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Photosynthesis and Ribulose 1,5-Bisphosphate Levels in Intact Chloroplasts[1]

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ABSTRACT

The response of ribulose 1,5-bisphosphate levels and CO_2 fixation rates in isolated, intact spinach chloroplasts to pyrophosphate, triose phosphates, DL-glyceraldehyde, O_2, catalase, and irradiance during photosynthesis has been studied. Within 1 minute in the light, a rapid accumulation of ribulose bisphosphate was measured in most preparations of intact chloroplasts, and this subsequently dropped as CO_2 fixation increased. Pyrophosphate, triose phosphates, and catalase increased CO_2 fixation and also the levels of ribulose bisphosphate. CO_2 fixation was inhibited by DL-glyceraldehyde and O_2 with corresponding decreases in ribulose bisphosphate. When the rate of photosynthesis decreased at limiting irradiances (low light), the level of ribulose bisphosphate in the chloroplast did not always decrease, suggesting that ribulose bisphosphate was not limiting CO_2 fixation under these conditions. When triose phosphates (fructose bisphosphate plus aldolase) were added to suspensions of chloroplasts at low irradiances, ribulose bisphosphate increased while CO_2 fixation decreased. These observations provide considerable evidence that high ribulose bisphosphate levels clearly are not solely sufficient to permit rapid rates of CO_2 fixation, but that factors other than ribulose bisphosphate concentration are overriding the control of photosynthesis.

Isolated chloroplasts are capable of using carbon reserves to produce considerable ribulose bisphosphate. Upon illumination in the absence of CO_2 and O_2, intact chloroplasts produced up to 13 millimolar ribulose bisphosphate.

RuBP[2] carboxylase-oxygenase occupies a central role in the biochemical regulation of photosynthetic CO_2 fixation in green plants (5). If the level of the substrate for carboxylation, RuBP, does not saturate CO_2 fixation, then the processes involved in its synthesis, such as electron transport, photophosphorylation, or the activity of enzymes of the reductive pentose-P pathway, are limiting for photosynthesis. Contrarily, if RuBP levels are saturating, then CO_2 fixation will be limited either by CO_2 availability or by the activity of RuBP carboxylase. Measurements of the RuBP level can be of value in determining where photosynthesis is rate-limited.

CO_2 acts as a positive effector of the RuBP carboxylase. It both activates and serves as a substrate for the enzyme in the chloroplast (1, 12). O_2 competes with CO_2 as a substrate for the RuBP carboxylase. The inhibition of photosynthesis by atmospheric O_2 in isolated chloroplasts has been attributed to a depletion of RuBP due to O_2-stimulated P-glycolate formation (15). A second explanation has been that O_2 competitively inhibits RuBP carboxylase with respect to CO_2 (7).

Here, we examine the effects of PPi, O_2, DL-glyceraldehyde, sugar phosphates, and irradiance on the level of RuBP and the rate of CO_2 fixation in isolated spinach chloroplasts. Experimental conditions where RuBP appeared either limiting or saturating for photosynthetic CO_2 fixation have been observed.

MATERIALS AND METHODS

Chloroplasts were isolated from 6-week-old spinach plants (*Spinacia oleracea* var. *Viroflay*) by previously described procedures (1, 13). In these preparations, 70 to 95% of the chloroplasts were intact as measured by ferricyanide-dependent O_2 evolution (17). Chloroplast RuBP was measured as described by Sicher *et al.* (24).

CO_2 fixation with isolated chloroplasts was performed in solution C (13) containing 0.33 M sorbitol, 0.05 M Hepes-NaOH (pH 7.8), 2.0 mM EDTA, 1.0 mM $MgCl_2$, 1.0 mM $MnCl_2$, 0.5 mM K_2HPO_4, 10 mM $NaHCO_3$ (0.5 μCi/μmol), and chloroplasts as indicated. The temperature was 25 C and the light intensity was 800 $\mu E/m^2 \cdot s$ as determined with a quantum photometer (Lambda Inst. Corp.). For the determination of $^{14}CO_2$ incorporation, 0.05-ml samples were injected into 8-ml glass scintillation vials containing 0.5 ml of 1.0 N HCl. The samples were then dried on a heating block at 90 C to remove unfixed $^{14}CO_2$ and counted by liquid scintillation.

RESULTS

Chloroplast RuBP and CO_2 Fixation. Metabolic studies with both algae (20) and intact isolated chloroplasts (14) indicated that RuBP carboxylase is active in the light and inactive in the dark. Chloroplasts in the light accumulate RuBP, which, in the 1st min of darkness, decreases to a constant but significant level as carboxylation is inactivated (4, 16).

Present studies confirmed these observations (Fig. 1). In the light there was a brief (15–30 s) initial lag followed by a period of linear CO_2 fixation. During this time the chloroplast RuBP pool increased. When the light was turned off, CO_2 fixation ceased and the RuBP level decreased. During the 1st min of a second light period, CO_2 fixation resumed and the RuBP level rose from below 10 to over 130 nmol/mg Chl. Before the dark period, the rate of CO_2 fixation was 94 μmol/mg Chl·h. This compares to a maximum rate of 64 μmol/mg Chl·h during the second light period, suggesting that RuBP availability was not limiting the rate of CO_2 fixation during that time.

Effects of PPi on Chloroplast RuBP levels and CO_2 Fixation. The effect of PPi on the rate of CO_2 fixation by isolated spinach chloroplasts is shown in Figure 2. During the 1st min of illumination there was an increase in chloroplast RuBP, which, in the absence of PPi, subsequently dropped to a steady-state level of 8 to 10 nmol/mg Chl. Addition of 1.25 mM PPi stimulated the rate of CO_2 fixation up to 10 min, but between 10 and 20 min both

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[2] Abbreviations: RuBP: ribulose 1,5-bisphosphate; FBP: fructose 1,6-bisphosphate; Ri5P: ribose 5-phosphate.

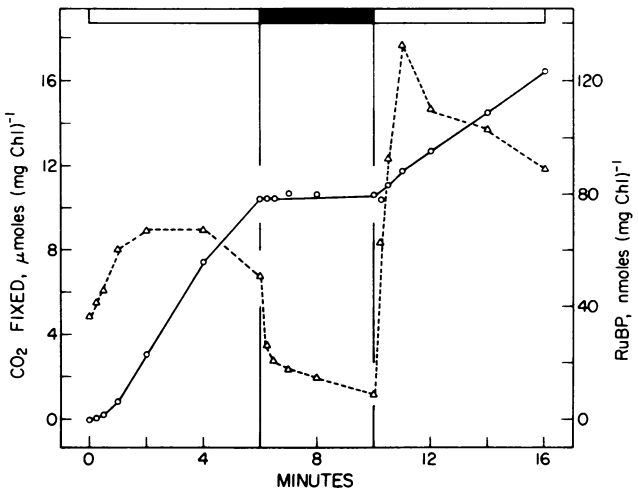

FIG. 1. Spinach chloroplast CO_2 fixation rates and levels of RuBP in light and dark. CO_2 fixation (○) was determined in solution C (13) plus PPi (1.7 mM), catalase (1,110 IU/ml), $NaH^{14}CO_3$ (10.4 mM, 0.5 μCi/μmol) and chloroplasts (0.095 mg Chl/ml). Samples for RuBP (△) were taken from a similar reaction mixture containing $NaH^{12}CO_3$ and diluted 10-fold into 25 mM Hepes-NaOH (pH 8.0), 20 mM $MgCl_2$, 0.6 mM 2,6-dichlorophenol-indophenol, 4.0 μM carbonyl cyanide 3-chlorophenylhydrazone, and 10 mM $NaH^{14}CO_3$ (5.0 μCi/μmol) (24).

rates of CO_2 fixation appeared about equal. Between 4 and 20 min, chloroplasts with PPi accumulated RuBP. This build-up occurred even as the rate of CO_2 fixation declined, suggesting that RuBP was not limiting CO_2 fixation during this period. By 30 min CO_2 fixation apparently failed to sustain the continual accumulation of RuBP.

Current evidence suggests that PPi interferes with the action of the phosphate translocator (2, 26). By preventing the export of sugar phosphates, PPi causes intermediates of the reductive pentose-P pathway to accumulate in the chloroplast, which could result in enhanced rates of CO_2 fixation (2, 18). In Figure 2 this may have been the case between 1 and 10 min.

In the light, isolated intact chloroplasts can accumulate large amounts of RuBP, especially in the absence of CO_2 and O_2. With 2 mM PPi, chloroplasts illuminated for 40 min in a N_2 atmosphere have been observed to accumulate over 320 nmol RuBP/mg Chl. The same chloroplasts without PPi generated over 100 nmol RuBP/mg Chl. The internal stroma volume of these chloroplasts was 25 μl/mg Chl, so that RuBP inside the chloroplast was 13 and 4 mM, respectively. The effect of PPi was not duplicated with the same amount of Pi. Since CO_2 was absent and there is not sufficient carbon in the total intermediates of the pathway (5), the carbon required for 13 mM RuBP probably came from starch breakdown.

Effects of O_2 and Catalase on Chloroplast RuBP and CO_2 Fixation. As previously observed (10), the uptake of CO_2 by isolated spinach chloroplasts was stimulated by the addition of catalase to the medium. In our hands the level of chloroplast RuBP was generally more than doubled when catalase (>1000 IU/ml) was included during photosynthesis, and this corresponded to a 2- to 4-fold increase in the rate of CO_2 fixation (data not shown). Isolated chloroplasts can reduce O_2 to the superoxide radical during photosynthesis and this radical is dismutated to H_2O_2 by superoxide dismutase, a chloroplast enzyme (9). The inhibition of photosynthesis by H_2O_2 could be caused by the oxidation of sulfydryl groups of chloroplast enzymes such as NADP-glyceraldehyde 3-P dehydrogenase (9) and FBP phosphatase (23) by H_2O_2.

The inhibiting effects of O_2 on CO_2 fixation have been extensively investigated (7, 15, 19, 22). This inhibition occurs by O_2 lowering chloroplast RuBP levels as well as by competing with CO_2 as a substrate for the RuBP carboxylase-oxygenase (15). With 70 to 80 μM CO_2, 100% O_2 inhibited chloroplast CO_2 fixation more than 70% compared to fixation at air levels of O_2 (Table IA). Added catalase and triose-P (FBP plus aldolase) stimulated the rate of CO_2 fixation 4- to 6-fold (Table IB). The photosynthetic rate with 100% O_2 was still half that with 21% O_2. RuBP amounts were increased by the presence of catalase and triose-P; however, the level was less with 100% O_2. This stimulation of photosynthesis suggests that the RuBP levels produced in chloroplasts without catalase and triose-P were limiting. Our experience suggests that levels of chloroplast RuBP greater than 15 to 20 nmol/mg Chl are most likely saturating for CO_2 fixation. Thus, the difference in CO_2 fixation between 21 and 100% O_2 in Table IB was probably due to the direct competitive inhibition of RuBP carboxylase-oxygenase by O_2 (7).

These observations are consistent with the findings of Gibbs and co-workers (3, 22) who demonstrated that catalytic amounts of FBP and Ri5P reduced O_2 inhibition of CO_2 fixation. Recently, Collatz (8), using *Chlamydomonas reinhardtii* and separated spinach leaf cells, reported that upon lowering O_2 from 21 to 3% there were corresponding increases in photosynthesis and levels of RuBP.

Effects of DL-Glyceraldehyde and Sugar Phosphates on Chloroplast RuBP and CO_2 Fixation. CO_2 fixation by isolated chloroplasts was completely inhibited by 15 mM DL-glyceraldehyde,

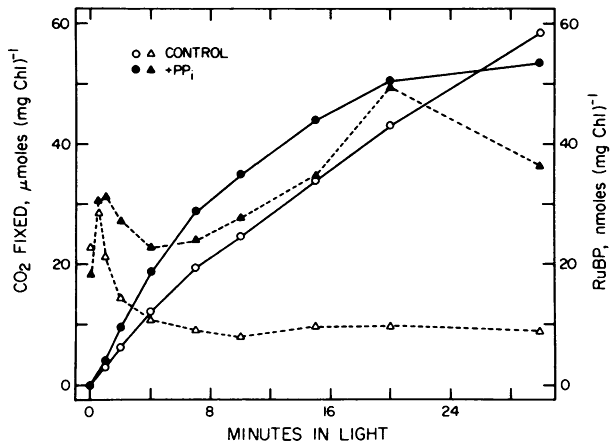

FIG. 2. Effect of PPi on CO_2 fixation and chloroplast levels of RuBP. CO_2 fixation (——) was determined with solution C plus $NaH^{14}CO_3$ (10.4 mM, 0.5 μCi/μmol) and chloroplasts (0.079 mg Chl/ml) in the presence (●, ▲) and absence (○, △) of PPi (1.25 mM). Chloroplast RuBP (- - -) was determined from similar reaction mixtures (24).

Table I. *Effect of O_2, Catalase and FBP-Aldolase on Rates of CO_2 Fixation and Levels of RuBP in Spinach Chloroplasts*

Samples (0.8 ml solution C) were purged for 5 min with CO_2-free air or O_2 prior to the addition of $NaH^{14}CO_3$ (3.33 mM, 0.5 μCi/μmol) and chloroplasts (0.046 mg Chl). RuBP was determined from similar reaction mixtures prepared with $NaH^{12}CO_3$ (24). Rates of CO_2 fixation and amounts of RuBP were measured after 5 min of photosynthesis. In experiment B catalase (1,000 IU/ml), FBP (1.0 mM), and aldolase (1.7 IU/ml) were added.

Experiment	CO_2 Fixation	RuBP
	μmol/mg Chl·h	*nmol/mg Chl*
	no additions	
A. 21% O_2	29.5	11.3
100% O_2	7.7	8.5
	plus FBP-aldolase and catalase	
B. 21% O_2	117.6	33.8
100% O_2	57.6	24.3

with a corresponding drop in RuBP to almost zero during the first 2 min of illumination (Fig. 3). Chloroplasts treated with DL-glyceraldehyde are unable to generate RuBP, most likely because the transketolase reaction of the photosynthetic carbon reduction pathway is inhibited (25). However, chloroplasts with 1 mM DL-glyceraldehyde had only 2 to 3 nmol less RuBP/mg Chl than chloroplasts without the inhibitor, yet CO_2 fixation was reduced more than 40%.

We have noted that 1 mM DL-glyceraldehyde does not affect catalysis by the purified RuBP carboxylase, but with illuminated intact chloroplasts it lowers the activation of the enzyme (1). DL-Glyceraldehyde may also be inhibiting the formation of more optimal Mg^{2+} or pH conditions in the chloroplast stroma (21, 27). Obviously, electron transport and photophosphorylation producing NADPH and ATP are not limiting when the RuBP levels are sufficient. The role of electron transport in maintaining the proper pH and Mg^{2+} gradient across the thylakoid membranes must be considered as a major factor in the regulation of activity of the RuBP carboxylase in the chloroplast.

Chloroplasts when incubated with FBP plus aldolase (to generate triose-P) or Ri5P produced more RuBP during the first 4 min in the light. There was no increase in the rate of CO_2 fixation (Fig. 4). Apparently with these chloroplasts, 22 to 24 nmol RuBP/mg Chl saturated CO_2 fixation during this period.

Effects of Irradiance on CO_2 Fixation and Chloroplast RuBP. At a limting irradiance (25 $\mu E/m^2 \cdot s$) the rate of CO_2 fixation between 1 and 4 min in the light was 18.9 μmol/mg Chl·h (Fig. 5). At saturating irradiance (800 $\mu E/m^2 \cdot s$) the same chloroplast preparation fixed CO_2 at 98.3 μmol/mg Chl·h during this period. Although the kinetics of the light-on RuBP formation were somewhat modified at the lower irradiance, the chloroplast RuBP pool size was almost identical during the 4- to 10-min period. In this experiment, the availability of RuBP was apparently not the cause of the limited rate of CO_2 fixation at the lower irradiance. This is not always the case as we have also noted with other chloroplast preparations and lower irradiances that the RuBP level can be correspondingly low and possibly limiting for CO_2 fixation (data not shown). However, with added FBP and aldolase, RuBP levels increased while CO_2 fixation decreased at lower irradiances (Fig. 6). Clearly, the drop in the rate of CO_2 fixation under these conditions must be due to a drop in activity of the RuBP carboxylase (1).

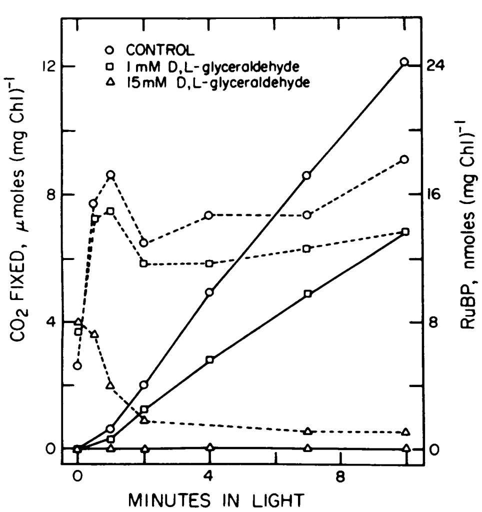

FIG. 3. Effect of DL-glyceraldehyde on CO_2 fixation and levels of chloroplast RuBP. CO_2 fixation (——) was determined with solution C plus PPi (1.0 mM), $NaH^{14}CO_3$ (8.3 mM, 0.5 μCi/μmol), chloroplasts (0.105 mg Chl/ml), and either no (○), 1.0 mM (□), or 15.0 mM (△) DL-glyceraldehyde. RuBP (- - -) was determined from similar media (24).

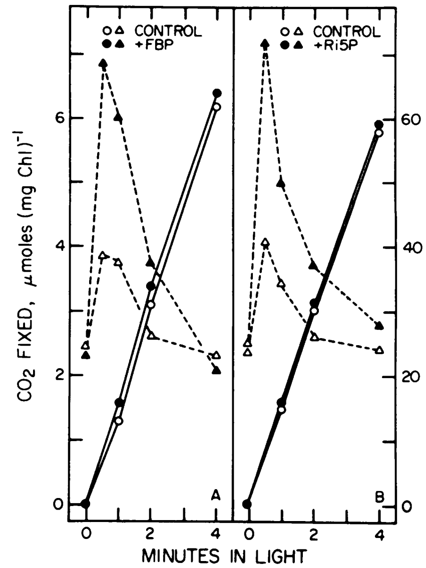

FIG. 4. Effect of FBP and Ri5P on CO_2 fixation and levels of chloroplast RuBP. CO_2 fixation (——) was determined with solution C plus PPi (1.0 mM), $NaH^{14}CO_3$ (10 mM, 0.5 μCi/μmol), chloroplasts (A, 0.082 mg Chl/ml or B, 0.065 mg Chl/ml) and (A) FBP (1.0 mM) and rabbit muscle aldolase (1.25 IU/ml) or (B) Ri5P (1.0 mM). Chloroplast RuBP (- - -) was determined from similar solutions having $NaH^{12}CO_3$ (24).

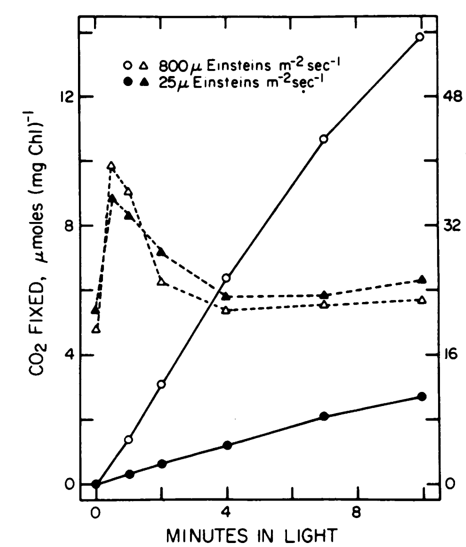

FIG. 5. Effect of irradiance on CO_2 fixation and levels of RuBP. The irradiance was either 800 (○, △) or 25 (●, ▲) $\mu E/m^2 \cdot s$. CO_2 fixation (——) was determined with solution C plus $NaH^{14}CO_3$ (9.2 mM, 0.5 μCi/μmol), PPi (0.9 mM) and chloroplasts (0.117 mg Chl/ml). RuBP (- - -) was determined from similar solutions containing $NaH^{12}CO_3$ (24).

DISCUSSION

Isolated chloroplasts often can be deficient in intermediates of the photosynthetic carbon reduction pathway (22). Consequently, CO_2 fixation in chloroplasts often responds to increased RuBP levels. The presence of PPi prevents the loss of intermediates from intact chloroplasts in the light (2, 26) and can increase CO_2 fixation by sustaining RuBP production. The addition of sugar phosphates, such as Ri5P or FBP and aldolase to make triose-P, will also increase chloroplast levels of RuBP. Addition of catalase to eliminate H_2O_2 produced in the light also increases CO_2 fixation and RuBP levels. O_2 reduces CO_2 fixation by lowering RuBP levels as well as by competing with CO_2 as a substrate for the RuBP carboxylase-oxygenase (Table I) (15).

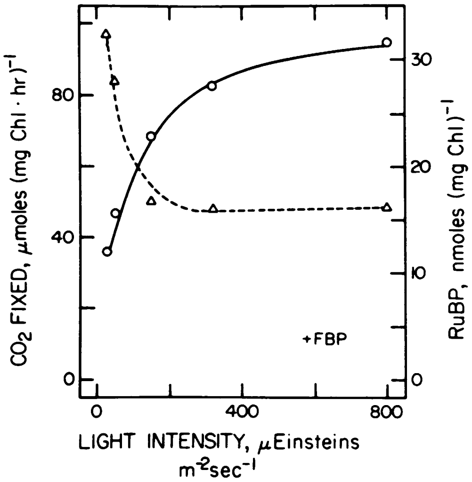

FIG. 6. CO_2 fixation and chloroplast RuBP levels as a function of irradiance in the presence of FBP. Rates of CO_2 fixation (○) were determined after 10-min illumination with solution C plus $NaH^{14}CO_3$ (10.4 mM, 0.5 µCi/µmol), FBP (1.0 mM), aldolase (1.25 IU/ml), and chloroplasts (0.077 mg Chl/ml). Samples for RuBP (△) were taken from similar reaction mixtures at 10 min (24).

Lower irradiances which result in lowered rates of CO_2 fixation can result in lowered chloroplast RuBP levels. This would be expected because CO_2 fixation is needed to provide carbon for RuBP production. If chloroplasts have sufficient carbon reserves to supply RuBP by conversion of triose-P or starch breakdown, RuBP levels in the chloroplast do increase at lower irradiances (Fig. 6). These observations that RuBP can be high when CO_2 fixation is decreasing strongly support the proposal that the RuBP carboxylase is a key metabolic regulator of photosynthesis (12).

Our results indicate that chloroplasts are capable of supplying RuBP faster than it is consumed by the RuBP carboxylase, especially if sufficient sources of fixed carbon are available (*i.e.* starch, triose-P, etc.). The intact leaf also possesses the photorespiratory glycolate pathway for recycling most of the carbon lost by the RuBP oxygenase back to the chloroplast. Therefore, the sensitivity to atmospheric O_2 as seen by RuBP depletion (Table I) in isolated chloroplasts might not be expected in the intact leaf (7). If RuBP were supplied sufficiently in a leaf, inhibition by O_2 might still occur and be explained by the competitive action of O_2 with CO_2 at the catalytic site of the enzyme (7).

When chloroplast CO_2 fixation was maximal (between 1 and 10 min), most of our measurements indicated that chloroplast RuBP levels below 15 to 20 nmol/mg Chl limited CO_2 fixation. Kirk and Heber (15) similarly found that 7 nmol/mg Chl in chloroplasts limited CO_2 fixation. The internal volume of our chloroplasts, measured as the difference between the ^3H_2O-permeable space and the [^{14}C]sucrose-impermeable space (11) was about 25 µl/mg Chl. With this volume, 15 nmol RuBP/mg Chl is equivalent to 0.6 mM RuBP. With 6 mg RuBP carboxylase/mg Chl having eight binding sites per mole of enzyme, there are 3 to 4 mM binding sites for RuBP in the chloroplast (12). Perhaps only half of the RuBP-binding sites are available as suggested by the effects of ionic strength on the purified enzyme (28). As the dissociation constant for the enzyme-RuBP complex is less than 30 µM (12), the presence of a large excess of carboxylase would bind almost all of the RuBP in the chloroplast. We find that intact leaves from these spinach plants usually contain 80 to 100 nmol RuBP/mg Chl, suggesting that the low levels found in intact chloroplasts may be an artifact of isolation.

The activities of Ru5P kinase, triose-P dehydrogenase, and FBP and sedoheptulose bisphosphate phosphatases are enhanced in the light (6, 16, 23). However, the evidence that high RuBP levels can exist in chloroplasts at limiting irradiances suggests that the activities of these enzymes may not limit photosynthetic CO_2 fixation. Light-driven photophosphorylation and electron transport are important to produce RuBP, but when RuBP is saturating, these parameters are not limiting photosynthesis. Other factors such as the role of electron transport in maintaining the proper pH and Mg^{2+} gradients across the thylakoid membranes must be considered as a major factor in regulating RuBP carboxylase activity and photosynthesis in the chloroplast (1, 21, 27). When RuBP levels saturate CO_2 fixation, the activity of RuBP carboxylase and its affinity for CO_2 must be the restraining point for photosynthesis.

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