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RATE-LIMITING FACTORS IN LEAF PHOTOSYNTHESIS

I. CARBON FLUXES IN THE CALVIN CYCLE

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Rates of photosynthesis of spinach leaves were varied by varying light intensity and CO_2 concentration. Metabolism of the leaves was then arrested by freezing them in liquid nitrogen. Chloroplasts were isolated by a nonaqueous procedure. In the chloroplast fractions, levels of intermediates of the carbon reduction cycle were determined and considered in relation to the photosynthetic flux situation of the leaves at the time before freezing. During induction of photosynthesis, ribulose 1,5-bisphosphate levels increased in parallel with CO_2 fixation. In the steady state, a similar relation between ribulose 1,5-bisphosphate levels and CO_2 uptake was observed at light intensities between 0 and 50 $W \cdot m^{-2}$. A further increase in light intensity increased CO_2 fixation rates but not ribulose 1,5-bisphosphate levels. Increasing the CO_2 concentration resulted in increased CO_2 uptake, whereas ribulose 1,5-bisphosphate levels decreased. Even under CO_2 saturation, ribulose 1,5-bisphosphate levels were about 100 nmol/mg chlorophyll corresponding to about 3.5 mM ribulose 1,5-bisphosphate in the chloroplast stroma. This suggests that even under CO_2 saturation, ribulose-1,5-bisphosphate carboxylase limits photosynhetic CO_2 uptake. Mass action ratios calculated from measured metabolite levels demonstrated that the thermodynamic gradient required for the regeneration of ribulose 1,5-bisphosphate from hexosephosphate and triosephosphate increased considerably as photosynthetic flux increased. Similar calculations revealed that the enzymatic apparatus responsible for the reduction of 3-phosphoglycerate to dihydroxyacetone phosphate is not displaced much from equilibrium even under maximum rates of photosynthesis at saturating CO_2. The same is true for aldolase. Fructose-1,6-bisphosphatase also did not limit Calvin cycle turnover. Only at very low light intensities and during the first minutes of the induction period was the ratio of fructose 1,6-bisphosphate to fructose 6-phosphate high. This observation was more readily explained in terms of fructose 1,6-bisphosphate binding to ribulose-1,5-bisphosphate carboxylase than by a rate limitation imposed by insufficient activation of fructose-1,6-bisphosphatase.

Introduction

Photosynthetic CO_2 uptake of leaves at constant water status and temperature is a function of both light intensity and CO_2. At very low light intensities, the increase in photosynthesis brought about by raising CO_2 from levels encountered in air to rate-saturating concentrations is insignificant in C_4 plants and very modest in C_3 plants. In the latter, it is attributed to suppression of photorespiratory reactions which consume energy and

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ribulose 1,5-bisphosphate, the substrate for CO_2 fixation. Apparently, under these conditions, photosynthesis is mainly limited by the availability of energy. Under illumination with high light intensities, increasing the CO_2 concentration from that encountered in air to saturation increases CO_2 fixation of C_3 species by a factor of 3–4 which is far more than could be accounted for by a suppression of photorespiration. It is commonly thought that under high light and in normal air, photosynthesis is limited by the activity of ribulose-1,5-bisphosphate carboxylase the k_m value of which is reported to be close to the CO_2 concentration in solutions equilibrated with air [1]. Since the CO_2 concentrations in the intercellular space of photosynthesizing leaves are lower than that of the surrounding air, the rate of light-saturated photosynthesis in air is usually considerably less than half saturated in respect to CO_2. Under CO_2 saturation, the rate of photosynthesis is generally believed to be limited by the capacity of the electron-transport chain to deliver reducing power to the energy-requiring reactions of the carbon-reduction cycle. At intermediate light intensities and in air, photosynthesis can be increased both by light and by CO_2. In this range, CO_2 and light obviously exercise fractional control on the rate of photosynthesis, and consequently, photosynthesis responds to both factors. We wished to know whether there are rate limitations in the biochemical machinery of photosynthesis in leaves of C_3 plants different from those imposed by the activity of ribulose-1,5-bisphosphate carboxylase or by the capacity of the electron-transport chain. In isolated chloroplasts, it is easy to establish conditions where photosynthesis is under pH control [2–4]. Several enzymes of the carbon reduction cycle are activated by light [5]. The extent of light activation has been shown to limit photosynthesis of isolated chloroplasts [6,7]. In particular, the activities of fructose-1,6-bisphosphatase, seduheptulose-1,7-bisphosphatase, phosphoribulokinase and NADP-dependent glyceraldehyde-phosphate dehydrogenase are modulated by light. It was of interest to know whether the reactions catalyzed by these enzymes also need to be considered as possible limiting factors in leaf photosynthesis and whether there exists pH control of photosynthesis in vivo beyond that described for light-dark transitions.

Materials and Methods

Spinach was grown in a greenhouse with additional light in an 10 h light/14 h dark period. Leaves were harvested after more than 6 h illumination. For measurements of photosynthesis in the induction phase, leaves were kept in the dark for at least 60 min. Photosynthesis was in the steady state after less than 20 min illumination at different light intensities or CO_2 concentrations. If not indicated otherwise, incident white light was 200 Wm^{-2}. The leaves were gassed in a cuvette with plexiglas cover which was cooled by water of 17°C. Gases were mixed by a Wösthoff pump, filled in polyethylene bags which then were used as gas source. Light intensity dependence and induction of photosynthesis were measured at 21°C. While still being exposed to dark or light, the leaves were plunged into liquid nitrogen to arrest metabolism. They were subsequently freeze-dried for 3 days at −30°C and further 4 days at −25 to −20°C. After homogenization of the leaves, chloroplasts were separated from a residual fraction by gradient and sedimentation centrifugations in CCl_4/petrol ether mixtures [8]. Gradients were established by first layering a CCl_4/petrol ether mixture (40 : 60, v/v) containing homogenized leaf material on top of a heavier mixture (between 74 : 76 and 78 : 22, v/v) and then swirling the two layers carefully within a limited range to produce a gradient. After separating material of different density by centrifugation at $2500 \times g$ for about 10 min, a contaminated chloroplast fraction was removed by suction. If required, this procedure was repeated. The chloroplast fraction was then sedimented in a light CCl_4/petrol ether mixture. Contamination by particles smaller than chloroplasts was removed by repeated short-time centrifugations (25–40 s at $1500–2000 \times g$) in CCl_4/petrol ether (40 : 60, v/v) which left contaminations of small particle sizes in the supernatant. Particles larger than chloroplasts were sedimented by very short centrifugations (5–10 s at about $600 \times g$) which left the chloroplasts in the supernatant.

Chlorophyll [9], the activity of pyruvate kinase [10] and the distribution of ribulose 1,5-bisphosphate and uridine diphosphoglucose [11] were taken to estimate the contamination of the chloroplast fraction by cytoplasm. Ribulose 1,5-bis-

phosphate and 3-phosphoglycerate [12], fructose 1,6-bisphosphate and dihydroxyacetone phosphate [13], fructose 6-phosphate and glucose 6-phosphate [14], ATP and ADP [15], phosphate [16] and $NADP^+$ [17] were determined after 30 min extraction of the chloroplasts in 6% $HClO_4$. NADPH was measured as the difference of total NADPH + $NADP^+$ (after oxidation of NADPH by methylphenazonium methosulphate) and $NADP^+$. After centrifugation, aliquots of the supernatant were neutralized with 0.35 $mol \cdot l^{-1}$ triethanolamine/5 $mol \cdot l^{-1}$ K_2CO_3. Measurements were performed in duplicate with an Aminco DW2 spectrophotometer in the dual wavelength mode at 340 and (reference beam) 400 nm. The assay temperature was 30°C.

The vacuolar contamination of the chloroplasts was determined by measuring α-D-mannosidase activity as described by Boller and Kende [18] who ascertained that α-D-mannosidase is exclusively located in the vacuole. Also, the anthocyanin content was measured in nonaqueous fractions of red beet (*Beta vulgaris*) leaves by measuring absorption in 6% $HClO_4$ at 540 nm. Both methods produced comparable results.

CO_2 gas exchange and scattering of a weak green measuring beam by leaves were measured as described earlier [19,20].

Enzymes were obtained from Sigma (uridine diphosphoglucose dehydrogenase) and Boehringer. Ribulose 1,5-bisphosphate carboxylase was prepared from a spinach leaf homogenate by ammonium sulfate and heat fractionation. The precipitate formed between 17 and 34% $(NH_4)_2SO_4$ saturation was collected and dissolved in buffer at pH 6.7; precipitate formed during 4 min heating to 62°C was removed. The carboxylase was, within limits of detection, free of dehydrogenase activity, and there was no reaction with ribose 5-phosphate when ATP was present.

Results and Discussion

Analysis of the metabolic state of chloroplasts in leaves

A rate limitation imposed at a particular point in a sequence of enzyme-catalyzed reactions is likely to result in the accumulation of a substrate and the depletion of a product. The reaction which limits flux will be displaced more from equilibrium after imposition of the limitation than before. The application of this simple relationship to an analysis of rate limitations in photosynthesis cannot be based on substrate measurements of whole leaves, since relevant metabolites may not only be present in the chloroplasts but also in other cellular compartments. Therefore, we have measured photosynthesis of leaves under different conditions and have rapidly stopped metabolism by freezing the leaves in liquid nitrogen. After freeze-drying the leaves, chloroplasts were isolated nonaqueously, and the chloroplast fraction was assayed for metabolites. The usefulness of the nonaqueous isolation procedure for an analysis of leaf cell compartmentation has been established before [8,21]. The contamination of the nonaqueous chloroplasts with cytosolic material was measured by measuring pyruvate kinase activity as previously described. In agreement with earlier data, about 10% of the total cellular activity of pyruvate kinase was found in the chloroplasts. It should be noted that some pyruvate kinase activity has been reported to be localized in the chloroplasts [22]. The chloroplast fraction should therefore be expected to be contaminated with less than 10% of cytosolic material. However, when uridine diphosphoglucose was measured in the chloroplast fraction of the leaf, about 12% of this metabolite was found in the chloroplast fraction. The contamination of the nonaqueous chloroplast fractions with vacuolar material as measured by the use of α-mannosidase or, in red beet, of antocyanin as vacuolar markers was about 15%. Although it is clear that the chloroplast fractions were not pure, the contaminations were not considered to be intolerably high. Therefore, metabolite levels measured in the chloroplast fraction could remain uncorrected for contaminations.

Ribulose 1,5-bisphosphate carboxylase

Chloroplasts isolated from predarkened leaves (60 min darkness) did not contain measurable levels of ribulose 1,5-bisphosphate. On illumination in air, ribulose 1,5-bisphosphate levels increased approximately in parallel with photosynthesis (Fig. 1). Steady-state ribulose 1,5-bisphosphate pool sizes were somewhat below or close to 200 nmol/mg chlorophyll corresponding

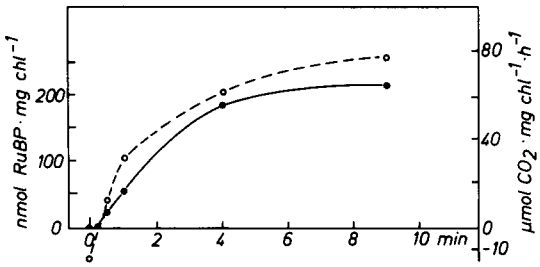

Fig. 1. CO_2 uptake (○) and ribulose 1,5-bisphoshate (RuBP) levels of leaves (●) during induction of photosynthesis. Light intensity, 200 Wm^{-2}; CO_2 concentration, 350 $\mu l \cdot l^{-1}$. Leaves had been kept at least 60 min in the dark before illumination.

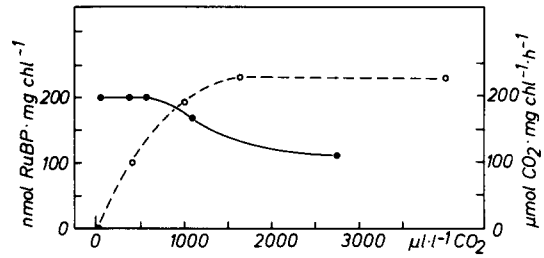

Fig. 3. CO_2 uptake (○) and ribulose 1,5-bisphosphate (RuBP) levels (●) as a function of the external CO_2 concentration. Light intensity, 200 Wm^{-2}; the cuvette was cooled by a water-bath of 17°C.

to 5–7 mmol $\cdot l^{-1}$ ribulose 1,5-bisphosphate in the chloroplast stroma. The calculation of concentrations from pool sizes is based on the assumption that the stroma space of chloroplasts contains 1 mg chlorophyll in 30 µl [23]. In other leaves and in protoplasts, even higher ribulose 1,5-bisphosphate levels have been reported (up to 350 nmol/mg chlorophyll; Refs. 24–26). Steady-state ribulose 1,5-bisphosphate levels were reached after 5–10 min illumination. When photosynthesis was measured in normal air in the steady state as a function of light intensity, the relationship shown in Fig. 2 was obtained. Again the ribulose 1,5-bisphosphate content of the chloroplasts increased with the rate of photosynthesis, but maximum levels were observed before photosynthetic CO_2 uptake attained its maximum rate in air. High ribulose 1,5-bisphosphate levels were also found under high intensity illumination in low CO_2 concentrations (close to the CO_2 compensation point). When the CO_2 content of the gas phase was raised above air levels, ribulose 1,5-bisphosphate levels declined, while photosynthetic CO_2 fixation increased (Fig. 3). Even the lowered ribulose 1,5-bisphosphate concentrations measured during CO_2-saturated photosynthesis amounted to 3 or 4 mmol $\cdot l^{-1}$ in the stroma space which is close to the concentration of the binding sites for ribulose 1,5-bisphosphate of the ribulose 1,5-bisphosphate carboxylase [27].

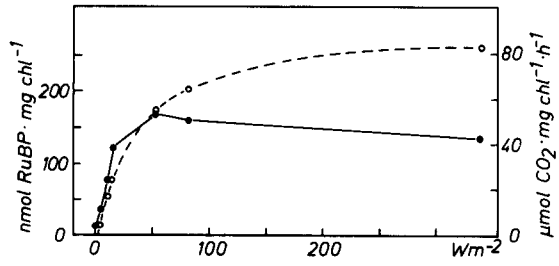

Fig. 2. Light intensity dependence of photosynthetic CO_2 uptake (○) and of ribulose 1,5-bisphosphate (RuBP) levels (●) at 350 $\mu l \cdot l^{-1}$ CO_2 at 21°C. Metabolism of the leaves was stopped after at least 20 min illumination.

In aqueously isolated chloroplasts, usually much lower levels of ribulose 1,5-bisphosphate (0.4 mmol $\cdot l^{-1}$, calculated from Ref. 28) are measured in the presence of high HCO_3^- concentrations, indicating that ribulose 1,5-bisphosphate regeneration limited the rate of photosynthesis in these chloroplast preparations possibly owing to the rapid export of triose-phosphate into the medium. The small discrepancy between increase in CO_2 uptake and increase in ribulose 1,5-bisphosphate pool sizes during induction of photosynthesis after 1 h darkness (Fig. 1) can be attributed to a slow light activation of enzymes involved in ribulose 1,5-bisphosphate regeneration, such as fructose 1,6-bisphosphatase, and to regulation of the ribulose-1,5-bisphosphate carboxylase activity. Sicher [29] has reported an overshooting activation during the first 2 min of illumination and a subsequent decrease in the activation state of the ribulose-1,5-bisphosphate carboxylase to an intermediate level between the dark state and the maximum reached after 2 min illumination. The extent of activation depends on light intensity and has been reported to be maximally 3-fold compared to the dark state [25].

The comparatively small decline of ribulose

1,5-bisphosphate levels seen on increasing CO_2 in the gas streams suggests that even in the presence of high CO_2 concentrations, ribulose-1,5-bisphosphate carboxylase may have remained a limiting factor in photosynthesis. It is significant in this context that maximum rates of photosynthesis observed in the presence of saturating CO_2 under high intensity illumination varied between 200 and 550 μmol CO_2 reduced per mg chlorophyll per h. Very high rates were obtained with field-grown and well-fertilized material in the spring (May) and in the fall (September and October).

3-Phosphoglycerate reduction and condensation of triosephosphates to fructose 1,6-bisphosphate

Reduction of 3-phosphoglycerate (PGA) to dihydroxyacetone phosphate (DHAP) involves three enzymes. Two intermediates, 1,3-diphosphoglycerate and glyceraldehyde 3-phosphate, are very difficult to measure. The overall reaction may be written as:

$$PGA + ATP + NADPH + H^+ \leftrightarrow DHAP + ADP + P_i + NADP^+ \quad (1)$$

Substrates and products of the reaction were measured in the nonaqueous chloroplast preparations obtained from spinach leaves which were illuminated with 200 $W \cdot m^{-2}$ white light in the presence of different CO_2 concentrations. The activities of phosphoglycerate kinase and of triosephosphate isomerase are known to be very high (for reviews see Refs. 27 and 30). There is reason to assume that none of these enzymes limited carbon flux from 3-phosphoglycerate to dihydroxyacetone phosphate. This focuses attention on glyceraldehyde-3-phosphate dehydrogenase, an enzyme known to be subject to light modulation [31]. Fig. 4 shows levels of 3-phosphoglycerate and dihydroxyacetone phosphate, and ATP/ADP ratios during induction of leaf photosynthesis. Illumination produced an increase in the chloroplast ATP/ADP ratio (and, not shown, also in the NADPH/$NADP^+$ ratio) which caused 3-phosphoglycerate reduction and dihydroxyacetone phosphate and fructose 1,6-bisphosphate accumulation (Figs. 4 and 6a). As photosynthesis accelerated, 3-phosphoglycerate became available from the carboxylation of ribulose 1,5-bisphosphate, and the ratio of 3-phosphoglycerate to dihydroxyacetone phosphate increased. While this occurred, the NADPH/$NADP^+$ ratio declined [32]. As has been outlined before [33], there is considerable freedom of adjustment of the individual components in the mass action ratio

$$R = \frac{(PGA)\cdot(ATP)\cdot(NADPH)\cdot(H^+)}{(DHAP)\cdot(ADP)\cdot(P_i)\cdot(NADP)}$$

which explains variable 3-phosphoglycerate to dihydroxyacetone phosphate ratios observed in different experiments. The deviation of R from the equilibrium constant of the reaction governs the flux in the overall reaction. The question may be raised whether mass action arguments are applicable to complex enzyme reactions in a flux situation whose analysis would require application of non-equilibrium thermodynamics. However, if the enzyme involved in the reduction of 3-phosphoglycerate were to become rate-limiting as photosynthesis approaches its maximum, this should be

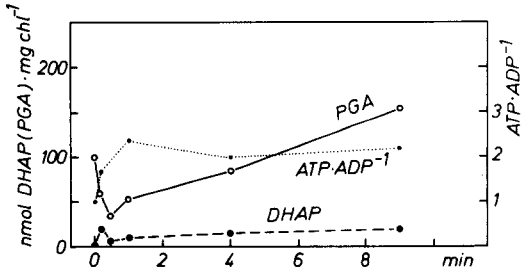

Fig. 4. ATP/ADP ratios (●·····●), dihydroxyacetone phosphate (DHAP) ●-----●) and 3-phosphoglycerate (PGA) (○——○) levels during induction of photosynthesis after at least 60 min dark adaptation. Light intensity = 200 $W \cdot m^{-2}$.

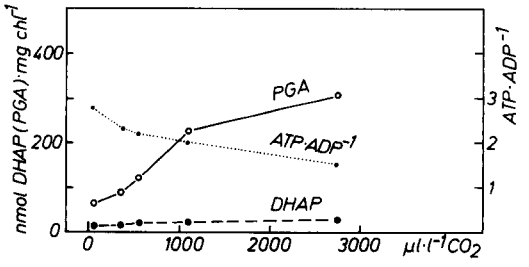

Fig. 5. ATP/ADP ratios (●·····●), dihydroxyacetone phosphate (DHAP) (●-----●) and 3-phosphoglycerate (PGA) (○——○) levels in dependence of the external CO_2 concentration. Light intensity = 200 $W \cdot m^{-2}$.

TABLE I

SUBSTRATE RATIOS AND VALUES OF THE MASS ACTION RATIO *R* OF THE 3-PHOSPHOGLYCERATE REDUCING SYSTEM OF CHLOROPLASTS

Ratios and values were measured under different photosynthetic flux situations caused by different CO_2 concentrations in the gas phase at constant high light intensity (200 $W \cdot m^{-2}$). 3-phosphoglycerate/dihydroxyacetone phosphate (PGA/DHAP) and ATP/ADP ratios were taken from Fig. 5. NADPH/NADP ratios and P_i contents were measured simultaneously. The H^+ content was assumed to be constant under these conditions.

CO_2 concentration ($\mu l \cdot l^{-1}$)	50	350	550	1100	2750
PGA/DHAP	5.3	5.0	6.0	10	11
NADPH/NADP	1.8	1.8	1.3	1.1	1.1
ATP/ADP	2.8	2.3	2.2	2.0	1.5
P_i (μmol/mg Chl)	1.12	0.73	0.74	0.72	0.6
H^+ (nmol/mg Chl ($\times 10^4$))	3	3	3	3	3
$\frac{PGA \cdot ATP \cdot NADPH \cdot H^+}{DHAP \cdot ADP \cdot NADP \cdot P_i} = R$ ($\times 10^6$)	7.2	8.5	7.0	9.2	9.1

reflected in a rise of *R*. Fig. 5 shows 3-phosphoglycerate and dihydroxyacetone phosphate levels and ATP/ADP ratios in chloroplasts as a function of CO_2 concentration. The light intensity was high and near light-saturation of photosynthesis even when CO_2 was high. As photosynthetic CO_2 uptake increased with increasing CO_2, ATP/ADP ratios decreased and 3-phosphoglycerate to dihydroxyacetone phosphate ratios increased. This reflects higher rates of 3-phosphoglycerate formation by ribulose 1,5-bisphosphate carboxylation and higher rates of phosphorylation of 3-phosphoglycerate to 1,3-diphosphoglycerate. Table I shows substrate ratios and P_i levels in chloroplasts under the different photosynthetic flux situations established by high intensity illumination in the presence of different CO_2 concentrations. The stroma pH was assumed to remain constant under these conditions. Evidence that the alkalization of the chloroplast stroma observed during a transition from dark to light is largely saturated at light intensities higher than 10 $W \cdot m^{-2}$ has been reported previously [2]. Although photosynthetic carbon flux at 50 ppm CO_2 (CO_2 compensation concentration) was only a small fraction of the flux at CO_2 saturation, the values of the mass action ratios *R* were comparable at different CO_2 concentrations. This suggests that in leaves the capacity of the enzymic system responsible for 3-phosphoglycerate reduction is very large and capable to cope easily even with unphysiologically high carbon fluxes observed under CO_2 saturation in high light.

However, a considerable problem is the measurement of P_i, because in well-fertilized spinach leaves, the main part of the cellular P_i resides in the large central vacuole of the mesophyll cells. Even a small vacuolar contamination of the chloroplast fraction could lead to a significant overestimation of chloroplast P_i levels. In aqueously isolated chloroplasts, stromal P_i concentrations have been reported to be close to 20 mM in the dark [34]. In the light, and with rate-saturating CO_2 present, they declined to about 6 mM, while phosphate ester concentrations increased. In the nonaqueous chloroplasts, stromal P_i concentrations calculated from measured levels were 37 mM at 50 ppm CO_2 and 20 mM at 2750 ppm CO_2. They are thus considerably higher than the concentrations reported for aqueously isolated chloroplasts. If the latter reflect the in vivo situation better than the measurements of P_i in the nonaqueous chloroplast fractions, values for the mass action ratio *R* would be higher than shown in Table I. In rate-saturating CO_2 (2750 ppm) they would be $60 \cdot 10^{-6}$ instead of $9.1 \cdot 10^{-6}$ as listed in Table I. Moreover, the mass action ratio would increase with increasing photosynthetic flux somewhat more steeply than shown in Table I (by a factor of 3 or 4 from 50 ppm CO_2 to 2750 ppm CO_2). Indeed, in aqueously isolated chloroplasts, *R* increased by a factor of 4 to 5 as photosynthetic fluxes increased during induction of photosynthesis [32]. This corresponds to an increase in the thermodynamic force driving reduction of 3-phosphoglycerate of less than 4 kJ/mol.

TABLE II

MASS ACTION RATIO OF THE 3-PHOSPHOGLYCERATE (PGA) REDUCTION REACTION

Values are based on calculated instead of measured (see Table I) dihydroxyacetone phosphate levels. Dihydroxyacetone phosphate (DHAP) levels were calculated from measured fructose 1,6-bisphosphate levels under the assumption that aldolase and triosephosphate isomerase reactions are in equilibrium.

CO_2 concentration ($\mu l \cdot l^{-1}$)	50	350	550	1100	2750
Measured DHAP (a) ($mmol \cdot l^{-1}$)	0.37	0.48	0.60	0.68	0.86
Calculated DHAP (b) ($mmol \cdot l^{-1}$)	0.58	0.72	0.77	1.06	1.36
a/b	0.64	0.67	0.78	0.64	0.63
Mass action ratio of PGA reduction (cf. Table I) ($\times 10^6$)	4.6	5.7	5.5	5.9	5.8

A comparison of dihydroxyacetone phosphate and fructose 1,6-bisphosphate levels in the non-aqueous chloroplasts revealed an inconsistency. Dihydroxyacetone phosphate is formed in photosynthesis from glyceraldehyde 3-phosphate by the action of triosephosphate isomerase. The activity of the triosephosphate isomerase is known to be so high that it appears to be justified to assume that this reaction is close to equilibrium in the chloroplast stroma. Glyceraldehyde 3-phosphate and dihydroxyacetone phosphate condense to form fructose 1,6-bisphosphate. The difficulty is that measured levels of fructose 1,6-bisphosphate were larger than expected if dihydroxyacetone phosphate, glyceraldehyde 3-phosphate and fructose 1,6-bisphosphate were in equilibrium. Quite obviously, either measured dihydroxyacetone phosphate levels were lower than actual levels, or measured fructose 1,6-bisphosphate levels included considerable proportions of bound fructose 1,6-bisphosphate which is thermodynamically inactive. Ribulose-1,5-bisphosphate carboxylase is known to be capable of binding fructose 1,6-bisphosphate [35,36]. Binding depends strongly on the ribulose 1,5-bisphosphate concentration of the stroma space. Under conditions of high intensity illumination, ribulose 1,5-bisphosphate levels were 3–6 mM, which should saturate most of the ribulose-1,5-bisphosphate carboxylase binding sites. Table II compares measured dihydroxyacetone phosphate levels with values calculated from measured fructose 1,6-bisphosphate levels under the assumption of enzymic equilibrium of the aldolase and the triosephosphate isomerase reactions. Interestingly, the ratios of measured to calculated dihydroxyacetone phosphate values did not differ much under the different photosynthetic flux situations produced by different CO_2 concentrations. They should have increased with increasing CO_2 levels if aldolase had become a limiting factor at very high rates of CO_2 fixation. Obviously, aldolase had as little difficulty in coping with carbon flux as the enzymes involved in reduction of 3-phosphoglycerate.

When mass action ratios R (see Table I) were computed with the calculated dihydroxyacetone phosphate levels (Table II), they differed only numerically from the values shown in Table I. Thus, the conclusion remains valid that the enzymic system reducing 3-phosphoglycerate to dihydroxyacetone phosphate is not a significant rate-limiting factor in photosynthesis.

It should be noted that the mass action ratios of Tables I and II differed surprisingly little from the equilibrium constant of reaction 1 which was calculated from the individual equilibrium constants of the reactions catalyzed by triosephosphate isomerase, glyceralde-phosphate dehydrogenase and phosphoglycerate kinase [10] to be $9.8 \cdot 10^{-6}$. The mass action ratios calculated on the basis of measured dihydroxyacetone phosphate levels were particularly close to the equilibrium constant.

Fructose-bisphosphatase

The chloroplast stroma is more alkaline in the light than in the dark, and a high stroma pH is maintained in the light by pumping protons into the intrathylakoid and the extrachloroplast spaces. Proton fluxes across the chloroplast envelope are

small compared with fluxes across the thylakoid membrane. Chloroplast fructose bisphosphatase is inactive in the dark. Its activation in the light requires a reduced thioredoxin system, substrates and an alkaline stroma pH [37]. Even the light-activated enzyme is strongly pH dependent. In aqueously isolated chloroplasts, the CO_2 dependence of photosynthesis exhibits an optimum. High CO_2 concentrations inhibit photosynthetic O_2 evolution [38,3]. Inhibition has been shown to be caused by a decrease in the activities of fructose-bisphosphatase and sedoheptulose-bisphosphatase which led to an increase in fructose 1,6-bisphosphate and sedoheptulose 1,7-bisphosphate and a decrease in sugar monophosphates [39]. We wished to know whether fructose-bisphosphatase and sedoheptulose-bisphosphatase can also assume a rate-limiting function in leaf photosynthesis. Fig. 6 shows levels of fructose 1,6-bisphosphate and fructose 6-phosphate (Fig. 6a) and ratios of fructose 1,6-bisphosphate to fructose 6-phosphate (Fig. 6b) during induction of leaf photosynthesis in air at a light intensity of 200 $W \cdot m^{-2}$. There was a rapid increase in the fructose 1,6-bisphosphate/fructose 6-phosphate ratio, followed by a decrease as the rate of photosynthetic CO_2 uptake became stationary. In the steady state, the ratio was strongly dependent on light intensity (Fig. 7). There was a maximum close to 10 $W \cdot m^{-2}$. In vitro, the alkalization of the chloroplast stroma is known to increase steeply between darkness and 10 $W \cdot m^{-2}$. At higher light intensities, it approaches saturation [2,40]. At light intensities above 10 $W \cdot m^{-2}$, the fructose 1,6-bisphosphate/fructose 6-phosphate ratio decreased although the rate of photosynthesis increased. If there is a rate limitation on photosynthesis imposed by fructose-bisphosphatase, the data of Fig. 7 suggest that it should be at low rather than at high light intensities. However, it is highly questionable whether the high ratios of fructose 1,6-bisphosphate to fructose 6-phosphate measured transiently during induction or permanently at very low light intensities really reflect corresponding ratios of the thermodynamically active species. Between darkness and 15 $W \cdot m^{-2}$, ribulose 1,5-bisphosphate levels rise from low (0–0.3 mM) to relative high values (greater than 4.0 mM). The binding site concentration of ribulose-1,5-bisphosphate carboxylase in the stroma space is high. Ribulose-1,5-bisphosphate carboxylase can effectively bind not only ribulose 1,5-bisphosphate but also substrates such as fructose 1,6-bisphosphate, NADPH and 3-phosphoglycerate. Since the K_d value of ribulose-1,5-bisphosphate carboxylase for ribulose 1,5-bisphosphate is very low (0.6 $\mu mol \cdot l^{-1}$), whereas the K_d values for fructose 1,6-bisphosphate (40 $\mu mol \cdot l^{-1}$), NADPH (70 $\mu mol \cdot l^{-1}$) and 3-phosphoglycerate (840 $\mu mol \cdot l^{-1}$) are about one order of magnitude higher, ribulose 1,5-bisphosphate displaces effectively other metabolites from the binding sites of the carboxylase. During the first 2 min of the induction phase of photosynthesis (Fig. 1) and at very low light intensities

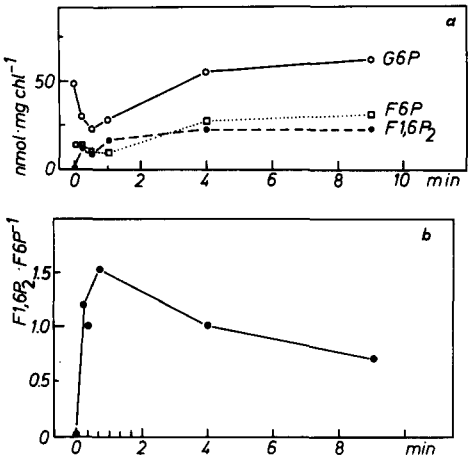

Fig. 6. Levels of glucose 6-phosphate (G6P) (○——○), fructose 6-phosphate (F6P) (□·····□) and fructose 1,6-bisphosphate ($F1,6P_2$) (●- - - - -●) during induction of photosynthesis (a) and corresponding fructose 1,6-bisphosphate/fructose 6-phosphate ratio (b). Light intensity, 200 Wm^{-2}; CO_2 concentration, 350 $\mu l \cdot l^{-1}$.

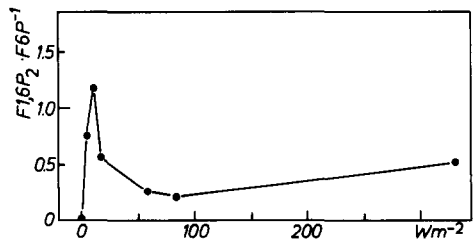

Fig. 7. Fructose 1,6-bisphosphate/fructose 6-phoshate ($F1,6P_2 \cdot F6P^{-1}$) ratios in spinach chloroplasts as a function of the light intensity. CO_2 concentration = 350 $\mu l \cdot l^{-1}$.

TABLE III

METABOLITE SEQUESTRATION BY RIBULOSE-1,5-BISPHOSPHATE CARBOXYLASE DURING STEADY-STATE PHOTOSYNTHESIS UNDER VARIOUS LIGHT REGIMES

Calculations are based on measured fructose 1,6-bisphosphate (FBP) and ribulose 1,5-bisphosphate (RuBP) levels and on the assumption that the concentration of active binding sites of ribulose-1,5-bisphosphate carboxylase is 3.5 mmol·l^{-1}. K_d values were taken from Ref. 39.

Light intensity (Wm^{-2})	5	10	15	55	80	320
RuBP concentration (mmol·l^{-1})	1.2	2.7	4.1	5.6	5.3	4.6
FBP concentration (mmol·l^{-1})	0.54	1.5	1.45	0.9	0.5	0.7
FBP-bound (mmol·l^{-1})	0.53	0.78	0.002	0.001	0.001	0.002
%FBP-free	2.2	48.4	99.9	99.9	99.8	99.7
%RuBP-free	0.02	0.07	14.7	37.5	34.0	24.0

(Fig. 2), ribulose 1,5-bisphosphate levels are below the binding site concentration of the carboxylase. Under these situations, binding of other substrates is likely to occur. Table III shows some calculations for binding of ribulose 1,5-bisphosphate and fructose 1,6-bisphosphate to ribulose-1,5-bisphosphate carboxylase under different photosynthetic flux situations. Binding of other substrates is neglected. It is evident that at very low light intensities where fructose 1,6-bisphosphate/fructose 6-phosphate ratios may be taken to suggest incomplete activation of fructose bisphosphatase (FIg. 7), a considerable part of the measured fructose 1,6-bisphosphate levels is bound to carboxylase [36,41]. Fig. 9 shows measurements of quantum requirements of CO_2 uptake by spinach leaves at 4, 8 and 12 W · m^{-2} red light in air or 2% oxygen. Under the latter conditions, photorespiratory energy consumption and competition between carboxylation and oxygenation of ribulose 1,5-bisphosphate are minimized. Even at very low light, quantum requirements were not independent of light intensities. Extrapolation of obtained data to zero light intensity yielded minimum quantum requirements usually close to 8–10 quanta/CO_2 absorbed. According to present views on the cooperation of two photosystems in photosynthesis, minimum quantum requirements should be 8, if linear electron transport were capable of providing 1.5 ATP molecules per 2 electrons, or 8–10, if a contribution of cyclic electron transport to ATP synthesis is necessary. The low quantum requirement of CO_2 reduction observed at light intensities, where fructose 1,6-bisphosphate/fructose 6-phosphate ratios were high, does not support the view, that fructose-bisphosphatase causes a substantial limitation of photosynthesis at low light intensities. Under high intensity illumination, the data of Fig. 7 do not reveal a rate-limiting func-

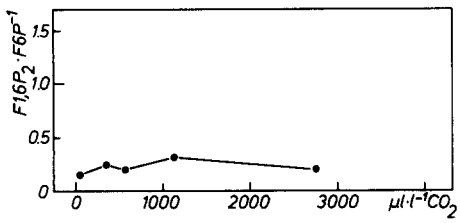

Fig. 8. Fructose 1,6-bisphosphate/fructose 6-phosphate ($F1,6P_2 \cdot F6P^{-1}$) ratios in dependence of the external CO_2 concentration. Light intensity = 200 W·m^{-2}.

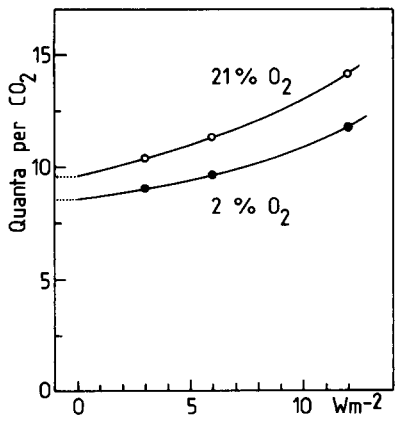

Fig. 9. Quanta of 671 nm light absorbed per molecule CO_2 removed from the gas stream after passage over a small spinach leaf (area 7 cm^2) in dependence of the light intensity and O_2 content of the air; extrapolation of the graphs to zero light intensity gave values of 9.6 quanta/CO_2 in 21% O_2 and of 8.5 quanta/CO in 2% O_2.

tion of fructose-bisphosphatase. Interestingly, this is even true when photosynthetic fluxes are very high in the presence of saturating concentrations of CO_2 which should be expected to decrease the stroma pH according to:

$$CO_2 + H_2O \leftrightarrow HCO_3^- + H^+ \quad (2)$$

Fig. 8 and Table IV show that the ratio of fructose 1,6-bisphosphate to fructose 6-phosphate remained significantly below 1 at very different photosynthetic fluxes. Even 1.6% CO_2 in the gas phase did not increase the chloroplast fructose 1,6-bisphosphate/fructose 6-phosphate ratio, but in 80% CO_2, the ratio increased to values higher than 4.0, indicating pH inhibition of photosynthesis (Table IV).

For a proper interpretation of the data, it should be noted that stomatal closure of leaves under high CO_2 concentrations produces differences between CO_2 in the internal and the external gas phase. In protoplasts, CO_2 inhibition of photosynthetic CO_2 uptake becomes significant at a CO_2 concentration equivalent to 2.5% [38] in air.

The data show that under high intensity illumination, fructose-bisphosphatase does not limit photosynthesis even in the presence of rate-saturating CO_2 levels. Obviously, the buffering capacity of the stroma is sufficient to tolerate the acidification caused by CO_2 concentrations which are much higher than those in air.

This demonstrates also that pH is not involved in the regulation of photosynthesis in leaves after illumination has increased the stroma pH and established transthylakoid and transenvelope proton gradients. In the transition from darkness to light, however, pH changes are important in conjunction with the reduction state of the thioredoxin system to activate chloroplast enzymes such as fructose-bisphosphatase [42,43,37].

TABLE IV

DEPENDENCY OF FRUCTOSE 1,6-BISPHOSPHATE (FBP) AND FRUCTOSE 6-PHOSPHATE (F6P) LEVELS AND OF THE FRUCTOSE 1,6-BISPHOSPHATE/FRUCTOSE 6-PHOSPHATE RATIO ON THE CO_2 CONCENTRATION OF THE GAS PHASE AT DIFFERENT LIGHT INTENSITIES

Light intensity ($W \cdot m^{-2}$)	CO_2 concentration	nmol/mg Chl		FBP/F6P
		FBP	F6P	
10	350 $\mu l \cdot l^{-1}$	9	12	0.8
	1.6%	13.5	18	0.8
	80%	13	3	4.3
30	350 $\mu l \cdot l^{-1}$	15	39	0.4
	1.6%	26.5	43	0.6
	80%	23	5.5	4.2
100	1.6%	22	61	0.4
	80%	25	5.5	4.5

Regeneration of ribulose 1,5-bisphosphate from triosephosphate and hexosephosphate

Ribulose 1,5-bisphosphate is regenerated in the carbon reduction cycle from fructose 6-phosphate and triosephosphate. Regeneration is complex and involves six enzymes. Two of them, sedoheptulose-bisphosphatase and phosphoribulokinase are known to be regulated by light. Several intermediates of the overall reaction are difficult to measure. Also, dephosphorylation of sedoheptulose is an irreversible step in the path to ribulose 1,5-bisphosphate. Analysis of a similar reaction, dephosphorylation of fructose 1,6-bisphosphate which is catalyzed by fructose-bisphosphatase, has shown that the ratio of fructose 1,6-bisphosphate to fructose 6-phosphate was constant over a wide range of CO_2 concentrations. Therefore, a similar assumption was made for sedoheptulose-bisphosphatase. With this assumption, it appeared to be permissible to approach the question of a possible rate limitation in ribulose 1,5-bisphosphate regeneration in a manner very similar to that used for analyzing the state of the chloroplast triosephosphate oxidation system. Regeneration of ribulose 1,5-bisphosphate may in a simplified form be written as:

$$F6P + 2\,GAP + DHAP + 3\,ATP \leftrightarrow 3\,RuBP + 3\,ADP + P_i \quad (3)$$

(for abbreviations see Table V) For the triosephosphate isomerase reaction, a state close to equilibrium is assumed. The concentration of other substrates has been measured. Table V lists concentrations of component metabolites and ATP/ADP ratios at different rates of photosynthesis brought about by different levels of CO_2. Calcula-

TABLE V

CALCULATION OF THE MASS ACTION RATIO OF THE REACTION SEQUENCE FROM FRUCTOSE 6-PHOSPHATE (F6P) AND TRISEPHOSPHATES TO RIBULOSE 1,5-BISPHOSPHATE (RuBP)

DHAP dihydroxyacetone phosphate; GAP, glyceraldehyde 3-phoshate.

CO_2 ($\mu l \cdot l^{-1}$)	50	350	550	1100	2750
DHAP ($mmol \cdot l^{-1}$)	0.37	0.48	0.60	0.68	0.86
GAP ($mmol \cdot l^{-1}$)	0.017	0.022	0.027	0.031	0.039
ATP/ADP	2.8	2.25	2.15	2.0	1.5
P_i ($mmol \cdot l^{-1}$)	35.4	23.1	22.5	22.5	19.2
F6P ($mmol \cdot l^{-1}$)	1.35	0.96	1.29	1.77	2.67
RuBP ($mmol \cdot l^{-1}$)	6.0	6.0	6.0	5.1	3.5
$\frac{DHAP \cdot GAP^2 \cdot F6P \cdot ATP^3}{P_i \cdot RuBP^3 \cdot ADP^3}$	$4.1 \cdot 10^{-7}$	$5.0 \cdot 10^{-7}$	$12.0 \cdot 10^{-7}$	$31.0 \cdot 10^{-7}$	$145.0 \cdot 10^{-7}$

tion of the mass action ratio

$$R = \left((F6P)(GAP)^2(DHAP)(ATP)^3\right)/\left((RuBP)^3(ADP)^3(P_i)\right) \quad (4)$$

shows, in contrast to the situation described for 3-phosphoglycerate reduction, a large increase of the mass action ratio as flux in photosynthesis increased with CO_2 concentration. This increase is even steeper than shown in Table V if a possible vacuolar contamination with phosphate of the nonaqueous chloroplast fraction is considered as outlined above for the enzymic system reducing 3-phosphoglycerate to dihydroxyacetone phosphate. Obviously, the free energy gradient necessary to drive regeneration of ribulose 1,5-bisphosphate increased far more than the free energy gradient for 3-phosphoglycerate reduction as the rate of photosynthesis increased. This reveals a rate-limiting function of ribulose 1,5-bisphosphate regeneration as photosynthesis approaches its limit.

Conclusion

The present analysis demonstrates that in spinach leaves the capacity of the 3-phosphoglycerate reducing system is so high that even very high rates of photosynthesis do not cause a large displacement of the component metabolites from the equilibrium state. A similar situation exists for chloroplast aldolase. Although, at first sight, high fructose 1,6-bisphosphate/fructose 6-phosphate ratios may suggest a rate-limiting function of fructose-bisphosphatase under some photosynthetic flux situations, close analysis reveals that, when this occurs, binding of fructose 1,6-bisphosphate to ribulose-1,5-bisphosphate carboxylase is responsible for high measured fructose 1,6-bisphosphate concentrations which increase calculated ratios. Thus, fructose-bisphosphatase does not limit photosynthesis within a rather wide range of CO_2 concentrations. Ribulose-1,5-bisphosphatase carboxylase, however, drastically limits photosynthesis in air when light intensities are high. The comparatively high level of ribulose 1,5-bisphosphate observed at CO_2 saturation of photosynthesis suggests that even under these conditions the enzyme may play a role in limiting flux in photosynthesis. Furthermore, mass action ratios are strongly suggestive of a further rate limitation in ribulose 1,5-bisphosphate regeneration, when rates of photosynthesis become very high.

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References

1 Yeoh, H.-H., Badger, M.R. and Watson, L. (1980) Plant Physiol. 66, 1110–1112
2 Enser, U. and Heber, U. (1980) Biochim. Biophys. Acta 592, 577–591
3 Werdan, K., Heldt, H.W. and Milovancev, M. (1975) Biochim. Biophys. Acta 396, 276–292

4 Flügge, U.I., Freisl, M. and Heldt, H.W. (1980) Planta 149, 48–51
5 Buchanan, B.B. (1974) Annu. Rev. Plant Physiol. 31, 341–374
6 Leegood, R.C. and Walker, D.A. (1982) Planta 156, 449–456
7 Heldt, H.W., Laing, W., Lorimer, G.H., Stitt, M. and Wirtz, W. (1981) in Photosynthesis (Akoyunoglou, G., ed.), Vol. 4, pp. 213–226, Balaban International Science Services, Philadelphia
8 Heber, U. and Willenbrink, J. (1964) Biochim. Biophys. Acta 82, 313–324
9 Arnon, D.J. (1949) Plant Physiol. 24, 1–15
10 Bergmeyer, H.U., Gawehn, K. and Grassl. M. (1974) in Methods of Enzymatic Analysis (Bergmeyer, H.U., ed.), Vol. 1, pp. 425–522, Academic Press, New York
11 Zalitis, J., Uram, M., Bowser, A. and Feingold, D.S. (1972) Methods Enzymol. 28, 430–435
12 Racker, E. (1974) in Methods of Enzymatic Analysis (Bergmeyer, H.U., ed.), Vol. 3, pp. 1362–1364, Academic Press, New York
13 Michal, G. and Beutler, H.-O. (1974) in Methods of Enzymatic Analysis (Bergmeyer, H.U., ed.), Vol. 3, pp. 1314–1319, Academic Press, New York
14 Lang, G. and Michal, G. (1974) in Methods of Enzymatic Analysis (Bergmeyer, H.U., ed.), Vol. 3, pp. 1238–1242, Academic Press, New York
15 Strehler, B.L. (1974) in Methods of Enzymatic Analysis (Bergmeyer, H.U., ed.), Vol. 4, pp. 2112–2126, Academic Press, New York
16 Ames, B.N. (1966) Methods Enzymol. 8, 115–116
17 Matsumura, H. and Miyachi, S. (!980) Methods Enzymol. 69, 465–470
18 Boller, T. and Kende, H. (1979) Plant Physiol. 63, 1123–1132
19 Heber, U. (1969) Biochim. Biophys. Acta 180, 302–319
20 Dietz, K.-J. and Heber, U. (1983) Planta 158, 349–356
21 Heber, U. (1974) Annu. Rev. Plant Physiol. 25, 393–421
22 Scheibe, R. and Beck, E. (1975) Planta 125, 63–67
23 Heldt, H.W. and Sauer, F. (1971) Biochim. Biophys. Acta 234, 83–91
24 Wirtz, W., Stitt, M. and Heldt, H.W. (1980) Plant Physiol. 66, 187–193
25 Perchorowicz, J.T. and Jensen, R.G. (1983) Plant Physiol. 71, 955–960
26 Perchorowicz, J.T., Raynes, D.A. and Jensen, R.G. (1981) Proc. Natl. Acad. Sci. USA 78, 2985–2989
27 Robinson, S.P. and Walker, D.A. (1981) in Biochemistry of Plants (Davies, D.D., ed.), Vol. 8, pp. 193–235, Academic Press, New York
28 Kirk, M.R. and Heber, U. (1976) Planta 132, 131–141
29 Sicher, R.C. (1982) Plant Physiol. 70, 366–369
30 Robinson, S.P. and Walker, D.A. (1979) Biochim. Biophys. Acta 545, 528–536
31 Müller, B. (1970) Biochim. Biophys. Acta 205, 102–109
32 Takahama, U., Shimizu-Takahama, M. and Heber, U. (1981) Biochim. Biophys. Acta 637, 530–539
33 Kobayashi, Y., Köster, S. and Heber, U. (1982) Biochim. Biophys. Acta 682, 44–54
34 Lilly, R. McC., Chon, C.J., Mosbach, A. and Heldt, H.W. (1977) Biochim. Biophys. Acta 460, 259–272
35 Ottaway, J.H. (1979) Biochem Soc. Trans. 7, 1161–1167
36 Ashton, A.R. (1982) FEBS Lett. 145, 1–7
37 Leegood, R.C., Kobayashi, Y., Neimanis, S., Walker, D.A. and Heber, U. (1982) Biochim. Biophys. Acta 682, 168–178
38 Kaiser, G. and Heber, U. (1983) Planta 157, 462–470
39 Purczeld, P., Chon, C.J., Portis, A.R., Heldt, H.W. and Heber, U. (1978) Biochem. Biophys. Acta 501, 488–498
40 Heldt, H.W., Werdan, K., Milovancev, M. and Geller, G. (1973) Biochim. Biophys. Acta 314, 224–241
41 Badger, M.R. and Lorimer, G.H. (1981) Biochemistry 20, 2219–2225
42 Kelly, G.J., Zimmermann, G. and Latzko, E. (1976) Biochem. Biophys. Res. Commun. 70, 193–199
43 Laing, W.A., Stitt, M. and Heldt, H.W. (1981) Biochim. Biophys. Acta 637, 348–359