytochrome c by Chloroplasts Mediated with Superoxide Anions—Cytochrome c is photoreduced by chloroplasts (17, 23–25). Recently, Nelson et al. observed a 50% inhibition of this reaction by superoxide dismutase (26).** Results in Figs. 1 and 2 confirm that with no autoxidizable additives the photoreduction of cytochrome c was inhibited more than 80% by superoxide dismutase. Half-inhibition occurred with about 5 nm spinach superoxide dismutase. The reduction rate of cytochrome c inhibited by 1 μM superoxide dismutase was between 12 to 24 μmoles per mg of chlorophyll per hour, depending on the chloroplast preparation. Since superoxide dismutase did not affect the photoreductions of dichlorodiphenol and NADP by spinach chloroplasts, we may assume that the depression of cytochrome c reduction by superoxide dismutase was not due to inhibition of the electron transport system by the enzyme.

The dependence on chloroplast concentration and the effect of cyanide are shown in Fig. 2. The slight increase in reduction by cyanide may be due to inhibition of superoxide dismutase contained in chloroplasts (22). The superoxide dismutase activity of erythrocyoubin is inhibited by cyanide (27). We also confirmed the inhibition of spinach superoxide dismutase by cyanide. In the presence of 1 mM cyanide, superoxide dismutase gave only a 35% inhibition of the photoreduction of cytochrome c. The effect of cytochrome c concentration on the reduction rate was the same as that reported by Keister and San Pietro (17). The concentration of 20 μM used here was near saturation. Half-saturation occurred at 5 μM.

The pH profile of the reduction rate in phosphate buffer is shown in Fig. 3. The highest rate occurred around pH 7.8. Inhibition by superoxide dismutase decreased at a low pH; at pH 6 the inhibition was only 40% even when 5 μM superoxide dismutase was added. When phosphate buffer was replaced by 50 mM Tricine, Tris, and N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid buffers at pH 7.8 or by 50 mM 2-(N-morpholino)ethanesulfonic acid buffer at pH 7.0, the respective inhibitions of photoreduction by 0.5 μM superoxide dismutase were 12, 35, 33, and 49%. However, in these organic buffers, the reduction rate was 2-fold that of phosphate buffer at pH 7.8. The low inhibition in the results of Nelson et al. (26) was probably due to the buffer used.

Adding spinach ferredoxin resulted in an enhancement of the photoreduction of cytochrome c, as previously observed (17, 24, 25). A further addition of 0.1 mM NADP had no effect on the reduction rate. In contrast to chloroplasts alone, the inhibition by superoxide dismutase was not remarkable (Fig. 4). In the presence of 1 to 3 μM ferredoxin, inhibition by superoxide dismutase was about 20%. This indicates that most of the photo-

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**Fig. 1.** Effects of superoxide dismutase on the photoreduction of cytochrome c by spinach chloroplasts. Standard reaction conditions were used except for the addition of spinach superoxide dismutase as indicated. The inset shows the time course for the photoreduction of cytochrome c in the absence and presence (S.D.) of 440 nM superoxide dismutase.

**Fig. 2 (left).** Effects of chloroplast concentration, cyanide, and superoxide dismutase on the photoreduction of cytochrome c. Standard reaction conditions were used except for varying chloroplast concentrations and, where indicated, adding 1 mM KCN or 530 nM spinach superoxide dismutase (S.D.).

**Fig. 3 (right).** Effects of hydrogen ion concentration on the photoreduction of cytochrome c by spinach chloroplasts in the presence and absence of superoxide dismutase. The reaction was carried out under standard conditions, except for the indicated pH of the phosphate buffer. Values shown for pH were determined at the end of the reaction. S.D. indicates the presence of 1.06 μM spinach superoxide dismutase.

**Fig. 4.** Effects of superoxide dismutase on the photoreduction of cytochrome c by spinach chloroplasts in the presence of varying concentrations of ferredoxin. The standard reaction mixture was used, except for the addition of spinach ferredoxin as indicated. S.D. shows the presence of 130 nM spinach superoxide dismutase.
ferredoxin and superoxide dismutase (Table II). Thus, hydrogen peroxide formed through the Mehler reaction did not in-
dismutase, with or without ferredoxin, the effect of the removal
reduction of cytochrome c, in either the presence or absence of
conditions may be due to direct electron transfer from a photo-
participation by oxygen. Table I shows the effects of oxygen on
was also not significantly affected under anaerobic conditions.
was also inhibited by superoxide dismutase.
reduction of cytochrome c proceeded through direct electron transfer
to cytochrome c from photoreduced ferredoxin with no
The photoreduction in the presence of ferredoxin
absence of oxygen. The low reduction rate under anaerobic
that cytochrome c was reduced by photoreduced phenazine
substrate: hydrogen peroxide and superoxide dismutase
The reaction rate was determined using a standard reaction
Conditions except for the addition of the compounds indicated.
PM
DCMU 3.2
10 µM PMS 97.8
1 µM PMS 62.7
1 µM PMS and 10 µM DCMU 3.2
0.1 mM benzyl viologen 33.0
0.1 mM methyl viologen 40.8
0.1 mM diquat 37.6
0.1 mM triquat 33.8

Effects of catalase, DCMU, PMS, and dipyridyl compounds on
The reaction was carried out using the standard reaction mixture
The reaction rate was determined using a standard reaction
Chloroplasts containing 10 µg of chlorophyll (---) and 530 nM superoxide dismutase (----) were added. Illumination conditions were the same as for the determination of cytochrome c reduction. Hydrogen peroxide was determined fluorometrically as described under "Materials and Methods."

Inhibition of cytochrome c reduction by superoxide dismutase
Inhibition of cytochrome c reduction by superoxide dismutase and cytochrome c on the production of hydrogen peroxide by illuminated spinach chloroplasts. The reaction mixture contained, in a total volume of 2 ml, 50 mM potassium phosphate, pH 7.8, 10 mM NaCl, and chloroplasts containing 10 µg of chlorophyll (O---O). In addition to the above reaction mixture, 20 µM cytochrome c (---), 20 µM cytochrome c and 530 nM superoxide dismutase (-----) and 530 nM superoxide dismutase (----) were added. Illumination conditions were the same as for the determination of cytochrome c reduction. Hydrogen peroxide was determined fluorometrically as described under "Materials and Methods."

Reduction of cytochrome c proceeded through direct electron transfer to cytochrome c from photoreduced ferredoxin with no participation by oxygen. Table I shows the effects of oxygen on the reduction of cytochrome c in the presence and absence of ferredoxin. The photoreduction in the presence of ferredoxin was also not significantly affected under anaerobic conditions. Without the addition of ferredoxin, the rates decreased in the absence of oxygen. The low reduction rate under anaerobic conditions may be due to direct electron transfer from a photoreductant in the chloroplasts. In the presence of superoxide dismutase, with or without ferredoxin, the effect of the removal of oxygen was negligible.

The addition of catalase and ethanol did not affect the photoreduction of cytochrome c, in either the presence or absence of ferredoxin and superoxide dismutase (Table II). Thus, hydrogen peroxide formed through the Mehler reaction did not in-

<table>
<thead>
<tr>
<th>Conditions and addition</th>
<th>Cytochrome c reduced</th>
<th>Cytochrome c reduced</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>~500 nm superoxide</td>
<td>+~500 nm superoxide</td>
</tr>
<tr>
<td></td>
<td>dismutase</td>
<td>dismutase</td>
</tr>
<tr>
<td></td>
<td>µmoles/mg chlorophyll/hr</td>
<td>µmoles/mg chlorophyll/hr</td>
</tr>
<tr>
<td>Aerobic................</td>
<td>33</td>
<td>9</td>
</tr>
<tr>
<td>Anaerobic.............</td>
<td>10</td>
<td>11</td>
</tr>
<tr>
<td>Aerobic, 3.5 µM ferredoxin</td>
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<td>93</td>
</tr>
<tr>
<td>Anaerobic, 3.5 µM ferredoxin</td>
<td>87</td>
<td>87</td>
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</table>

Effects of oxygen and superoxide dismutase on photoreduction of cytochrome c by spinach chloroplasts in presence and absence of ferredoxin

Table I also shows the effect of dichlorophenylidimethyl urea, phenazine methosulfate, and several dipyridyl compounds on cytochrome c reduction. Inhibition by dichlorophenylidimethyl urea clearly indicates that the electron transport system of the chloroplasts was responsible for this reaction. Phenazine methosulfate and dipyridyl compounds stimulated the photoreduction severalfold. In the presence of phenazine methosulfate reduction was inhibited by dichlorophenylidimethyl urea, but it was not significantly affected by superoxide dismutase. This suggests that cytochrome c was reduced by photoreduced phenazine methosulfate with no participation by the oxygen radical. The dipyridyl compound-stimulated reduction of cytochrome c was, however, inhibited by superoxide dismutase.

Inhibition of cytochrome c reduction by superoxide dismutase and the decrease in its rate under anaerobic conditions strongly suggests that this reduction was mediated by superoxide anion radicals formed through the univalent reduction of molecular oxygen in illuminated chloroplasts. Superoxide anions are spontaneously or with superoxide dismutase catalytically disproportionate to molecular oxygen and hydrogen peroxide. Therefore, if superoxide anions donated electrons to cytochrome c a cessation in the formation of hydrogen peroxide should occur. Fig. 5 shows that this is the case. In the absence of any additives, hydrogen peroxide was formed at 8 µmoles per mg of chlorophyll per hour (a). Dark controls or reaction mixtures containing 800 units of catalase showed no formation of hydrogen peroxide in the samples of Fig. 5. In the presence of cytochrome c, however, no hydrogen peroxide was formed. Under the same conditions, this chloroplast preparation reduced cytochrome c at 17 µmoles per mg of chlorophyll per hour (b) and in the pres-
ence of 1 mM cyanide the reduction rate was 21 μmoles per mg per hour (b'). When superoxide dismutase was present in addition to cytochrome c, hydrogen peroxide was again produced at the same rate as with chloroplasts alone. Under these conditions, the reduction rate of cytochrome c was decreased to 2.8 μmoles per mg of chlorophyll per hour (c).

The addition of dipyrindyl compounds to chloroplasts increased the production of hydrogen peroxide (data not shown). As expected from the results of Table II the addition of cytochrome c resulted in the cessation of the formation of hydrogen peroxide. When cytochrome c reduction was inhibited by superoxide dismutase, hydrogen peroxide was again produced. This is additional evidence for the formation of superoxide radicals during the autoxidation of reduced dipyrindyl compounds.

Adding superoxide dismutase alone to chloroplasts did not enhance the production of hydrogen peroxide. About 30 s was required between turning off the light and measuring the fluorescence intensity. This ineffectiveness of superoxide dismutase was probably due to the spontaneous disproportionation of superoxide anions during this time. The value for the spontaneous reaction rate of superoxide anions (28) is also a basis for this assumption. The superoxide dismutase-inhibited reduction rate of cytochrome c by the chloroplasts used in Fig. 5 was estimated as (b)–(c) or (b')–(c), i.e. 14.6 or 18.2 μeq per mg of chlorophyll per hour. The reduction rate of molecular oxygen determined from the formation of hydrogen peroxide was 2-fold (a), i.e. 16 μeq per mg per hour. The fairly good agreement between these values substantiates our supposition that the formation of superoxide radicals in chloroplasts is a part of the Mehler reaction and that hydrogen peroxide is formed only from superoxide radicals.

Photooxidation of Epinephrine by Chloroplasts—It has been shown that epinephrine oxidation is induced by superoxide radicals and is developed by free radicals (29, 30). To obtain additional evidence for the light-induced generation of superoxide radicals in chloroplasts we conducted experiments on the photooxidation of epinephrine.

The typical time course for epinephrine oxidation by the chloroplasts in Fig. 6 shows the dependence of the reaction on light. The dark oxidation rate was very low with chloroplasts from winter spinach. However, chloroplasts from spring spinach had a high dark rate, but the light minus dark rate was comparable to that of chloroplasts from winter spinach. A high dark oxidation was observed even with sucrose density centrifugation-purified chloroplasts from spring spinach. This dark oxidation disappeared completely in the presence of 1 mM potassium cyanide with no inhibition of photooxidation (Fig. 6).

Although the reason for this seasonal variation is not known, cyanide inhibition of dark oxidation suggests the possible participation of chloroplast-bound phenol oxidase (31, 32) in the dark reaction. Results presented here are those for chloroplasts from winter spinach.

The reaction rate was proportional to the chloroplast concentration up to 20 μg of chlorophyll per ml (Fig. 7). In boiled and Tris-treated chloroplasts and in the presence of dichlorophenylindothiazole the oxidation rate was very low, showing that the photooxidized reaction by chloroplast pigments was not responsible for this oxidation (Table III). The effect of epinephrine concentration is shown in Fig. 8. The apparent K<sub>m</sub> for epinephrine was estimated as 0.03 mM.

Only a slight stimulation of photooxidation was observed under 100% oxygen as compared with that under air. This agrees with the results of Heber and French who showed saturation of the Mehler reaction at low oxygen concentrations (6). Under anaerobic conditions epinephrine was not oxidized (Table IV). Inhibition of this oxidation by superoxide dismutase clearly shows the involvement of superoxide radicals in the reaction (Figs. 6 and 9). Half-inhibition occurred at 35 μM spinach superoxide dismutase. Thus, the possible donation of electrons from epinephrine to the oxidizing sides of Photosystem I or II was excluded.

Under standard conditions, the superoxide dismutase-inhibited rate of adrenochrome formation was between 30 to 75 μmoles per mg of chlorophyll per hour, depending on the chloroplast preparation. A higher rate than that for the photoreduction of cytochrome c probably indicates the occurrence of a chain reaction.

### Table III

<table>
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<tr>
<th>Experiments</th>
<th>Treatments or additions</th>
<th>Adrenochrome formed</th>
<th>Dichloroindophenol reduced</th>
</tr>
</thead>
<tbody>
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<td>1</td>
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<td>48.1</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Boiled chloroplasts</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>5 μM DCMU</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>None</td>
<td>75.8</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Tris-chloroplasts</td>
<td>19.9</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>None</td>
<td>37.8</td>
<td>62.3</td>
</tr>
<tr>
<td>7</td>
<td>12.5 μM histone</td>
<td>33.2</td>
<td>43.6</td>
</tr>
<tr>
<td>8</td>
<td>25 μg histone</td>
<td>16.9</td>
<td>27.7</td>
</tr>
<tr>
<td>9</td>
<td>37.5 μg histone</td>
<td>6.8</td>
<td>24.1</td>
</tr>
<tr>
<td>10</td>
<td>50 μg histone</td>
<td>2.0</td>
<td>20.9</td>
</tr>
</tbody>
</table>

*DClMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.*
tion even at pH 7.8. The photooxidation rate increased at an elevated pH. At pH 9.0 the rate was about 2-fold that at pH 7.8 and at pH 6.5 the rate dropped to about half. The apparent increase in oxidation at a high pH may be indicative of an extension of chain length, as reported by Misra and Fridovich (30).

Table IV summarizes the effects of several compounds on the photooxidation of epinephrine. In addition to the inhibition by superoxide dismutase which catalyzed the formation of hydrogen peroxide, the ineffectiveness of catalase and ethanol led us to assume that hydrogen peroxide did not induce the oxidation of epinephrine. Ferredoxin did not stimulate photooxidation, and by trapping reduced ferredoxin with NADP the oxidation rate was decreased only about 10%. Phenazine methosulfate, however, inhibited photooxidation even in the presence of ferredoxin, probably because it stimulated cyclic electron flow. All this suggests that in chloroplasts superoxide radicals are mainly formed through the univalent reduction of molecular oxygen by a bound electron carrier other than ferredoxin.

To see if photoreduced cytochrome c-reducing substance (34) is able to reduce univalently molecular oxygen, its effect on the photooxidation of epinephrine was tested. Cytochrome c-reducing substance was isolated from spinach chloroplasts according to the procedures of Fujita and Myers (34) with sonication, acetone precipitation, and gel filtration. As shown in Table IV, this substance did not appreciably increase photooxidation.

Treatment of chloroplasts with basic proteins bring about blockage of electron transport around Photosystem I (35). As shown in Table III, the photoreduction rate for dichloroindophenol was decreased to about one-third by histone treatment as Brand et al. also observed (35). However, under those conditions, the photooxidation of epinephrine was almost completely inhibited. This suggests that the probable site of the univalent reduction of oxygen was Photosystem I rather than Photosystem II.

**DISCUSSION**

Besides the initiation of the photooxidation of sulfite (12), the following observations support the supposition that spinach chloroplasts univalently reduce molecular oxygen on illumination and form superoxide anions. (a) Illuminated chloroplasts reduced cytochrome c and oxidized epinephrine, without the addition of autoxidizable compounds. Both reactions have been shown to be induced by superoxide anion radicals (29, 30, 36). (b) Both were inhibited due to superoxide dismutase removing superoxide radicals from the reaction mixture by disproportionating them to hydrogen peroxide and oxygen. Catalase,
however, did not affect the reactions. (c) Both were dependent on molecular oxygen. The requirement of oxygen for both reactions excluded the possibility of the formation of superoxide anions on the oxidizing side of Photosystem II as an intermediate in the photo-oxidation of water in chloroplasts; at least in the free state.

The average rate of superoxide anion formation by illuminated spinach chloroplasts was estimated at about 15 μmoles per mg of chlorophyll per hour, with no addition of autoxidizable compounds, from the superoxide dismutase-inhibited rate of cytochrome c photoreduction. This rate increased severalfold in the presence of dipyrrolid compounds (Table II). Since dipyrrolid compounds are photoreduced in chloroplasts (37–39) and the reduced forms of these compounds are autoxidizable free radicals (40), superoxide anions might be formed during their autoxidation. Stanclifte and Irvine (41) have shown that superoxide anions are formed during autoxidation of diquat-free radicals.

Formation of hydrogen peroxide was completely stopped by cytochrome c and, on an equivalent basis, the superoxide dismutase-inhibited rate of cytochrome c photoreduction was the same as that of hydrogen peroxide formation (Fig. 5). These results strongly suggest that hydrogen peroxide is formed through the disproportionation of superoxide anions produced by the univalent reduction of oxygen and the absence or the very low rate, if any, of the divalent reduction of oxygen in chloroplasts. Thus, the univalent reduction of oxygen was a partial reaction of the Mehler reaction and hydrogen peroxide was formed only from superoxide anions in the chloroplasts. Disproportionation would proceed spontaneously or catalyze with superoxide dismutase in chloroplasts (22). The spontaneous disproportionation rate was reported as $2 \times 10^6$ mol s$^{-1}$ at pH 7.7 (28).

Inhibition of superoxide anion-induced reaction by dihydroxyphenylamine, Tris treatment, or by boiling chloroplasts clearly indicates the participation of the electron transport system in the univalent reduction of oxygen (Table II and III). Although flavins univalently reduce oxygen under light (26, 42) the formation of superoxide anions by this mechanism could be eliminated because illumination in our experiments was given with a red light.

Adding spinach ferredoxin to chloroplasts did not stimulate the photooxidation of epinephrine, although the formation of superoxide anions (43, 44) and hydrogen peroxide (45) was shown during the autoxidation of reduced ferredoxin. On the other hand, the photoreduction of cytochrome c was enhanced by ferredoxin, but ferredoxin-stimulated reduction was not inhibited by superoxide dismutase, as was also observed by Nelson et al. (26). Furthermore, the ferredoxin-stimulated photoreduction of cytochrome c was not affected by the removal of oxygen (Table I). This suggests that the univalent reduction rate of oxygen due to exogenously added ferredoxin was very low compared with the rate due to the chloroplast-bound photo- reductant. Cytochrome c-reducing substance added exogenously also did not appreciably stimulate the photooxidation of epinephrine. However, the present results do not exclude the possibility that cytochrome c-reducing substance bound to chloroplast lamellae functioned as a reductant of oxygen.

In the presence of phenazine methosulfate photoreduction of cytochrome c was stimulated, but it was not inhibited by superoxide dismutase. In contrast, the photooxidation of epinephrine was inhibited by phenazine methosulfate (Tables II and IV). Honeycutt and Krogmann (8) showed that the photoreduction of oxygen on Anabaena lamellae was inhibited by pyocyanin or phenazine methosulfate. Inhibition of oxygen reduction by a cofactor of cyclic photophosphorylation suggests that oxygen and the cofactor are competing for a photoreductant bound to chloroplasts and that the reactivity for phenazine methosulfate was much higher than that for oxygen. Although superoxide anions are formed during the autoxidation of reduced phenazine methosulfate (46), lack of stimulation by phenazine methosulfate in the formation of superoxide anions in chloroplasts must be due to the fact that photoreduced phenazine methosulfate immediately donated electrons to the oxidizing side of Photosystem I.

All the evidence indicates that the major reducing site of oxygen was neither the ferredoxin nor the cytochrome c-reducing substance. Inhibition of the photooxidation of epinephrine due to histone treatment of the chloroplasts suggests that the reducing site of oxygen was Photosystem I rather than Photosystem II (Table III). Inhibition by dichlorophenyldimethylylurea (Tables II and III) also indicates a different site for the reduction of oxygen than that between Photosystem II and the site of action of dichlorophenyldimethylylurea. Fluorescence studies of chloroplasts under aerobic and anaerobic conditions have resulted in the hypothesis that Photosystem I is the reducing site of oxygen (6, 47, 48). Taking into account the effect of phenazine methosulfate, we propose that the primary electron acceptor of Photosystem I is the major univalent reductant of molecular oxygen in spinach chloroplasts.

From spectrophotometric evidence the primary electron acceptor of Photosystem I, P-430, has been proposed (49). Some evidence has also been presented to show that P-430 is a bound form of ferredoxin in chloroplasts (49–52). The ferredoxin-reducing substance is also a candidate for the primary electron acceptor (53). Identification of the reductant of oxygen and the determination of the function of superoxide dismutase in chloroplasts are interesting future projects because of the pronounced affect of oxygen on photorespiration (54).

Acknowledgment—We wish to express our sincere thanks to Professor Z. Kasai for his encouragement.

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