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O_2-Dependent Inhibition of Photosynthetic Capacity in Intact Isolated Chloroplasts and Isolated Cells from Spinach Leaves Illuminated in the Absence of CO_2

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Abstract. When isolated intact chloroplasts or cells from spinach (*Spinacia oleracea* L.) leaves are incubated in the light in the absence of CO_2, their capacity for subsequent CO_2-dependent photosynthetic oxygen evolution is drastically decreased. This inhibition is light and oxygen-dependent and can be prevented by addition of bicarbonate. It is concluded that the normal dissipation of photosynthetic energy by carbon assimilation and in processes related to photorespiration is an essential condition for the physiological stability of illuminated intact chloroplasts and cells.

Key words: Chloroplasts – Leaf cells – Photoinhibition – Photorespiration – Photosynthesis.

Introduction

Light energy trapped by chloroplasts in green cells of higher plants is predominantly utilized by concurrent carbon metabolism. In leaf cells of C_3 plants in the normal atmosphere (330 ppm CO_2, 21% O_2) a large part of recently assimilated carbon is channeled through the photorespiratory carbon oxidation cycle which is integrated with the photosynthetic carbon reduction cycle (Lorimer et al., 1978). At lower CO_2 concentrations in air an increasing proportion of carbon is diverted to the photorespiratory oxidation cycle as a consequence of the kinetic properties of ribulose-1,5-bisphosphate carboxylase/oxygenase. At the CO_2 compensation point (about 50 ppm CO_2 in 21% O_2 at 25° C) the endogenous generation of CO_2 by the photorespiratory oxidation cycle exactly balances CO_2 fixation by the photosynthetic reduction cycle. The photorespiratory oxidation cycle itself has a high demand for photosynthetic energy and appears to lower the cellular energy state (Krause et al., 1978).

If the light energy trapped by the chloroplast is not used in carbon metabolism, it is conceivable that the excess energy could cause damage to the light harvesting reactions of the photosynthetic apparatus. Such damage, termed photoinhibition, has been extensively studied in chloroplast fragments (i.e. isolated thylakoid systems) incapable of complete carbon assimilation, but also in algal cells and higher plants (see Satoh, 1970a, b; Björkman, 1973; Takahama and Nishimura, 1975). The results obtained from studies with isolated thylakoid membranes are, by and large, difficult to interpret and to relate to functional intact chloroplasts or cells of higher plants growing under natural illumination and atmospheric conditions. It is generally believed, for example, that the thylakoid membranes are protected against photoinhibition by pigments (carotenoids), chemical constituents (ascorbate, glutathione, and tocopherol) and enzymes, such as superoxide dismutase, which dismute superoxide radicals or quench singlet oxygen, the chemical species thought to be responsible for photoinhibition (Halliwell, 1978).

In this report we show that functional intact chloroplasts and isolated cells of spinach leaves show a rapid inhibition of photosynthetic capacity when illuminated in the absence of CO_2. The provision of CO_2 externally, or the internal generation of CO_2 via the photorespiratory oxidation cycle, seem to be essential to the stability of these illuminated photosynthetic systems.

Materials and Methods

Intact chloroplasts were isolated from spinach leaves as described by Heber (1973). The precentage of intact chloroplasts in each

Abbreviation: chl = chlorophyll

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preparation was determined by the ferricyanide reduction test (Heber and Santarius, 1970). Photosynthetic capacity of the isolated chloroplasts was determined by measuring light and CO_2-dependent O_2 evolution in an assay medium containing 0.33 mol l^{-1} sorbitol, 1 mmol l^{-1} $MgCl_2$, 1 mmol l^{-1} $MnCl_2$, 2 mmol l^{-1} EDTA, 10 mmol l^{-1} NaCl, 0.5 mmol l^{-1} KH_2PO_4 and 40 mmol l^{-1} HEPES, pH 7.6. The medium was purged with CO_2-free air at low pH to remove dissolved CO_2 and the pH adjusted with CO_2-free NaOH. Photosynthetic O_2 evolution was entirely dependent on added $KHCO_3$ (2 mmol l^{-1}). All assays contained 20 µg/ml catalase (cf. Egneus et al., 1975) which in contrast to a recent report by Allen (1977) routinely increased the rate of CO_2-dependent O_2 evolution. The details of each experiment, as well as the photosynthetic capacity and percentage of intact chloroplasts are given in the legends to each table and figure in the following section.

Intact cells of spinach leaves were isolated by the procedure of Nishimura and Akazawa (1975), with the following modifications. After stripping the lower epidermis, 1 × 1 cm pieces of leaf were vacuum-infiltrated in a solution of 0.7 mol l^{-1} mannitol, 1% (mass/vol.) potassium dextran sulphate (Meito Sangyo Co.) and 0.5% (mass/vol.) macerase (Calbiochem), pH 5.8 (KOH). Batches of 1 g leaf material in 10 ml of this maceration medium were incubated for 4–5 h at 23° C under gentle shaking. Cells liberated during this time had low photosynthetic capacity and were removed by decantation. The remaining leaf material was agitated with a magnetic stirrer for 5 min at 4° C with 10 ml of a solution of 0.7 mol l^{-1} mannitol and 20 mmol l^{-1} HEPES, pH 7.0 (KOH). The resulting slurry, containing a high proportion of single cells, was filtered through nylon mesh, centrifuged for 3 min at about 100 *g*, resuspended in 5 ml of the same medium and stored at 0° C. Photosynthetic capacity of these cells was assayed after gently centrifuging 2 ml of the above suspension and resuspending the cells in 5 ml of CO_2-free assay medium containing 0.6 mol l^{-1} mannitol and 40 mmol l^{-1} HEPES adjusted to pH 8.0 with KOH. A 2.5 ml aliquot of this solution was incubated in the cell of a Rank oxygen electrode. Light-dependent O_2 evolution was entirely dependent on the addition of $KHCO_3$ (4 mmol l^{-1}). The pH used was optimal and the addition of $CaCl_2$ (2 mmol l^{-1}), KH_2PO_4 (0.5 mmol l^{-1}) or catalase had no effect on the rate of O_2 evolution.

Pretreatments of chloroplasts or cells in the absence of CO_2 at different O_2 concentrations or light intensities were carried out in the O_2-electrode vessel which was thermostatically controlled to the temperatures specified. White light was provided by a 240 W–24 V iodine lamp and was filtered through a 10 cm water layer and an infra-red absorbing filter Calflex C (1 mm) from Balzers, Liechtenstein. For red and blue light the following additional filters were used: RG 630 cutoff filter (3 mm) from Schott & Gen., Mainz; filter 4–72 (3 mm), half band width about 380 to 535 nm, from Corning, New York.

Results

a) Isolated Intact Chloroplasts

When intact spinach chloroplasts were illuminated in the absence of CO_2, their capacity for CO_2-dependent photosynthetic oxygen evolution was substantially decreased. Figure 1 shows the time course of such photoinhibition. During irradiation with a high intensity of white light (870 W m^{-2}) the oxygen level in the medium was 9%. Pretreatment under the same

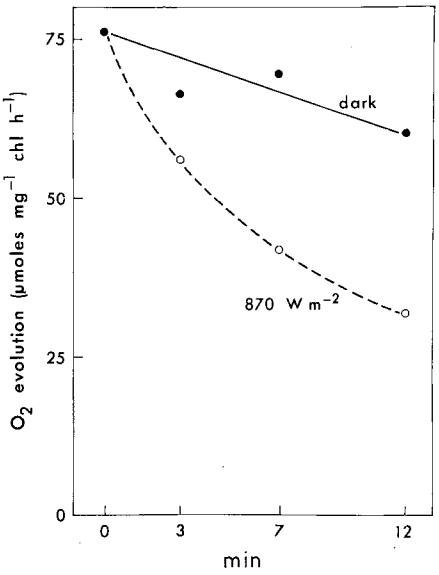

Fig. 1. Time-course of change in the photosynthetic capacity of isolated chloroplasts incubated in the light (870 W m^{-2} white light, (○) or dark (●) in CO_2-free buffer equilibrated with 9% O_2 at 15° C. At the end of the incubation 4 mmol l^{-1} $KHCO_3$ was added and the rate of O_2 evolution measured with 255 W m^{-2} white light. Chloroplasts equivalent to 33 µg chl/ml assay medium, 68% intact, were used and rates were corrected for broken chloroplasts

conditions, but in the dark, resulted in a much smaller decrease in the capacity of CO_2-dependent O_2 evolution. Addition of dithiothreitol (3 mmol l^{-1}) to the assay medium, or addition of ascorbate (2 mmol l^{-1}) to the stock suspension of chloroplasts had no apparent effect on photoinhibition or inactivation during incubation in the dark.

The extent of photoinhibition depended on the oxygen concentration in the medium, as shown in Figure 2. The isolated chloroplasts were pretreated for 7 min in the dark or the light (420 W m^{-2}) at different levels of oxygen, before bicarbonate was added and rates of light-dependent oxygen evolution determined. The results are shown as percent of control samples preincubated in the dark. In the experiment of Figure 2, the apparent K_i of oxygen was about 12% O_2.

Figure 3 shows the light-dependency of the inhibition. Isolated chloroplasts were incubated in the dark or light for 7 min in the absence of CO_2 at the air level of oxygen (21%). The rate of light-dependent O_2 evolution was then measured with 2 mmol l^{-1} $KHCO_3$ at 500 W m^{-2} red light. Rates are given as percent of dark-incubated controls. It may be seen that the photoinhibition was not light-saturated at the highest intensities applied. Although the type of response shown in Figure 3 has been observed in most experiments, it is worth mentioning that occa-

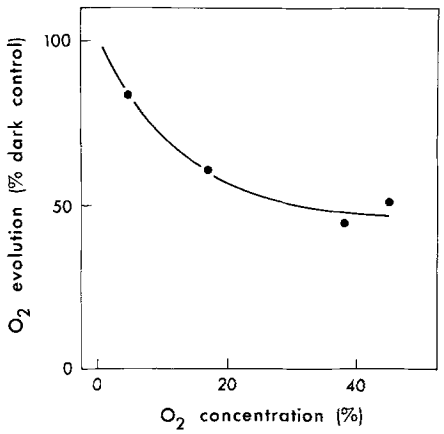

Fig. 2. Effect of O_2 concentration on the inhibition of photosynthetic capacity of isolated chloroplasts incubated for 7 min in the light (420 W m^{-2}) in CO_2-free buffer at 20° C. The rates are expressed as a percentage of the rates obtained following 7 min incubation in the dark under the same conditions. At the end of each incubation 4 mmol l^{-1} $KHCO_3$ was added and O_2 evolution measured with 420 W m^{-2} white light. The chloroplasts used (33 µg chl/ml) were 94% intact and corrected rates of O_2 evolution in controls were 170 µmol mg^{-1} chl h^{-1}

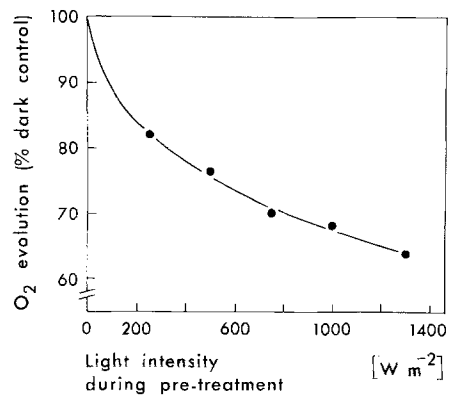

Fig. 3. Effect of intensity of white light on inhibition of photosynthetic capacity of isolated chloroplasts incubated for 7 min in CO_2-free buffer equilibrated with 21% O_2 at 20° C. At the end of the incubation 2 mmol l^{-1} $KHCO_3$ was added and the rate of O_2 evolution was measured in 500 W m^{-2} red light. The rates are expressed as a percentage of rates obtained with controls incubated for 7 min in the dark under the same conditions. Chloroplasts equivalent to 50 µg chlorophyll in 3 ml assay medium, 81% intact, were used. The corrected rates of O_2 evolution in the dark incubated controls ranged from 247 to 198 µmol mg^{-1} chl h^{-1} from the beginning to the end of the experiment

sionally damage was almost proportional to light intensity, and that in rare cases low-intensity light was scarcely injurious, while high-intensity light was destructive.

Illumination in the presence of CO_2 had no inhibitory effect on the photosynthetic capacity of intact chloroplasts, except for extremely high light intensities. Figure 4 shows a typical experiment in which 7 min pretreatment in the light in the presence of 2 mmol l^{-1} $KHCO_3$ at 25% O_2 resulted in faster rates of O_2 evolution than 7 min pretreatment in dark. If CO_2 was absent during the pretreatment, photosynthetic capacity was reduced by about 40%. Table 1 confirms this response in several other experiments and shows that red or blue light was equally effective in inhibiting the photosynthetic capacity of isolated chloroplasts in the absence of CO_2. No inhibition was observed following preillimination in the presence of 2 mmol l^{-1} $KHCO_3$. The presence of bicarbonate during incubation in the dark had no effect on subsequently measured rates.

b) Isolated Leaf Cells

When isolated spinach leaf cells are illuminated in the light in CO_2-free buffer, the capacity for subsequent CO_2-dependent O_2 evolution is reduced (Table 2). Substantial inhibition is observed following 20 min illumination with 500 n mol photons cm^{-2} s^{-1} white light. The inhibition is light-dependent and can be entirely prevented by bicarbonate; if 4 mmol l^{-1} $KHCO_3$ is present during illumination, stable rates of O_2 evolution can usually be observed for at least 30 min. In the experiments shown in Table 2 it is evident that the inhibition of photosynthetic capacity of illuminated leaf cells purged with CO_2-free air shows a dependence on O_2 concentration which is similar to that of isolated chloroplasts.

This response is complicated by the fact that these leaf cells are presumably capable of internal CO_2 production via the photorespiratory oxidation cycle which is itself dependent on O_2 concentration (Servaites and Ogren, 1978). That internal CO_2 production may partially protect the photosynthetic capacity of illuminated spinach leaf cells purged with CO_2-free air is indicated by preliminary studies with the inhibitor isonicotinyl hydrazide. These studies (Caers, Osmond, Inoue and Akazawa, unpublished) show that pretreatment with 10 mmol l^{-1} isonicotinyl hydrazide substantially inhibits (about 85%) CO_2 production from externally supplied glycine and further increases the inhibition of photosynthetic capacity of spinach leaf cells illuminated in CO_2-free buffer. Evidently both the provision of external CO_2 (Table 2) or the internal generation of CO_2 are important in the maintenance of photosynthetic capacity of illuminated leaf cells.

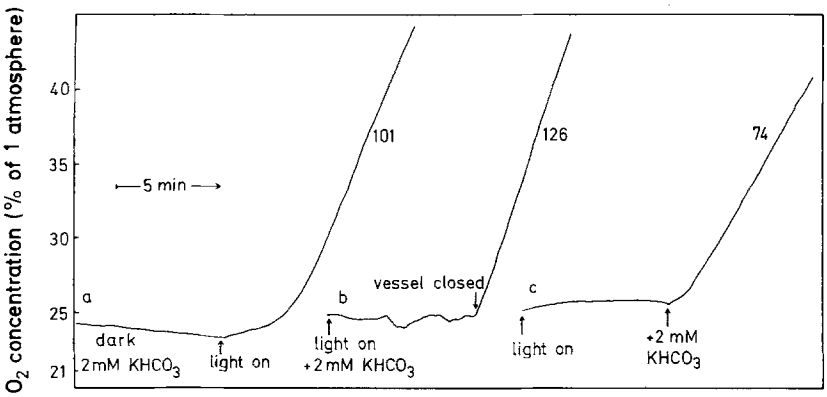

Fig. 4a–c. Recorder tracings of O_2 evolution by isolated chloroplasts following incubation in the presence or absence of $KHCO_3$ (2 mmol l^{-1}) in the light or dark in buffer equilibrated with 25% O_2 at 20° C. Chloroplasts equivalent to 100 µg chlorophyll in 3 ml assay medium, 84% intact, were used. The rates of O_2 evolution (µmol mg^{-1} chl h^{-1}) are shown for each trace.
a 7 min incubation with $KHCO_3$ in the dark;
b 7 min incubation with $KHCO_3$ in white light (500 W m^{-2});
c 7 min incubation without $KHCO_3$ in white light (500 W m^{-2})

Table 1. Photoinhibition of CO_2-dependent O_2 evolution of isolated intact chloroplasts by visible light of different spectral composition

Pretreatment conditions[a]	Rate (µmol mg^{-1} chl h^{-1})	Inhibition (%)
Red light 500 W m^{-2}		
2 mmol l^{-1} $KHCO_3$	126	–
$-KHCO_3$	62	51
Dark, 2 mmol l^{-1} $KHCO_3$	107	15
Red light 250 W m^{-2}		
2 mmol l^{-1} $KHCO_3$	182	–
$-KHCO_3$	119	35
Blue light 250 W m^{-2}		
2 mmol l^{-1} $KHCO_3$	160	–
$-KHCO_3$	113	29
White light 250 W m^{-2}		
2 mmol l^{-1} $KHCO_3$	127	–
$-KHCO_3$	86	32

[a] In all experiments pretreatment period was 7 min at 20° C and 21% O_2. In the high light pretreatment chloroplasts equivalent to 100 µg chlorophyll in 3 ml assay medium were used and were 86% intact. In the low light pretreatments chloroplasts equivalent to 50 µg chlorophyll per 3 ml were used and were 70% intact. All rates were determined in 500 W m^{-2} red light

Table 2. Photoinhibition of CO_2-dependent O_2 evolution of isolated spinach leaf cells preilluminated in white light

Pretreatment conditions	Expt[a]	Rate (µmol mg^{-1} chl h^{-1})	Inhibition (%)
Control			
4 mmol l^{-1} $KHCO_3$, 21% O_2	1	52	–
4 mmol l^{-1} $KHCO_3$, 21% O_2	2	64	–
$-KHCO_3$, 21% O_2	1	17	67
$-KHCO_3$, 21% O_2	2	31	52
$-KHCO_3$, 7,5% O_2	2	45	30
$-KHCO_3$, 5% O_2	1	39	25
$-KHCO_3$, 2% O_2	2	55	14
$-KHCO_3$, 1% O_2	1	49	6

[a] Experiment 1, pretreatment in 500 n mol photons $cm^{-2}s^{-1}$ white light; photosynthetic O_2 evolution measured in rate-limiting red light; 100 n mol photons $cm^{-2}s^{-1}$, 22° C. Experiment 2, pretreatment and assay of photosynthetic capacity measured in 500 n mol photons $cm^{-2}s^{-1}$, white light, 22° C

Discussion

The experiments reported above show that the capacity of photosynthetic CO_2-dependent O_2 evolution is reduced when intact isolated chloroplasts or leaf cells are preilluminated with visible light in the absence of CO_2 but presence of O_2. The oxygen dependence of these responses indicates that oxidative processes rather than pure photochemical reactions are involved in the decrease of photosynthetic capacity. The inhibition is not caused by partial inactivation of ribulose bisphosphate carboxylase due to CO_2 deprivation. Such inactivation has been studied in isolated chloroplasts by Bahr and Jensen (1978) and was found to be rapidly reversed by addition of bicarbonate. Accordingly, in our experiments CO_2-dependent photosynthetic O_2 evolution was not affected by the absence of CO_2 during a preceding dark-incubation. Further experiments are required to establish whether the lowered photosynthetic capacity observed in intact chloroplasts and cells following the above treatment results from inhibition of photosynthetic electron transport and/or photophosphorylation, described in a number of earlier reports. Chloroplast fragments show an O_2 and temperature dependent photoinhibition when illuminated in the absence of electron acceptors (Satoh, 1970a, b) and this inhibition appears to depend on the generation of superoxide and/or singlet oxygen by a Mehler type reaction of O_2 with reduced components of photosystem I (Asada et al., 1974; Takahama and Nishimura, 1975). If these processes are also involved in the inhibition of photosynthetic capacity of intact cells and chloroplasts illuminated in CO_2-free air it seems that the mechanisms thought to protect thylakoid membranes against photoinhibition (Halliwell, 1978) are not particularly effective in vivo.

The above experiments suggest that the presence of CO_2 and the orderly dissipation of photosynthetic energy is essential to the stability of illuminated intact chloroplasts and cells. We suggest that the protective effect of CO_2 is based on the utilization of photosyn-

thetic energy in reactions of assimilatory and photorespiratory carbon metabolism, which would prevent an over-energization of the thylakoid system in the light.

It is possible that when intact chloroplasts are illuminated in the absence of CO_2, the orderly dissipation of photosynthetic energy may continue to some extent if photosynthetic substrates and reserves are available. Such compounds can be converted via ribulose-1.6-bisphosphate to phosphoglycolate and 3-phosphoglycerate by the oxygenase reaction (Kirk and Heber, 1976; Krause et al., 1977). While glycolate is excreted, the phosphoglycerate is photosynthetically reduced. Reserve carbohydrates in the chloroplast may also be mobilised and enter the reduction cycle where they would be converted to ribulose-1.6-bisphosphate and subsequently to glycolate by ATP and NADPH requiring reactions. The variable concentration of these compounds in different chloroplast preparations presumably accounts for some variability in observed responses, particularly in the light intensity experiments described above.

The preliminary indication that internal CO_2 production from the photorespiratory oxidation cycle of intact cells can provide some protection to illuminated leaf cells purged with CO_2-free air requires further investigation. It is consistent with recent observations (Cornic, 1976; Powles and Osmond, unpublished) that illumination of intact leaves in CO_2-free air under conditions which prevent photorespiration results in a substantial decrease both in light saturated photosynthetic capacity and in the efficiency of light utilization (i.e. in quantum yields). These observations support the view that CO_2 production by the photorespiratory carbon oxidation cycle can function both to prevent CO_2 depletion in the cell and to provide a mechanism for the dissipation of excess photochemical energy under conditions of limited supply of exogenous CO_2 (Osmond and Björkman, 1972; Kirk and Heber 1976; Krause et al., 1978). The similarities and the differences between the apparent photoinhibition demonstrated in these isolated and intact systems remain to be assessed, as does the relationship between these processes and those described in chloroplast membrane fragments.

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