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REDUCTION OF OXYGEN BY THE ELECTRON TRANSPORT CHAIN OF CHLOROPLASTS DURING ASSIMILATION OF CARBON DIOXIDE

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SUMMARY

In photosynthetically competent chloroplasts from spinach the quantum requirements for oxygen evolution during CO_2 reduction were higher, by a factor often close to 1.5, than for oxygen evolution during reduction of phosphoglycerate. Mass spectrometer experiments performed under rate-limiting light indicated that an oxygen-reducing photoreaction was responsible for the consumption of extra quanta during carbon dioxide assimilation. Uptake of $^{18}O_2$ during reduction of CO_2 was considerably higher than could be accounted for by oxygen consumption during glycolate formation and by the Mehler reaction of broken chloroplasts which were present in the preparations of intact chloroplasts. The oxygen reducing reaction occurring during CO_2 assimilation resulted in the formation of H_2O_2. This was indicated by a large stimulation of CO_2 reduction by catalase, but not of phosphoglycerate reduction. Catalase could be replaced as a stimulant of photosynthesis by dithiothreitol or ascorbate, compounds known to react with superoxide radicals. There was no effect of dithiothreitol and ascorbate on phosphoglycerate reduction. A main effect of superoxide radicals and/or H_2O_2 was shown to be at the level of phosphoglycerate formation. Evidence for electron transport to oxygen was also obtained from $^{14}CO_2$ experiments. The oxidation of dihydroxyacetonephosphate during a dark period or after addition of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone in the light was studied. The results indicated a link between the chloroplast pyridine nucleotide system and oxygen. Oxygen reduction during photosynthesis under conditions where light is rate limiting is seen as important in supplying the ATP which is needed for CO_2 reduction but is not provided during electron transport to NADP. A mechanism is discussed which would permit proper distribution of electrons between CO_2 and oxygen during photosynthesis.

INTRODUCTION

For the photosynthetic reduction of CO_2 in the Calvin cycle, ATP and NADPH are required at a ratio of at least 1.5/1. The corresponding ratio in current

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proposals on the mechanism of C_4-photosynthesis is 2.5/1 [1]. The ratio of ATP produced during the transport of two electrons from water to NADP is, after 20 years of work, still a matter of controversy. Values ranging from 1 to 2 have been proposed [2–6]. If ATP production during NADP reduction in functional chloroplasts is insufficient to supply the ATP necessary for photosynthesis, as substantial evidence indicates, then where does the missing ATP come from? Early work with broken chloroplasts suggests two processes capable of supplying the extra ATP. Cyclic photophosphorylation, which has been observed at high rates only in broken chloroplasts supplied with unphysiological cofactors or in the absence of oxygen, does not lead to a gas exchange reaction. In pseudocyclic photophosphorylation ATP is synthesized during electron transport from water to oxygen. While there is an oxygen exchange, its net balance is zero. Evidence for a physiological role of either process in photosynthesis is mostly negative or unavailable [7]. Mass spectrometric gas exchange measurements performed with intact cells have indicated oxygen uptake during photosynthesis [8–12]. Isolated broken chloroplasts can also reduce oxygen during photosynthetic electron transport [13, 14]. However, such results are very difficult to evaluate. A complicating factor in work with intact cells is the dark respiratory system. In addition, photorespiratory processes causing oxygen uptake, such as the formation of glycolate and its oxidation during photosynthesis, are difficult to account for quantitatively in a balance sheet trying to sort out individual processes which contribute to the observed oxygen uptake in intact cells in the light. We have therefore undertaken to investigate oxygen uptake during photosynthesis of isolated chloroplasts, which are functionally intact and are capable of very high rates of photosynthesis, but lack the complications of secondary metabolism as they do not possess a functional glycolate pathway.

MATERIALS AND METHODS

Chloroplast preparation

Chloroplasts were isolated from greenhouse- or field-grown spinach (*Spinacea oleracea* L) in a modification [2] of Jensen and Bassham's method [15]. The chloroplasts were washed once in Jensen and Bassham's solution A and often a second time in solution B. Ascorbate was omitted from solution C. The percentage of chloroplasts in the preparations without intact outer membranes (broken chloroplasts) was determined according to Heber and Santarius [16]. Preparations containing between 75 and 95 % intact chloroplasts were used in most experiments, except in the mass spectrometer experiments where more than 85 % of the chloroplasts used were intact. The stability of the chloroplasts was good. For example, after 10 h storage at O °C the percentage of intact chloroplasts decreased in one preparation from 95 % to 77 %. CO_2-dependent oxygen evolution of the chloroplasts was high. All chloroplast preparations used reduced more than 100 μmol $CO_2 \cdot mg^{-1}$ chlorophyll $\cdot h^{-1}$ at light saturation. Phosphoglycerate was reduced at rates higher than 150 μmol $\cdot mg^{-1}$ chlorophyll $\cdot h^{-1}$ at light saturation. The assay medium used in the experiments had the following composition: sorbitol 0.33 M, $MgCl_2$ 1 mM, $MnCl_2$ 1 mM, sodium EDTA 2 mM, NaCl 10 mM, KH_2PO_4 0.15 mM, *N*-2-hydroxypiperazine-*N'*-2-ethanesulfonic acid 50 mM, pH adjusted to 7.6. Substrate concentrations were 2 mM. At these concentrations inhibition of photosynthesis by oxygen was negligible. When

dithiothreitol or catalase was added the concentrations were 3 mM and 1500–3000 international enzyme units/ml, respectively. The chlorophyll concentrations were usually 33 μg/ml assay medium. In the quantum requirement and mass spectrometer experiments 66 μg/ml and 100 μg/ml assay medium were used. Chlorophyll was determined according to Arnon [17].

Oxygen measurements

To distinguish between oxygen uptake appearing simultaneously with oxygen evolution a mass spectrometer was used (Varian, MAT CH7). A special adapter with direct access to the irradiation chamber of the mass spectrometer was constructed according to Hoch et al. [8]. The mass spectrometer was operated at an electron energy of 70 eV. The emission current was 100 μA, and the temperature 150 °C. The mass numbers between 24 and 46 were continously recorded in a cycle of 15–17 s. The mass peaks of the two oxygen isotopes $^{16}O_2$ and $^{18}O_2$ are a direct measure of the concentration of oxygen in the samples. The $^{18}O_2$ was obtained from Kernforschungszentrum, Karlsruhe. The assay solution was gassed with nitrogen before adding the $^{18}O_2$. After the addition of the isotope the total amount of oxygen was determined with an oxygen electrode. The samples containing chloroplasts, $^{16}O_2$ and $^{18}O_2$ were stirred in the dark in the adapter for about 10 min and then irradiated for 10 min.

CO_2- and phosphoglycerate-dependent oxygen evolution were determined with a Clark oxygen electrode. The samples were stirred in all oxygen experiments. The quantum requirements of CO_2 and phosphoglycerate reduction were measured with an oxygen electrode according to Heber [2].

^{14}C measurements

Products formed during CO_2 and phosphoglycerate reduction were measured using radioactive $Na_2{}^{14}CO_3$ or [^{14}C]phosphoglycerate. These compounds were added to the chloroplasts in the dark. The chloroplasts were than irradiated, under the same light conditions as in corresponding oxygen experiments. Samples were taken out at different times after the start of the irradiation and either killed in methanol for paper chromatography, or added to a small volume of acetic acid on planchets for determining CO_2 fixation. In the last case the aliquots were dried and counted in a windowless methane gas flow counter (Frieseke and Hoepfner). The methanol samples were analyzed by paper chromatography and radioautography according to Pedersen et al. [18]. The radioactivity of the various spots was determined with the methane flow counter. In order to be certain that the ^{14}C left on the origin after chromatography was starch several of the spots were hydrolyzed by sealing in glass tubes containing 0.5 ml 1 M HCl and digesting in a boiling water bath for 1 h. On re-chromatography the resulting hydrolysate gave only one radioactive spot which co-chromatographed with glucose.

Light sources

For the quantum requirements a 674 nm interference filter (half-band width 12 nm), in combination with a Calflex C heat filter, a 3 mm 630 nm cut-off filter and an 8 cm water filter, transmitted parallel light to the samples, whose absorption was measured in a large Ulbricht sphere. In the mass spectrometer experiments the same filter combination was used with the difference that the water filter was 2 cm. The

light was transmitted to the sample in the adapter of the mass spectrometer through a light pipe. In all other experiments a 3 mm 630 nm cut-off filter or a broad band interference filter (Balzers Filtraflex K 6, halfband width 50 nm) in combination with a Calflex C heat filter and an 8 cm water filter, was used. Light intensities were recorded with silicon photodiodes which had been calibrated against a compensated thermopile (Kipp and Zonen, Delft). Saturation of CO_2 or phosphoglycerate reduction was observed at 100 $W \cdot m^{-2}$.

RESULTS

1. Quantum requirements

The NADPH requirements for the evolution of oxygen during the reduction of CO_2 and of phosphoglycerate are the same. The quantum requirements for oxygen evolution by the two reactions should therefore be identical if the differing ATP requirements can be met by ATP produced during electron transport from water to NADP.

In earlier work [2], high and variable quantum requirements were found for the evolution of oxygen during CO_2 reduction, while oxygen evolution during phosphoglycerate reduction had a quantum requirement of about 8. The addition of dithiothreitol removed a rate-limiting step of CO_2 reduction and lowered its quantum requirement to 12 [2]. As will be shown later, catalase also serves to increase the efficiency of CO_2 reduction. The quantum requirements of phosphoglycerate- and CO_2-dependent oxygen evolution by intact chloroplasts in the presence of an excess of catalase, under conditions where light was strictly rate limiting, is shown in Fig. 1.

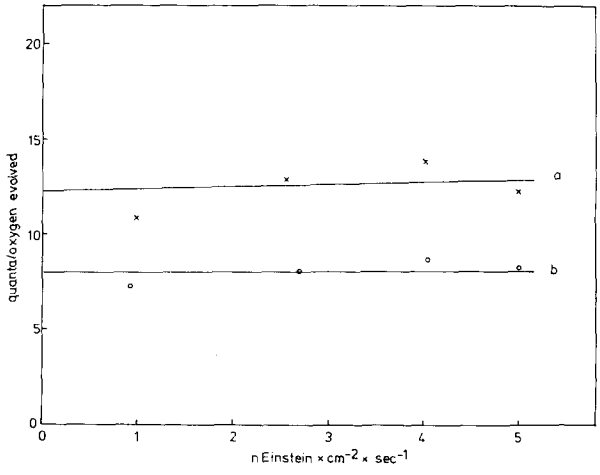


Fig. 1. The quantum requirements of CO_2- and phosphoglycerate-dependent oxygen evolution at 674 nm as a function of the light intensity. (a) CO_2 reduction (× — ×) (b) Phosphoglycerate reduction (○—○).

The quantum requirement was always higher for CO_2 reduction than for phosphoglycerate reduction. In Fig. 1 it is seen that approx. 50 % more light energy was required for CO_2-dependent oxygen evolution than for phosphoglycerate-dependent oxygen evolution. However, the situation varied somewhat with different chloroplast

preparations. In one extreme case, not shown, the quantum requirement of CO_2 reduction was only about 20 % higher than the corresponding value for phosphoglycerate reduction. At the substrate concentrations used, there was no measurable inhibition of photosynthesis by oxygen which could lower the quantum yield. Since phosphoglycerate and CO_2 reduction differ energetically only by the higher ATP requirement of the latter reaction, and since light was the rate-limiting factor in these measurements, we interpret the results as showing an extra energy requirement for the production of ATP.

2. Mass spectrometer experiments

Evolution of one molecule of oxygen from water indicates a transport of 4 electrons to an acceptor molecule. Current schemes of photosynthetic electron transport envisage 2 excitation steps per transferred electron [6]. A quantum requirement of 8 for phosphoglyceratedependent oxygen evolution (Fig. 1) therefore shows that phosphoglycerate is a highly efficient electron sink. Electrons generated in rate-limiting light will reduce physiological substrates such as phosphoglycerate as long as no secondary limitations force the electrons to reduce other acceptors which may be present in the system. One such acceptor is oxygen. The higher quantum requirement of CO_2-dependent oxygen evolution suggests that CO_2 is not the only electron acceptor during photosynthesis. It therefore appeared possible that oxygen is not only evolved but also reduced during CO_2 reduction. To test this, rates of exchange of $^{16}O_2$ and $^{18}O_2$ during photosynthesis were measured in a mass spectrometer. The light-dependent oxygen exchange catalyzed by intact chloroplasts in the presence of bicarbonate or phosphoglycerate is shown in Figs 2 and 3. The incident light energy was approx. 9 W · m^{-2}. At this intensity the reduction of CO_2 and phosphoglycerate

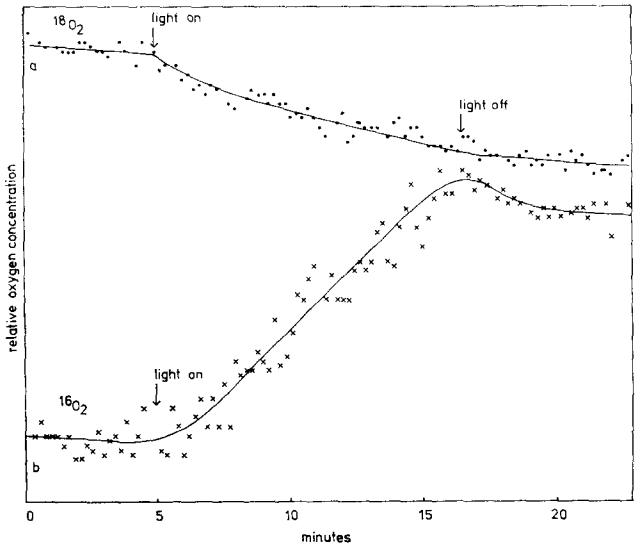


Fig. 2. Oxygen exchange from isolated chloroplasts of spinach under CO_2-reducing conditions, measured with a mass spectrometer, as a function of the irradiation time. Intensity of 674 nm light 9.2 W · m^{-2}. Percentage of intact chloroplasts 95. (a) $^{18}O_2$ (○—○) (b) $^{16}O_2$ (×—×).

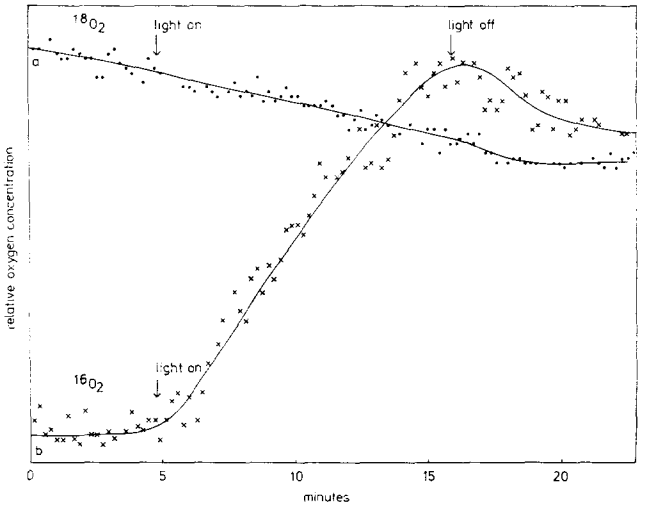


Fig. 3. Oxygen exchange from isolated chloroplasts of spinach under phosphoglycerate-reducing conditions, measured with a mass spectrometer, as a function of the irradiation time. Otherwise as in Fig. 2.

was proportional to light. In the dark, the concentration of both $^{16}O_2$ and $^{18}O_2$ decreased due to diffusion from the stirred samples to the mass spectrometer. Upon irradiation an oxygen evolution was observed in both the CO_2- and phosphoglycerate-reducing chloroplasts. This was caused by the oxidation of $H_2{}^{16}O$ and appeared as an increase in the concentration of the $^{16}O_2$ isotope only (Figs 2b and 3b). In contrast, the concentration of the $^{18}O_2$ isotope decreased faster in the light than in the dark. This shows that oxygen was taken up during CO_2 and phosphoglycerate reduction. However, the oxygen uptake was always much larger in the chloroplasts reducing CO_2 than in those reducing phosphoglycerate (Fig. 2a versus 3a). As the total oxygen concentration and the isotope composition in the samples was known, the rate of oxygen evolution and oxygen uptake could be calculated (Table I). The calcula-

TABLE I

OXYGEN EXCHANGE FROM ISOLATED SPINACH CHLOROPLASTS AS MEASURED WITH THE MASS SPECTROMETER

The percentage of intact chloroplasts varied between 87 and 95. Intensity of 674 nm light 9.2–9.5 $W \cdot m^{-2}$.

Expt	CO_2-dependent oxygen exchange ($\mu mol \cdot mg^{-1}$ chlorophyll $\cdot h^{-1}$)		Phosphoglycerate-dependent oxygen exchange ($\mu mol \cdot mg^{-1}$ chlorophyll $\cdot h^{-1}$)	
	Net O_2 evolution	O_2 uptake	Net O_2 evolution	O_2 uptake
1	10.3	5.1	17.4	0.9
2	10.8	7.9	20.3	1.9
3	12.1	4.9		
4	15.6	5.7	20.5	1.8
5	15.9	7.0	24.9	3.7
6	16.5	2.5		

tions were done under the assumption that $^{16}O_2$ is taken up simultaneously with $^{18}O_2$ in proportion to the partial pressure of the two oxygen isotopes. The oxygen uptake during CO_2 reduction was in most cases about 40 % of the net oxygen evolution. Owing to the simultaneous presence of dissolved $^{16}O_2$ and $^{18}O_2$ in the sample, this cannot be seen immediately in Fig. 2. In contrast, oxygen uptake during phosphoglycerate reduction was smaller, generally by a factor of about 10, than oxygen evolution. These experiments were done with chloroplasts which were washed only once, but no catalase was added.

3. Oxygen-consuming reactions in chloroplasts

Oxygen reduction as measured by $^{18}O_2$ uptake may be brought about by different reactions. Broken chloroplasts are known to reduce slowly oxygen to hydrogen peroxide [13], which may secondarily be decomposed into water and oxygen. By measuring the $^{18}O_2$ uptake by 100 % broken chloroplasts in the same system and under the same conditions which were used for phosphoglycerate and CO_2 reduction, the uptake due to broken chloroplasts could be accounted for. It was close to 0.5 μmol · mg^{-1} chlorophyll · h^{-1}.

Another reaction which leads to the uptake of oxygen is the formation of glycolate in intact chloroplasts. To get an estimate of this oxygen uptake, the distribution of the reaction products formed during CO_2 or phosphoglycerate reduction was determined. The conditions were the same as in the mass spectrometer experiments. During CO_2 reduction, phosphoglycerate, dihydroxyacetonephosphate and glycolate were major products (Fig. 4). Other labelled compounds were sugar mono- and diphosphates, and starch. During the illumination period glycolate accounted for less than 5 % of the incorporated carbon (Fig. 4).

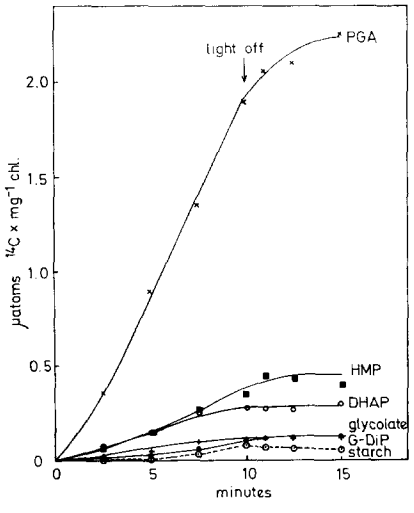


Fig. 4. Products formed by isolated chloroplasts of spinach from $Na_2{}^{14}CO_3$ as a function of the irradiation time. Intensity of 674 nm light 9.2 W · m^{-2}. Percentage of intact chloroplasts 86. $^{14}CO_2$-fixation 17.6 μmol · mg^{-1} Chlorophyll · h^{-1}. PGA = phosphoglycerate, SMP = sugar monophosphates, DHAP = dihydroxyacetone phosphate, SDP = sugar diphosphates.

Dominant reaction products of phosphoglycerate reduction were starch, which became unusually heavily labelled, dihydroxyacetone phosphate and sugar diphosphates. Calculated on a carbon basis, less than 5 % of the reaction products were represented by glycolate after 10 min of illumination.

Assuming a stoichiometry of 1 : 1 between glycolate formation and oxygen uptake [19], the contribution of glycolate formation to the total oxygen uptake observed in the mass spectrometer experiments could be calculated. When the oxygen uptake due to glycolate formation and the reactions in the broken chloroplasts are deducted from the total oxygen uptake, a balance sheet of oxygen consumption is obtained (Table II). It is evident that in addition to the two oxygen consuming reactions considered above a third type of oxygen uptake is present in the chloroplasts. This oxygen uptake was of considerable magnitude in the chloroplasts reducing CO_2 and was much smaller, or not present, in the chloroplasts reducing phosphoglycerate.

TABLE II

A BUDGET OF THE OXYGEN UPTAKE OBTAINED FROM THE MASS SPECTROMETER EXPERIMENTS

Mehler reaction: oxygen uptake due to hydrogen peroxide formation in broken chloroplasts [13] assuming that 10 % of the chloroplasts were broken. Light was less than half-saturating for photosynthesis. Otherwise see text and Table I. Oxygen uptake measured as $\mu mol \cdot mg^{-1}$ chlorophyll $\cdot h^{-1}$.

CO_2-reducing conditions				Phosphoglycerate reducing conditions			
Total O_2 uptake (A)	Mehler reaction of broken chloroplasts (B)	O_2-uptake due to glycolate formation (C)	A−(B+C) uptake	Total O_2 uptake (A)	Mehler reaction of broken chloroplasts (B)	O_2-uptake due to glycolate formation (C)	A−(B+C)
7.9	0.5	0.4	+7.0	3.7	0.5	1.9	+1.3
7.0	0.5	0.6	+5.9	1.9	0.5	1.6	−0.2
5.7	0.5	0.6	+4.6	1.8	0.5	1.6	−0.3
5.1	0.5	0.4	+4.2	0.9	0.5	1.3	−0.9
4.9	0.5	0.5	+3.9				
2.5	0.5	0.6	+1.4				

4. Effects of catalase, dithiothreitol and ascorbate on CO_2- and phosphoglycerate-dependent oxygen evolution.

To test the possibility that the unknown oxygen uptake reaction, which is apparent from Table II, was caused by a "physiological" Mehler reaction reducing oxygen to H_2O_2 in intact chloroplasts during photosynthesis, catalase was added to well-washed chloroplasts reducing CO_2 or phosphoglycerate. It was found to stimulate oxygen evolution. A typical recorder trace of CO_2-dependent oxygen evolution before and after an addition of catalase is shown in Fig. 6, curve a, for a low light intensity, an extreme response in curve b for a high light intensity. The intensity factor is not responsible for the large differences in the response of traces a and b, since the extent of the stimulation by catalase did not increase very much with light

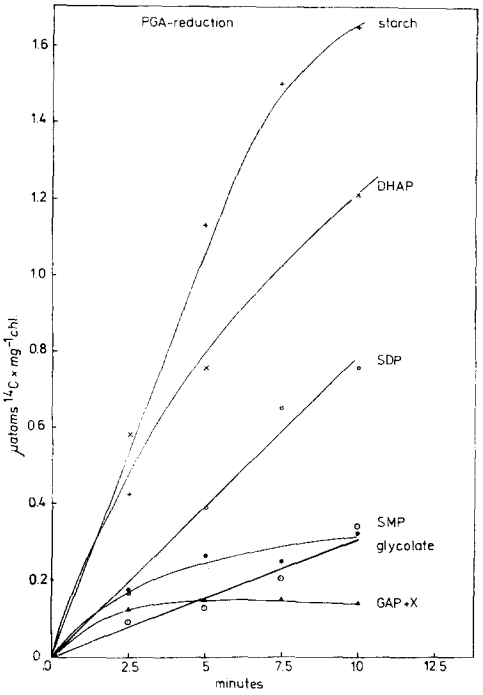


Fig. 5. Products formed by isolated chloroplasts of spinach from [^{14}C]phosphoglycerate as a function of the irradiation time. Intensity of 674 nm light 9.5 W · m^{-2}. Percentage of intact chloroplasts 86. Phosphoglycerate reduction 31.2 μmol · mg^{-1} chlorophyll · h^{-1}. GAP = glyceraldehyde phosphate, X = unknown, other abbreviations as in Fig. 4.

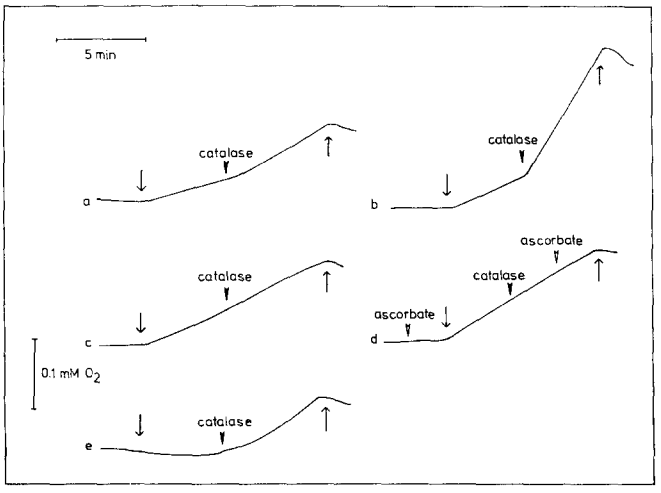


Fig. 6. CO_2- and phosphoglycerate-dependent oxygen evolution from intact chloroplasts of spinach. Light intensity (a, c, d) 25 W · m^{-2} (b, e) 200 W · m^{-2}. Percentage of intact chloroplasts 78. Catalase (1500 units/ml) added as marked (a, b) CO_2-dependent oxygen evolution. (c) phosphoglycerate-dependent oxygen evolution. (d) CO_2-dependent oxygen evolution. Chloroplasts had been incubated with 2 mM ascorbate for 5 min in the dark before the irradiation. (e) CO_2-dependent oxygen evolution; ascorbate and cysteine were omitted from the chloroplast isolation medium. ↓, light on; ↑, light off.

TABLE III

THE EFFECT OF CATALASE ON CO_2-DEPENDENT OXYGEN EVOLUTION IN ISOLATED SPINACH CHLOROPLASTS

Values expressed as $\mu mol \cdot O_2 \cdot mg^{-1}$ chlorophyll $\cdot h^{-1}$.

Light intensity ($W \cdot m^{-2}$)	Percentage of intact chloroplasts	−Catalase	+Catalase	$\frac{+Catalase}{-Catalase}$
32*	92	24.4	58.8	2.4
24.4*	74	22.5	45	2.0
23.4*	82	31.5	62.5	2.0
26**	85	22	38.9	1.8
25**	78	10	25.2	2.5
19.5**	78	18.7	29.3	1.6
16.5**	84	14.5	26.5	1.8

* Light of 674 nm.

** Light obtained by using a 630 nm cut-off filter in combination with a broad-band interference filter (Balzers K_6); half-band width 50 nm. Catalase concentration 1500 units/ml.

intensity. Some representative values of the catalase effect in different chloroplast preparations under different light intensities are shown in Table III. The ratio of CO_2-dependent oxygen evolution before and after catalase addition usually varied between 1.3 and 2.7, but occasionally much larger ratios were obtained (up to 6). The variation is attributed to the fact that even careful and extensive washing did not remove all endogenous catalase activity from the chloroplast preparations. When catalase was added to the chloroplasts there usually was a smooth increase of oxygen evolution indicating that hydrogen peroxide did not accumulate much during illumination. Only in some experiments an oxygen gush was observed on addition of catalase (Fig. 6 e). Heated catalase was ineffective in stimulating CO_2-dependent oxygen evolution. The effect of catalase on CO_2 fixation is shown in Fig. 7, curve a. It is

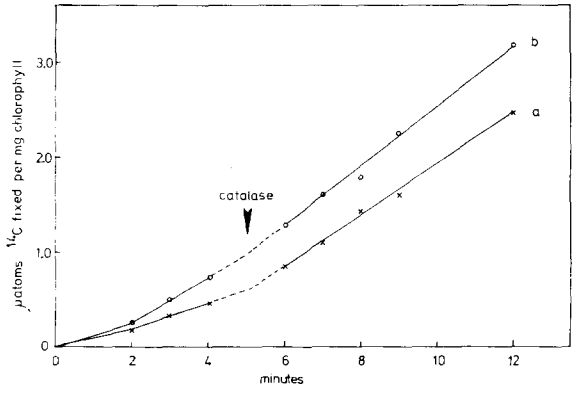


Fig. 7. The effect of catalase on CO_2-fixation in isolated chloroplasts of spinach. (a) no ascorbate added, (b) intact chloroplasts treated with 2 mM ascorbate 5 min before the start of the irradiation. Light intensity 25 $W \cdot m^{-2}$, obtained with a broad band interference filter. Percentage of intact chloroplasts 83. Catalase (1500 units/ml) added as marked.

evident that catalase not only stimulated CO_2-dependent oxygen evolution, but also CO_2 fixation. The extent of stimulation was similar in the two cases. In contrast to CO_2-dependent oxygen evolution, phosphoglycerate-dependent oxygen evolution did not increase much on addition of catalase (Fig. 6, curve c) or, more typically, no increase at all was observed. The chloroplast preparations used contained between 74 and 92 % intact chloroplasts (Table III). A possible explanation of the catalase stimulation of CO_2 reduction is that it is due to the decomposition of H_2O_2 formed by the Mehler reaction [13] of the broken chloroplasts which were present in the preparations. From Table 3 it is clear that no such relationship exists.

Dithiothreitol has been shown to stimulate CO_2-dependent oxygen evolution [2]. The effect of dithiothreitol was similar to that of catalase in that a stimulation of oxygen evolution became apparent immediately on addition to the chloroplasts. If dithiothreitol was present, catalase was ineffective in stimulating CO_2-dependent oxygen evolution. Catalase-stimulated chloroplasts were not further activated by dithiothreitol. In some experiments, dithiothreitol did not stimulate but slightly inhibited CO_2-dependent oxygen evolution. Ascorbate is also known to stimulate CO_2 fixation in isolated chloroplasts [20]. In contrast to dithiothreitol and catalase, ascorbate added to irradiated chloroplasts did not immediately affect oxygen evolution. A preincubation in the dark for 3–5 min was required. Addition of catalase to ascorbate-stimulated chloroplasts did not much increase oxygen evolution (Fig. 6, curve d) or CO_2 fixation (Fig. 7, curve b), indicating that ascorbate, like dithiothreitol, could replace catalase as a stimulant. Experiments were performed to test whether the omission of ascorbate and cysteine from the chloroplast isolation medium could affect photosynthesis. An extreme example of the absence of ascorbate and cysteine during chloroplast isolation is shown in Fig. 6e. In this case there was some oxygen uptake on illumination instead of oxygen evolution, which ensued only after catalase was added to the chloroplasts. In other experiments, oxygen evolution was observed on illumination, but its rate was lower compared to chloroplasts prepared in the absence of ascorbate and cysteine.

In contrast to CO_2-dependent oxygen evolution, phosphoglycerate-dependent oxygen evolution was not affected by the addition of ascorbate or of dithiothreitol.

The catalase experiments show that H_2O_2 is formed during CO_2-reduction of isolated well-washed chloroplasts. Obviously there is insufficient catalase present in the preparations to dismutate the H_2O_2 which is formed in the light. Since catalase stimulates photosynthesis, one would expect H_2O_2 to be inhibitory. This was indeed seen in experiments where both CO_2-dependent oxygen evolution and $^{14}CO_2$ fixation were measured. 10^{-5} M H_2O_2 produced an almost complete inhibition of photosynthesis. After addition of catalase, CO_2-dependent oxygen evolution reappeared.

Phosphoglycerate-dependent oxygen evolution was also inhibited by low concentrations of H_2O_2, but was less sensitive than CO_2-dependent oxygen evolution. To localize the H_2O_2 effect, the experiment shown in Fig. 8 was performed. Well-washed chloroplasts were permitted to photosynthesize in the presence of 2 mM $H^{14}CO_3^-$ for 5 min before catalase was added. After its addition phosphoglycerate increased dramatically, but the response of sugar phosphates and glycolate was small. Consequently, the ratio of phosphoglycerate to dihydroxyacetonephosphate increased drastically. In a control experiment, in which ascorbate was present from the beginning to eliminate rate limitations within the carbon cycle, labelling of all sugar

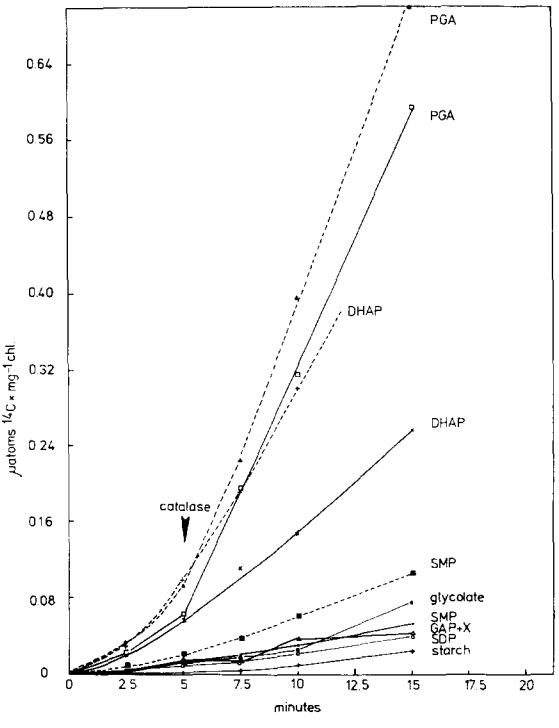


Fig. 8. Products formed by isolated chloroplasts of spinach from $Na_2{}^{14}CO_3$ as a function of the irradiation time. The dotted curves represent an experiment in which the chloroplasts were incubated with 2 mM ascorbate for 5 min before the start of their radiation. Light intensity 25 $W \cdot m^{-2}$, obtained with a broad band interference filter. Catalase (1500 units/ml) added as marked. Percentage of intact chloroplasts 75. Otherwise see Fig. 5.

phosphates and of phosphoglycerate was increased as compared with the experiment in which ascorbate was absent. Only labelling of starch was the same with and without ascorbate (not shown). There was only a small break in the curve when catalase was added in addition to ascorbate. The ratio of phosphoglycerate to dihydroxyacetonephosphate was lowered by the presence of ascorbate.

The experiment shows that destruction of H_2O_2 by catalase removes a rate-limitation in the formation of phosphoglycerate. The ratio of phosphoglycerate to dihydroxyacetonephosphate, which increased faster in the absence than in the presence of ascorbate after catalase addition, further indicates that the phosphoglycerate reducing system in chloroplasts which have been exposed to H_2O_2, encounters difficulties in handling the flux between phosphoglycerate and dihydroxyacetonephosphate. This agrees with the sensitivity of phosphoglycerate reduction to the addition of H_2O_2 and suggests that there are effects of this compound on the phosphoglycerate reducing system which are less readily reversible than the rate-limitation on phosphoglycerate formation.

Triosephosphate oxidation by intact chloroplasts

The incorporation of ^{14}C into dihydroxyacetonephosphate and phosphoglycerate during photosynthesis of intact chloroplasts in the presence of $H^{14}CO_3^-$ is

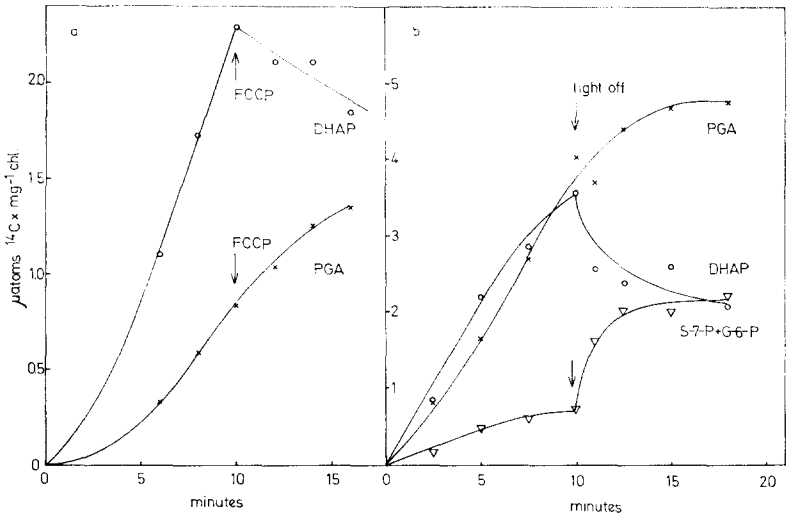


Fig. 9. The effect of FCCP (a) and a dark period (b) on some of the products formed by isolated intact spinach chloroplasts during the fixation of $Na_2{}^{14}CO_3$. (a) percentage of intact chloroplasts 62, CO_2-dependent oxygen evolution 117 μmol · mg^{-1} chlorophyll · h^{-1}, light intensity 100 W · m^{-2}. FCCP 10^{-5} M added as marked. (b) percentage of intact chloroplasts 86, CO_2-dependent oxygen evolution 113 μmol · mg^{-1} chlorophyll · h^{-1}, light intensity 130 W · m^{-2}. FCCP = carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, DHAP = dihydroxyacetone phosphate, PGA = phosphoglycerate, S-7-P = sedoheptulose 7-phosphate, G-6-P = glucose 6-phosphate.

shown in Fig. 9a. After 10 min of illumination, 10 μM carbonyl cyanide *p*-trifluorophenylhydrazone was added which uncoupled phosphorylation from electron transport and stopped CO_2 fixation. CO_2-dependent oxygen evolution by the chloroplasts was replaced by an oxygen uptake as seen in oxygen electrode measurements (Fig. 10). However, even though CO_2 fixation had completely ceased, [^{14}C]phospho-

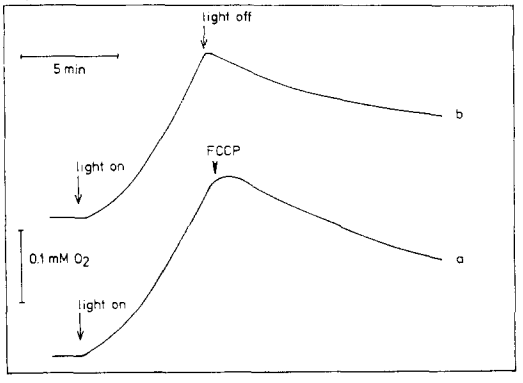


Fig. 10. The effect of FCCP (a) and a dark period (b) on CO_2-dependent oxygen evolution by isolated spinach chloroplasts. Catalase (1500 units/ml) was added 5 min before the start of the illumination. FCCP 10^{-5} M added as marked. Percentage of intact chloroplasts 80, CO_2-dependent oxygen evolution 125 μmol · mg^{-1} chlorophyll · h^{-1}. Light intensity 200 W · m^{-2}. FCCP = carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

glycerate continued to rise. This rise occurred at the expense of dihydroxyacetonephosphate, which decreased under continuous illumination about as much as phosphoglycerate increased. Assuming that phosphoglycerate had the same specific activity as the added $H^{14}CO_3^-$, the extent of phosphoglycerate accumulation after photosynthesis had stopped could be calculated. It was more than 150 nmol phosphoglycerate · mg^{-1} chlorophyll in 6 min. Formation of phosphoglycerate from triosephosphate gives rise to stoichiometric amounts of reduced pyridine nucleotides and ATP. However, available amounts of pyridine nucleotides and adenylates in the chloroplasts were much smaller than needed for the reaction to proceed without further turnover. Intact chloroplasts from spinach contain 60–90 nmol adenylates, 10–20 nmol NADP(H) and 5–15 nmol NAD(H) per mg of chlorophyll (Heber and Kirk, unpublished and ref. 16). Moreover, in the presence of the uncoupler, chloroplast pyridine nucleotides are kept largely reduced by light and are thus unavailable as electron acceptors. In the face of this situation, triosephosphate oxidation to phosphoglycerate can be understood only if there is turnover of both pyridine nucleotides and ATP. In fact, ATP must be rapidly split under the uncoupling conditions used. This shifts the phosphoglycerate kinase/glyceraldehydephosphate dehydrogenase reaction toward phosphoglycerate and permits triosephosphate oxidation even in the presence of largely reduced pyridine nucleotides. Still the reaction would not be able to proceed without reoxidation of reduced pyridine nucleotides. The pattern of carbon compounds labeled with ^{14}C during photosynthesis did not indicate that any reductive reactions took place after the uncoupler was added. Only oxygen was available as electron acceptor while triosephosphate oxidation proceeded. It was indeed seen to be taken up after addition of the uncoupler (Fig. 10). The experiment thus demonstrates that there is a link in intact chloroplasts between the chloroplast pyridine nucleotide system and oxygen. This is even apparent in the light/dark transition after photosynthetic CO_2 reduction by intact and fully coupled chloroplasts (Fig. 9b). After the light was turned off, dihydroxyacetonephosphate decreased drastically while sugar monophosphates and phosphoglycerate increased. The extent of phosphoglycerate accumulation varied in different experiments. In Fig. 9b it was much too large to be accounted for by carboxylation of available ribulose diphosphate. Under bicarbonate saturation the pool of ribulose diphosphate was very small in intact chloroplasts and usually did not exceed 10 nmol · mg^{-1} chlorophyll. Rather phosphoglycerate formation in the dark appears to reflect triosephosphate oxidation, while sugar monophosphates may be formed from starch and from dihydroxyacetonephosphate via the reactions of aldolase and fructose diphosphatase. Triosephosphate oxidation in the dark appears to be similar to that observed in the light in the presence of carbonyl cyanide *p*-trifluorophenylhydrazone and may be possible as long as ATP formed in this reaction is removed by secondary reactions such as ATPase action. Darkening the chloroplasts after photosynthetic CO_2 reduction under high intensity illumination indeed caused transient oxygen uptake similar to that seen in the light after uncoupling (Fig. 10).

DISCUSSION

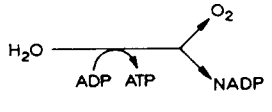
The effects of catalase, dithiothreitol and ascorbate on photosynthetic CO_2 reduction may best be explained by assuming that oxygen can be reduced to the

superoxide radical ion $O_2^{\cdot -}$ during photosynthesis. The radical ion can give rise to other radicals [21] or is dismutated to H_2O_2 by superoxide dismutase, which is a chloroplast enzyme [22]. The radicals or H_2O_2 inhibit photosynthesis, probably by oxidizing SH-groups of enzymes. Dithiols and ascorbate are known to react with radicals [21, 23]. In the presence of chloroplasts they also rapidly reduce H_2O_2. It is suggested that catalase, ascorbate and dithiothreitol stimulate photosynthesis by preventing the formation or accumulation of H_2O_2. Restoration of partially inhibited photosynthesis by these agents is thought to be brought about by SH-group regeneration, either directly (dithiothreitol) or through an endogenous reducing system.

The electron transport from water to oxygen, which results in H_2O_2 formation is either a physiological reaction or, alternatively, an electron leakage caused by inefficient NADP reduction thus forcing electrons to oxygen. Patterson and Myers [24], who recently reported on light-dependent H_2O_2 formation in *Anacystis*, favoured the latter view. Our results lead to a different conclusion. The low quantum requirement of phosphoglycerate reduction [2] (Fig. 1) shows that NADP reduction in isolated chloroplasts is highly efficient. In agreement with the high quantum yield of phosphoglycerate reduction in chloroplasts reducing phosphoglycerate under rate-limiting light, we observed little $^{18}O_2$ uptake (Fig. 3a), which could not be accounted for by glycolate formation or by the action of broken chloroplasts (Table II). Since phosphoglycerate reduction was also found to be sensitive to H_2O_2, but, in contrast to CO_2 reduction, was only slightly or not at all stimulated by catalase, little H_2O_2 must have been formed.

The quantum requirement of CO_2 reduction was higher than that of phosphoglycerate reduction (Fig. 1), suggesting the existence of another photoreaction besides NADP reduction during assimilation of CO_2. Considerable oxygen was taken up during CO_2 reduction (Fig. 2a and Table II). That this oxygen uptake is not caused entirely by the ribulose diphosphate oxygenase reaction is seen from the experiments designed to show the contribution of glycolate formation in intact chloroplasts, nor can it be attributed to broken chloroplasts in the preparation (Figs 4, 5 and Table II).

It must be emphasized that relevant data were obtained under conditions where light was strictly rate-limiting for CO_2 reduction. Under these conditions there is no extra electron pressure to force reduction of oxygen in a "safety valve" reaction. In view of the high quantum efficiency of NADP reduction it must be concluded that oxygen reduction under low light is a regulated process. It has long been known that oxygen reduction by the electron transport chain is accompanied by ATP formation [25]. As we have observed ATP/2 e ratios in intact chloroplasts which are well below 1.5 [26], yet CO_2 reduction requires at least 1.5 molecules of ATP per NADPH, we suggest the following scheme to account for electron flow in chloroplasts during CO_2 reduction under low light.



NADP is, for affinity reasons, the preferred electron acceptor in chloroplasts. Sufficient ATP is formed during NADP reduction to permit phosphoglycerate reduction.

As a consequence, there is little or no flow of electrons to oxygen. During CO_2 reduction less ATP becomes available during the electron transport to NADP than is needed to drive the conversion of CO_2 into sugars if the ATP/e ratio is below 1.5. As the availability of ATP limits NADPH oxidation during reduction of CO_2, the NADP pool is depleted and NADPH accumulates even under light which is rate-limiting for CO_2 reduction. This has indeed been observed in intact chloroplasts [2]. When NADP levels are low, electrons are diverted to oxygen. This results in ATP formation and relieves the ATP deficiency thus permitting NADPH oxidation. As the NADP level increases electrons are directed back to NADP. In this model the distribution of electrons between NADP and oxygen is regulated by the coupling efficiency of the thylakoid membranes. Additional evidence that a link between oxygen and the pyridine nucleotide system exists in the chloroplasts was obtained from uncoupling experiments (Figs 9a and 10). This has been discussed under results and will not be considered again. A high coupling efficiency, which is expressed by high ATP/e ratios, would permit little reduction of oxygen and would lead to a high quantum yield of photosynthesis. A low efficiency increases oxygen reduction and lowers the quantum yield of photosynthesis. Both low and high quantum yields have been observed during photosynthesis of intact organisms [27] or of isolated chloroplasts (cf. p. 255). Values as high as 0.125, which would leave little room for oxygen reduction, are extremely rare. We conclude that under most conditions oxygen is a necessary electron acceptor in photosynthesis.

The proposed model, which accounts for electron flow under low light intensities, does not consider cyclic electron transport as a way of supplying energy for photosynthesis. Light scattering measurements performed with intact leaves have shown that, for affinity reasons, cyclic electron flow becomes possible only after the electron flow to oxygen has been largely saturated [28]. The capacity of the reaction reducing oxygen in intact chloroplasts is at the present unknown. It is, as the above measurements show, high enough to supply needed energy during photosynthesis under low light. Depending on the capacity of the oxygen reduction mechanism, cyclic electron flow may or may not be able to contribute to the energy balance of chloroplasts under high intensity illumination. It appears that green cells are admirably equipped with self-regulatory devices capable of distributing electrons to different acceptors according to their energy needs under different light regimes.

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