

# Protein-Bound Ribulose Bisphosphate Correlates with Deactivation of Ribulose Bisphosphate Carboxylase in Leaves<sup>1</sup>

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## ABSTRACT

Previous reports indicate that ribulose 1,5-bisphosphate (RuBP) binds very tightly to inactive ribulose bisphosphate carboxylase (rubisco) *in vitro*. Therefore, we decided to investigate whether there was evidence for tight binding of RuBP associated with deactivation of rubisco *in vivo*. We modified a technique for rapidly separating 'free' metabolites from those bound to high molecular compounds. *Arabidopsis thaliana* plants were illuminated at various irradiances before freezing the leaves in liquid N<sub>2</sub> and assaying rubisco activity and RuBP. The percentage activation of rubisco varied from 37% at low irradiance (45 micromoles quanta per square meter per second) to 100% at high irradiance (800 micromoles quanta per square meter per second). The total amount of RuBP did not vary much with irradiance, but bound RuBP changed from 36% of the total at low irradiance to none at high irradiance. Bound RuBP was significantly correlated with the estimated number of inactive rubisco sites, with a ratio of about 1:1. After a step increase in irradiance, rubisco activation increased and total RuBP increased transiently, but steady levels of both occurred by 10 minutes. The amount of bound RuBP decreased with a similar time course to the estimated decrease in inactive rubisco sites. After a step decrease in irradiance, rubisco deactivated slowly for at least 25 minutes. Bound RuBP increased gradually but did so more slowly than the estimated increase in inactive rubisco sites.

The enzyme rubisco<sup>2</sup> catalyses photosynthetic CO<sub>2</sub> fixation with the substrates RuBP and CO<sub>2</sub> and photorespiratory oxygenation with the substrates RuBP and O<sub>2</sub>. Binding of CO<sub>2</sub> and Mg<sup>2+</sup> is required before the isolated enzyme is active *in vitro* (13, 14). Activation of higher plant rubisco *in vitro* is severely inhibited if RuBP is present (6, 10, 13, 16), and it was demonstrated that RuBP excludes activator CO<sub>2</sub> from the enzyme (10). Inactive rubisco (without CO<sub>2</sub> and Mg<sup>2+</sup>) binds RuBP very tightly. *K<sub>d</sub>* values of 1 μM (30) and 0.021 μM (10) were reported for spinach rubisco, whereas the *K<sub>m</sub>* (RuBP) for activated enzyme was 2 to 3 orders of magnitude higher (10). The extent to which RuBP inhibited activation *in vitro* was attributed to the low *K<sub>d</sub>* (RuBP) of inactive rubisco and also to the small value of the dissociation rate constant (10).

When describing rubisco kinetics mathematically, von Caemmerer and Farquhar (4) concluded that rubisco could not function effectively *in vivo* with the low *K<sub>d</sub>* values quoted above.

It was reported recently (21) that activation of rubisco *in vitro* is possible in the presence of RuBP if a stromal protein called rubisco activase is present. ATP is also required (28) but the mechanism of activation is not yet known.

A number of environmental conditions cause partial deactivation of rubisco in leaves, including low irradiance (5, 15, 20, 29). However, despite the studies on interactions between rubisco and RuBP *in vitro*, it is not known whether RuBP binds to inactivate rubisco *in vivo*. In the present study, we modified a technique for rapidly separating 'free' metabolites from those tightly bound to high mol wt compounds and applied it to leaf extracts from *Arabidopsis* plants frozen under different irradiances. The aim was to find whether changes in rubisco activation state *in vivo* were associated with changes in the amount of tightly bound RuBP.

## MATERIALS AND METHODS

*Arabidopsis thaliana* plants were grown in 50 ml tubes containing a 1:1:1 mixture of vermiculite, perlite, and peat. Day-length was 12 h at 250 μmol quanta m<sup>-2</sup> s<sup>-1</sup> and 25°C; nights were 17°C. Plants were thinned to 1 per tube at 2 to 3 weeks after germination and were watered regularly with Hoagland solution 1 (12). Plants were used in experiments when the leaf area was at least 9 cm<sup>2</sup>.

**Photosynthesis.** Photosynthesis was measured in an open gas-exchange system. All calculations were done according to von Caemmerer and Farquhar (3). Humidified CO<sub>2</sub>-free air was mixed with 3% CO<sub>2</sub> using mass flow controllers (models FC-260 and FC-261, Tylan Corp.,<sup>3</sup> CA). Flow into the plant chamber was measured with a mass flow meter (Tylan, model FM-360). The CO<sub>2</sub> partial pressure in the system and the change in partial pressure caused by the plant were measured with an IR gas analyzer (model 225 MK 3, Analytical Development Co., Hoddesdon, England) fitted with ice traps. The change in water vapor was measured with a chilled mirror hygrometer (model Dew-10, General Eastern Corp., Mass.). Light was from a 500 W quartz-halogen lamp. A water bath and IR filter were placed between the lamp and the chamber. Irradiance was varied with copper or cheesecloth screens. The plant chamber had a temperature-controlled water jacket and leaf temperature was measured with a copper-constantan thermocouple (American gauge 40). The tube in which the plant was growing was sealed over the soil and around the hypocotyl and was inserted halfway into the chamber through the glass floor. At the required sampling time, photosynthesis was measured, and then the plant was pulled rapidly

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<sup>2</sup> Abbreviations: rubisco, ribulose 1,5-bisphosphate carboxylase/oxygenase; RuBP, ribulose 1,5-bisphosphate; PGA, 3-phosphoglycerate.

<sup>3</sup> Mention of a trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the United States Department of Agriculture and does not imply its approval to the exclusion of other products or vendors that may also be suitable.

through the bottom opening into a container of liquid N<sub>2</sub> just below the chamber.

**Rubisco.** Half the leaves of each plant were used to assay rubisco activity (the remainder was used for RuBP measurements). Leaves were ground to a fine powder in a mortar and pestle containing liquid N<sub>2</sub>, the powder was quickly mixed into 100 mM Tricine (pH 8.1) with 10 mM MgCl<sub>2</sub>, and an aliquot was assayed immediately. Another aliquot was assayed after activating with 10 mM HCO<sub>3</sub><sup>-</sup>, 10 mM MgCl<sub>2</sub>, and 0.05 mM gluconate-6-P for 40 min on ice. The 0.5 ml assay contained 100 mM Tricine (pH 8.1), 10 mM MgCl<sub>2</sub>, 10 mM [<sup>14</sup>C]NaHCO<sub>3</sub> (0.5 Ci mol<sup>-1</sup>), and 0.4 mM RuBP. RuBP was synthesized according to Jordan and Ogren (9) with minor modifications. After 30 s at 25°C, the assays were ended by injecting 0.1 ml of 4 N HCOOH plus 1 N HCl. Samples were dried, redissolved in 1 ml of 0.1 N HCl, and mixed with 4 ml of Optifluor (Packard Instrument Co., Ill.) to measure acid-stable radioactivity.

At high irradiance, rubisco activity measured immediately after extraction was somewhat greater than that measured after activating *in vitro* (*i.e.* percentage activation was >100%), perhaps due to some proteolysis during incubation. Using these values to calculate the amount of inactive rubisco would result in negative numbers. We therefore increased all the activated values by the proportion necessary to give a mean of 100% activation at high irradiance. This seemed justified since the activated values showed no significant variation with irradiance. The adjusted values were then used for all calculations of percentage activation of rubisco, and amount of inactive rubisco. The number of inactive rubisco sites was calculated by subtracting the initial rubisco activity from the total activated activity and assuming a specific activity of 2 mol CO<sub>2</sub> mol sites<sup>-1</sup> s<sup>-1</sup> (equivalent to 1.75 μmol CO<sub>2</sub> min<sup>-1</sup> mg rubisco<sup>-1</sup>). Reports indicate that C<sub>3</sub> species vary in rubisco specific activity (*e.g.*, 26 and references cited therein), but there are no published values for *Arabidopsis*. The value we used is within the range of published values when these are adjusted to 25°C.

**RuBP.** In order to separate bound from free RuBP, we modified the method of Penefsky (19). A thin layer of glass wool was placed in the bottom of 5 ml syringe tubes. The tubes were filled with Sephadex G-50-150 which was equilibrated with 100 mM Tricine (pH 8.1) and then suspended in 15 ml centrifuge tubes. These were centrifuged for 20 s at 900 g and then kept on ice until just before loading the leaf extract. After grinding in liquid N<sub>2</sub>, the frozen leaf powder was mixed into cold 100 mM Tricine (pH 8.1). One 0.9 ml aliquot was injected into 0.9 ml of 7 N HCOOH and used to determine the total RuBP concentration. Another 0.9 ml aliquot was pipetted onto the top of a cold Sephadex column and centrifuged for 45 s at 900 g. Approximately 10 s elapsed between mixing the leaf powder in Tricine and starting the centrifuge. The eluent (containing the high mol wt compounds) was quenched with 1.15 ml of 7 N HCOOH in the bottom of the centrifuge tube. The formic acid extracts were centrifuged for 3 min at 17,000g. The supernatant was retained, dried under vacuum, and resuspended in 200 mM Tricine (pH 8.1). RuBP assays were in 0.5 ml containing 200 mM Tricine (pH 8.1), 10 mM MgCl<sub>2</sub>, 10 mM [<sup>14</sup>C]NaHCO<sub>3</sub> (1 Ci mol<sup>-1</sup>), and 25 μg rubisco. Rubisco was purified according to Salvucci *et al.* (24). Assays were ended after 45 min at room temperature by injecting 0.1 ml of 4 N HCOOH plus 1 N HCl. Acid-stable radioactivity was determined as for rubisco assays.

Chl per leaf area and in Tricine extracts was measured according to Arnon (1).

## RESULTS

**Testing the Technique.** The technique did not yield a complete separation of rubisco from free RuBP, *i.e.* eluents did not contain 100% of the rubisco and 0% of free RuBP. When rubisco alone

(1 mg ml<sup>-1</sup> or 14 μM sites) was added to the columns, 88.7% was obtained in the eluent (mean of 4 samples). With RuBP alone, less than 10% appeared in the eluent (Tables I and II).

Deactivated rubisco was incubated with RuBP at twice the concentration of binding sites. After centrifuging, 33% of the total RuBP occurred in the eluent, and this percentage was not significantly altered if activated rubisco was added immediately before centrifuging (Table I), nor did such addition of activated rubisco affect the small percentage of free RuBP occurring in the eluent (Table I). Thus, it appears that no significant metabolism of either bound or free RuBP occurred during the time required for separation.

When RuBP was added to deactivated rubisco immediately before centrifuging, 29.0% of the added RuBP appeared in the eluent (Table I). This indicates that significant binding of free RuBP to purified rubisco can occur during centrifuging. If deactivated rubisco was incubated with PGA prior to adding RuBP, less binding of RuBP occurred during centrifuging (Table I).

To test whether binding of free RuBP occurred during centrifugation of leaf extracts, we added RuBP to extracts from leaves sampled under different irradiances. Such binding seemed most likely to be detected in extracts from darkened leaves since these contained deactivated rubisco (mean activation was 45%) but no endogenous RuBP (which might also bind during centrifuging). The percentage of added RuBP that was obtained in the eluent (endogenous RuBP was subtracted) was no greater with leaf extracts than with RuBP alone and was not significantly (*P* < 0.05) affected by the irradiance level at which the leaves were sampled (Table II). We conclude that free RuBP did not bind significantly to high mol wt compounds during centrifuga-

Table I. Testing the Separation of Free and Bound RuBP using Purified Rubisco

Column 1 indicates various mixtures that were incubated for 30 min on ice, column 2 indicates any additions that were made immediately before centrifuging, and column 3 shows the percentage of total RuBP that was obtained in the eluent. ECM is rubisco incubated with 10 mM HCO<sub>3</sub><sup>-</sup> and 10 mM Mg<sup>2+</sup> and E is rubisco with no HCO<sub>3</sub><sup>-</sup> or Mg<sup>2+</sup>. Specific activities were 1.4 μmol mg<sup>-1</sup> min<sup>-1</sup> (25°C) for the former and 1% of this for the latter. Final solutions added to the columns all contained 100 mM Tricine (pH 8.1), 0.1 mM EDTA, and 28 μM RuBP. Rubisco site concentration was 14 μM (1 mg ml<sup>-1</sup>) or 28 μM when ECM was added to E. PGA concentration was 56 μM.

Incubation	Addition	% RuBP in eluent (SE)	Sample No.
E + RuBP		33.2 (2.2)	4
E + RuBP	ECM	36.9 (0.1)	2
E	RuBP	29.0 (2.5)	2
RuBP		6.4 (1.1)	2
RuBP	ECM	7.6 (0.4)	2
E + PGA	RuBP	20.9 (2.0)	4

Table II. Testing Separation of Free and Bound RuBP with Leaf Extracts

Added RuBP in the eluent is given as a percentage of that added to the top of the column. All solutions were in 100 mM Tricine (pH 8.1). Frozen leaf powder was mixed into buffer with and without added RuBP in order to measure and subtract the amount of endogenous RuBP in the eluent. Leaves in the dark had no detectable RuBP. The estimated rubisco site concentration in leaf extracts was about 4 to 5 μM.

Solution Added to Column	% in Eluent (SE)	Sample No.
9 μM RuBP	9.0 (0.5)	2
9 μM RuBP + high light leaves	6.8 (1.5)	3
+ low light leaves	7.4 (2.4)	3
+ dark leaves	8.3 (1.3)	6

tion of leaf extracts. Subsequent determinations of free and 'protein-bound' RuBP in leaf extracts were calculated with the assumption that the eluent contained 88.7% of the rubisco and 7.1% of the free RuBP (mean of high and low irradiance samples in Table II).

**Measurements on Leaves under Constant Irradiance.** Figure 1 shows the effect of irradiance on  $\text{CO}_2$  assimilation rate and the total amount of RuBP in *Arabidopsis* leaves. In this experiment the plants were kept for 45 min at each irradiance before taking measurements. As irradiance was increased from 45 to  $800 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$ , there was a large increase in the  $\text{CO}_2$  assimilation rate, whereas the amount of RuBP did not vary greatly over this range.

The percentage activation of rubisco declined from 100% at high irradiance to 37% at  $45 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$  (Fig. 2). The total activity (measured after activation *in vitro*) did not change

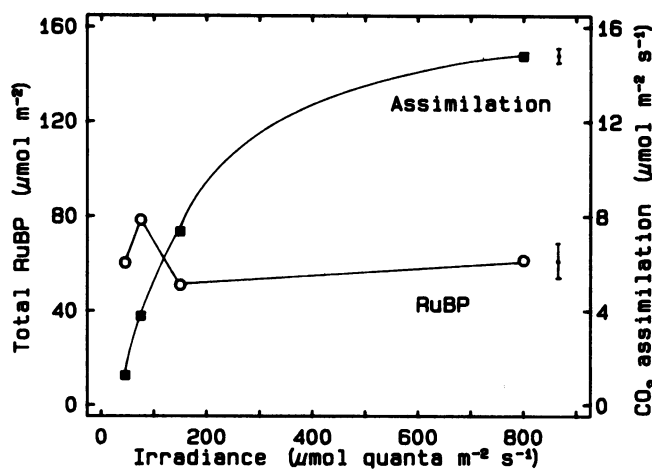


FIG. 1. Effects of irradiance on  $\text{CO}_2$  assimilation rate and total RuBP. Plants were at each irradiance for 45 min before taking measurements. Leaf temperature was  $25^\circ\text{C}$ , external  $\text{CO}_2$  was  $340 \mu\text{bar}$ , and  $\text{O}_2$  was 21%. Each point is the mean of 5 plants. The SE for each variable was calculated by analysis of variance and is indicated by the bars on the figures.

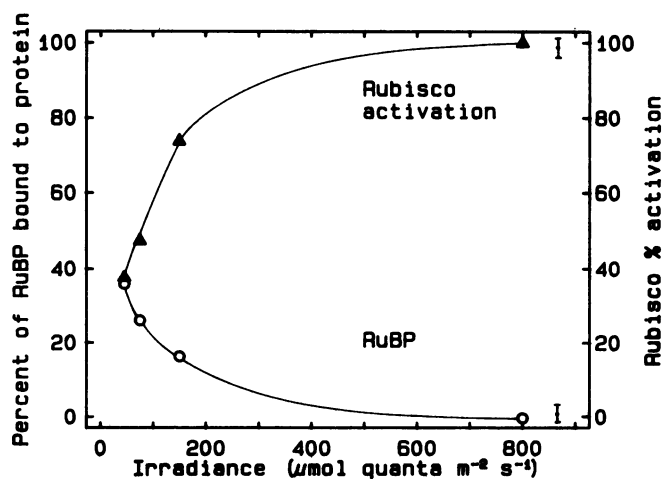


FIG. 2. Effects of irradiance on rubisco activation and protein-bound RuBP. Plants were at each irradiance for 45 min. The values of rubisco percentage activation were adjusted to give a maximum of 100% (see "Materials and Methods"); the actual highest value was 112%. Protein-bound RuBP is that which separated with the high mol wt compounds after centrifuging through Sephadex. Points are the means of 5 plants; bars are as in Figure 1.

significantly with irradiance (data not shown). The proportion of protein-bound RuBP is also shown in Figure 2. At high irradiance, there was no bound RuBP, but as irradiance and rubisco activation decreased, the amount of bound RuBP increased and was 36% of the total RuBP at  $45 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$ .

In Figure 3 the actual amounts of protein-bound RuBP are plotted against the estimated number of active sites on rubisco. There was a significant correlation between bound RuBP and inactive rubisco with a ratio of about 1:1.

**Measurements after a Change in Irradiance.** In these experiments, we compared the time course of changes in rubisco activation and bound RuBP after a step change in irradiance. Changes in irradiance had no effect on the rubisco activity measured after activating *in vitro* (data not shown).

When the irradiance was decreased from 700 to  $35 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$  the total amount of RuBP decreased dramatically in the first 2 min and then gradually increased again, whereas rubisco activation decreased continuously over the whole 25 min of measurements (Fig. 4a). The rate of  $\text{CO}_2$  assimilation dropped rapidly to below zero at 3 min then increased slightly and remained constant from 5 min onwards (data not shown).

Conversely, when irradiance was suddenly increased the total RuBP concentration increased transiently, but by 10 min it had decreased to a steady level somewhat higher than the initial value. Rubisco activation increased from 35% initially to 95% after 5 min and remained at 100% from 10 min onward (Fig. 5a). The rate of  $\text{CO}_2$  assimilation increased with a time course similar to the changes in rubisco activation (data not shown).

After the step increase in irradiance, the amount of protein-bound RuBP and the estimated number of inactive rubisco sites both decreased, and their changes showed very similar time courses (Fig. 5b). After the step decrease in irradiance, the amounts of inactive rubisco and protein-bound RuBP both gradually increased, but the latter remained less than the former during the 25 min of measurements (Fig. 4b).

## DISCUSSION

After centrifuging through Sephadex, extracts from leaves frozen under low irradiance had more RuBP in the eluent (con-

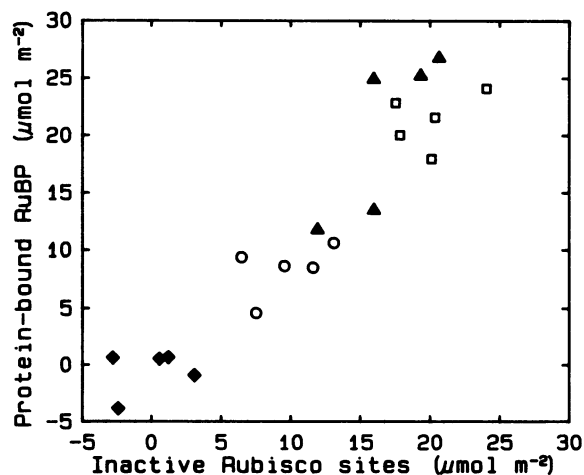


FIG. 3. Amounts of RuBP separating with the high mol wt compounds plotted against the number of inactive rubisco sites. The latter numbers were estimated from the measured rubisco activities and assuming a specific activity of  $2 \text{ mol CO}_2 \text{ mol sites}^{-1} \text{ s}^{-1}$ . The total of active and inactive rubisco sites was estimated as  $34 \mu\text{mol m}^{-2}$  (mean of all data). Symbols represent irradiances of 800 ( $\blacklozenge$ ), 150 ( $\circ$ ), 75 ( $\blacktriangle$ ), and 45 ( $\square$ )  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ . Plants were at each irradiance for 45 min. Linear regression of the data was significant, with a correlation coefficient of 0.94 and a slope of 1.1.

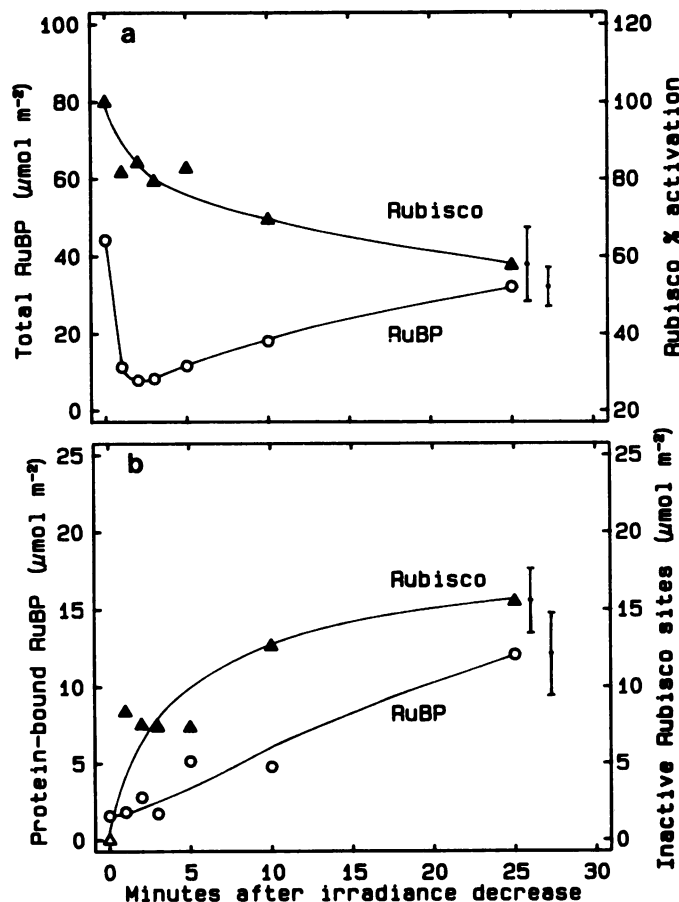


FIG. 4. Effects of a step decrease in irradiance on RuBP and rubisco. Plants were at  $700 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$  for 35 min before decreasing the irradiance to  $35 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$  (at time 0). Points are the means of 2 plants. Bars are as in Figure 1. The total of active and inactive rubisco sites was estimated as  $40 \mu\text{mol m}^{-2}$  (mean of all data). a, Changes in total RuBP and in rubisco activation. Values of percentage activation were adjusted to give a maximum of 100% (see "Materials and Methods"). The actual highest value was 121%. b, The amounts of protein-bound RuBP and inactive rubisco were calculated as in Figure 3.

taining the high mol wt compounds) than did extracts from leaves sampled at high irradiance. In order to determine whether this was a result of RuBP binding to inactive rubisco during the centrifugation process, we added free RuBP to extracts from leaves sampled at different irradiances and found no evidence for such binding (Table II). However, RuBP did bind to purified deactivated rubisco during centrifugation (Table I). Two possible reasons for this difference come to mind. First, since many chloroplast metabolites can bind to rubisco *in vitro* (see "Introduction"), little of the rubisco in leaves may be present in the E (Table I) form. If most of the deactivated rubisco in leaves has some bound metabolite (perhaps PGA or gluconate-6-P in darkened leaves), this may retard the binding of added RuBP. Second, free phosphorylated metabolites in the leaf extract may competitively inhibit binding of RuBP. Incubation of purified deactivated rubisco with PGA did partially prevent binding of RuBP during centrifuging (Table I), and this may be due to the two suggestions given above. Whatever the reason, the results in Table II indicate that free RuBP in leaf extracts did not bind to high mol wt compounds during centrifuging. We therefore conclude that the significant effect of irradiance on the proportion of endogenous RuBP obtained in the eluent (Fig. 2) was a result of RuBP binding tightly to some high mol wt compound(s) *in vivo*.

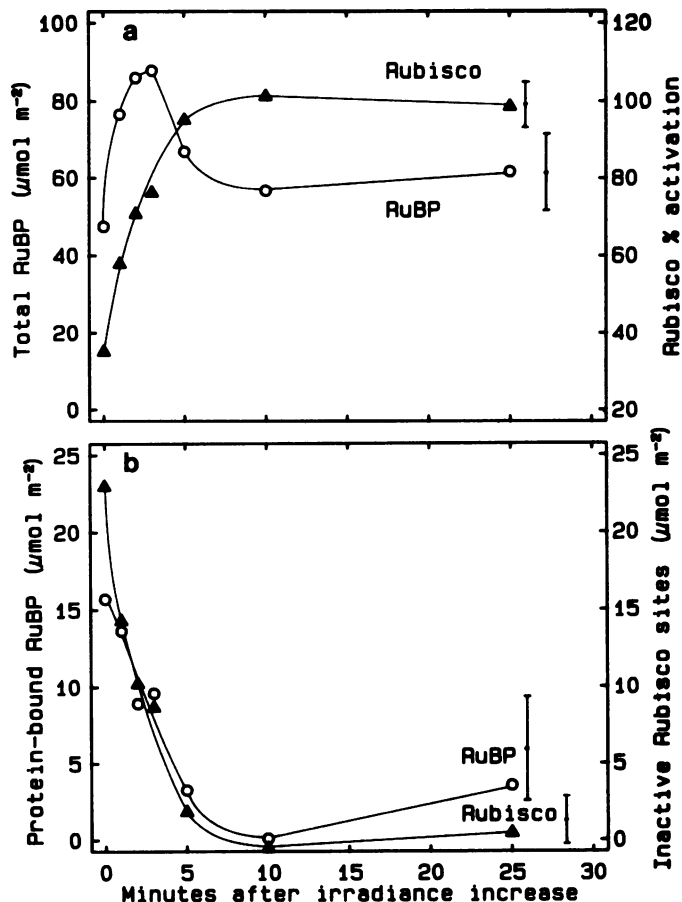


FIG. 5. Effects of a step increase in irradiance on RuBP and rubisco. Plants were at  $35 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$  for 35 min before increasing the irradiance to  $700 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$  (at time 0). Points are the means of 2 plants. Bars are as in Figure 1. The total of active and inactive rubisco sites was estimated as  $37 \mu\text{mol m}^{-2}$  (mean of all data). a, Changes in total RuBP and in rubisco activation. Values of percentage activation were adjusted to give a maximum of 100% (see "Materials and Methods"). The actual highest value was 116%. b, The amounts of protein-bound RuBP and inactive rubisco were calculated as in Figure 3.

Although we have no direct evidence that this bound RuBP was associated with rubisco, it seems likely because of the significant correlation between the amount of bound RuBP and the amount of inactive rubisco shown in Figure 3. These data indicate that all the inactive sites may be bound with RuBP. However, the exact slope in this figure depends on the value of specific activity used in the calculations (a lower specific activity would give more sites than bound RuBP) and on whether any bound RuBP dissociated during centrifugation, thus underestimating the amount of bound RuBP. Measurements by Jordan and Chollet (10) indicated that dissociation of RuBP bound to purified inactive spinach rubisco was extremely slow as more than 90% of the label in RuBP eluted with the protein after a 12 min gel filtration (Sephadex G-75) at 2°C. Using the data in Table I and assuming 8 binding sites per holoenzyme, we calculated that only 64% of the bound RuBP separated with the protein after centrifuging through Sephadex. If 6.5 binding sites per holoenzyme are assumed, as found by Jordan and Chollet (10), then the percentage would be 79%. However, since association of RuBP and deactivated rubisco during centrifugation was different for purified enzyme than for leaf extracts (see above discussion), dissociation may also differ. Therefore, we did not attempt to make a correction for dissociation in the data from leaf extracts.

In theory the procedure could be used to determine other bound metabolites, but recovery is likely to be poor with compounds that dissociate rapidly relative to the time required for separation.

The method indicated no RuBP bound to protein at high irradiance (Fig. 2). However, RuBP obviously does bind to active rubisco *in vivo* since photosynthesis occurs. Evidently, RuBP bound to active rubisco either dissociated or was converted to products during the centrifugation through Sephadex, and only that bound to inactive rubisco appeared in the eluent.

As mentioned in the "Introduction," activation of rubisco *in vitro* requires binding of CO<sub>2</sub> and Mg<sup>2+</sup> to form a carbamate. Some studies of rubisco *in vitro* indicate that inactive forms of carbamylated rubisco may exist (18) (J Pierce, personal communication; D Edmondson, J Andrews, personal communication) but it is not known if such forms exist *in vivo*. Measurements on isolated chloroplasts (2) indicated that the proportion of active rubisco corresponded directly to the amount of carbamylated enzyme, but no such measurements have been made on leaves. Therefore, we cannot be sure whether the RuBP bound to inactive rubisco in leaves is bound to carbamylated or to decarbamylated enzyme, although the latter seems most likely.

Various authors have used measurements of RuBP in leaves to determine what factors limit photosynthesis under various conditions. The current data are pertinent to this question because if a significant proportion of RuBP is tightly bound to inactive rubisco then measurements of total leaf RuBP will not be indicative of the amount of RuBP available for catalysis. Furthermore, von Caemmerer and Farquhar (4) have suggested that some RuBP may be chelated with stromal Mg<sup>2+</sup> and hence also unavailable for catalysis.

When irradiance was constant, the ratio of bound RuBP to inactive rubisco was about 1:1 (Fig. 3). A similar ratio held as both declined after irradiance was suddenly increased (Fig. 5b). However, after a step decrease in irradiance the amount of bound RuBP remained less than that of inactive rubisco for most of the time course (Fig. 4b). The total amount of RuBP dropped dramatically (an 80% reduction after 2 min, Fig. 4a), and so there was much less available to bind. Nevertheless, at no point during the time course was all this RuBP bound. Bound RuBP was only 16% of the total RuBP after 1 min and remained between 20 to 40% from 2 min onwards (data not shown), even though the amount of inactive rubisco was greater than this. Apparently, not all the RuBP in the leaf could bind to inactive rubisco under these conditions. Some of the RuBP is undoubtedly bound to active rubisco *in vivo*. It is also possible that some RuBP is chelated with Mg<sup>2+</sup> in the stroma. von Caemmerer and Edmondson (5) estimated that about 40% of the total RuBP may be bound to Mg<sup>2+</sup> under high irradiance and atmospheric [CO<sub>2</sub>]. The amount depends *inter alia* upon the stromal [Mg<sup>2+</sup>] and the [PGA] (since PGA also binds to Mg<sup>2+</sup>).

Another possibility is that some metabolite(s) other than RuBP were bound to some of the inactive rubisco sites. Many phosphorylated stromal metabolites can bind to inactive rubisco *in vitro* with varying affinities (11). *In vivo*, the most likely candidate is PGA since it is present in relatively high concentrations and has quite a high affinity for inactive rubisco ( $K_d = 25 \mu\text{M}$ ; 11). Previous reports found that after a step decrease in irradiance the amounts of most phosphorylated metabolites decreased transiently, whereas the amount of PGA showed a transient increase (22, 27) and doubled in less than 1 min (22). Since RuBP decreased markedly (Fig. 4a) the ratio of PGA/RuBP must have changed even more, and it seems possible that some of the inactive rubisco sites were bound with PGA. Such binding of PGA seems unlikely during constant irradiance or after a step increase in irradiance because RuBP appeared to fill all the inactive rubisco sites (Figs. 3 and 5b).

Deactivation of rubisco after a step decrease in irradiance was very slow and appeared to be continuing even after 25 min at low irradiance (Fig. 4a). A similar slow deactivation has been reported by others (22, 25). Presumably this slow deactivation allowed the slow increase in total RuBP which occurred after the initial rapid decline. Similar changes in total RuBP were reported previously (17, 22), and, in fact, Mott *et al.* (17) show RuBP still increasing up to 60 min after a step decrease in irradiance.

In contrast, activation of rubisco after a step increase in irradiance was almost complete after 5 min, and the total RuBP pool reached a constant size by at least 10 min (Fig. 5a). Rubisco activase was found to be required for significant activation to occur *in vitro* in the presence of RuBP (21). The time course of such activation (21, 28) was similar to that found in leaves (Fig. 5a). The rate of deactivation *in vitro* was not affected by activase (21) but was more rapid than the rate found in leaves (Fig. 4a; Refs. 22, 25). Phosphorylated effectors bind to both the active and inactive forms of rubisco. Since binding or release of the activating CO<sub>2</sub> and Mg<sup>2+</sup> occurs only with effector-free enzyme, these effectors inhibit the rates of activation and of deactivation, but the latter rate was found to be inhibited more than the former by several effectors (11). Hence, it is possible that the rate of deactivation *in vivo* after a step decrease in irradiance (Fig. 4a) is retarded by the presence of some phosphorylated effector(s) bound to the activated rubisco.

What causes the differences in the final activation state of rubisco under different irradiances (Fig. 2) (5, 15, 20, 29)? Two possibilities are changes in pH or [ATP]. Rubisco activation is affected by pH, whether incubated in the presence of activase and RuBP (21) or without activase (14, 16). Measurements of light scattering from leaves indicate indirectly that the trans-thylakoid pH gradient changes over a considerable range of irradiances (8, 25). Changes in stromal pH are likely to be less than this because of the buffering capacity of the stroma; however, stromal pH has never been measured in leaves.

Activation of rubisco with activase *in vitro* increased with increasing [ATP] (28) and there was a strong correlation between rubisco activation and [ATP] in isolated chloroplasts (23). Measurements in leaves showed a transient drop in [ATP] or ATP/ADP after a decrease in irradiance (22, 27), but Dietz and Heber (7) found that the steady state levels in atmospheric CO<sub>2</sub> did not appear to vary greatly with irradiance. We conclude that, at present, the effect of irradiance on rubisco activation remains unexplained. More extensive measurements of ATP and rubisco activation in leaves under various conditions may help to resolve this question.

**Note Added in Proof.** Measurements by Keith Mott (personal communication) using a different method also indicate the presence of tightly bound RuBP *in vivo*. When leaves were transferred from various irradiances into darkness, there was a pool of RuBP that was only slowly metabolized, and this pool correlated with the amount of inactive rubisco.

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