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STOICHIOMETRY OF REDUCTION AND PHOSPHORYLATION DURING ILLUMINATION OF INTACT CHLOROPLASTS

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SUMMARY

1. Intact chloroplasts which had retained their envelopes and were capable of photoreducing CO_2 at rates ranging from 70 to 240 μmoles $\cdot$ mg^{-1} chlorophyll $\cdot$ h^{-1} showed a large increase in their endogenous levels of ATP and NADPH on illumination in the absence of electron acceptors. At low light intensities, addition of 3-phosphoglycerate, whose reduction consumes ATP and NADPH in an equimolar ratio, drastically decreased the levels both of chloroplast ATP and NADPH. Only at light intensities not far from light saturation for 3-phosphoglycerate reduction did ATP become available in excess of NADPH, as revealed by the different response of the endogenous levels of ATP and NADPH to addition of 3-phosphoglycerate.

2. Addition of bicarbonate to preilluminated chloroplasts or reduction of the light intensity during photosynthetic CO_2 reduction decreased only the ATP, not the NADPH levels, indicating a rate limitation of CO_2 reduction by ATP.

3. Quantum requirements for the reduction of 3-phosphoglycerate or oxaloacetate at low light intensities were consistently close to 4. For the reduction of CO_2 they were never significantly below 12 and usually much higher. As the reduction of CO_2 requires twice as much reduced pyridine nucleotide as that of 3-phosphoglycerate or oxaloacetate, this indicates that the ATP generated during pyridine nucleotide reduction is insufficient to drive CO_2 reduction.

4. Dithiothreitol improved significantly quantum yields of CO_2 reduction, but not of 3-phosphoglycerate reduction. It also stimulated the reduction of oxygen (Mehler reaction) by intact or broken chloroplasts.

5. The light-dependent increase of both chloroplast NADPH and ATP was inhibited by 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) at concentrations which are ineffective in suppressing Photosystem I-dependent cyclic photophosphorylation of broken chloroplasts.

6. From the data it is concluded that not much more than one molecule of ATP is formed in intact chloroplasts during the transport of two electrons from water to $NADP^+$. Coupling of phosphorylation to electron transport appeared to be flexible, not tight. The extra ATP needed for the reduction of CO_2 was produced by an independent photochemical reaction presumably reducing oxygen.

Abbreviation: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

INTRODUCTION

It is widely accepted that the reduction of CO_2 in photosynthesis requires ATP and NADPH. Chloroplasts have been shown *in vitro* to be capable of reducing $NADP^+$ and of phosphorylating ADP at high rates[1]. *In vivo* the phosphorylation potential ([ATP]/[ADP] $[P_i]$) is increased by light and the redox ratio of the NADP system is shifted to the reduced side[2,3]. ATP and NADPH are consumed at ratios higher than 1.5 or 2.5 during the formation of sugars or polysaccharides from CO_2 along the metabolic routes of the Calvin cycle[4] or of the Hatch–Slack pathway[5] and of saccharide synthesis. *In vitro* ATP is synthesized during noncyclic electron transport from water to an acceptor molecule or in the presence of suitable catalysts during cyclic electron transport. Many experimental values for the ratio of transported electrons to ATP formation are close to 2 (ref. 6). Accordingly, during reduction of 1 molecule of $NADP^+$ *in vivo* only 1 molecule of ATP should be formed. The question as to the origin of the extra ATP required for sugar synthesis is unresolved. There are three possibilities:

(1) Cyclic photophosphorylation provides the needed ATP[7,8]. However, evidence to the contrary has been presented by Tanner *et al.*[9]. High rates of cyclic photophosphorylation have been observed only *in vitro* in the presence of suitable cofactors such as phenazine methosulfate. While cyclic electron transport furnishing ATP does occur *in vivo* under nitrogen[10], we have reported evidence that in the presence of oxygen it is suppressed and replaced by pseudo-cyclic electron transport[11].

(2) As ATP coupled to NADPH formation in a 1:1 ratio becomes limiting for the reduction of CO_2, electrons are diverted to oxygen[11] producing ATP in a pseudo-cyclic type of photophosphorylation. This would relieve the limitation of CO_2 reduction and would constitute a self-regulatory system capable of adjusting to changing ATP requirements.

(3) The stoichiometry between phosphorylation and $NADP^+$ reduction is not 1 but 2 (ref. 12). In fact, ATP/2 *e* ratios substantially in excess of 1 have been reported[13–16]. Two coupling sites have been suggested to exist in the electron transport chain of chloroplasts[14,17]. However, a coupling ratio of 2, while leading to an ATP surplus in the Calvin cycle, would still not meet the ATP requirement of the Hatch–Slack pathway.

Experiments on the stoichiometry between phosphorylation and electron transport have been performed only with fragmented chloroplasts, since a permeability barrier prevents entry of added ADP and $NADP^+$ or related electron acceptors to intact chloroplasts. Therefore, no data on chloroplasts capable of reducing CO_2 at high rates are available. In this communication attempts are described to decide whether the ATP formed during electron transport to $NADP^+$ is sufficient to maintain CO_2 reduction in functionally active chloroplasts of spinach.

MATERIAL AND METHODS

Leaves were cut from rapidly growing spinach and used within 1 h for chloroplast isolation. Before grinding for 10 s in a Waring blendor they were preilluminated for 5 min to 10 min at 0 °C with a 500-W incandescent lamp placed 80–100 cm from the leaves. The isolation procedure was essentially that described by Jensen and

Bassham[18], except that nitrate and pyrophosphate were omitted from the media, the NaCl concentration was 10^{-2} M and that the centrifugation steps were modified[19]. As a protective agent the grinding medium contained 3 mM cysteine. Debris was removed by centrifugation for 1 min at $200 \times g$ and the chloroplasts were washed once in Medium A[18] before transferring them to Medium B. Care was taken to remove semi-fluid layers containing disproportionate amounts of broken chloroplasts from the chloroplast pellets. The percentage of broken chloroplasts in the preparations was determined by the ferricyanide reduction procedure[19]. The preparations used for the experiments contained between 70 and 99% intact chloroplasts. Oxygen evolution or consumption was measured in a Clark type electrode. The medium for 3-phosphoglycerate, oxaloacetate, and CO_2 reduction contained 0.33 M sorbitol, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, 10 mM NaCl, 0.8 mM phosphate and 50 mM *N*-2-hydroxypiperazine-*N'*-2-ethanesulfonic acid, pH 7.6. The oxygen concentration was usually reduced to about 0.12 mM by flushing with nitrogen. When it was desirable to remove dissolved bicarbonate and CO_2, flushing was performed at a low pH. The chlorophyll concentration was 33 μg/ml. Substrate concentrations were 2 mM for 3-phosphoglycerate and oxaloacetate and 4 mM for $NaHCO_3$. $NADP^+$ and NADPH were determined by enzymic cycling according to Slater and Sawyer[20], ATP by the luciferin–luciferase assay by Strehler[21]. A Packard liquid scintillation counter was used to record the kinetics of light emission. The calibration curve for ATP was sigmoid, with a considerable proportion of it approaching linearity. Procedural details for the pyridine nucleotide determinations have been described elsewhere[3]. For the measurements of quantum yields, the absorption of 674 nm light (interference filter 674, half band width 12 nm; 1 mm Calflex C, Balzers, Liechtenstein; 3 mm RG 630, Schott and Gen., Mainz; and 8 cm water) by the chloroplast suspension was measured in a large Ulbricht sphere. The chloroplast suspension was in the same lucite reaction cuvette that was used for recording oxygen evolution. During the reactions the light intensity was recorded by a silicone photodiode, which was calibrated by a compensated thermopile (Kipp and Zonen, Delft). Reactions were performed at 20 °C. The activities were corrected for broken chloroplasts which absorbed light without contributing to the reactions. For measuring oxygen reduction during the Mehler reaction[22] catalase (EC 1.11.1.6) was poisoned by adding 1 mM KCN. To measure the ratio between CO_2 uptake and oxygen evolution during photosynthesis a small amount of $NaH^{14}CO_3$ was injected into the sample compartment of the oxygen electrode during the linear phase of photosynthetic CO_2 reduction. After 5 min CO_2 reduction was stopped by injection of trichloroacetic acid. Aliquots were dried on planchets and counted. The amount of CO_2 fixed as determined from the known specific activity of the bicarbonate in the sample was related to oxygen evolution as recorded by the electrode.

RESULTS AND DISCUSSION

(*1*) *3-Phosphoglycerate reduction*

The reduction of 3-phosphoglycerate in chloroplasts in the light is catalyzed by endogenous glyceraldehyde phosphate dehydrogenase (EC 1.2.1.13) and phosphoglycerate kinase (EC 2.7.2.3) and consumes ATP and NADPH in an equimolar ratio. In contrast to chloroplasts *in situ*, intact isolated chloroplasts contained in the dark

only a very low level of ATP (usually less than 5 nmoles·mg^{-1} chlorophyll) and a rather high level of AMP (up to 30 nmoles·mg^{-1} chlorophyll) owing to the combined action of ATPase (EC 3.6.1.3) and adenylate kinase (EC 2.7.4.3)[19]. Adenylate pools differed rather considerably in different preparations of intact chloroplasts, probably due to occasional adenylate leakage which, however, did not significantly affect biochemical activities of the chloroplasts. In contrast, $NADP^+$ pools were of comparable size in different preparations. About 5 to 15% of the endogenous $NADP^+$ was reduced in the dark. On illumination, the levels of ATP and of NADPH rose rapidly until a new steady state was attained. Light saturation of the increase in the endogenous pools of NADPH and ATP was reached at low intensities of red light (below 5 kergs·cm^{-2}·s^{-1}) in the absence of added electron acceptors. If ATP formation is coupled to $NADP^+$ reduction at a ratio much higher than 1, then addition of 3-phosphoglycerate should disturb the steady state and result at appropriate light intensities during continuous 3-phosphoglycerate reduction in a significant decrease of the NADPH level only. ATP levels should be maintained high. Conversely, if the coupling ratio is close to one and if there is no other source of ATP formation, the levels of both ATP and NADPH should be decreased. Fig. 1 shows the increase of ATP and NADPH in chloroplasts containing a high adenylate level during illumination in the absence of an added electron acceptor and the response to addition of 3-phosphoglycerate. The light intensity was not very far from saturation for 3-phosphoglycerate reduction, and 3-phosphoglycerate-dependent oxygen evolution corresponded to a reduction of about 150 μmoles 3-phosphoglycerate·mg^{-1}

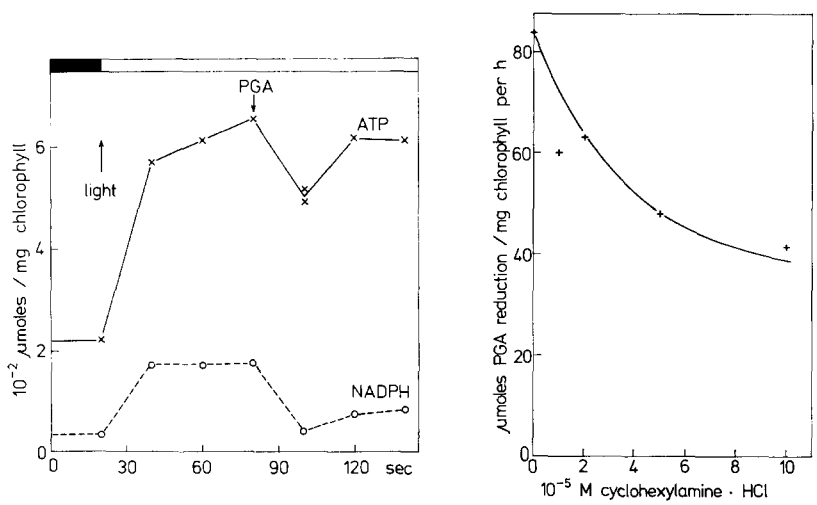

Fig. 1. Changes in the levels of NADPH and of ATP in intact chloroplasts in a dark–light transition and on adding $2 \cdot 10^{-3}$ M 3-phosphoglycerate (PGA) in the light phase. Intensity of red light (half band width from 636 to 715 nm) was 60 kergs·cm^{-2}·s^{-1}. 79% of the chloroplasts in the preparation were intact, *i.e.* had retained their outer envelope. 3-Phosphoglycerate reduction at light saturation was 190 μmoles·mg^{-1} chlorophyll·h^{-1}, at the intensity used in the experiment approx. 150 μmoles·mg^{-1} chlorophyll·h^{-1}.

Fig. 2. Inhibition by cyclohexylamine of 3-phosphoglycerate (PGA) reduction by intact chloroplasts. Intensity of red light (half band width from 636 to 715 nm) was 33 kergs·cm^{-2}·s^{-1}. 3-Phosphoglycerate reduction at light saturation was 135 μmoles·mg^{-1} chlorophyll·h^{-1}. 94% of the chloroplasts in the preparation were intact.

chlorophyll·h^{-1}. Addition of 3-phosphoglycerate caused a comparable decrease in both ATP and NADPH which was followed by a slow return to intermediate levels. The latter is assumed to be caused by mass action forces, according to the quasi-equilibrium ([NADPH] [ATP] [3-phosphoglycerate]/ [$NADP^+$] [P_i] [ADP] [glyceraldehyde phosphate]) $\approx K$. The accumulation of reaction products causes the NADP system to swing back to a more reduced state and the adenylate system to a higher phosphorylation potential.

These and similar data are considered as evidence against a coupling ratio of 2:1 between ATP formation and $NADP^+$ reduction. This conclusion is supported by a simple experiment. If the coupling ratio were 2:1, then careful partial uncoupling by an amine should at appropriate light intensities, which are still rate limiting, but high enough to give a low quantum yield, stimulate the rate of 3-phosphoglycerate reduction before more drastic uncoupling would decrease it, since in fully coupled chloroplasts a high phosphorylation potential would restrict reduction of $NADP^+$. Addition of varying amounts of the uncoupler cyclohexylamine to chloroplasts evolving oxygen in the light during 3-phosphoglycerate reduction decreased oxygen evolution already at low uncoupler concentrations (Fig. 2). This would be expected from a coupling ratio of 1:1, but not from a ratio of 2:1. The same concentrations of uncoupler, that decreased 3-phosphoglycerate reduction, stimulated reduction of oxaloacetate.

Even more evident than in Fig. 1 is the strong depression of the ATP level by 3-phosphoglycerate in chloroplasts which had a lower adenylate content (Fig. 3). At very low intensities addition of 3-phosphoglycerate to intact chloroplasts first caused an almost complete oxidation of NADPH which was followed by a transient rise of its level (Fig. 3A). At the same time ATP was decreased almost to the dark

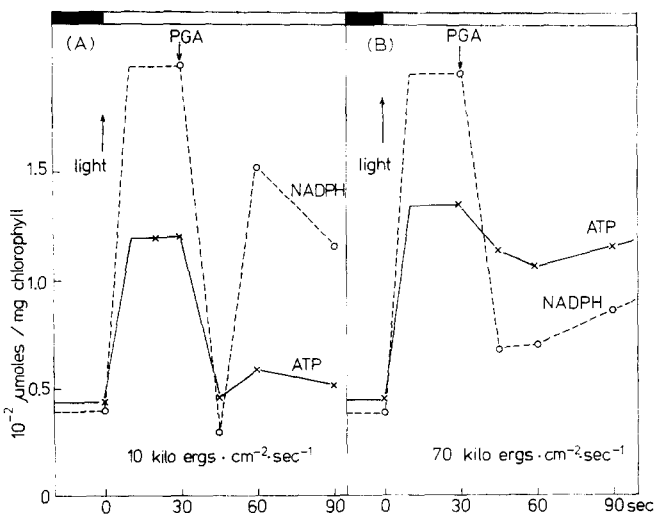

Fig. 3. Changes in the levels of NADPH and of ATP in intact chloroplasts in a dark–light transition and on adding $2 \cdot 10^{-3}$ M phosphoglycerate in the light phase. Illumination with a broad band of red light, A. 10 kergs·cm^{-2}·s^{-1} B. 70 kergs·cm^{-2}·s^{-1}. 86% of the chloroplasts in the preparation were intact. Phosphoglycerate reduction was 117 μmoles·mg^{-1} chlorophyll·h^{-1} at 70 kergs·cm^{-2}·s^{-1} and 51 μmoles·mg^{-1} chlorophyll·h^{-1} at 10 kergs·cm^{-2}·s^{-1}.

state and remained there. This behaviour is also inconsistent with a production of NADPH and ATP in a ratio of 1:2. The strong depression of the ATP level as compared with that of NADPH may have been caused by some ATP consumption in the phosphoribulokinase (EC 2.7.1.19) reaction which fills the ribulose diphosphate pool. In contrast to the picture seen at low light intensities, at intensities approaching saturation, 3-phosphoglycerate depressed the endogenous level of NADPH more strongly than that of ATP (Fig. 3B). The latter returned at sufficiently high intensities to values very similar to those observed before addition of 3-phosphoglycerate, while NADPH remained at a decreased level after slowly swinging back from a strongly oxidized state. The different response of ATP and NADPH at low and increased light intensities is interpreted to mean that a second reaction capable of providing ATP comes into play when the "electron pressure" is raised by more intense illumination. With oxygen present as a possible electron acceptor in addition to 3-phosphoglycerate either cyclic or pseudo-cyclic photophosphorylation are candidates for a second ATP-generating reaction. A well-known property of cyclic electron transport is its insensitivity to DCMU[23] which blocks noncyclic and pseudo-cyclic electron transport between Photosystems II and I (ref. 24). Fig. 4 shows that not only the light-induced rise of NADPH but also that of ATP was effectively blocked by concentrations of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) which do not inhibit cyclic photophosphorylation. Even addition of ascorbate did

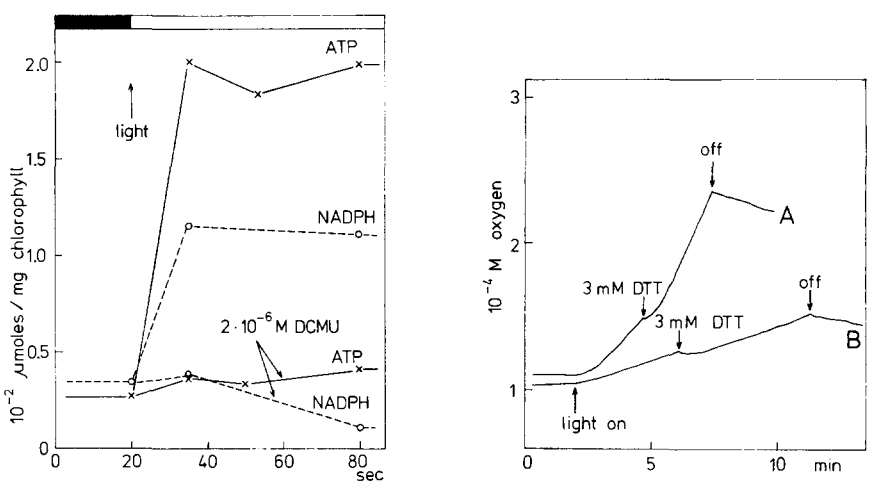

Fig. 4. Changes in the levels of NADPH and of ATP in intact chloroplasts in a dark–light transition as influenced by $2 \cdot 10^{-6}$ M DCMU. Intensity of red light was 75 kergs·cm^{-2}·s^{-1}. 95% of the chloroplasts in the preparation were intact. The unpoisoned chloroplasts reduced CO_2 at 88 and phosphoglycerate at 170 µmoles·mg^{-1} chlorophyll·h^{-1} at light saturation (250 kergs·cm^{-2}·s^{-1}). Reduction by poisoned chloroplasts was too low to be measured.

Fig. 5. Effect of illumination of leaves immediately before chloroplast isolation on capability of isolated chloroplasts to photoreduce CO_2. (A) Leaves preilluminated for 5 min by a 500-W incandescent lamp. CO_2 reduction of chloroplasts in the absence of dithiothreitol 75, with dithiothreitol (DTT) 160 µmoles·mg^{-1} chlorophyll·h^{-1}. (B) Same leaves as in A kept for 30 min in the dark before chloroplast isolation. CO_2 reduction of chloroplasts in the absence of dithiothreitol 18, with dithiothreitol (DTT) 20 µmoles·mg^{-1} chlorophyll·h^{-1}.

not increase endogenous ATP levels. This points to pseudo-cyclic electron transport as a source of extra ATP.

(2) CO_2 reduction

After a lag phase of usually 1 to 2 min (ref. 25) intact chloroplasts may evolve oxygen in a CO_2-dependent reaction. Illumination of leaves prior to chloroplast isolation[26] and addition of dithiothreitol (Fig. 5) resulted in rates of oxygen evolution of the chloroplasts comparable and occasionally perhaps superior to those of the parent leaves. Contrary to the situation with 3-phosphoglycerate, addition of bicarbonate to preilluminated chloroplasts did not result in a decrease of NADPH (Fig. 6). Only ATP was decreased. This decrease was clearly seen only when the lag phase of CO_2 reduction was short or absent after preillumination. In the experiment shown in Fig. 7, chloroplasts were illuminated in the presence of bicarbonate at an intermediate light intensity. Sudden reduction of the light intensity left the level of NADPH unaffected, but decreased drastically the ATP level. These results clearly show that in rate-limiting light not $NADP^+$ reduction but phosphorylation limits CO_2 reduction.

It has been proposed that the reduction of CO_2 to the sugar level proceeds *via* the reductive splitting of the 6-carbon carboxylation product[27]. Presumably this reaction would not require ATP. In a complete turn of the carbon reduction cycle

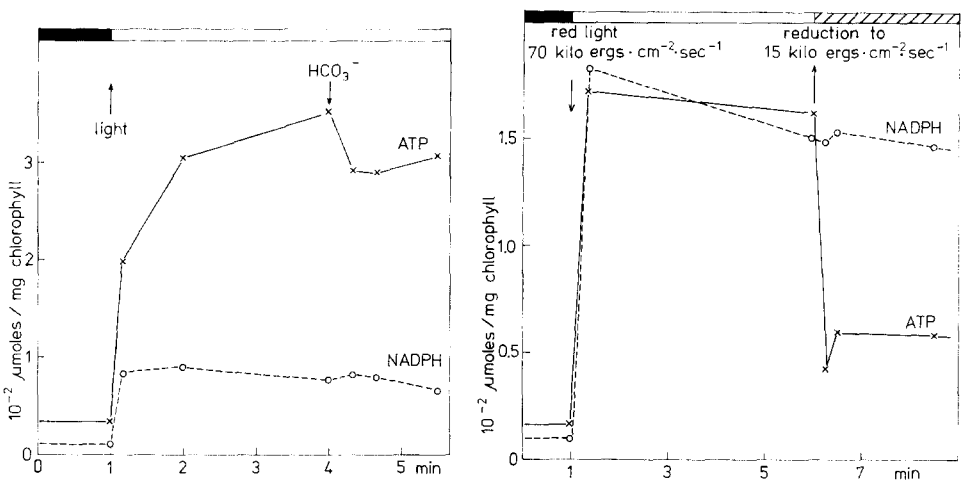

Fig. 6. Changes in the levels of ATP and of NADPH in intact chloroplasts in a dark–light transition and on adding $4 \cdot 10^{-3}$ M $NaHCO_3$ in the light phase. Intensity of red light was 70 kergs · cm^{-2} · s^{-1}. 84% of the chloroplasts in the preparation were intact. Oxygen concentration was approx. $0.27 \cdot 10^{-3}$ M. Steady-state CO_2 reduction at light saturation was 118 µmoles · mg^{-1} chlorophyll · h^{-1}.

Fig. 7. Changes in the levels of NADPH and of ATP in intact chloroplasts in a dark–light transition and on lowering the light intensity from 70 to 15 kergs · cm^{-2} · s^{-1} during photosynthetic CO_2 reduction. Illumination with a broad band of red light. 88% of the chloroplasts in the preparation were intact. $NaHCO_3$ was present throughout the experiment at a concentration of $4 \cdot 10^{-3}$ M; oxygen concentration was approx. $0.12 \cdot 10^{-3}$ M. Steady-state photosynthetic oxygen evolution at 70 kergs · cm^{-2} · s^{-1} was 55 µmoles · mg^{-1} chlorophyll · h^{-1}, at 15 kergs · cm^{-2} · s^{-1} 16 µmoles · mg^{-1} chlorophyll · h^{-1}.

therefore only 2 ATP molecules would be needed in addition to 4 reducing equivalents. This would imply that a coupling ratio of 1 : 1 between phosphorylation and reduction of a 2 *e* acceptor would be sufficient to maintain photosynthesis in isolated chloroplasts. However, an identical requirement for ATP and NADPH of the reduction of either 1 molecule of added CO_2 or 2 molecules of added 3-phosphoglycerate is inconsistent with the observation of different rate limitations for these processes under intermediate light intensities. Under these conditions NADPH was rate limiting for 3-phosphoglycerate reduction (Figs 1 and 3) and ATP for CO_2 reduction (Figs 6 and 7).

An excess of reducing equivalents during CO_2 reduction was also revealed by addition of oxaloacetate to chloroplasts reducing CO_2 in the light. Oxaloacetate enters chloroplasts and is reduced in the light to malate. Reduction is indicated by oxygen evolution[28]. Addition of oxaloacetate to chloroplasts photoreducing 3-phosphoglycerate at low light intensities did not increase the rate of oxygen evolution indicating that no excess of reducing equivalents was available (Table I). In contrast, chloroplasts photoreducing CO_2 under rate-limiting light usually responded to addition of oxaloacetate with a significant stimulation of oxygen evolution. As obviously the availability of reducing equivalents was not the limiting step, only ATP could have been rate limiting.

TABLE I

OXYGEN EVOLUTION BY INTACT CHLOROPLASTS IN THE PRESENCE OF BICARBONATE (4 mM) OR 3-PHOSPHOGLYCERATE (2 mM) WITH AND WITHOUT OXALOACETATE (2 mM)

Energy of incident red light ($kergs \cdot cm^{-2} \cdot s^{-1}$)	*μmoles oxygen evolution* $\cdot mg^{-1}$ *chlorophyll* $\cdot h^{-1}$ *in the presence of*			
	Bicarbonate	*Bicarbonate + oxaloacetate*	*3-Phosphoglycerate*	*3-Phosphoglycerate + oxaloacetate*
23	51	59	58	62
15			31	27
5	17	20	21	20

(3) Quantum requirements of oxygen evolution during the reduction of 3-phosphoglycerate, oxaloacetate and CO_2 by intact chloroplasts

Half maximal reduction of CO_2 by intact chloroplasts was observed at intensities of red light between 10 and 18 $kergs \cdot cm^{-2} \cdot s^{-1}$. Lower intensities (between 7 and 10 $kergs \cdot cm^{-2} \cdot s^{-1}$) were sufficient to half saturate 3-phosphoglycerate reduction. As maximal oxygen evolution on addition of either bicarbonate or 3-phosphoglycerate was comparable, this suggested different quantum requirements for the production of oxygen during CO_2 or 3-phosphoglycerate reduction. Fig. 8 shows the number of quanta of 674 nm light required for the reduction of 1 molecule of 3-phosphoglycerate by intact chloroplasts at different light intensities as measured by oxygen evolution. Corrections were applied for broken chloroplasts incapable of photoreducing added 3-phosphoglycerate. Extrapolation of the data to zero intensity indicates a minimal quantum requirement of 4. Table II lists results also from other experiments. The

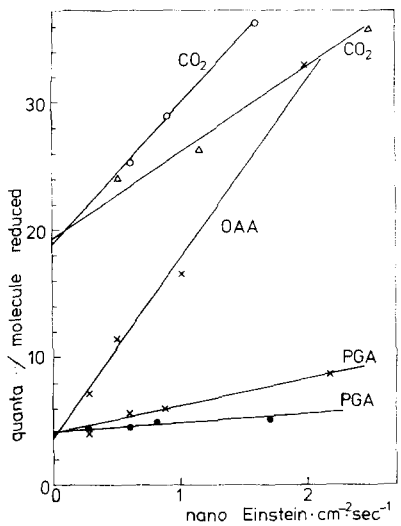

Fig. 8. Number of quanta required for the reduction of 1 molecule of CO_2, 3-phosphoglycerate (PGA) or oxaloacetate (OAA) by intact chloroplasts at different incident energies of 674 nm light. Values are corrected for 100% intact chloroplasts. ×—×, chloroplast preparation contained 88% intact chloroplasts; △—△, chloroplast preparation contained 92% intact chloroplasts; ○—○, chloroplast preparation contained 64% intact chloroplasts.

TABLE II

QUANTUM REQUIREMENTS OF OXYGEN EVOLUTION DURING CO_2, PHOSPHOGLYCERATE, AND OXALOACETATE REDUCTION BY INTACT CHLOROPLASTS

Values are corrected for 100% intact chloroplasts and extrapolated to zero light intensity. Oxygen concentration was approx. $0.1 \cdot 10^{-3}$ M.

Expt No.	*Additive*	*Number of quanta required for the reduction of 1 molecule* of*		
		CO_2	*3-Phospho-glycerate*	*Oxaloacetate*
1	3 mM dithiothreitol	12.3	3.6	4.4
2	3 mM dithiothreitol	11.6	4	—
3	—	19	4.2	—
4	—	17.6	5.2	5
5	—	22	3.5	3.5
6	—	15.7	5.4	—
7	—	21	3.7	—
8	—	20	4.1	4.2
Av.	3 mM dithiothreitol	12	3.8	—
	—	19	4.4	4.2

* Assuming a stoichiometry between substrate reduction and oxygen evolution of 1 for CO_2 and 2 for 3-phosphoglycerate and oxaloacetate.

obtained values agree with published data on the quantum requirement of NADP reduction by broken chloroplasts[29,30] and are close to the value postulated by the sequential scheme of photosynthesis involving two light reactions. At saturating light intensities added 3-phosphoglycerate was reduced at rates between 140 and 300 μmoles $\cdot$ mg^{-1} chlorophyll $\cdot$ h^{-1}.

At very low light intensities the quantum requirement for the reduction of oxaloacetate by intact chloroplasts also approached 4. With increasing light intensity the quantum yield of oxaloacetate reduction decreased more than that of 3-phosphoglycerate reduction (Fig. 8). This is considered to be an expression of the constraint on pyridine nucleotide reduction exerted by a high phosphorylation potential. Oxaloacetate consumes during its reduction only reducing equivalents, not ATP. Since ATP synthesis is coupled to NADP reduction, accumulation of ATP controls NADPH formation lowering the quantum yield. Control of oxaloacetate reduction by the phosphorylation potential is also indicated by the stimulation of oxaloacetate reduction on addition of the uncoupler cyclohexylamine[28]. The data show that in contrast to mitochondria phosphorylation in chloroplasts is loosely coupled permitting reduction to occur at reduced rates even in the absence of ATP consumption.

The quantum requirement of CO_2 reduction was high in the absence of added dithiothreitol. The lowest values indicated by extrapolation to zero intensity were 16 quanta/molecule of oxygen evolved at a low oxygen concentration. Usually values were higher and occasionally reached 30 quanta/CO_2. Rates of CO_2 reduction at saturating light were between 70 and 240 μmoles $\cdot$ mg^{-1} chlorophyll $\cdot$ h^{-1}. The surprisingly high quantum requirement of CO_2 reduction as compared with that of 3-phosphoglycerate or oxaloacetate reduction is additional evidence that the ATP produced during $NADP^+$ reduction is insufficient to maintain photosynthesis. Obviously a second reaction, presumably providing additional ATP, contributes to CO_2 reduction increasing the quantum requirement.

(4) Effect of dithiothreitol on the reduction of CO_2 and of oxygen

Dithiothreitol stimulated the rate of oxygen evolution during photosynthetic CO_2 reduction (Fig. 5). This stimulation was occasionally not seen or even replaced by inhibition at rate-saturating light, but was always observed under rate-limiting illumination. In the presence of dithiothreitol the quantum requirement of CO_2 reduction, but not that of 3-phosphoglycerate reduction was decreased. Values for the quantum requirement of CO_2 reduction in the presence of 3 mM dithiothreitol are listed in Table II. As cysteine was found to be inhibitory, the effect of dithiothreitol appeared to be rather specific. Since the quantum yield of $NADP^+$ reduction as revealed by the quantum requirement of 3-phosphoglycerate reduction is high, dithiothreitol obviously increased the effectiveness of the second reaction which participates in CO_2 reduction. There was the possibility that the quantum requirement of CO_2 reduction in the presence of dithiothreitol was even lower than 12 as indicated in Table II on the basis of oxygen evolution, since dithiothreitol has been shown to donate electrons to Photosystem II in broken chloroplasts (Trebst, A., personal communication). This reaction, while contributing to CO_2 fixation, would not lead to oxygen evolution. However, even in the presence of dithiothreitol the ratio of CO_2 uptake to oxygen evolution did not exceed 1 (Table III). In its absence it was significantly lower indicating that the quantum requirement for CO_2 fixation was

TABLE III

STOICHIOMETRY BETWEEN CO_2 FIXATION AND OXYGEN EVOLUTION DURING PHOTOSYNTHESIS OF INTACT CHLOROPLASTS IN THE PRESENCE AND IN THE ABSENCE OF 3 mM DITHIOTHREITOL

Expt No.	*Intensity of red light* (*kergs·cm*$^{-2}$·*s*$^{-1}$)	CO_2/O_2	
		Without dithiothreitol	*With dithiothreitol*
1	20	0.66	0.96
	40	0.74	0.94
2	20	0.73	0.97
	40	0.71	0.85

actually higher than calculated in Table II on the basis of an assumed 1:1 stoichiometry between CO_2 reduction and oxygen evolution.

In a previous communication comparative data on the fluorescence and photo-induced shrinkage of isolated chloroplasts and of chloroplasts *in situ* were reported[31]. These data indicated that isolated chloroplasts react with oxygen less readily than chloroplasts in the leaf. The effect of dithiothreitol on photosynthetic CO_2 reduction suggested that it might act on noncyclic electron transport from water to oxygen making it more effective. Fig. 9 shows the effect of dithiothreitol on oxygen uptake

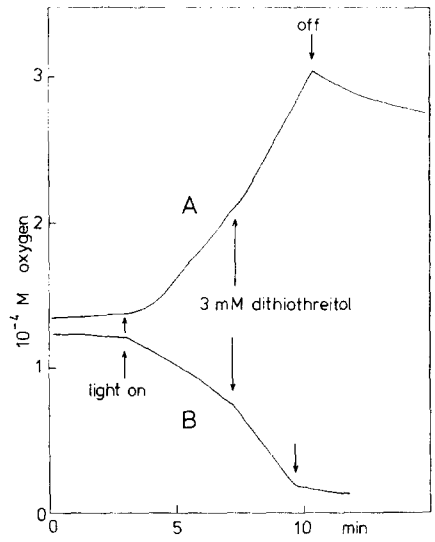

Fig. 9. Effect of 3 mM dithiothreitol on photosynthesis and on oxygen uptake of isolated chloroplasts. (A) Oxygen evolution during photosynthetic CO_2 reduction with $4 \cdot 10^{-3}$ M $NaHCO_3$ as substrate. (B) Oxygen uptake in the Mehler reaction. 10^{-3} M KCN served to inhibit decomposition of H_2O_2. Intensity of red light 210 kergs·cm^{-2}·s^{-1} (A) and 240 kergs·cm^{-2}·s^{-1} (B). Rate of oxygen evolution without dithiothreitol 78 and with dithiothreitol 191 μmoles·mg^{-1} chlorophyll·h^{-1}; rate of oxygen uptake without dithiothreitol 17 and with dithiothreitol 39 μmoles·mg^{-1} chlorophyll·h^{-1}.

in the Mehler reaction of isolated chloroplasts as compared with the effect on CO_2 reduction. The autoxidation of dithiothreitol was negligible. A large stimulation of oxygen uptake by intact and also by osmotically shocked chloroplasts was observed. The results are interpreted to indicate that the Mehler reaction has a physiological function *in vivo*. It is thought to be the reaction which supplies the extra ATP needed for CO_2 reduction.

(5) *Regulation of phosphorylation during photosynthetic electron transport*

It has previously been reported that chloroplast shrinkage of leaves induced by far-red light under nitrogen is completely inhibited by either oxygen or CO_2 (ref. 11). Half maximal inhibition was observed at a concentration of 1000 ppm oxygen or 10 to 20 ppm CO_2 in nitrogen. Shrinkage induced under nitrogen by far-red light, which excites Photosystem I, is caused by cyclic electron flow and is assumed to be indicative of a high phosphorylation potential[17]. Its inhibition by CO_2 and oxygen shows that these electron acceptors have a higher affinity for electrons of the electron transport chain than the acceptor mediating transfer of electrons in the cyclic pathway. Together with data on the inhibition of the light-induced ATP rise by DCMU (Fig. 4) and on the stimulation of both the reduction of oxygen and CO_2 in intact chloroplasts by dithiothreitol (Fig. 9) these results suggest that reduction of oxygen is a physiological process during photosynthesis which provides the extra ATP needed for CO_2 reduction. The lower affinity of oxygen as compared with CO_2 for electrons of the electron transport chain ensures that oxygen reduction cannot compete with CO_2 reduction for electrons. Only when NADPH oxidation during CO_2 reduction is limited by the ATP supply and NADPH levels rise are electrons diverted to oxygen increasing the ATP level and permitting NADPH oxidation again to occur. As the affinity data show, cyclic electron flow can only contribute to the ATP supply of chloroplasts when the reaction reducing oxygen becomes saturated.

It should be noted that the observations of an ATP deficiency of CO_2 reduction and of a coupling ratio between ATP synthesis and $NADP^+$ reduction which is not far from 1 in intact chloroplasts, are not in contradiction to the findings of the maximal coupling ratio of 2 in thylakoid systems[13,16]. As shown by their capability to photoreduce oxaloacetate intact chloroplasts, in contrast to mitochondria, are not tightly but loosely coupled. A necessary consequence of loose coupling is that maximal coupling ratios can only be maintained under optimal cofactor and substrate conditions and at a low phosphorylation potential. *In vivo* coupling should be expected to be flexible, as ATP synthesis is under the restraint of an increased and changing phosphorylation potential. In addition, substrate and cofactor conditions are not likely to be optimal and other processes such as direct or indirect proton leakage compete with phosphorylation to degrade the chemical potential[32,33] of a proton gradient across thylakoid membranes. Indirect proton leakage can indeed be seen in leaves and intact chloroplasts as photoinduced chloroplast shrinkage and the competitive nature of shrinkage and phosphorylation has been established[11,34,35].

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