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Limitation of CO_2 Assimilation and Regulation of Benson-Calvin Cycle Activity in Barley Leaves in Response to Changes in Irradiance, Photoinhibition, and Recovery

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ABSTRACT

The response of the Benson-Calvin cycle to changes in irradiance and photoinhibition was measured in low-light grown barley (*Hordeum vulgare*) leaves. Upon the transition from the growth irradiance (280 micromoles per square meter per second) to a high photoinhibitory irradiance (1400 micromoles per square meter per second), the CO_2 assimilation rate of the leaves doubled within minutes but high irradiance rapidly caused a reduction in quantum efficiency. Following exposure to high light the activities of NADP-malate dehydrogenase and fructose-1,6-bisphosphatase obtained near maximum values and the activation state of ribulose-1,5-bisphosphate carboxylase increased. The activity of the latter remained constant throughout the period of photoinhibitory irradiance, but the increase in the activities of fructose-1,6-bisphosphatase and NADP-malate dehydrogenase was transient decreasing once more to much lower values. This suggests that immediately following the transition to high light reduction and activation of redox-modulated enzymes occurred, but then the stroma became relatively oxidized as a result of photoinhibition. The leaf contents of glucose 6-phosphate and fructose 6-phosphate increased following exposure to high light but subsequently decreased, suggesting that following photoinhibition sucrose synthesis exceeded the rate of carbon assimilation. The ATP content attained a constant value much higher than that in low light. During photoinhibition the glycerate 3-phosphate content greatly increased while ribulose-1,5-bisphosphate decreased. The fructose-1,6-bisphosphate and triose phosphate contents increased initially and then remained constant. During photoinhibition CO_2 assimilation was not limited by ribulose-1,5-bisphosphate carboxylase activity but rather by the regeneration of the substrate, ribulose-1,5-bisphosphate, related to a restriction on the supply of reducing equivalents.

Carbon assimilation in the chloroplast may be limited by the supply of light, CO_2, or phosphate (8, 9, 26, 28). With regard to irradiance, efficient utilization of light energy is required at low photon flux densities. As photosynthesis becomes light-saturated it becomes limited by the flux through the Benson-Calvin pathway and in particular by the activity of RuBP[1] carboxylase (8, 9). When photosynthesis approachs light saturation, more light is absorbed than can be used effectively to drive photosynthesis, and dissipation of excess energy is necessary because excessive excitation of the photosystems, particularly PSII, leads to damage and loss of function. Photoinhibition occurs when the capacity to make effective use of absorbed light energy is low or impaired (20, 22) and is frequently observed when plants grown in a low light environment are exposed to high irradiance or when plants normally acclimated to high light are exposed to environmental stress (20). Exposure to high light elicits changes in the thylakoid membrane that decrease the efficiency of PSII and protect the photosynthetic machinery from further damaging effects of high light such as photooxidation. Regulation of quantum yield, qE, and protein phosphorylation may all serve to prevent overreduction of the electron transport system and photoinhibitory damage (5–7, 15, 16, 30). Quantum yield is decreased when photosynthesis is limited by the reactions of carbon metabolism and following photoinhibition (5, 16, 30). Photoinhibition is most likely to occur as a result of damage to the PSII reaction center caused by overreduction of the acceptor pools.

In respiratory systems the term 'respiratory control' is used to describe the restriction of electron transport by high ΔpH such that energy is conserved when the ATP supply is adequate. In photosynthetic systems an analogous term, 'photosynthetic control,' has evolved and this describes the restrictions placed on thylakoid electron flow in order to coordinate the synthesis of ATP and NADPH with the rate at which these metabolites can be used in carbon metabolism. At high irradiances when carbon assimilation limits the overall rate of photosynthesis (14), ATP and NADPH production are likely to exceed demand. In this situation photosynthetic control of electron transport would be expected to occur. However, the classical description of photosynthetic control in terms of regulation of the rate of electron flow via the restriction of the rate of plastoquinol oxidation, when trans-thylakoid ΔpH is high, is itself a photoinhibitory condition since it would favor overreduction of PSII and over-oxidation of PSI. Thus, photosynthetic control itself may lead to a reduction in PSII function and a loss of quantum efficiency. In this regard it is difficult to distinguish between regulation of electron flow resulting from photosynthetic control processes and a true limitation of electron flow caused by loss of PSII activity.

Studies on the effects of photoinhibition on the activity of the Benson-Calvin cycle in intact isolated chloroplasts and

[1] Abbreviations: RuBP, ribulose-1,5-bisphosphate; F_o, fluorescence level when all the PSII centers are open; F_m, maximum fluorescence level; Fru-6-P, fructose-6-phosphate; Fru-1,6-bisP, fructose-1,6-bisphosphate; F_v, variable fluorescence level; Glu-6-P, glucose-6-phosphate; Gly-3-P, glycerate-3-phosphate; qE, energy-dependent Chl fluorescence.

leaves suggested that the rate of regeneration of RuBP was limiting carbon assimilation and that this was caused at least in part by increased rates of oxidation of redox-modulated enzymes (3, 13, 18, 22). In the present study, we demonstrate that following photoinhibition the stroma becomes overoxidized, resulting in decreased activities of thiol-modulated enzymes and a reduced capacity for Gly-3-P metabolism.

MATERIALS AND METHODS

Plant Material

Hordeum vulgare var cytris was grown in pots in a growth chamber with a 16 h photoperiod and a temperature of 22°C in the light and 16°C in the dark. The plants were grown in a low light regime (280 μmol m^{-2} s^{-1}) until the fourth leaf was well developed (28 ± 1 d old). In all the following experiments the fourth leaf only was used.

Carbon Assimilation and Fluorescence Measurement

Net CO_2 assimilation was measured using infrared gas analysis (ADC 225 MK III and Heraeus), and the transpiration rate was assessed with a hygrodynamic sensor (General Eastern). Leaves were allowed to equilibrate in air for 2 h in the assimilation chamber at the growth irradiance and temperature. The plants were than exposed to high irradiance (1400 μmol m^{-2} s^{-1}) for the periods of time stated in the text. All other parameters were maintained constant. Fluorescence measurements were made at 77 K with an apparatus similar to that described by Powles and Bjorkman (21) except that the light source was a 24 V Osram lamp. Leaf samples were taken at the times indicated and given a 5 min dark treatment prior to freezing. After 2 min equilibration at 77 K, leaf samples were illuminated (0.5 μE m^{-2} s^{-1} at the surface) through an interference filter (470 nm). PSII fluorescence was measured at 690 nm. For the fluorescence measurements the photoinhibitory treatment was given in a leaf disc O_2 electrode (LD2 Hansatech) in air.

Metabolite and Enzyme Determinations

Metabolite and enzyme measurements were made on samples of whole leaves which were incubated in the CO_2 assimilation chamber. At the times indicated the leaves were pulverized in liquid nitrogen with constant conditions of irradiance. The leaf powder in liquid nitrogen was divided into fractions for enzyme and metabolite analysis.

For metabolite measurements the frozen samples were ground with frozen $HClO_4$ (1 M). Metabolite measurements were made following neutralization with K_2CO_3. RuBP, PGA, triose phosphate, and Fru 1-6 bisP were measured as described previously (12). ATP was measured spectrophotometrically by the increase in absorbance at 340 nm associated with NADP reduction in a coupled reaction system (1 mL) containing 0.2 M Hepes-KOH buffer (pH 7.8), 20 mM $MgCl_2$, 0.2 mM NADP, 20 μg glucose, and glucose 6-phosphate dehydrogenase (3.5 units mL^{-1}) and hexokinase (2 units mL^{-1}). Fru-6-P and Glu-6-P were estimated as described previously (12).

For the enzyme assays, the frozen leaf samples were ground with frozen extraction buffer consisting of 0.1 M Tricine-KOH buffer (pH 8.0) containing 1 mM dithiothreitol, 10 mM $MgCl_2$, 50 mM KCl, 1 mM EDTA, and 0.1% Triton X-100. Enzymes were assayed immediately after the sample had thawed. NADP-malate dehydrogenase was measured spectrophotometrically following the oxidation of NADPH in a reaction mixture containing leaf extract, 0.2 mM NADPH, 0.5 mM oxaloacetate, 10 mM $MgCl_2$, 1 mM EDTA, and 0.1 M Tricine-KOH buffer (pH 8.0). Maximum activity of NADP malate dehydrogenase was assayed following incubation of the leaf extract in a medium containing 0.1 M Tricine-KOH (pH 8.3), 10 mM $MgCl_2$, and 100 mM dithiothreitol for 20 min at 20°C. This medium had been degassed and bubbled with N_2 prior to use. The activation was confirmed by taking samples over a time course until no more increase in activity could be observed. Fru-1,6-bisPase was assayed spectrophotometrically at 340 nm in a reaction mixture containing 100 mM Tricine-KOH buffer (pH 8.0), 10 mM $MgCl_2$, 10 mM dithiothreitol, 0.2 mM NADP, 0.1 mM fructose-1,6-bisphosphate, and phosphoglucose isomerase (2.5 units mL^{-1}) and glucose 6-phosphate dehydrogenase (3.5 units mL^{-1}) in a total reaction volume of 1 mL. RuBP carboxylase was measured as described by Parry *et al.* (19).

Chl and Phaeophytin Estimations

Total Chl was assayed by the method of Arnon (1) and total phaeophytin (in acid-treated extracts) was estimated by the method of Vernon (29).

RESULTS

CO_2 Fixation and Chl *a* Fluorescence

When attached barley leaves were exposed to an irradiance (1400 μmol m^{-2} s^{-1}) far in excess of that experienced by the plants during their development (280 μmol m^{-2} s^{-1}), light-induced loss of thylakoid function was observed (Table I). Photoinhibition was measured by the decrease in the ratio of Fv to Fm and was accompanied by a decrease in the quantum yield of CO_2 assimilation (Table I). The F_v/F_m ratio and quantum yield both declined within minutes following the

Table I. *Effect of High Light (1400 μmol m^{-2} s^{-1}) on Chl a Fluorescence and the Quantum Yield of Carbon Assimilation in Barley Leaves*

Duration of Light-Induced Inhibition	Fluorescence Parameters				Quantum Yield
	F_0	F_m	F_v/F_m	F_v/F_0	
min					
0	18.50	92.4	0.80	3.99	0.095
10	19.55	88.0	0.78	3.50	0.093
30	20.10	68.1	0.70	2.32	0.071
45	19.5	64.0	0.69	2.28	0.073
60	19.3	57.0	0.66	1.95	0.055
90	20.7	58.0	0.64	1.80	0.052
105	19.5	57.0	0.66	1.92	0.052
120	20.3	53.0	0.62	1.61	
150	19.2	52.0	0.63	1.70	0.048
180	20.1	53.0	0.62	1.64	0.046

transition to high irradiance, reaching a minimum value after 1 h 30 min exposure. The F_v/F_m ratio and quantum yield subsequently remained at the constant lower level throughout the remaining period of high irradiance (Table I). The reduction in quantum yield resulted in a significant loss of capacity for CO_2 assimilation at low irradiance. Rates of CO_2 assimilation at high irradiance were much less effected (Fig. 1). The inhibition of photosynthesis at low irradiance paralleled the loss of quantum efficiency and reached a minimum after 1 h 30 min (Fig. 1; Table I).

The effects of transitions in irradiance, involving photoinhibitory loss to electron transport capacity, on the activity of the Benson-Calvin cycle were assessed by arresting metabolism in leaf samples throughout a time-course where the following regime was applied. Barley leaves were allowed to photosynthesize in the assimilation chamber in air at the growth irradiance (280 μmol m^{-2} s^{-1}) until steady-state photosynthesis had been attained (Fig. 2). The light intensity was then abruptly changed to 1400 μmol m^{-2} s^{-1}. CO_2 assimilation increased rapidly within the initial minutes of high irradiance and attained a maximum value. Subsequently, the rate of photosynthesis at high irradiance declined slowly over the remaining period of high irradiance (Figs. 1 and 2). After 2 h

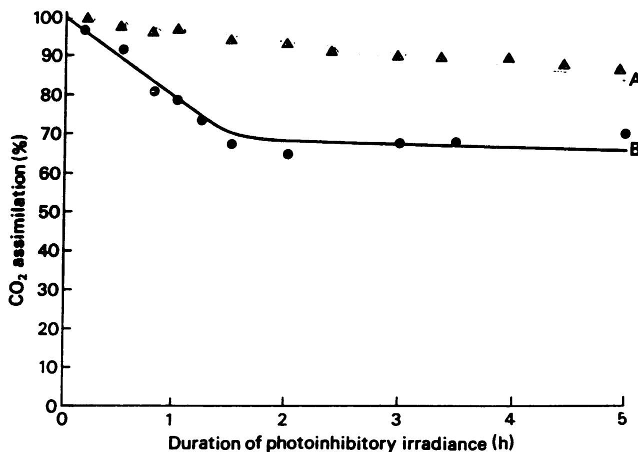

Figure 1. Effect of photoinhibitory irradiance on CO_2 assimilation measured at high light 1400 μmol m^{-2} s^{-1} (▲) and low light 280 μmol m^{-2} s^{-1} (●). CO_2 assimilation was 100% at 20 μmol CO_2 m^{-2} s^{-1}.

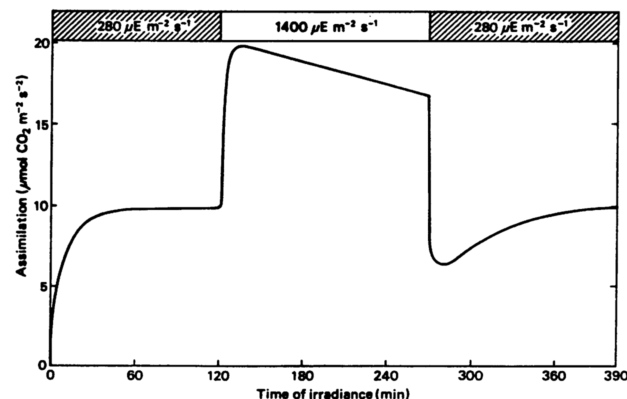

Figure 2. Time-course of CO_2 assimilation with respect to changes in irradiance.

30 min at high irradiance, 1 h after photoinhibition had reached a maximum steady-state value (Table I), the light intensity was abruptly decreased to 280 μmol m^{-2} s^{-1}. The rate of CO_2 assimilation fell to a value 25 to 35% lower than the steady-state rate attained prior to the transition to high irradiance (Figs. 1 and 2). CO_2 assimilation at low irradiance subsequently recovered the original value within a 1 h 30 min period (Fig. 2).

Metabolite Levels

Following the transition in irradiance from 280 to 1400 μmol m^{-2} s^{-1}, the RuBP pool and triose-P pools of the leaf rapidly increased within a period of minutes (Fig. 3). In this time there was a small increase in the Gly-3-P content of the leaves. In the following period when photoinhibitory damage ensued (Table I), the RuBP content of the leaves decreased gradually to a level lower than that attained in low light. The triose-P pool remained relatively constant at the higher value attained initially at high irradiance (Fig. 3). The Gly-3-P pool increased considerably over the initial 1 h 30 min of photoinhibitory light and then increased more slowly to the end of the high light treatment (Fig. 3). Upon the subsequent transition to low light, the RuBP, triose phosphate, and PGA pools rapidly declined to minimum values but subsequently rapidly recovered to higher values (Fig. 3). However, whereas the Gly-3-P and triose-P pools returned to levels similar to those obtaining before the initial transition to high irradiance, the RuBP pool attained a steady-state value somewhat lower than that found in the initial period of low irradiance.

The transition from low to high light caused a sharp increase in the Glu-6-P and Fru-6-P contents of the leaf (Fig. 4). These pools then increased more gradually over the following 1 h 30 min; however, they then decreased over the following 1 h period suggesting that the rate of hexose-P utilization exceeded the rate of synthesis. The fru-1,6-bisP pool decreased sharply upon the transition from low to high light but subsequently increased once more to a higher constant value during photoinhibitory treatment. Following the transition from high to low light, the Glu-6-P, Fru-6-P, and Fru-1,6-bisP pools decreased rapidly. These pools subsequently increased once more and then gradually decreased to attain steady-state values (Fig. 4).

Enzyme Activities, ATP Content and Redox State

The activity of RuBP carboxylase measured at 280 μmol m^{-2} s^{-1} was 390 to 450 $\mu mol \cdot h^{-1} \cdot mg^{-1}$ Chl. The activation state of RuBP carboxylase increased from 60 to 65% active at 280 μmol m^{-2} s^{-1} to 75 to 80% at 1400 μmol m^{-2} s^{-1} (Fig. 5). The higher activation state achieved following the transition to high irradiance persisted throughout the period of photoinhibitory irradiance. Following the subsequent transition from high to low light, RuBP carboxylase activity initially rose to 100% activation before the activation state decreased again to a lower value (Fig. 5).

The ATP content of the leaves increased sharply following the transition from low to high irradiance. However, the rise was transient and sharply offset when the ATP level plunged rapidly. This decline was also only short-lived as the ATP

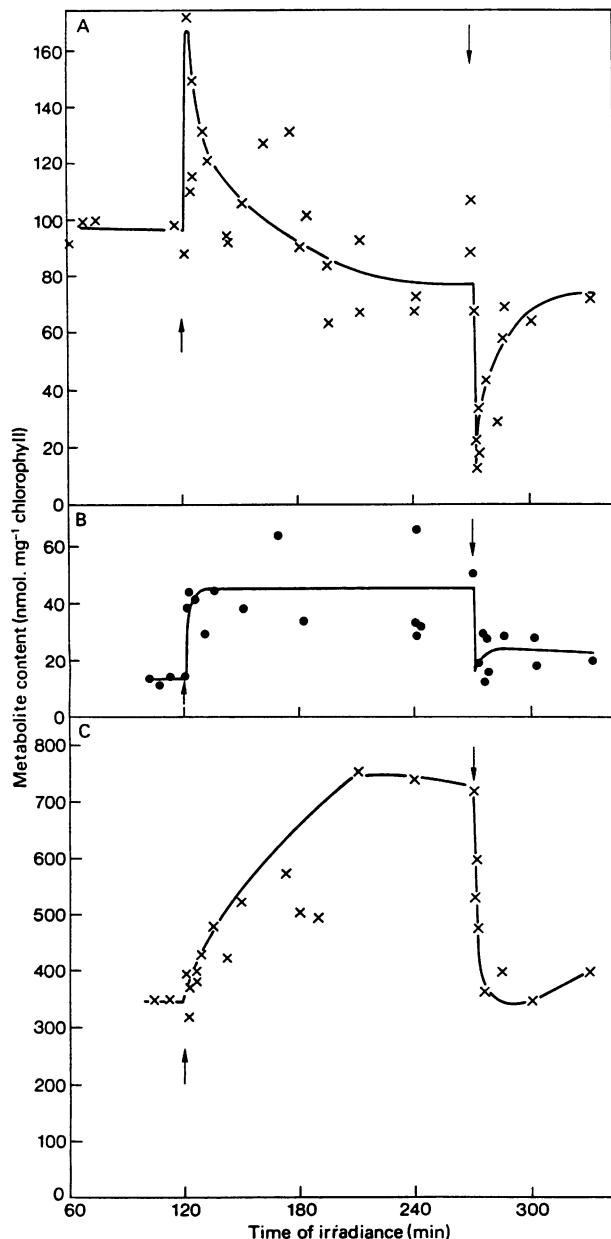

Figure 3. Changes in barley leaf metabolite pools in response to changes in irradiance and photoinhibition. Transitions in irradiance were from 280 μmol m^{-2} s^{-1} to 1400 μmol m^{-2} s^{-1} (↑) and from 1400 μmol m^{-2} s^{-1} to 280 μmol m^{-2} s^{-1} (↓). Ribulose-1,5-bisphosphate, A; triose phosphate, B; glycerate 3-phosphate, C.

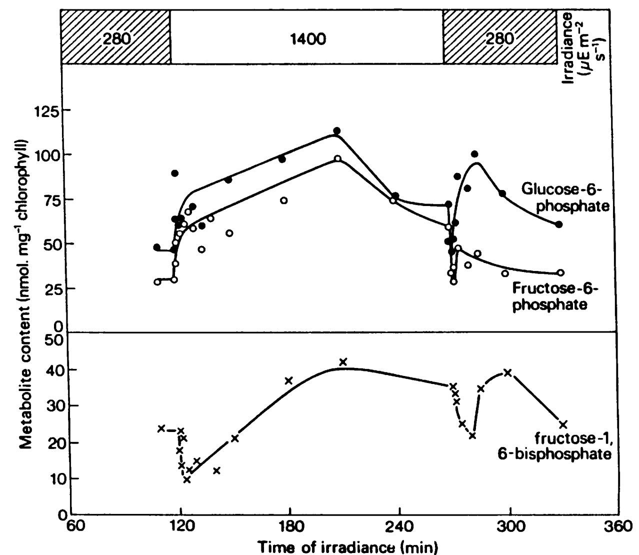

Figure 4. Changes in barley leaf metabolite contents in response to changes in irradiance and photoinhibition.

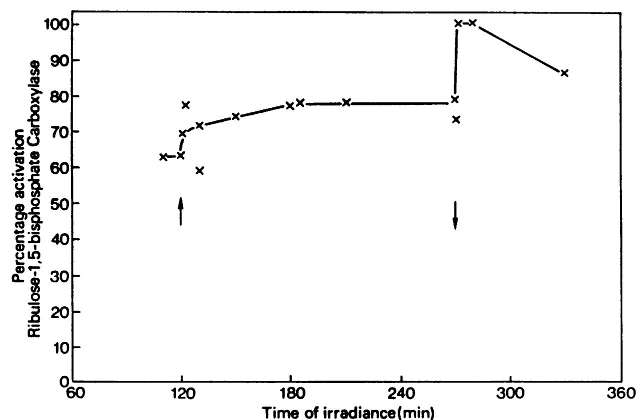

Figure 5. Effect of changes in irradiance and photoinhibition on the activation state of RuBP carboxylase. Barley leaves were illuminated at 280 μmol·m^{-2}·s^{-1}. The light intensity was then changed to 1400 μmol m^{-2} s^{-1} (↑) and maintained at this photoinhibitory irradiance for 2 h 30 min. Subsequently, the light intensity was decreased once more to 280 μmol m^{-2} s^{-1} (↓).

level increased more slowly, once more achieving a high constant steady-state value (Fig. 6).

The activation state of the light-activated enzyme NADP-malate dehydrogenase was used as a sensitive physiological indicator of the redox state of the chloroplast stroma (10, 25). NADP-malate dehydrogenase activity increased to almost maximum (95%) following the transition to high irradiance (Fig. 6). However, the activity soon decreased once more to a lower value. This would suggest that there was an initial large increase in the redox-state of the stroma following the onset of high irradiance but as photoinhibitory damage to the thylakoids occurred the redox state of the stroma and the associated activities of light-modulated enzymes decreased. This is corroborated by the changes in the activity of the Benson-Calvin cycle enzyme Fru-1,6-bisPase. The pattern of activation of this enzyme in relation to transitions in irradiance and photoinhibitory treatment was similar to that of NADP-malate dehydrogenase (Fig. 6). Fru-1,6-bisPase activity was extremely high following the onset of high irradiance. However, this high activation state was not maintained and as photoinhibition of electron flow occurred, enzyme activity also fell to a much lower steady-state value (90–100 μmol h^{-1} mg^{-1} Chl).

DISCUSSION

In low-light-grown barley leaves, exposure to high irradiance resulted in photoinhibition and decrease in quantum

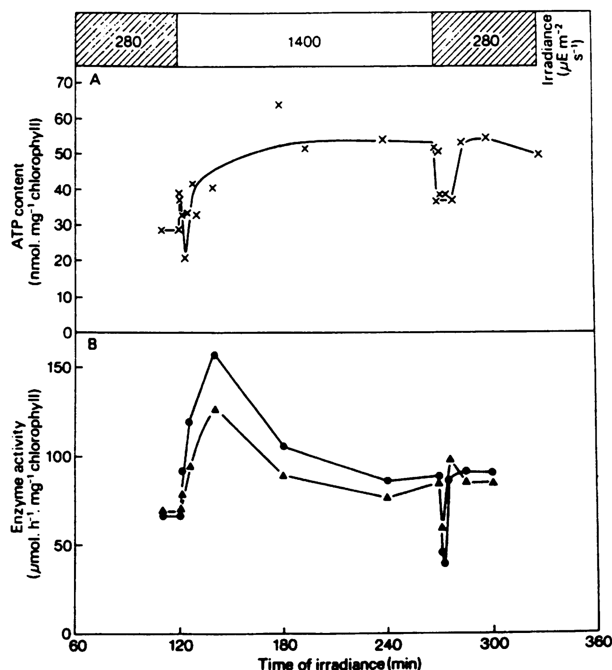

Figure 6. Response of the leaf ATP pool (A) and leaf activities (B) of NADP malate dehydrogenase (▲) and fructose-1,6-bisphosphatase (●) to changes in irradiance.

yield that was also evidenced by a significant loss of photosynthetic capacity at low irradiance. However, the decrease in the rate of photosynthesis measured at high irradiance was relatively small. We have examined the effects of changes in irradiance and photoinhibition on photosynthetic metabolite pool and stromal enzyme activities in order to assess the basis for restriction of Benson-Calvin cycle activity resulting from photoinhibitory treatments.

The abrupt transition from low to high irradiance caused changes in the level of activation of key enzymes of the Benson-Calvin cycle and in the pool sizes of photosynthetically active metabolites that were necessary in order to accommodate the higher rate of photosynthesis. Immediately following the transition to high irradiance, Fru-1,6-bisPase activity increased and the Fru-1,6-bisP content of the leaves decreased. NADP-malate dehydrogenase activity was also increased attaining almost maximum activity immediately following exposure to high light. These observations suggest that initially the transition to high light caused the stroma to become highly reduced. However, highly reducing conditions predispose the photosynthetic membranes to photoinhibition (2, 17). As photoinhibitory loss of thylakoid function occurred, a progressive loss in the capacity for noncyclic electron flow was evidenced by a decline in the activity of redox-modulated enzymes. The decrease in the activation state of NADP malate dehydrogenase suggests that the stroma was becoming much more oxidized during this period. Partial inhibition of the light activation of chloroplast enzymes resulting from photoinhibition has been shown in both isolated chloroplasts (13, 18) and leaves (3, 26). Miginiac-Maslow *et al.* (18) reported decreases in the activity of NADP-malate dehydrogenase in isolated intact spinach chloroplasts fixing CO_2 following high light treatment. Similarly, Giersch and Robinson (13) showed loss of Fru-1,6-bisPase activity of spinach chloroplasts resulting from photoinhibition. The results presented here confirm that strong inhibitory effects on both enzymes are observed in leaves exposed to photoinhibitory irradiance. It is apparent that it is failure to maintain the reduced and active forms of thiol-modulated enzymes that leads to loss of enzyme activity during photoinhibition. H_2O_2-mediated reversal of thiol-activation as suggested by Giersch and Robinson (13) can be discounted since the ascorbate-gluthathione cycle for H_2O_2 detoxification is fully active throughout the photoinhibitory treatment (11). We are drawn to the conclusion that photoinhibition results in oxidation of the stroma and in this situation there is a strong competition for a limited supply of reducing power.

The abrupt change from low to high light caused the ATP pool to oscillate for some minutes following the transition. However, the ATP pool rapidly reached a new steady-state level in high light, much higher than that maintained in low light and the ATP content of the leaf subsequently remained constant throughout the period of photoinhibition. This implies that ATP synthesis is not limiting for CO_2 assimilation at photoinhibitory irradiance and that the ability to synthesize ATP is not impaired by photoinhibition. Situations of high light generate a high transthylakoid ΔpH. This together with the high ATP content would provide metabolic conditions where photosynthetic control electron transport would be expected to occur. Such a situation would favor a decrease in the ratio of noncyclic to cyclic electron flow (18). This is evidenced by the changes that occur in photosynthetically active metabolites following photoinhibitory treatment. Immediately following the transition to high light, the RuBP content of the leaves was greatly increased, the triose-P content increased while the Gly-3-P level was little changed. As photoinhibitory damage progressed the RuBP pool rapidly fell while the Gly-3-P pool increased threefold. This suggests that the carboxylation rate was greater than the rate of Gly-3-P reduction in these circumstances. The level of triose-P remained relatively constant throughout the high light period; thus, the ratio of Gly-3-P to triose-P was increasing as a consequence of photoinhibition. This increase in the ratio of Gly-3-P to triose-P is caused by a failure to sustain rates of Gly-3-P reduction. Since ATP is available to drive the first reaction of the reductive phase of the Benson-Calvin cycle, the conversion of Gly-3-P to Gly-1,3-bisP, the subsequent reduction of Gly-1,3-bisP to triose-P must be limiting the metabolism of Gly-3-P. We must conclude that it is either the activity of NADP-glyceraldehyde 3-P dehydrogenase or the supply of NADPH that limits the reduction of Gly-3-P. NADPH production as a result of noncyclic electron flow has been found to be inhibited prior to a decrease in the activation state of NADP-malate dehydrogenase (18) in isolated chloroplasts. These observations support the suggestion that as a result of photoinhibition, photosynthetic carbon assimilation is limited by the supply of reducing power.

The measured activation state of RuBP carboxylase confirms this view. The activity and activation state of this enzyme increase following the transition to high light. In parallel, the initially high level of RuBP produced by the light

transition progressively decreases. Thus, the activity of RuBP carboxylase is not limiting the assimilation of CO_2 during photoinhibition but rather it is the regeneration of the substrate, RuBP, that limits Benson-Calvin cycle activity. The activation state of RuBP carboxylase has been shown to be related to the ATP content of the stroma via the mediation of RuBP carboxylase activase (4, 19, 24) such that increased ATP levels favor increased activation states of RuBP carboxylase. The results presented here show that during photoinhibition both the leaf ATP content and the RuBP carboxylase activity are high suggesting that activation of RuBP carboxylase is favored in these circumstances. Surprisingly, the RuBP carboxylase activation state is increased further immediately following the transition from photoinhibitory irradiance to low light in spite of the fact that the ATP content transiently falls. This further oxidation of the system following the transition to low light apparently stimulates the system to produce maximum activation of RuBP-carboxylase.

The changes in metabolic pool size that accompany simple transitions in irradiance are well characterized (23, 27). Following the transition from photoinhibitory irradiance to low light, the changes observed in metabolite levels are more complex than those occurring following the change from high to low light in the absence of photoinhibition. The electron transport chain operates at maximum efficiency only when values of assimilatory force are low and phosphorylation and electron flow become less efficient as assimilatory force increases to high levels (14). Assimilatory force is lowest when the Benson-Calvin cycle is operating at maximum efficiency and the redox-modulated enzymes are activated (14). During photoinhibitory irradiance, the Gly-3-P/triose-P ratio is high, suggesting that assimilatory force is low and thus carbon assimilation is limited by electron transport. The transition from photoinhibitory irradiance to low light causes little change in the steady state Gly-3-P/triose-P ratio (16.7–18.9) because at low light electron transport is still limiting for carbon assimilation. Surprisingly, the Gly-3-P content of the leaf rapidly decreased following the transition to low light and did not increase as would be predicted from previous work (23, 27). A decrease in irradiance should, in most circumstances, result in the accumulation of Gly-3-P rather than a loss of this metabolite (23, 27). This change in response to the light transition may result from changes in the relationship between carbon assimilation and electron transport during exposure to photoinhibitory irradiance relative to that obtaining in high light without photoinhibitory damage.

We must conclude that during photoinhibition the Benson-Calvin cycle is limited by the capacity for production of reductant and this situation results in a reduction in assimilatory force as determined by the Gly-3-P/triose-P ratio. Whether this oxidation of the stroma is the result of photosynthetic control exerted over thylakoid electron flow by a large transthylakoid ΔpH or simply by loss of PSII function limiting the capacity for noncyclic electron flow cannot be determined. Clearly, a complex balance in the relationship between limitation and regulation exists during photoinhibition. Photoinhibitory loss of PSII capacity may be viewed as a manifestation of the operation of photosynthetic control processes occurring when electron transport is restricted by high ΔpH.

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