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Photosynthesis and Light Activation of Ribulose 1,5-*bis*phosphate Carboxylase in the Presence of Starch

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ABSTRACT

Limitation of photosynthesis and light activation of ribulose, 1,5-*bis*phosphate carboxylase (RuBPCO) were examined in the 5th leaf of seedlings of red clover (*Trifolium pratense* L. cv. Renova) for 5 d following an increase in photosynthetic photon flux density (*PPFD*) from 200 to 550 μmol quanta m^{-2} s^{-1}. Net photosynthesis and its stimulation at 2·0 kPa O_2, initial activity of rapidly extracted RuBPCO, standard activity of RuBPCO after incubation of the extracts in the presence of CO_2, Mg^{2+}, and inorganic phosphate and contents of soluble protein, starch, soluble sugars, and various photosynthetic metabolites were determined. Photosynthesis decreased and starch content increased. No decrease in photosynthesis was found if, when *PPFD* was increased, all leaves except the investigated 5th leaf were removed, suggesting that the decrease in photosynthesis was due to accumulated carbohydrates. The stimulation of photosynthesis at 2·0 kPa O_2 did not decrease and the ratio of the total foliar steady-state contents of triose phosphate to 3-phosphoglycerate increased suggesting that the decrease in photosynthesis was not due to limiting inorganic phosphate in chloroplasts. Intercellular CO_2 partial pressure and RuBP content were not decreased. Nevertheless, the ratio of photosynthesis to initial RuBPCO activity decreased, suggesting that the catalysis per active RuBPCO site was decreased. The increase in *PPFD* in the growth cabinet and the *PPFD* at which leaves were preconditioned for 1 h, affected not only initial activity but also the standard activity of RuBPCO. The results suggest that a varying proportion of RuBPCO was bound to membranes and was contained in the insoluble fraction of the extracts. A comparison of photosynthesis with extracted RuBPCO activity suggested that membrane bound RuBPCO did not contribute to photosynthetic CO_2 fixation and that the binding and release to and from membranes modulated actual RuBPCO activity *in vivo*.

Key words: Photosynthesis, ribulose 1,5-*bis*phosphate carboxylase, starch.

INTRODUCTION

Leaves of perennial ryegrass (Woledge, 1978) and red clover (Joggi, Hofer, and Nösberger, 1983) are shaded during early development if they are growing vegetatively in canopies. The low light intensity during the early stages of growth appears to be the reason for decreased photosynthetic capacity during later stages of growth when leaves reach the upper canopy layers and are fully illuminated. The decreased photosynthetic capacity seems to be due partly to the adaptation of the photosynthetic apparatus to low light intensity (Björkman, 1981). On the other hand, the photosynthetic capacity decreases steadily following the increase in light intensity, an effect which cannot be explained as a phenomenon of adaptation (Joggi *et al.*, 1983). It seems to be due to product inhibition of photosynthesis after increased production of photo-assimilates at increased light intensity. Product inhibition of photosynthesis is usually attributed to the deficiency of inorganic phosphate in chloroplasts. Phosphate is then bound in sugar phosphates and is not available for the synthesis of ATP. Deficiency in inorganic phosphate was found to be associated with the decreased stimulation of photosynthesis at 2·0 kPa O_2, with decreased triose-phosphate/3-phosphoglycerate and ATP/ADP ratios (Leegood and Furbank, 1986) and with decreased initial activity of rapidly extracted ribulose 1,5-*bis*phosphate carboxylase/oxygenase (RuBPCO) (Schnyder, Mächler, and Nösberger, 1986).

In this study, red clover seedlings were grown at

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200 μmol quanta m^{-2} s^{-1} photosynthetic photon flux density (*PPFD*) and photosynthesis and RuBPCO activity were investigated after an increase in *PPFD* to 550 μmol quanta m^{-2} s^{-1}. The results show that the decrease in photosynthetic capacity at increased *PPFD* is not due to a deficiency in inorganic phosphate in chloroplasts and suggest that it is due to the properties of RuBPCO.

MATERIALS AND METHODS

Red clover (*Trifolium pratense* L. cv. Renova) was grown from seeds in pots filled with soil which were placed in a growth cabinet. The photoperiod was 16 h with light being provided by a bank of fluorescent tubes and incandescent bulbs. The *PPFD* was 200 μmol quanta m^{-2} s^{-1} at plant height during the first 7 weeks after sowing and was then increased to 550 μmol quanta m^{-2} s^{-1}. Temperature was 18/13 °C and humidity 70/85% (day/night). Plants were irrigated every 2 d with deionized water. Rhizobia (RCR 5) were added twice during early growth. All investigations were made on the attached 5th leaf of the main shoot. The initial appearance of the petiole was considered to be the date of leaf appearance. Fifth leaves were 12-d-old when *PPFD* was increased.

CO_2 exchange and transpiration rates of leaves were measured using an IRGA and a dew point hygrometer in an open system as described by Hofer, Schnyder, Mächler, and Nösberger (1986). Single attached leaves were placed into temperature controlled cuvettes of metal and perspex. A small fan in each cuvette kept boundary layer resistance of the leaf at a minimum. Light was provided by mercury vapour lamps separated from the cuvettes by an 8·0 cm water layer. *PPFD* was adjusted by changing the distance between the light source and the leaf. Leaf temperature, as determined by a thermocouple pressed to the lower surface, was 18 °C. Gas exchange measurements were taken after 45 min preconditioning. Intercellular CO_2 partial pressure was calculated according to Fick's law. The gas mixture containing 2·0 kPa O_2 was prepared using Wösthoff pumps.

Following measurements of gas exchange, the single leaves were removed from the cuvettes, rapidly extracted and tested for initial carboxylase activity of RuBPCO as described earlier (Mächler and Nösberger, 1980). Leaves were homogenized in a mortar with 3·0 g sand and 4·0 cm^3 of 20 mol m^{-3} Tris–HCl (pH 8·2), 20 mol m^{-3} $MgCl_2$ and 10 mol m^{-3} mercaptoethanol. The homogenate was diluted with 10 cm^3 of the same solution and filtered through a nylon net (20 μm mesh) by placing an open-ended rubber cylinder with the net attached to the bottom end into the homogenate. Twenty mm^3 of the filtrate which rose up inside the cylinder were tested for carboxylase activity according to Lorimer, Badger, and Andrews (1977). Mortar, sand, and solutions were precooled in ice. Assay temperature was 10 °C in order to preserve the activation of the enzyme in its initial state. The entire extraction procedure was completed within less than 1 min. Part of the extract was incubated for 10 min at 25 °C in the presence of 10 mol m^{-3} $NaHCO_3$, 20 mol m^{-3} $MgCl_2$, and 5·0 mol m^{-3} K_2HPO_4 and then assayed for standard activity. Soluble protein content was determined by the Bio-Rad method with BSA as a reference.

Enzymatic CO_2 fixation, as determined according to the procedure described above, did not correspond directly with photosynthetic CO_2 fixation in intact leaves since temperature of the assay mixture was 10 °C and CO_2 partial pressure was saturating, whereas in leaves, temperature was 18 °C and intercellular CO_2 partial pressure was about 29 Pa. Additionally, photosynthetic CO_2 fixation was decreased due to photorespiration. Therefore, enzyme activities were multiplied by a constant correction factor $K = 1{\cdot}35$. This correction factor includes 3 components, $K = K_1 \times K_2 \times K_3$.

The component K_1 corrected for the difference in temperature between the enzyme assay and the leaf and was calculated according to the Arrhenius equation.

where v_{10} and v_{18} are the reaction velocities of carboxylation at 10 °C and 18 °C, respectively and A is the activation energy ($A = 90$ kJ mol^{-1}, as determined for purified wheat RuBPCO by Mächler, Keys, and Cornelius, 1980).

The component K_2 corrected for the difference in CO_2 partial pressure between enzyme assay and leaf and for competitive inhibition of carboxylation by O_2 and was calculated according to the modified Michaelis Menten equation.

where v_c is the carboxylation velocity under leaf conditions, V_c is the maximum carboxylation velocity as determined in the enzyme assay, K_c and K_o are the Michaelis constants for carboxylation and oxygenation, respectively ($K_c = 15{\cdot}9$ Pa CO_2, $K_o = 35{\cdot}3$ kPa O_2, as interpolated for 18 °C from spinach data as determined by Jordan and Ogren, 1984), O is oxygen partial pressure in air and C_i is intercellular CO_2 partial pressure in leaves ($C_i = 29{\cdot}1 \pm 0{\cdot}8$ Pa ($\pm$s.e., $n = 15$)).

The component K_3 corrected for the decrease in CO_2 fixation in leaves due to photorespiration and was calculated according to equation 3.

Equation 3 is derived from the equation for the substrate specificity of RuBPCO, $S = v_c O / v_o C$ (Laing, Ogren, and Hageman, 1974), and from the equation for the stoichiometry of photorespiratory CO_2 release, $F = v_c - 1/2\ v_o$, where v_c and v_o are reaction velocities of carboxylation and oxygenation, O and C are partial pressures of O_2 and intercellular CO_2, F is net photosynthesis and S is the substrate specificity of RuBPCO. ($S = 3061$ Pa/Pa, as interpolated for 18 °C from spinach data as determined by Jordan and Ogren, 1984).

The percentage of RuBPCO in protein extracts was quantitated electrophoretically according to Bauwe (1979).

Leaves for the determination of starch and for the electron micrographs were sampled in the growth cabinet 4–6 h after the beginning of the light period. Starch was determined after extraction with DMSO and HCl following the procedure of Beutler (1984). Sucrose, glucose, and fructose were determined after extraction in boiling water according to the procedure of Outlaw and Tarczinski (1984).

Leaves for the determination of total foliar steady-state contents of photosynthetic metabolites were sampled in the growth cabinet 4–6 h after the beginning of the light period using freeze stop tongs and were extracted in 1 M $HClO_4$ as described by Leegood and Furbank (1986). After centrifugation, the supernatants were neutralized with 5 M KOH. Ribulose 1,5-*bis*phosphate contents were determined according to Holtum, Gibbs, and Latzko (1984). Extracts for the determination of the contents of triosephosphate, 3-phospho-glycerate, fructose*bis*-phosphate, fructose-6-phosphate, and glucose-6-phosphate were treated with charcoal (30 mg cm^{-3}) and then assayed using a fluorimeter according to Lowry and Passonneau (1972). Adenylates were determined by the luciferase method with LKB

chemicals, following the procedure of Lundin, Rickardsson, and Thore (1980).

RESULTS

Photosynthesis and RuBPCO activity

Photosynthesis of leaves, if they were preconditioned and measured at 550 μmol quanta m^{-2} s^{-1} *PPFD*, was about 18 μmol CO_2 m^{-2} s^{-1} as long as *PPFD* in the growth cabinet was 200 μmol quanta m^{-2} s^{-1} and decreased by 30% as soon as *PPFD* in the growth cabinet was increased (Fig. 1). Photosynthesis of leaves preconditioned and measured at 200 μmol quanta m^{-2} s^{-1} was about 10 μmol CO_2 m^{-2} s^{-1} and was not affected by the change in *PPFD* in the growth cabinet.

Initial activity of RuBPCO rapidly extracted from leaves, if they were preconditioned at 550 μmol quanta m^{-2} s^{-1} *PPFD*, was about 18 μmol CO_2 m^{-2} s^{-1} as long as *PPFD* in the growth cabinet was 200 μmol quanta m^{-2} s^{-1} and decreased by 18% when *PPFD* in the growth cabinet was increased (Fig. 1). Initial RuBPCO activity of leaves preconditioned at 200 μmol quanta m^{-2} s^{-1} was 10 μmol CO_2 m^{-2} s^{-1} at the beginning of the experiment and increased by 30% when *PPFD* in the growth cabinet was increased.

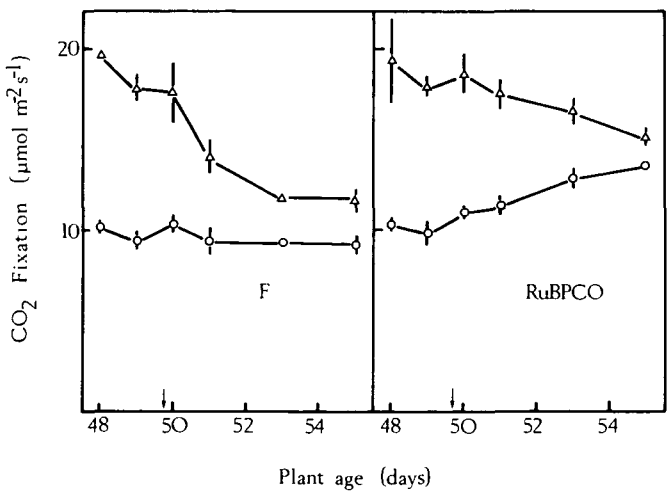

FIG. 1. Photosynthesis (*F*) and initial carboxylase activity of RuBPCO after rapid extraction. Plants were taken before and after an increase in *PPFD* in the growth cabinet (indicated by arrow, 200 μmol quanta m^{-2} s^{-1} before, 550 μmol quanta m^{-2} s^{-1} afterwards) and 5th leaves were preconditioned and measured at 200 (○) and 550 (△) μmol quanta m^{-2} s^{-1} *PPFD*. Means of three determinations ± s.e. are shown. Absence of error bars indicates that s.e. was smaller than symbol size.

The ratio of photosynthesis to RuBPCO activity was about 1·0 as long as *PPFD* in the growth cabinet was 200 μmol quanta m^{-2} s^{-1} and decreased to about 0·8 when *PPFD* in the growth cabinet was increased to 550 μmol quanta m^{-2} s^{-1} (Table 1). The PPFD during preconditioning and gas exchange measurement had no effect on the ratio of photosynthesis to RuBPCO activity.

The results show that the decrease in photosynthesis at 550 μmol quanta m^{-2} s^{-1} *PPFD* was partly due to a decrease in RuBPCO activity. However, the decrease in RuBPCO activity did not fully account for the decrease in photosynthesis. There was also a decrease in the ratio of photosynthesis to RuBPCO activity, suggesting that a factor other than RuBPCO activity contributed to the decrease in photosynthesis. Surprisingly, the decrease in the ratio of photosynthesis to RuBPCO activity was also found when the plants were preconditioned and measured at 200 μmol quanta m^{-2} s^{-1} *PPFD*, suggesting that it was independent of the actual carbon flux.

No similar decrease in photosynthesis was found when, at the beginning of the period with high *PPFD*, all leaves except the investigated 5th leaf were removed (Fig. 2). Under these conditions, photosynthesis decreased transiently during the first day only and increased after 5 d to values which were higher than before the period of high *PPFD*. Thus, the decrease in photosynthesis, following an increase in *PPFD* in the growth cabinet, ended when the increased source/sink ratio at increased *PPFD* was alleviated by the removal of leaves. This suggests that the decrease in photosynthesis was due to increased production and accumulation of photo-assimilates.

Figure 1 shows that the increase in *PPFD* in the growth cabinet affected the activation properties of RuBPCO. Photosynthesis and light activation of RuBPCO were,

TABLE 1. *Ratio of photosynthesis* (F) *to initial activity of RuBPCO after rapid extraction before and after an increase in* PPFD *in the growth cabinet*

Leaves were preconditioned and measured at either 200 or 550 μmol quanta m^{-2} s^{-1} *PPFD*. Means of three replications ± s.e. are shown.

Plant age (d)	*PPFD* in growth cabinet (μmol m^{-2} s^{-1})	*F*/RuBPCO	
		Determined at 200 μmol m^{-2} s^{-1}	Determined at 550 μmol m^{-2} s^{-1}
48	200	1·09 ± 0·043	1·15 ± 0·141
49	200	1·07 ± 0·024	1·10 ± 0·019
50	550	1·06 ± 0·075	1·06 ± 0·037
51	550	0·92 ± 0·050	0·90 ± 0·106
53	550	0·82 ± 0·029	0·80 ± 0·043
55	550	0·76 ± 0·052	0·87 ± 0·021

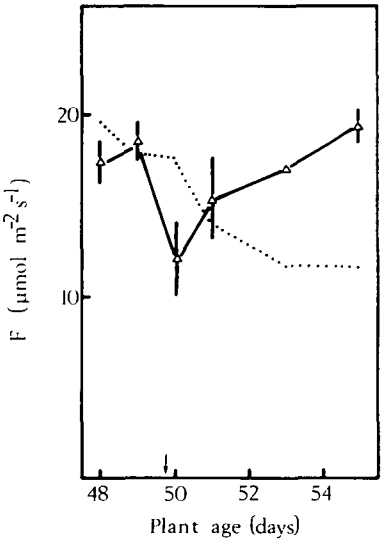

FIG. 2. Effect of the removal of all leaves except the investigated 5th leaf on photosynthesis as measured at 550 μmol quanta m^{-2} s^{-1} *PPFD*. Leaves were removed when *PPFD* was increased from 200 to 550 μmol quanta m^{-2} s^{-1} (indicated by arrow). Photosynthesis of control plants is indicated by the dotted line. Means of three determinations ± s.e. are shown. Absence of error bars indicates that s e. was smaller than symbol size.

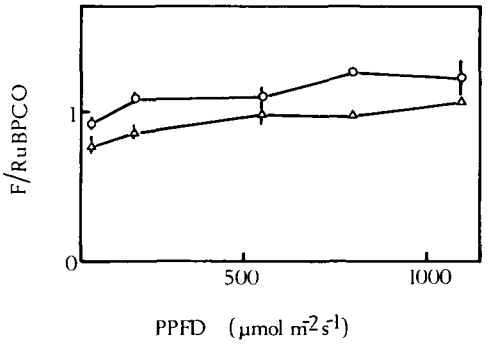

FIG. 4. The response to *PPFD* of the ratio of photosynthesis (*F*) to initial carboxylase activity of RuBPCO after rapid extraction. For explanations see legend to Fig. 3.

therefore, studied in more detail in a further experiment with plants taken immediately before and 5 d after the increase in *PPFD* in the growth cabinet.

The increase in *PPFD* in the growth cabinet had no effect on photosynthesis at low *PPFD* but decreased light saturated photosynthesis by 15% (Fig. 3). Initial activity of RuBPCO at low *PPFD* was considerably increased, whereas light saturated RuBPCO activity was not affected in this experiment. The ratio of photosynthesis to initial RuBPCO activity decreased significantly when *PPFD* in the growth cabinet was increased (Fig. 4). The decrease in the ratio was independent of the *PPFD* at which leaves were preconditioned and measured. This is consistent with the data in Table 1 and confirms that photosynthesis was affected by a factor other than RuBPCO activity and suggests that this factor was independent of the actual carbon flux. Stomatal conductance was decreased after the change in light conditions in the growth cabinet but did not decrease intercellular CO_2 partial pressure decisively (Table 2).

Standard activity of RuBPCO (activity after incubation of the extracts in the presence of CO_2, Mg^{2+}, and inorganic phosphate) and soluble protein content (Fig. 5) were affected by the *PPFD* during preconditioning of the leaves. Highest standard activity and protein content were found after preconditioning at low *PPFD*. The light response was affected by the increase in *PPFD* in the growth cabinet. Standard activity and protein content at low *PPFD* were increased after the change in light conditions in the growth cabinet, whereas no effect was found at saturating *PPFD*. The ratio of initial to standard activity showed a similar light response before and after the increase in *PPFD* in the growth cabinet (Fig. 5). Only

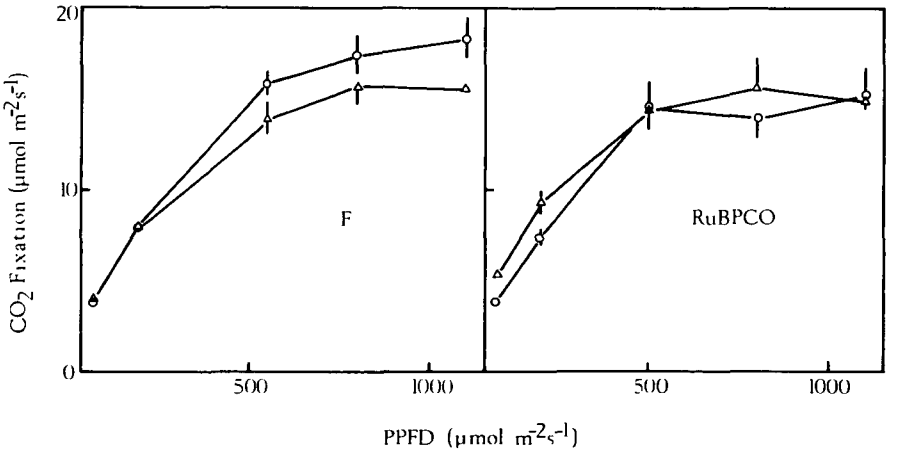

FIG. 3. The responses to *PPFD* of photosynthesis (*F*) and initial carboxylase activity of RuBPCO after rapid extraction. Plants were taken immediately before (○) and 5 d after (△) an increase in *PPFD* in the growth cabinet from 200 to 550 μmol quanta m^{-2} s^{-1} and 5th leaves were preconditioned and measured at various *PPFD*s. Means of three replications ± s.e. are shown. Absence of error bars indicates that s.e. was smaller than symbol size.

TABLE 2. *Light response of stomatal conductance* (k_g) *and intercellular* CO_2 *partial pressure* (C_i) *as determined in the experiment shown in Fig. 3*

Plants were taken immediately before (A) and 5 d after (B) the increase in *PPFD* in the growth cabinet. For further explanations see legend to Fig. 3. Means of three replications ± s.e. are shown.

PPFD (μmol m^{-2} s^{-1})	k_g (mmol CO_2 m^{-2} s^{-1})		C_i (Pa)	
	A	B	A	B
80	125 ± 24·6	75 ± 16·9	31·1 ± 0·81	29·1 ± 1·14
200	161 ± 20·8	158 ± 31·2	30·4 ± 0·69	30·3 ± 0·98
550	244 ± 45·4	182 ± 52·4	27·6 ± 1·18	25·6 ± 2·00
800	219 ± 27·9	177 ± 23·4	26·6 ± 0·83	24·3 ± 0·38
1100	252 ± 35·7	195 ± 28·2	26·8 ± 0·78	26·5 ± 0·82

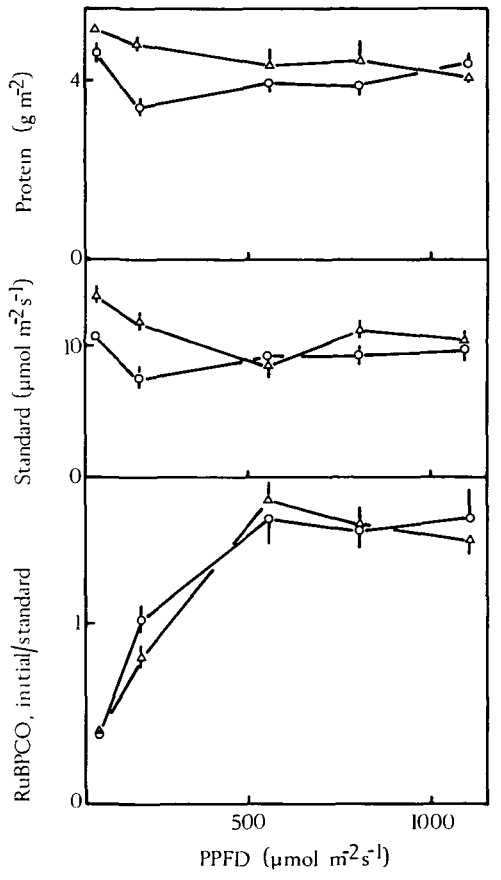

FIG. 5. The responses to *PPFD* of the leaf contents of soluble protein and RuBPCO standard activity and of the ratio of initial to standard activity. For explanations see legend to Fig. 3.

at 200 μmol quanta m^{-2} s^{-1} was the ratio higher before than afterwards.

The decrease in standard activity and protein content with increasing *PPFD* during preconditoning was due to incomplete extraction of RuBPCO. This was shown with plants taken before the change in the light conditions in the growth cabinet. Leaves were preconditioned at 200 μmol quanta m^{-2} s^{-1} and then extracted in the presence of various $MgCl_2$ concentrations. The extractable amount of standard activity and of RuBPCO protein increased as $MgCl_2$ concentration was decreased, suggesting that RuBPCO was partly associated with membranes (McNeil and Walker, 1981). The decrease in the extractable amount of RuBPCO in the presence of $MgCl_2$ did not occur if the leaves were preconditioned in the dark (Table 3).

% stimulation of photosynthesis at 2·0 kPa O_2

The % stimulation of photosynthesis at 2·0 kPa O_2 was determined at 550 μmol quanta m^{-2} s^{-1} *PPFD* immediately before and 3 d after the increase in *PPFD* in the growth cabinet (Table 4). The decrease in photosynthesis as measured at 550 μmol quanta m^{-2} s^{-1} after the increase in *PPFD* in the growth cabinet was not associated with a decrease in the % stimulation of photosynthesis at 2·0 kPa O_2, suggesting that the decrease in photosynthesis was not due to a limitation of inorganic phosphate in the photosynthetic chloroplasts.

Carbohydrate contents

Leaf dry weight increased by about 25% when *PPFD* in the growth cabinet was increased. The increase was mostly due to starch. Starch content in the 5th leaves was low before the increase in *PPFD* and increased rapidly as *PPFD* was increased (Fig. 6). Following the increase in *PPFD* (days 50–55), photosynthesis and starch content

TABLE 3. *Effect of* $MgCl_2$ *concentration in the extraction buffer on the amount of extracted standard RuBPCO activity and soluble protein*

The proportion of RuBPCO in the protein was determined electrophoretically after Bauwe (1979). Plants were taken before the increase in *PPFD* in the growth cabinet and 5th leaves were pre-incubated at 200 μmol quanta m^{-2} s^{-1} or overnight in the dark. The low standard activity in the dark appeared to be due to a tight binding inhibitor. Means of three replications ± s.e. are shown.

PPFD (μmol m^{-2} s^{-1})	200	200	200	0
$MgCl_2$ (mol m^{-3})	0	2	20	20
Standard activity (μmol CO_2 m^{-2} s^{-1})	13·8 ± 2·0	14·8 ± 0·8	10·7 ± 0·9	8·8 ± 0·2
Protein (g m^{-2})	5·0 ± 0·30	5·5 ± 0·23	4·3 ± 0·39	4·9 ± 0·21
RuBPCO (%)	66 ± 2·8	67 ± 4·3	53 ± 3·7	68 ± 6·0
RuBPCO (g m^{-2})	3·3 ± 0·28	3·7 ± 0·38	2·3 ± 0·24	3·3 ± 0·34

TABLE 4. *Stimulation of photosynthesis at 2·0 kPa O_2 of 5th leaves of red clover seedlings as measured at 550 μmol quanta m^{-2} s^{-1} PPFD*

Plants were taken immediately before and 3 d after the increase in *PPFD* in the growth cabinet. Means of three determinations ± s.e. are shown.

	Before the increase in *PPFD*	3 d after the increase in *PPFD*
Photosynthesis at 21 kPa O_2 (μmol CO_2 m^{-2} s^{-1})	15·7 ± 1·8	12·5 ± 0·4
Photosynthesis at 2·0 kPa O_2 (μmol CO_2 m^{-2} s^{-1})	20·8 ± 2·0	16·5 ± 0·2
Increase at 2·0 kPa O_2 (%)	33 ± 3·5	32 ± 3·1

showed a negative linear correlation ($r = -0·97$). Electron micrographs were taken immediately before (Fig. 7A) and 5 d after the increase in *PPFD* (Fig. 7B). The micrographs show the formation of large starch granules in the chloroplast stroma after the increase in *PPFD* and suggest that the stromal space was considerably reduced. Sucrose content increased to a lesser extent and glucose and fructose were not affected (Fig. 6).

Photosynthetic metabolites

The total foliar steady-state contents of photosynthetic metabolites in the 5th leaves, as sampled in the growth cabinet, were studied in two independent experiments. Experiment I concentrated on triose phosphate and 3-phosphoglycerate (Fig. 8). The triose phosphates increased considerably after the increase in *PPFD* in the growth cabinet. The ratio of triose phosphate to 3-phosphoglycerate increased accordingly suggesting increasing availability of products from the light reactions (Dietz and Heber, 1986). Photosynthesis and the 3-phosphoglycerate/triose phosphate ratio, as determined after the increase in *PPFD* (days 50–55), were linearly correlated ($r = 0·99$). This result confirms that the decrease in photosynthesis was not due to a limitation by inorganic phosphate in the chloroplasts.

In Experiment II, the increase in triose phosphate and in the triose phosphate/3-phosphoglycerate ratio was also apparent but less pronounced (Table 5). Ribulose 1,5-*bis*phosphate was not changed, suggesting that the decrease in photosynthesis at 550 μmol quanta m^{-2} s^{-1} *PPFD* was not due to a decrease in the content of this metabolite. 3-Phosphoglycerate, fructose*bis*phosphate, fructose 6-phosphate, and glucose 6-phosphate tended to increase. The ATP/ADP ratio was not affected.

DISCUSSION

The decrease in photosynthesis of red clover leaves following an increase in *PPFD* was associated with an increase in carbohydrate content. A decrease in photosynthesis associated with an increase in carbohydrate content has been found in spinach, barley, soybean, ryegrass, millet, and alfalfa when plants had been illuminated continuously for 10–48 h (Foyer, 1988; Travis and Prendergast, 1987; Wardlaw and Eckardt, 1987) and in wheat and cotton following CO_2 enrichment (Azcón Bieto, 1983; Sasek, Delucia and Strain, 1985). The decrease in photosynthesis was attributed to inhibition as a result of accumulated photosynthetic carbohydrates.

Product inhibition of photosynthesis after treatment of the plants in continuous light, at increased light intensity, at increased CO_2 partial pressure or at decreased temperature was found to be associated with a lack of stimulation of photosynthesis at 2·0 kPa O_2 (Schnyder, Mächler, and Nösberger, 1984, 1986; Sharkey, 1985). Leegood and Furbank (1986) found that ATP/ADP and triosephosphate/3-phosphoglycerate ratios were decreased under these conditions and that stimulation at 2·0 kPa O_2 was restored when leaves were treated with inorganic phosphate. This showed that the insensitivity of

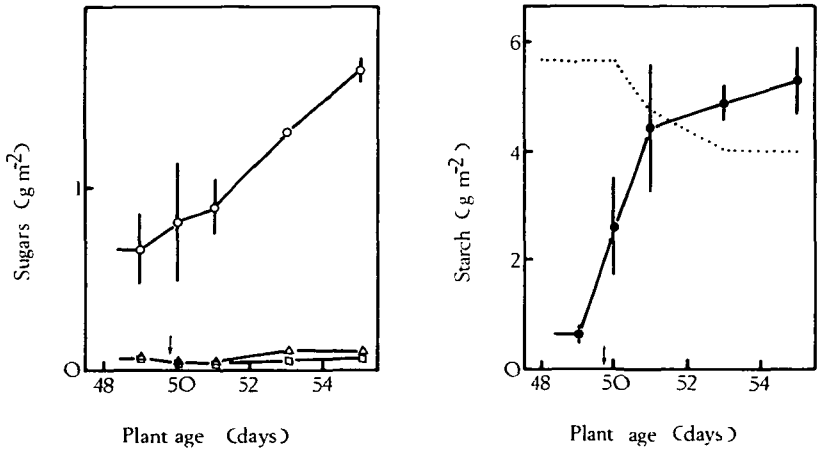

FIG. 6. Leaf contents of sucrose (○), glucose (△), fructose (□), and starch (●) in the 5th leaves before and after an increase in *PPFD* in the growth cabinet (indicated by arrow, 200 μmol quanta m^{-2} s^{-1} before, 550 μmol quanta m^{-2} s^{-1} afterwards). Leaves were taken 4 to 6 h after the beginning of the light period. Means of five determinations ± s.e. are shown. Absence of error bars indicates that s.e. was smaller than symbol size. The dotted line shows photosynthesis at 550 μmol quanta m^{-2} s^{-1} in relative units.

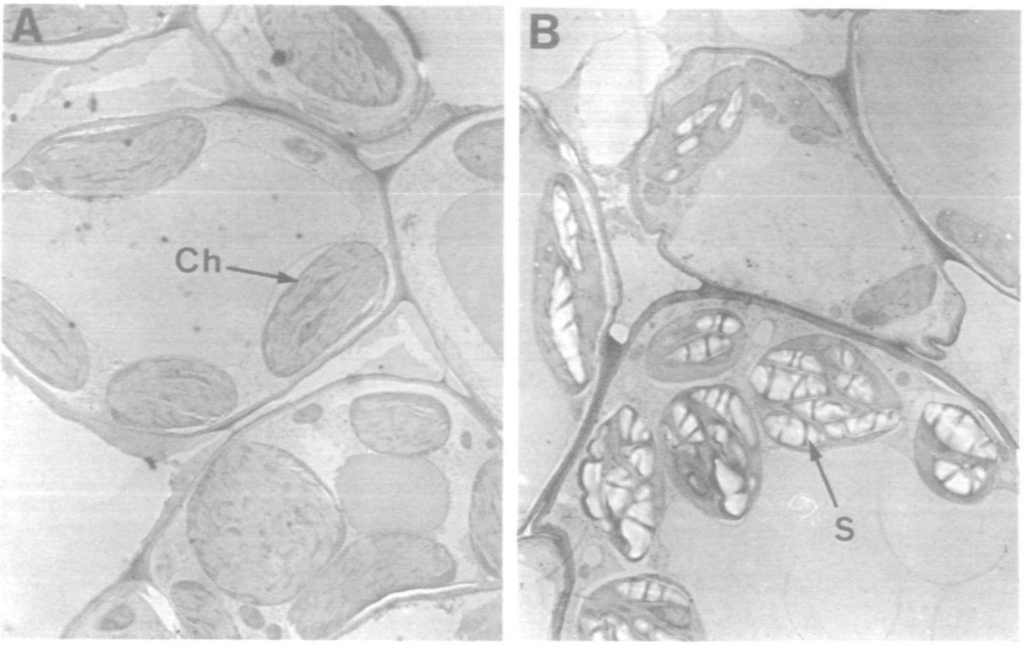

FIG. 7. Electron micrographs of chloroplasts in 5th leaves immediately before (A) and 5 d after (B) an increase in *PPFD* in the growth cabinet. Leaves were taken 4 to 6 h after the beginning of the light period. Enlargement 5000×. Ch: chloroplast; S: starch. (Micrographs by M. Müller, Institut für Zellbiologie, Eidgenössische Technische Hochschule, Zürich.)

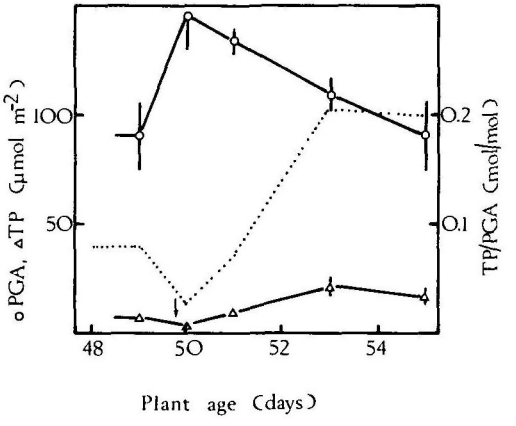

FIG. 8. Total foliar steady-state contents of 3-phosphoglycerate (PGA) and triose phosphate (TP) and molar ratio of TP to PGA (dotted line) in 5th leaves before and after an increase in *PPFD* in the growth cabinet (indicated by arrow, 200 μmol quanta m^{-2} s^{-1} before, 550 μmol quanta m^{-2} s^{-1} afterwards). Leaves were sampled in the growth cabinet. Means of five determinations ± s.e. are shown. Absence of error bars indicates that s.e. was smaller than symbol size.

photosynthesis to O_2 was due to a deficiency in inorganic phosphate. Thus, the generally accepted mechanism for product inhibition of photosynthesis is the limitation of ATP synthesis in chloroplasts due to sequestration of inorganic phosphate in sugar phosphate (Walker, Leegood, and Sivak, 1986).

Our study is not in agreement with this concept. O_2 insensitivity of photosynthesis and decrease in ATP/ADP and triosephosphate/3-phosphoglycerate ratios were not found, suggesting that photosynthesis was not limited by inorganic phosphate. Nevertheless, the decrease in photosynthesis appeared to be due to the accumulation of carbohydrates. The formation of large starch granules in the chloroplasts suggested a decrease in the stromal space and an increase in the concentration of stromal components (e.g. organic and inorganic phosphates). Inorganic and various organic phosphates are activators of RuBPCO. However, they decrease the catalysis of CO_2 fixation per active enzyme site (Hatch and Jensen, 1980). Our study shows that the ratio of actual CO_2 fixation in the leaves to RuBPCO activity was decreased when, after an increase in *PPFD*, starch was accumulated although intercellular CO_2 partial pressure and RuBP content were not affected, suggesting that actual catalysis per active enzyme site was decreased. This decrease in catalysis was independent of the actual light intensity and thus it appeared to be independent of the actual carbon flux. At low *PPFD*, the decrease in catalysis appeared to be compensated for by an increase in RuBPCO activity, thus enabling unreduced CO_2 fixation. At high *PPFD*, the decrease in catalysis resulted in a decreased photosynthesis.

The variation of standard activity of RuBPCO was due to varying proportions of membrane bound enzyme and raised the question as to whether or not membrane bound RuBPCO contributed to photosynthetic CO_2 fixation. Akazawa (1978) suggested that closely co-ordinated interactions may exist between the light-induced events in the thylakoid membranes and the carboxylase reaction also

TABLE 5. *Total foliar steady-state contents and molar ratios of photosynthetic metabolites in 5th leaves of red clover seedlings immediately before, 3 h after and 4 d after the increase in* PPFD *in the growth cabinet*

Leaves were sampled in the growth cabinet. Means of five replications ± s.e. are shown.

	Before the increase in *PPFD*	3 h after the increase in *PPFD*	4 d after the increase in *PPFD*
		μmol m^{-2}	
Ribulose 1,5-*bis*phosphate	26·1 ± 4·5	23·0 ± 2·0	23·4 ± 2·8
3-Phosphoglycerate (PGA)	77·6 ± 6·6	83·3 ± 8·2	96·6 ± 10·1
Triosephosphate (TP)	6·9 ± 0·7	9·2 ± 1·3	10·8 ± 2·5
Fructose*bis*phosphate	2·1 ± 0·2	2·3 ± 0·5	4·5 ± 0·6
Glucose 6-phosphate	26·9 ± 2·8	30·5 ± 4·7	31·0 ± 4·1
Fructose 6-phosphate	14·1 ± 1·3	22·2 ± 3·1	21·6 ± 2·5
		mol/mol	
TP/PGA	0·09 ± 0·01	0·11 ± 0·008	0·11 ± 0·013
ATP/ADP	2·2 ± 0·25	2·0 ± 0·12	2·0 ± 0·29

taking place in the membrane system. However, in this study, extracted initial RuBPCO activity appeared to account for photosynthetic CO_2 fixation and a contribution of unextracted membrane-bound enzyme seemed not to be needed. A study of the effect of the decrease in standard activity with increasing *PPFD* on the ratio of photosynthesis to initial RuBPCO activity was instructive. Before the change in the light conditions in the growth cabinet, standard activity decreased by 30% when PPFD was increased from 80 to 200 μmol quanta m^{-2} s^{-1}. Following the change, standard activity decreased by 38% when *PPFD* was increased from 80 to 550 μmol quanta m^{-2} s^{-1} (Fig. 5). Assuming a contribution of membrane bound RuBPCO to photosynthetic CO_2 fixation, it would be expected that the decreases in standard activity would increase the ratio of photosynthesis to initial RuBPCO activity by 43% and 61%, respectively. However, the respective increases in the ratio of photosynthesis to initial RuBPCO activity were only 15% and 22% (Fig. 4). This discrepancy between expected and actual increases in the ratio of photosynthesis to initial RuBPCO activity with increasing *PPFD* appeared not to be due to RuBP concentration, which is not expected to decrease with increasing *PPFD*. Neither was it due to intercellular CO_2 partial pressure (Table 2), suggesting that the contribution of membrane bound RuBPCO to photosynthetic CO_2 fixation was low. Thus, our findings favour the view that the binding and release of RuBPCO to and from membranes contribute to the modulation of RuBPCO activity *in vivo*.

Standard activity at 200 μmol quanta m^{-2} s^{-1} was lower before than after the increase in *PPFD* in the growth cabinet (Fig. 5). This could be due to the decrease in the stromal space in the presence of starch, an increase in the concentration of organic and inorganic phosphates in the stroma, a sequestration of Mg^{2+} by these phosphates, and a concomitant release of the membrane bound enzyme. The increase of active RuBPCO in the stroma was possibly the reason for down regulation of the degree of activation under these conditions (Fig. 5). The degree of activation of extracted RuBPCO, as estimated from the ratio of initial to standard activity, increased as *PPFD* during preconditioning of the leaves was increased, suggesting that regulation of RuBPCO activity occurred by carbamylation and decarbamylation of the enzyme. However, after a dark period of 14 h, standard activity of RuBPCO was decreased by 34% suggesting that regulation by a tight binding inhibitor also occurred. The ratio of initial to standard activity was greater than unity at high *PPFD*. This is a peculiarity of our method and is due to high initial activities which fully account for photosynthetic CO_2 fixation as shown in this study. Incubation of the extracts in the presence of bicarbonate decreases the activity to a stable standard activity (Mächler and Nösberger, 1988). Standard activity is, therefore, not maximum activity as is often wrongly assumed and the calculation of % activation is unreasonable.

CONCLUSIONS

The properties of RuBPCO were affected by an increase in *PPFD*, probably due to accumulated starch. Effects on RuBPCO activity appeared to be due to the binding and release of the enzyme to and from membranes and to changes in the degree of activation. The catalysis of CO_2 fixation per active RuBPCO site appeared to be affected also, leading to decreased photosynthetic capacity.

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