Photoinhibitory Damage to Chloroplasts under Phosphate Deficiency and Alleviation of Deficiency and Damage by Photorespiratory Reactions

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Z. Naturforsch. **44c**, 524–536 (1989); received February 3, 1989

Dedicated to Professor Achim Trebst on the occasion of his 60th birthday

Photosynthesis, Photoinhibition, Photorespiration, Electron Transport, Phosphate Deficiency, Phosphate Homeostasis

Effects of Pi deficiency on photosynthesis of isolated spinach chloroplasts were examined. The following observations were made:

(1) Chloroplasts isolated in Pi-free media evolved oxygen in the light in the absence of added Pi until acid-extractable P_i in the chloroplasts had decreased to 1 to 2.5 mM. This P_i was unavailable for photophosphorylation as shown by the inability of the chloroplasts to respond by oxygen evolution to the addition of PGA. In the state of P_i-deficiency, stromal ATP to ADP ratios were in the light close to or below ratios observed in the dark. In the presence of 2 mM PGA, addition of 20 μM Pi was insufficient to increase ATP to ADP ratios, but sufficient for appreciable oxygen evolution.

(2) More Pi was available for oxygen evolution of phosphate-deficient chloroplasts at low levels of CO_2 than at high levels. This was due mainly to the suppression of oxygenation of RuBP by high CO_2 levels which prevented formation of phosphoglycolate and the subsequent release of Pi into the chloroplast stroma.

(3) More oxygen was produced by phosphate-deficient chloroplasts at a low light intensity than at a high light intensity. This was due to increased availability of endogenous Pi under low light and to photoinhibition of the chloroplasts by high light. The main product of photosynthesis of phosphate-deficient chloroplasts in the presence of a high bicarbonate concentration was starch, and the main soluble product was PGA.

(4) After phosphate-deficient chloroplasts had ceased to evolve oxygen in the light, they became photosensitive. Part of the loss of the capacity for oxygen evolution is attributed to leakage of PGA, but the main reason for loss of function is photoinactivation of electron transport. Both photosystems of the electron transport chain were damaged by light.

(5) Protection against photoinactivation was provided by coupled electron transport. Photoinactivation of phosphate-deficient chloroplasts was less extensive in the presence of low CO_2 concentrations which permitted oxygenation of RuBP than at high CO_2 concentrations. Electron transport to CO_2 and other physiological electron acceptors and to the herbicide methylviologen was also protective. However, electron transport to oxygen in the Mehler reaction failed to provide appreciable protection against high light intensities, because oxygen reduction is slow and reactive oxygen species produced in the light contribute to photoinactivation.

Introduction

Productivity in photosynthesis depends on the availability of light and carbon dioxide. Also, levels of photosynthetic intermediates and cofactors must be adequate to ensure optimum performance of the photosynthetic apparatus. All intermediates of the photosynthetic carbon cycle are phosphorylated, and phosphorylation of ADP requires Pi. Thus, the availability of Pi can be a factor in restricting photosynthesis [1, 2].

Paradoxically, light is not only used by chloroplasts to reduce CO_2 to sugars but also to oxidize sugars to CO_2 [3]. At the expense of ATP and NADPH formed in the light, isolated chloroplasts effectively oxidize sugar phosphates such as DHAP to phosphoglycolate which is then hydrolyzed liberating Pi in the chloroplast stroma. Glycolate is the sole soluble end product of photosynthesis of isolated chloroplasts, when CO_2 is absent [4]. When it is present, DHAP and PGA are synthesized from CO_2 and Pi. They are exported from the chloroplasts to be further processed in the cytosol of mesophyll cells

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DHAP, dihydroxyacetone phosphate; PGA, 3-phosphoglycerate; P_i, inorganic phosphate; PS I, II, photosystem I, II; RuBP, ribulose-1,5-bisphosphate.

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0341–0382/89/0500–0524 $ 01.30/0

to sucrose and other products. When phosphoglycolate is produced during oxygenation of ribulose-1,5-bisphosphate (RuBP), the Pi liberated by its hydrolysis remains in the chloroplasts and glycolate is released. Under evolution of CO_2, it is metabolized in a sequence of cytosolic reactions which constitute the photorespiratory pathway. The glycerate formed is returned to the chloroplasts. After its phosphorylation, it enters the carbon cycle (for review see [5]).

In terms of productivity, photorespiration is a wasteful process. It consumes energy and decreases net photosynthesis of C_3-plants [6]. There is the question why it has withstood evolutionary pressures which are directed towards optimizing the performance of biological systems. In this report, we like to show that the release of Pi inside the chloroplasts, when phosphoglycolate is hydrolyzed, may partially alleviate phosphate deficiency problems of photosynthesis. Moreover, the coupled electron flow accompanying the photorespiratory oxidation of sugars prevents over-reduction of the photosynthetic electron transport chain [7] and protects chloroplasts against photoinhibitory damage by permitting energy-dependent dissipation of excess light energy in the form of heat.

Materials and Methods

Plants

Spinach was grown in the greenhouse. Additional illumination was provided by 400 W HQI lamps (Schreder, Winterbach, Germany). The daily illumination period was 10 (summer) or 11 (winter) hours.

Chloroplast isolation

Intact chloroplasts were isolated by a procedure similar to that used by Jensen and Bassham [8]. Leaves were briefly blended in a medium containing 300 mM sorbitol, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM ethylenediamine tetraacetate, 3.3 mM cysteine, 1.25 mM ascorbate, 30 mM KCl and 50 mM 2-(N-morpholino)-ethane sulfonate (MES), pH 6.1. The pellet obtained after a first centrifugation of the filtrate (50 sec, $2000 \times g$) was resuspended in the same medium and centrifuged again. In the experiments with isolated chloroplasts were no Pi was added to the incubation medium, chloroplasts were resuspended and centrifuged one more time. The resulting chloroplast fraction was suspended in a small volume of reaction medium which contained 300 mM sorbitol, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM ethylenediamine tetraacetate, 30 mM KCl and 50 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonate (HEPES), pH 7.6, and stored on ice until used. The percentage of chloroplasts which had retained intact envelopes was determined by the ferricyanide method [9]. Usually, 70 to 95% of the chloroplasts in the preparations were intact.

Chloroplast reactions

CO_2-dependent oxygen evolution by the chloroplasts was measured in the electrode described by Delieu and Walker [10]. Catalase was added to the reaction medium (80 μg ml^{-1}). The chlorophyll concentration was usually 150 μg ml^{-1}. The chloroplasts were illuminated with red light (halfbandwidth from 610 to 720 nm). The cylindric cuvette had a diameter of 1 cm. In the presence of 2 mM bicarbonate and 0.2 mM Pi, light-saturated CO_2-dependent oxygen evolution was between 100 and 150 μmol per mg chlorophyll and hour.

To measure the distribution of photosynthetic products, chloroplasts were illuminated in reaction medium with 4.5 mM $NaH^{14}CO_3$ (specific activity 56 mCi $mmol^{-1}$). Aliquots were removed after different illumination times and killed in hot ethanol. Products were separated by two-dimensional chromatography on cellulose plates. Solvents were phenol/acetic acid/water/EDTA (1478 g/20 ml (96% v/v)/521 ml/5 ml (0.5 M)) for the first dimension and a freshly prepared mixture of *n*-butanol/propionic acid/water (1850 ml/900 ml/1225 ml) for the second dimension. Radioactive spots were located by autoradiography. They were removed from the plates and measured in a scintillation counter.

The capacity of thylakoids for electron transport was measured after rupturing intact chloroplasts by the addition of 9 volumes of water. Double strength medium was added after a short time so as to reestablish isotonic conditions. Light-dependent ferricyanide reduction by the thylakoids was measured in the presence of 5 mM NH_4Cl in a home-built cross-beam spectrophotometer at 420 nm (chlorophyll concentration about 5 μg ml^{-1}). Photosystem I activity was measured in the presence of 5 mM NH_4Cl as light-dependent oxygen uptake after adding 1 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). The methanol concentration was 2% (v/v) and catalase was present at 4 μg ml^{-1}. Electron donors were

5 mM ascorbate and 0.1 mM dichlorophenolindophenol, electron acceptor was 0.2 mM methylviologen. For measurements of chlorophyll fluorescence, suspensions of chloroplasts containing 150 μg chlorophyll ml^{-1} reaction medium were made anaerobic by adding 10 mM glucose, glucose oxidase (83 U ml^{-1}) and catalase (80 μg ml^{-1}). Modulated chlorophyll fluorescence was recorded using a PAM fluorometer of Walz, D-8521 Effeltrich, Germany [11]. The detecting device was insensitive to unmodulated actinic illumination.

Substrate determinations

For the measurement of Pi, PGA, ATP and ADP, perchloric acid was added to chloroplasts suspensions either in the light or in the dark (0.6 M final concentration). Acid was neutralized with K_2CO_3, and $KClO_4$ and debris were removed. ATP and ADP were determined by the luciferin/luciferase method [12, 13], PGA enzymatically [14] and Pi by a molybdate method [15].

Results and Discussion

Photosynthesis of phosphate-deficient spinach chloroplasts at different CO_2 concentrations

Fig. 1 shows photosynthetic oxygen evolution of chloroplasts which were isolated from spinach leaves in a Pi-free medium. Even after careful washing, the chloroplasts contained Pi. When they were illuminated in the presence of CO_2, oxygen was evolved after a brief lag phase. Both the rate of oxygen evolution and the amount of oxygen evolved were low at high levels of CO_2 and high at low levels of CO_2. With 20 mM bicarbonate, which corresponds at 20 °C to about 3.5% CO_2 in the gas phase (pK of CO_2 is 6.37), oxygen evolution declined after a short time and finally approached zero. Addition of 2 mM PGA remained without effect on oxygen evolution indicating that the stromal Pi supply had been exhausted by the photosynthetic formation of phosphate esters. Pi is needed for the reduction of PGA. In its presence, chloroplasts are known to evolve oxygen according to

$$H_2O + NADP^+ + ADP + P_i \rightarrow NADPH + H^+ + ATP + 1/2\,O_2. \quad (1)$$

ATP and NADPH are consumed acording to

$$PGA + ATP + NADPH + H^+ \rightarrow DHAP + ADP + P_i + NADP^+. \quad (2)$$

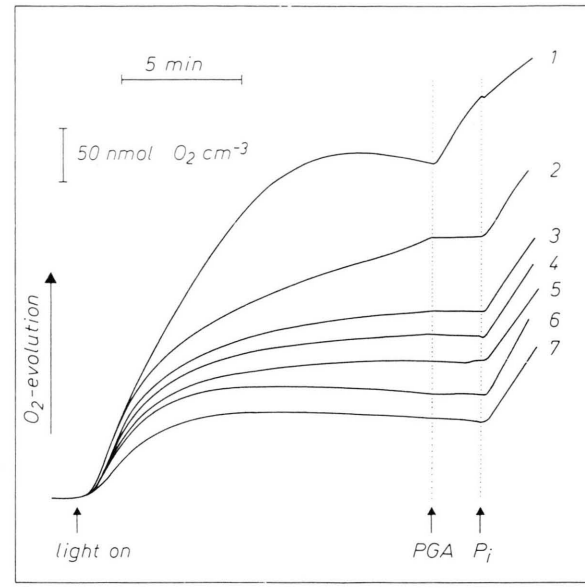

Fig. 1. Light-dependent oxygen evolution by intact spinach chloroplasts in the presence of different concentrations of bicarbonate. The chloroplasts had been isolated and washed in media which were free of Pi, and the reaction medium was also free of Pi. Two light sources (500 Wm^{2-} each) illuminated the chloroplast suspension from two sides. Bicarbonate concentrations were as follows: (1) 0.1 mM bicarbonate added*, (2) 0.25 mM bicarbonate added, (3) 0.5 mM bicarbonate added, (4) 1 mM bicarbonate added, (5) 2 mM bicarbonate added, (6) 10 mM bicarbonate added, (7) 20 mM bicarbonate added.

Indeed, levels of acid-extractable Pi as measured by complex formation with molybdate [15] ranged from 10 to 25 mM in different preparations of darkened spinach chloroplasts and decreased to 1 to 2 mM in chloroplasts which had been illuminated until oxygen evolution had ceased. Similar results have been described by Robinson and Giersch [16] and Furbank *et al.* [17] who have determined stromal Pi levels by a colorimetric method and by ^{32}P chromatography. Approximately, 1 mM Pi was found to be associated with thylakoid membranes, and more Pi appeared to be bound to other chloroplast constituents. Bound Pi is unavailable for photosynthetic reactions. It cannot be used to phosphorylate ADP.

As shown in Table I, the ATP/ADP ratio in darkened chloroplasts was about 0.3. It increased in the

* The total bicarbonate concentration was higher by about 0.25 mM than indicated by the additions because the reaction medium had not been freed of CO_2/bicarbonate.

Table I. Rates of light-dependent oxygen evolution of isolated chloroplasts in the presence of 20 mM $NaHCO_3$, and ATP/ADP ratios in the chloroplast stroma. The chloroplasts were isolated and suspended in a Pi-free medium. The intensity of red light was 500 Wm^{-2}.

	O_2 evolution μmol (mg chlorophyll h)$^{-1}$	Ratio of ATP/ADP
Dark	0	0.34
Light: 2 min	50	0.84
7 min	0	0.33
8 min		
(2 mM PGA added after the 7th min)	0	0.24
11 min		
(20 μM Pi added after the 9th min)	15	0.28
11 min		
(200 μM Pi added after the 9th min)	72	1.00

light while oxygen was evolved and then decreased approximately to and sometimes even distinctly below the dark level as oxygen evolution ceased. Addition of 20 μM Pi to the chloroplasts was sufficient to restart oxygen evolution, but failed to increase the ATP/ADP ratio appreciably. Apparently, ATP was consumed for PGA reduction as fast as it was produced, and import of Pi limited the rate of oxygen evolution. It is known that PGA is a competitive inhibitor of Pi transport into the chloroplasts [18]. Indeed, when 0.2 mM Pi was added to the chloroplasts, both oxygen evolution and ATP/ADP ratios increased considerably.

When the bicarbonate concentration was not 20 mM, but only 10 mM (Fig. 1), both the initial rate of oxygen evolution and the total amount of oxygen evolved increased. The increased amount of oxygen shows that more Pi had become available for carbon reduction. After oxygen evolution had ceased, PGA was again unable to restart oxygen production until Pi was added. Further reductions in the bicarbonate concentration resulted in further increases in the rate of oxygen evolution and in the total amount of oxygen evolved. Again it must be concluded that Pi availability for carbon reduction had increased as availability of CO_2 had decreased. Interestingly, at intermediate bicarbonate concentrations, oxygen evolution no longer ceased completely after the initial fast rate had declined. It continued slowly, but stopped when PGA was added. Phosphate was required for restarting. At the lowest concentrations of bicarbonate, however, the level of oxygen declined after it had reached a maximum. The decline shows that carbon reduction was replaced as a dominant reaction by carbon oxidation (4) which may be written in a simplified form as

$$2\ DHAP^{2-} + 1.5\ O_2 \rightarrow 3\ glycolate^- + 2\ HPO_4^{2-} + 3\ H^+ \quad (3)$$

and

$$2\ PGA^{3-} + 0.5\ O_2 \rightarrow 3\ glycolate^- + 2\ HPO_4^{2-} + H^+. \quad (4)$$

Equations (3) and (4) sum up a sequence of individual reactions of the Calvin cycle during which ATP and NADPH are consumed. Phosphate is liberated. Indeed, when PGA was added after the oxygen concentration was beyond its maximum, oxygen uptake was replaced by oxygen evolution showing that, in this instance, Pi was not lacking. As a matter of fact, the addition of Pi did not increase oxygen evolution as it did at higher CO_2 concentrations. Rather, it was inhibitory.

Two questions must be answered. i. Why is photosynthesis faster at low than at high CO_2? ii. Why is Pi availability greater at low than at high CO_2?

i. The inhibition of photosynthesis by high levels of CO_2 is explained by the pH dependence of photosynthesis [19, 20]. In solution, bicarbonate equilibrates with CO_2. Only the latter penetrates the chloroplast envelope readily. In the chloroplast stroma, it reacts according to

$$CO_2 + OH^- \rightarrow HCO_3^- \quad (5)$$

decreasing the stroma pH when its concentration is sufficiently high. This inhibits fructose-1,6-bisphosphatase and sedoheptulose-1,7-bisphosphatase. Iso-

lated chloroplasts are much less efficient in regulating their stroma pH than chloroplasts *in situ*. Photosynthesis of spinach leaves is not inhibited by 5% CO_2 in the gas phase (data not shown), although the lag phase seen after long darkening, and displayed for isolated chloroplasts in Fig. 1, is prolonged.

ii. The increased availability of Pi at decreased CO_2 levels is caused by release of Pi from phosphate esters during starch synthesis and during hydrolysis of phosphoglycolate. RuBP carboxylase is capable of either carboxylating or oxygenating RuBP. At high CO_2 concentrations, oxygenation is competitively suppressed. RuBP can only be carboxylated. This gives rise to 2 molecules PGA per RuBP. At low concentrations of CO_2, oxygenation is possible and phosphoglycolate is formed together with PGA. Its hydrolysis liberates Pi which serves to phosphorylate ADP. The ATP formed makes reduction of PGA to DHAP and evolution of oxygen possible (see equations (1) and (2)). From DHAP, RuBP is synthesized which, once again, can be either carboxylated or oxygenated. Oxygenation becomes the predominant reaction only when CO_2 is depleted. In the experiment of Fig. 1, such depletion is observed at the lowest bicarbonate concentration. It results in net oxygen uptake and leads to the oxidation of phosphate esters and carbohydrates, which had accumulated during the phase of reduction, and to the liberation of Pi. In such a case, not only the addition of PGA can restart oxygen evolution in the absence of added Pi as shown in Fig. 1 (top trace), but also addition of bicarbonate (data not shown).

Products of photosynthesis of phosphate-deficient chloroplasts at a low and at a high light intensity

When spinach chloroplasts isolated in a Pi-free medium were illuminated in the absence of P_i, with 20 mM bicarbonate present, O_2 evolution was predictably slower at a low than at a high light intensity. Surprisingly, however, whereas O_2 evolution ceased soon under high intensity illumination because the supply of P_i was exhausted, it continued in low light until the amount of oxygen evolved exceeded that evolved in high light. Increased O_2 evolution under light limitation was due to increased starch synthesis. Fig. 2 shows the distribution of ^{14}C-label in different products of photosynthesis. Chloroplasts were illuminated with 4.5 mM $NaH^{14}CO_3$ at a high light intensity (1900 Wm^{-2}) either for a full period of 22 min (Fig. 2A) or for only 5 min (Fig. 2B). At this time, oxygen evolution was already declining (see Fig. 1). The light intensity was then reduced from 1900 to 100 Wm^{-2} which, at the chlorophyll concentration of the sample, was below light saturation. Fig. 2 shows that starch was the dominant product of photosynthesis both under high intensity illumination and in low light. However, there was a difference in that ^{14}C-labelling of starch increased continuously in low light until 2 mM unlabelled PGA was added, whereas it reached saturation after about 10 min under continuing high intensity illumination. Among the soluble products of photosynthesis, PGA was dominant both under high and low intensity illumination. DHAP was only a minor component. This is not surprising in view of very low ATP/ADP ratios (Table

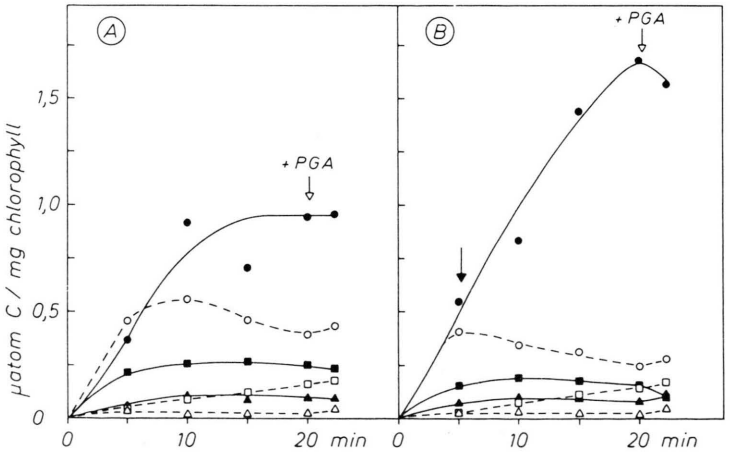

Fig. 2. Distribution of labelled carbon in different products during photosynthesis of chloroplasts in Pi-free reaction medium. The concentration of $NaH^{14}CO_3$ was 4.5 mM. The chloroplasts were either continuously illuminated with 1900 Wm^{-2} white light (A) or, after an initial period of 5 min at 1900 Wm^{-2}, with a reduced light intensity of 100 Wm^{-2} (B). The solid arrow indicates the time when the light intensity was reduced. 2 mM unlabelled PGA was added as indicated. ● Labelled starch, ○ labelled PGA, ■ labelled sugar monophosphates, □ labelled maltose, ▲ labelled sugar bisphosphates, △ labelled DHAP.

I). Sugar monophosphate levels were higher than sugar bisphosphate levels. Furbank *et al.* [17] have shown that RuBP was a major sugar bisphosphate even after oxygen evolution had ceased and CO_2 levels were high. Its persistence in the presence of high CO_2 levels is explained by inhibition of RuBP carboxylase under phosphate deficiency [21]. Labelled glycolate was not measured in the experiment of Fig. 2. However, it was present, when bicarbonate concentrations were low (data not shown), and it was the major soluble product of photosynthesis under CO_2 deficiency (4). Maltose is a degradation product of starch. Its concentration increased with the time of illumination.

The dominance of starch among the products of photosynthesis shown in Fig. 2 was unexpected, because starch synthesis requires ATP which is low under Pi deficiency (Table I). Phosphate is liberated in starch synthesis only when ATP is present. A key enzyme in the regulation of starch synthesis is ADP-glucose pyrophosphorylase. This enzyme is inhibited by Pi and activated by PGA [22]. Its activation by high PGA/Pi ratios permitted starch synthesis to occur even though ATP levels were low. However, the importance of ATP is illustrated by increased starch synthesis under low intensity illumination. Fig. 2 shows that the concentration of phosphate esters was lower under low intensity illumination than under high intensity illumination. In consequence, the level of Pi that is available for ATP synthesis was increased at the low light intensity. Increased ATP/ADP ratios explain increased DHAP/PGA ratios and increased starch synthesis in low light. It is a very remarkable fact that a decrease in light intensity can be instrumental in increasing photosynthesis under conditions of Pi deficiency.

Leakage of phosphoglycerate from the chloroplasts

During experiments with phosphate-deficient chloroplasts such as shown in Fig. 1 it was noticed that Pi added after different times of illumination stimulated photosynthetic oxygen evolution more after short times of illumination than after longer times (Fig. 3). This may have different reasons. i. Chloroplasts may break during the experiment. Only intact chloroplasts are capable of CO_2- or PGA-dependent oxygen evolution. ii. Chloroplasts might age. iii. Photosynthetic intermediates or cofactors may get lost from the chloroplasts. iv. Enzymes or electron carriers may become inactivated.

Measurements of chloroplast intactness with the ferricyanide method [9] failed to reveal significant chloroplast breakage (Table II). Chloroplast ageing could not be responsible for the progressive inhibition of oxygen evolution shown in Fig. 3 because

Table II. Intactness of isolated chloroplasts as a function of time of exposure of phosphate-deficient chloroplasts to high-intensity illumination. The chloroplast suspension was illuminated with two light sources of 500 Wm^{-2} each.

Time of illumination [min]	Chloroplasts with intact envelopes [%]
0	92
3	94
6	95
10	93
15	88
20	88

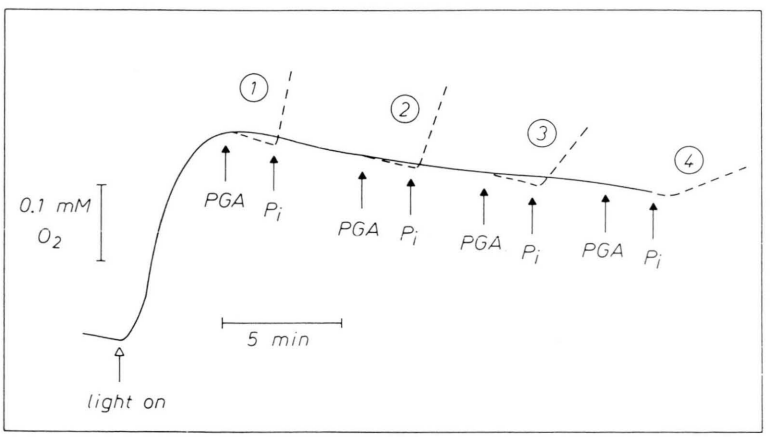

Fig. 3. Photosynthetic oxygen evolution of chloroplasts in Pi-free reaction medium. The bicarbonate concentration was 20 mM and the light intensity 1000 Wm^{-2} (2 lamps, 500 Wm^{-2} each). (1) After 5 min illumination, 2 mM PGA was added, and after further 2 min, 0.2 mM Pi; (2) same as (1) except that PGA was added after 10 min; (3) same as (1) except that PGA was added after 15 min; (4) same as (1) except that PGA was added after 20 min.

Table III. Concentration of PGA in the chloroplast stroma, and percentage of the total PGA which was found in the medium outside illuminated chloroplasts. It is assumed that the stroma volume of chloroplasts containing 1 mg chlorophyll is 25 µl.

	Dark	Light: 8 min	12 min	16 min
Concentration of PGA in stroma (mM)	1.4	2.05	1.3	0.95
% of total PGA in supernatant	37	72	81	88

inhibition was strictly light-dependent. However, loss of photosynthetic intermediates was observed. Table III shows enzymic measurements of PGA in chloroplasts which had been illuminated in the presence of 20 mM $NaHCO_3$. It is not surprising that PGA was found even in darkened chloroplasts, as the medium used for chloroplast isolation was Pi-free so that internal PGA could not be exchanged by the phosphate translocator of the chloroplast envelope for external Pi, when the leaves were homogenized. However, the stromal PGA concentration did not increase much on illumination (Table III), although available Pi was consumed as demonstrated by the lack of oxygen response after addition of PGA (Fig. 3) and the concentration of DHAP was very low (Fig. 2). After 8 min illumination, the stromal PGA concentration was only 2 mM. It declined later on and PGA appeared outside the chloroplasts. Its appearance cannot be explained by exchange against external Pi, as the media used for chloroplast isolation and washing had been free of Pi. Moreover, any Pi liberated from broken chloroplasts and taken up in exchange for exported PGA would have given rise to oxygen evolution. However, no oxygen evolution was observed in the presence of 20 mM bicarbonate after endogenous Pi had been consumed by the chloroplasts (see Fig. 1). Apparently PGA leaked into the medium. The rate of unidirectional leakage was approximately 0.2 µmol per mg chlorophyll and hour in the experiment of Table III. Although this may appear to be a very low rate, it can decrease the stromal concentration of PGA (or of Pi, from which PGA is synthesized) by 8 mM within one hour. Thus, the commonly accepted idea of phosphate and phosphate ester homeostasis in chloroplasts, which is based on the anion exchange characteristics of the phosphate translocator [23], requires modification.

Light-dependent inactivation of electron transport under phosphate deficiency

In the experiment of Fig. 3, Pi-dependent oxygen evolution in the presence of bicarbonate and PGA decreased drastically during illumination. Although leakage of the stromal metabolite PGA was appreciable (Table III), it is insufficient to explain the extent of loss of the capacity for oxygen evolution of the chloroplasts. In the experiment shown in Fig. 4, phosphate-deficient chloroplasts were illuminated in the presence of 20 mM bicarbonate at two different temperatures. The kinetics of oxygen evolution were as depicted in Fig. 1 (lowest trace). After different times of illumination, samples were removed and chloroplasts were ruptured osmotically. The capacity of the thylakoid membranes for electron transport was then measured in saturating light in the presence of NH_4Cl. Ferricyanide served as acceptor of electrons liberated during water oxidation. This electron transport involved both photosystems (PS I + II). The activity of photosystem I alone was measured

Table IV. Light-saturated electron transport to ferricyanide in uncoupled thylakoids from phosphate-deficient chloroplasts, which had been illuminated with red light (two light sources of 500 Wm^{-2} each) in the presence of 50 µM, 290 µM or 620 µM oxygen (corresponding to 4, 22 or 48% oxygen in the gas phase).

Illumination time [min]	Electron transport, µmol ferricyanide reduced/mg chlorophyll per hour		
	Low oxygen	Intermediate oxygen	High oxygen
0	1020	920	–
10	790	395	260
15	740	240	180
20	652	180	135

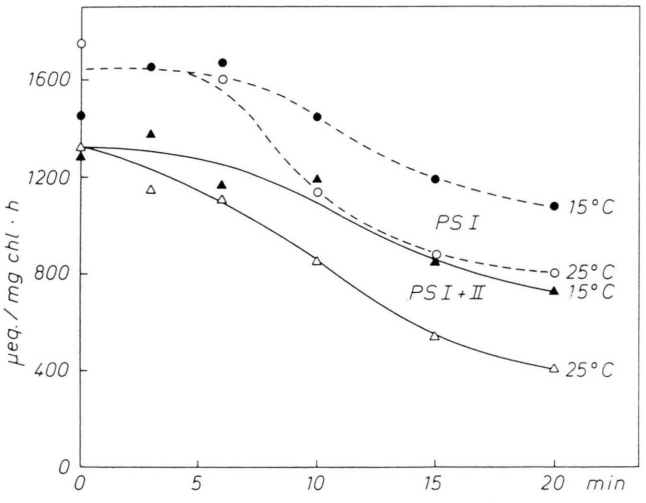

Fig. 4. Light-saturated electron transport of thylakoids which were uncoupled by NH_4Cl. Temperature was 20 °C. The thylakoids had been liberated from intact chloroplasts after the latter were illuminated with red light (two light sources of 500 Wm^{-2} each) for the indicated times at 15 or 25 °C in a reaction medium which contained 20 mM bicarbonate, but no Pi. Photosystem I: Ascorbate was electron donor and methylviologen acceptor of photosystem I-dependent electron flow. Photosystem II was inactivated by DCMU. The concentration of NH_4Cl was 15 mM. Photosystem I + II: Water as electron donor and ferricyanide as acceptor. The concentration of NH_4Cl was 5 mM.

with methylviologen as electron acceptor. Electron transport to photosystem II was inhibited by the herbicide DCMU. Electron donors were in this case ascorbate and 2,6-dichlorophenolindophenol. During the first 5 min of illumination, little change in electron transport capacity was observed. However, after endogenous Pi was exhausted and oxygen evolution had ceased, the chloroplasts became sensitive to illumination. Within 20 min in the light, electron transport mediated by photosystem I decreased by 50% at 25 °C. Electron transport mediated by both photosystems was somewhat slower even at the beginning of illumination than the photosystem I reaction. It decreased even more than the latter, by 70% during a 20 min illumination period at 25 °C. Interestingly, inactivation of electron transport was slower when the chloroplasts were illuminated at 15 °C than when they were illuminated at 25 °C. This shows that a thermal reaction was involved in inactivation.

In the experiment of Fig. 5, a suspension of phosphate-deficient chloroplasts which contained 10 mM glucose was made anaerobic by adding glucose oxidase and catalase prior to illumination (A). Another preparation (B) was first illuminated for 20 min, before glucose oxidase and catalase were added. The

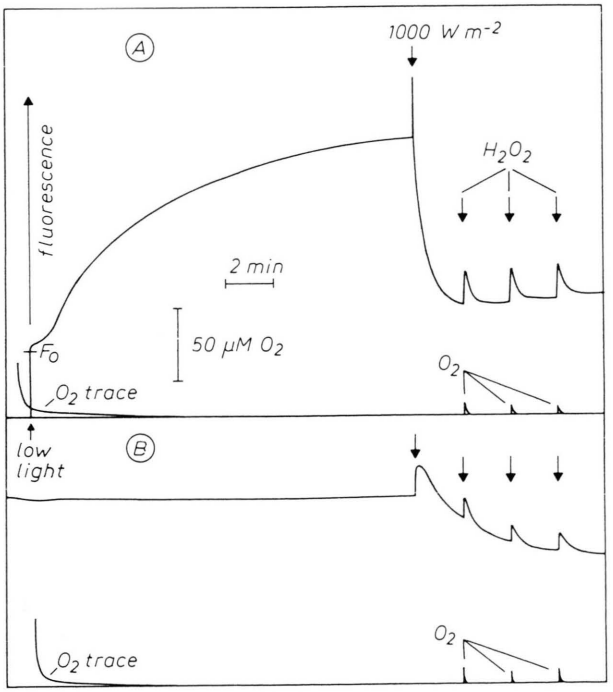

Fig. 5. Chlorophyll fluorescence from photosystem II under anaerobic conditions. (A) Chloroplasts suspended in Pi-free reaction medium (20 mM bicarbonate present) were made anaerobic by glucose, glucose oxidase and catalase without prior illumination. (B) Chloroplasts suspended in Pi-free reaction medium (20 mM bicarbonate present) were made anaerobic after a 20 min illumination period (light intensity 2×500 Wm^{-2}). As oxygen was removed by the enzymic oxygen trap, chlorophyll fluorescence increased under the influence of a low intensity measuring beam in (A) indicating reduction of electron carriers. In (B), carriers had been reduced during preillumination. After near-maximum fluorescence was reached in (A), actinic illumination first increased and then decreased fluorescence. Oxidation of reduced pheophytin is indicated by oxygen-dependent fluorescence increases. Oxygen was liberated from H_2O_2 by catalase as shown on the oxygen trace.

oxygen traces in Fig. 5 show rapid consumption of oxygen by the combined action of both enzymes. Chlorophyll fluorescence of the suspensions was excited by a modulated 650 nm measuring beam (intensity 2.5 Wm^{-2}) and measured by a photodiode which was sensitive only to modulated fluorescence, not to the fluorescence excited by an unmodulated actinic beam of 1000 Wm^{-2} [11]. Low intensity illumination caused a slow increase in chlorophyll fluorescence which is emitted by the pigment system of photosystem II (Fig. 5A). It is indicative of the reduction of electron carriers of the electron transport chain [24]. The maximum fluorescence emitted from the preilluminated chloroplast suspension (B) was 35% below that emitted from the untreated control (A). In the latter case, the slow rise in fluorescence seen in Fig. 5A was absent because the electron transport chain had been reduced during preillumination. Loss of so-called variable fluorescence [11] which is shown by decreased maximum fluorescence in Fig. 5B is commonly interpreted as loss of functional reaction centers. Actinic illumination with a high light intensity produced in both samples a transient fluorescence increase. It was followed by strong fluorescence quenching. This quenching is unrelated to that produced as a consequence of the formation of a *trans*-thylakoid proton gradient [25]. As the chloroplasts were in a state of over-reduction, formation of a large proton gradient was impossible. Part of the fluorescence quenching is caused by the photoaccumulation of reduced pheophytin in the reaction centers of photosystem II [26]. Reduced pheophytin is a strong quencher of chlorophyll fluorescence. It is rapidly oxidized in the presence of oxygen. When H_2O_2 was added from which oxygen is liberated by catalase, fluorescence increased indicating pheophytin oxidation [26]. After oxygen was removed by the enzymic oxygen trap (see oxygen traces of Fig. 5), fluorescence returned to the quenched state. The kinetics of quenching were faster in the control than in the preilluminated sample both when the actinic beam was turned on and after oxygen liberated from H_2O_2 had been removed. The fluorescence increase brought about by oxygen was larger in the control than in the preilluminated sample indicating that less pheophytin was photoreduced after preillumination than before. The fluorescence data and the observations recorded in Fig. 4 show that not only photosystem I but also photosystem II had suffered photoinhibitory damage after phosphate-deficient chloroplasts had ceased to evolve oxygen in the light. Similar damage was observed when not Pi but CO_2 limited photosynthesis of isolated chloroplasts [7, 27].

The dependence of photoinhibition of Pi-deficient chloroplasts on the intensity of incident light is shown in Fig. 6. Damage increased with increasing light intensity.

Protection of the electron transport chain against photoinactivation

In the experiment of Fig. 7, phosphate-deficient chloroplasts were either illuminated in the presence of 20 mM bicarbonate (A) or only with the low concentration of bicarbonate present in the isotonic reaction mixture (B). This low concentration was complemented during the experiment by two additions of 0.1 mM bicarbonate each (as indicated). Light-dependent oxygen evolution with either 20 mM bicarbonate or without added bicarbonate is shown in the inset in a parallel experiment in order to demonstrate that Pi is available only in the latter case. This is shown by PGA-dependent oxygen evolution.

After the times indicated, aliquots were removed from the illuminated cuvette, the chloroplasts were ruptured osmotically and uncoupled electron transport was assayed. As shown already in Fig. 4, electron transport was progressively inactivated in the presence of high CO_2 levels (*i.e.* 20 mM bicarbonate) which suppressed oxygenation of RuBP. Inactivation was also observed in the sample illuminated in the presence of a low CO_2 concentration, but it was slower and less extensive. Apparently, the oxygenation of RuBP which permitted increased oxygen evolution in this sample was protective, although the rate of electron transport facilitated by the Pi released from phosphoglycolate was very low (see inset). It should be noted that under the low-Pi conditions of the experiment ribulose-1,5-bisphosphate carboxylase was partially inactivated [17, 21] so that electron transport to the products of carboxylation/oxygenation was much slower than it would have been *in vivo*, when the enzyme is fully active.

As a matter of fact, the protective effect of oxygenation of RuBP on electron transport is not specific. Full protection of the electron transport chain against photoinactivation is provided by the unphysiological electron acceptor methylviologen at a concentration as low as 20 μM [7]. Methylviologen permits slow coupled electron transport. Protection

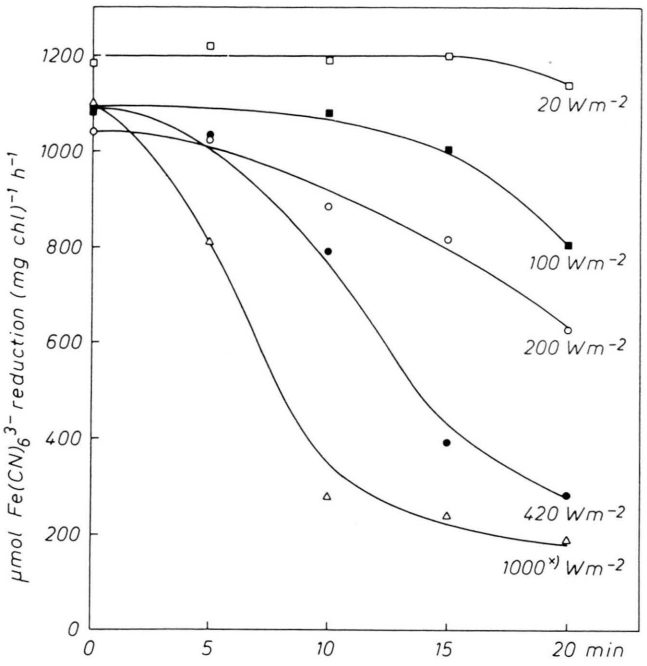

Fig. 6. Light-saturated electron transport from water to ferricyanide of thylakoids which were uncoupled by NH_4Cl. The thylakoids had been liberated from intact phosphate-deficient chloroplasts after the latter were illuminated with different light intensities for the times indicated. The reaction medium contained 20 mM bicarbonate and the temperature was 20 °C. The chloroplast preparation used for the 20 Wm^{-2}-experiment was different from that used for the higher light intensities. $^{x)}$ To produce 1000 Wm^{-2}, two lamps were used.

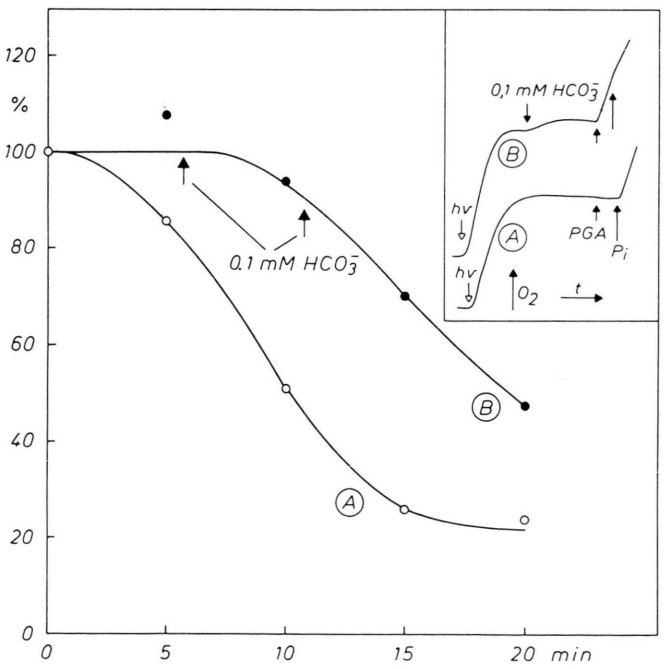

Fig. 7. Light-saturated electron transport to ferricyanide of thylakoids which were uncoupled by 5 mM NH_4Cl. The rate indicated by 100% was 1150 µmol (mg chlorophyll)$^{-1}$ h^{-1}. The thylakoids had been liberated from intact chloroplasts after the latter were illuminated at 20 °C in a Pi-free reaction medium either with only a low concentration of endogenous bicarbonate (B) or with 20 mM added bicarbonate (A). Ordinate: Time of illumination of the intact chloroplasts with 1000 Wm^{-2} red light. The inset shows oxygen evolution of intact chloroplasts in a parallel experiment with the same chloroplast preparation. Note PGA-dependent oxygen evolution in (B), but not in (A). It indicates liberation of Pi from phosphoglycolate.

by methylviologen is completely lost in the presence of 10 mM NH_4Cl. Apparently, formation of a proton gradient across the thylakoids is needed to protect the electron transport chain against damage [28]. Under physiological conditions, the proton gradient triggers changes in the pigment bed of the membranes which lead to the efficient dissipation of excess excitation energy as heat. Formation of zeaxanthin from violaxanthin appears to play a role in this process [29]. Non-radiative dissipation of excess light energy is indicated by the decrease of fluorescence commonly called energy-dependent fluorescence quenching [30]. Any condition which permits sufficient coupled electron flow for the formation of a large *trans*-thylakoid proton gradient should therefore be protective. Fig. 8 shows that indeed a variety of electron acceptors are capable of preventing or at least reducing photoinactivation of electron transport. It also shows that photoinhibitory damage to the chloroplasts is indicated by an increase in the fluorescence emitted by the chloroplasts at very low light intensities, when the electron transport chain is oxidized. The ratio of this so-called F_0 fluorescence before and after high-intensity illumination (F_0/F_0' in Fig. 8) is decreased after the chloroplasts have suffered photoinhibition.

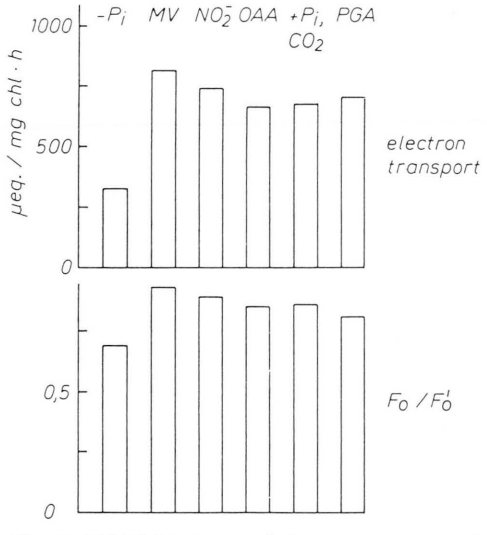

Fig. 8. (A) Light-saturated electron transport of uncoupled thylakoids, after phosphate-deficient chloroplasts had been illuminated with 2×500 Wm^{-2} red light for 20 min in air-saturated reaction medium in the presence of different electron acceptors. The concentration of methylviologen (MV) was 20 μM and that of Pi (where added) 200 μM. CO_2 was in equilibrium with 2 mM bicarbonate. The concentrations of nitrite (NO_2^-), oxaloacetate (OAA) and PGA were also 2 mM. $-P_i$: no electron acceptor added. (B) The ratio of chlorophyll fluorescence emitted from the chloroplasts whose plastoquinone pool was oxidized as measured before and after 20 min of illumination (2 lamps, 500 Wm^{-2} red light each). Care was taken to ascertain that the light intensity exciting fluorescence was so low as not to cause reduction of the acceptor Q_A of the reaction center of photosystem II.

Whereas *in vivo* the availability of several electron acceptors including CO_2 is subject to large variations, oxygen is an electron acceptor which is always present. It is reduced by chloroplasts in the light in the Mehler reaction. Electron transport to oxygen is often called pseudocyclic electron transport, and the photophosphorylation accompanying it pseudocyclic photophosphorylation. The rate of electron transport to oxygen is low, when physiological electron acceptors are available, and it is faster, when they are exhausted. The experiments shown in Fig. 4 and 6 to 8 were performed under aerobic conditions. Still, oxygen did not prevent damage. As a matter of fact, damage became only apparent after consumption of Pi had made regeneration of $NADP^+$ impossible and oxygen was the only available electron acceptor. Photoinactivation of photosystem II (but not of photosystem I) is fast in the absence of oxygen (*cf.* [31]). A protective effect of low concentrations of oxygen on electron transport has been reported [31, 32] and was also observed in the present investigation. However, electron transport to oxygen is not fast enough to protect the chloroplasts at the light intensities used for the experiments of Fig. 4, 6 and 7. Moreover, oxygen species produced by illumination are highly reactive [34]. As a matter of fact, at increased concentrations, oxygen increased damage (Table IV). Apparently, it plays a dual role [32, 34]. In its function as electron acceptor it is protective, but its reaction products are damaging. The O_2^- formed during oxygen reduction is converted to H_2O_2 by superoxide dismutase which is a chloroplast enzyme. In the presence of ascorbate, ascorbate peroxidase reduces H_2O_2, and light-dependent electron transport regenerates ascorbate from the resulting dehydroascorbate [33]. All experiments shown were performed in the presence of catalase which served to destroy any H_2O_2 formed in the light. Exposure of Pi-deficient chloroplasts to light in the presence of 10 mM ascorbate instead of catalase produced photoinhibition very similar to that observed in the presence of catalase.

Conclusions

Under phosphate deficiency, isolated intact chloroplasts become sensitive to light particularly when high CO_2 concentrations prevent oxygenation of RuBP. The rapid inhibition of photosynthetic reactions observed under high intensity illumination is explained in part by leakage of PGA, but mainly by photoinhibition of electron transport. Both photosystems suffer extensive damage within less than 20 min of high intensity illumination.

When CO_2 concentrations are so low as to permit oxygenation of RuBP in addition to carboxylation, damage to the photosystems is prevented or decreased. This effect is caused by the liberation of Pi from phosphoglycolate which can be recycled permitting electron transport to proceed. Coupled electron transport is a prerequisite for the efficient dissipation of excess excitation energy as heat. Phosphate is also recycled during starch synthesis. However, electron transport supported by starch synthesis is too slow in isolated chloroplasts under imposed phosphate deficiency to provide much protection against photoinhibition. When oxygen supports electron flow by reacting with RuBP, it is protective. However, it is surprisingly ineffective in providing protection as a Hill oxidant in the Mehler reaction. Actually, at high concentrations, its direct interaction with the electron transport chain is damaging (Table IV). Physiological electron acceptors compete successfully with oxygen for electrons thereby preventing or decreasing the formation of reactive oxygen species such as O_2^-.

Under field conditions, electron transport to CO_2 will normally be mainly responsible for creating the high energy condition of thylakoid membranes which is necessary for the degradation of excess light into heat. However, under water stress stomata of leaves close and restrict entry of CO_2. Under these conditions, coupled electron transport is maintained by the interplay between photorespiratory CO_2 production and the photosynthetic refixation of the liberated CO_2 [7]. In the experiment of Fig. 7, only part of this electron transport capacity was realized. Phosphate limitation had decreased the activation state of RuBP carboxylase. Still, partial protection was observed. Under *in vivo* conditions, photorespiration is effective in preventing or minimizing photoinactivation of electron transport when no other source of CO_2 is available [7]. The idea that photorespiration is involved in the protection of leaves against photoinactivation of light-dependent electron transport was first expressed by Björkman and Osmond [35]. It has received scattered support [7, 36–39]. An excellent review summarizing work on photoinhibition has been published by Powles [40].

Acknowledgements

This work was supported within the research performed by the Forschergruppe "Ökophysiologie" of the Deutsche Forschungsgemeinschaft. Cooperation with the Estonian Academy of Sciences was made possible by the exchange agreement between the Deutsche Forschungsgemeinschaft and the USSR Academy of Sciences and by the Gottfried-Wilhelm-Leibniz-Program of the Deutsche Forschungsgemeinschaft. T. M. is indebted to the A. v. Humboldt Foundation for a research fellowship. The technical assistance of Mrs. Trumpfheller is gratefully acknowledged.

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