The Effect of Irradiance on the Activation State of Ribulose Bisphosphate Carboxylase in Soybean Leaves

by

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(ABSTRACT)

Effect of irradiance on both initial and total ribulose bisphosphate carboxylase (RuBP Case) activities was investigated in soybean (Glycine max cv. Will) leaves. Initial RuBP Case activity (IA), present in a leaf extract prepared at 0°C and assayed without prior activation, was used as an estimate of in vivo activity. Total activity (TA) was measured in leaf extracts after full activation in high levels of CO_2 and Mg^{2+}.

Observed large variations in RuBP Case IA and TA in preliminary study, which prompted the necessity for optimizing conditions for extracting and assaying the enzyme in soybean. In a series of experiments optimal assay pH, RuBP levels, Mg^{+} levels, and CO_2 levels were determined. Stability of RuBP Case in leaves and extracts was also investigated; observed storage of leaf tissue or extract at 0°C maintained stability of RuBP Case activation state for hours.

Modified assay procedure was used to measure RuBP Case IA and

TA in leaves of soybean plants grown in 4 different irradiances: 20%, 53%, 70% and 100% full sun (FS). TA expressed on a leaf area basis was significantly reduced with lower irradiance, the result of a reduction in leaf volume. Percent activation (%act, IA/TA) was similar for the higher irradiance treatments (approx. 94%), but lower (74%) in leaves of the 20% FS grown-plants. However, percent activation in the 20% and 100% FS grown-plants was similar when leaves were exposed briefly to similar light conditions. Concluded %act to be dependent on leaf irradiance level at the time of leaf removal and extraction and not influenced by irradiance level during growth.

Diurnal response of RuBP Case IA and TA also investigated in soybean plants growing in field, greenhouse and growth chamber. In all 3 environments, both IA and TA increased with increasing irradiance. Diurnal changes in TA were not due to changes in level of Fraction 1 protein (RuBP Case). Concluded light may also have a role in increasing TA as well as IA of RuBP Case.

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INTRODUCTION

Ribulose bisphosphate carboxylase (RuBP Case) is the most abundant protein in nature, comprising up to 50% of the soluble protein of plant leaves and is believed to be a major regulatory enzyme of photosynthesis. The protein catalyzes the incorporation of CO_2 to RuBP to form 2 molecules of phosphoglycerate (PGA; Weissbach et al, 1956). The protein also has an oxygenase function (Ogren and Bowes, 1971), whereby PGA and phosphoglycolate are formed from one molecule of RuBP and O_2. This latter reaction occurs as a result of competition between CO_2 and O_2 at the enzyme's catalytic site. Significant progress has been made in recent years in elucidating the mechanisms of activation and catalysis of purified RuBP Case. However, the physiological and environmental factors which regulate RuBP Case functioning in vivo are not completely understood. Such information could be significant, because there is evidence that dramatically increased crop yield would result from increased photosynthetic carbon uptake (Hardy et al, 1978; Zelitch, 1975). RuBP Case is of particular interest in this respect, because it functions inefficiently in fixing CO_2 at atmospheric conditions and therefore may be a major limiting

factor in photosynthesis. One other characteristic of the enzyme that needs further study is the oxygenase reaction. Oxygenation of RuBP results in the formation of phosphoglycolate, which is further metabolized in the photorespiratory carbon cycle with the consequent release of CO_2 at the expense of ATP and reducing power generated by the light reactions. This process is believed (Zelitch, 1975) to be responsible for the loss of up to 50% of the photosynthetically fixed CO_2 fixed daily in C3 plants (plants which primarily use RuBP Case as the CO_2-fixing enzyme).

Purified RuBP Case is activated in a two-step process involving first the addition of CO_2 to an activation site on the enzyme in a rate-limiting step, followed secondly by the rapid addition of a Mg^{2+} ion (Lorimer, 1981; Laing and Christseller, 1976). The final Enzyme-CO_2-Mg^{2+} (ECM) complex is believed to be the active form of the enzyme. The $K_{act}(CO_2)$ for this activation process has been estimated to be much higher than CO_2 levels in the atmosphere (Laing and Christseller, 1976). Thus, RuBP Case may exist in a partial state of activation *in vivo*. Evidence to support this possibility has been shown in measurements of the enzyme activity *in vivo*. Perchorowicz *et al*. (1981) measured RuBP Case activity in an extract of wheat leaf tissue without

prior activation, and observed a lower activity than that which was observed in extract allowed to fully activate in CO_2 and Mg^{2+} prior to assay. The lower activity was believed to represent the *in vivo* amount of RuBP Case activity in the leaf (termed "initial" activity), and the higher activity represented the total amount of activatable RuBP Case. The difference between initial and total activity represented the amount of RuBP Case which was inactive *in vivo*. In several different plant species, a substantial amount of the total RuBP Case in the leaf was found to be inactive. Perchorowicz *et al* (1981, 1982) estimated the percent activation (initial activity /total activity) to be 60% for chamber-grown wheat, 70% for chamber-grown spinach, and 80% for greenhouse-grown soybeans. Therefore only a part of the total RuBP Case is active *in vivo*, and could represent a potential for increasing photosynthesis rate.

Light is believed to play a major role in regulating the activity of RuBP Case *in vivo*. Upon illumination, photosynthetic electron transport induces an efflux of Mg^{2+} from the thylakoids into the stroma in exchange for H+ ions resulting in an increase in stromal Mg^{2+} concentration as well as an increase in stromal pH from 7.0 to 8.0. These changes in stromal H+ and

Mg^{2+} concentrations would favor activation of RuBP Case. Hence, RuBP Case in vivo activity could be regulated irradiance level. Studies by Perchorowicz et al (1981, 1983) and Sicher (1982) in which photosynthetic CO_2-fixation rate and RuBP Case initial activity were measured simultaneously under different irradiances showed that RuBP Case initial activity and photosynthesis both increased with increasing irradiance, presumably as a result of increased stromal Mg^{2+} and pH.

Irradiance level during growth is known to strongly influence plant growth and development (for a review, see Boardman, 1977), yet little is known of the effect of altering irradiance during growth on the percent activation of RuBP Case. Plant species adapted to growing in full sun exhibit drastic alterations of leaf anatomy, photosynthetic capacity, chloroplast structure and in the amount and activity of RuBP Case (Bjorkman, 1971, Bowes et al, 1972; Blenkinsop and Dale, 1975; Crookston et al, 1975; Gauhl, 1975; Singh et al, 1974). Earlier studies which measured the effect of low irradiance during growth on RuBP Case activity were conducted prior to the time that the mechanism for activation of RuBP Case was fully understood. Therefore, these studies are difficult to interpret in considering the effect of altered irradience during growth on

the *in vivo* state of activation of RuBP Case. Although the total amount of Fraction-1 protein (RuBP Case) decreased with irradiance during growth (Gauhl, 1977; Blenkinsop and Dale, 1975), RuBP Case percent activation may not necessarily follow that same pattern. Reduced irradiance during growth results in an increase in the chloroplast granal / stromal volume ratio (Bjorkman, 1971; Crookston, 1975). This effect may result in relatively higher Mg^{2+} and pH in the stroma of low irradiance-grown plants, resulting in conditions which favor for the activation of RuBP Case.

The work presented here consists of two main parts. The first (Chapter 1) describes our efforts to optimize conditions for extraction and assay of RuBP Case initial and total activity, in terms of pH, Mg^{2+}, HCO_3-, RuBP levels and preincubation time. In addition, the effect of storage time on the stability of RuBP Case activity in both leaf tissue and after extraction was studied. This study was prompted by a previous attempt (Appendix A) to measure RuBP Case initial and total activities in 4 different soybean cultivars during a growing season using an assay procedure based on those in the current literature (McCermitt *et al.*, 1982; Perchorowicz *et al.*, 1981, 1982). Variations in both initial and total activities were observed

and initial activity actually exceeded total activity throughout the second half of the growing season (Fig. 10, Appendix A) These results may have been due to the conditions under which RuBP Case activity was assayed.

Chapter 2 describes our investigation of the effect of irradiance during growth on initial and total RuBP Case activity in leaves of greenhouse- grown soybean plants, using our modified assay procedure (Chapter 1).

CURRENT STATUS OF RESEARCH

MOLECULAR STRUCTURE AND PHYSICAL PROPERTIES OF RUBP CASE

RuBP Case is the major component of Fraction 1 protein, the name given to the large homogenous protein that was first isolated from green leaves by Wildman and Bonner (1947) and observed to comprise roughly half of the leaf's soluble protein. Estimations of its molecular size came from sedimentation coefficient values, which placed it between 480,000 to 560,000 daltons (see Kawashima and Wildman, 1970). Early studies of the quaternary structure of RuBP Case, using high resolution electron microscopy, described it as having a cubic structure of 24 equal-sized subunits (Haselkorn et al, 1955). A later study (Steer et al., 1968), suggested an octahedral structure, still having 24 subunits. These models were later proven incompatable when it was discovered that the protein contained two different sized subunits (Kawashima, 1969; Rutner et al, 1967; Sugiyama and Akazawa, 1967). Based on this information, and the use of more advanced analytical techniques, a more recent study (Baker et al, 1975) proposed that

the RuBP Case molecule of eukaryotic origin is an oligomer consisting of 8 large subunits (MW ea. = 50,000) and 8 small subunits (MW ea. = 12,000). The large subunit has been shown to contain the catalytic site for CO_2/O_2 fixation (Akazawa, 1979). Studies investigating the mechanism for RuBP Case synthesis and assembly have shown that the large subunit is synthesized on the chloroplast ribosomes but the small subunit is coded on the nuclear DNA and transcribed on the cytoplasmic ribosomes (Ellis, 1981). The small subunit is transported into the chloroplast via a chloroplast envelope carrier mechanism (Highfield and Ellis, 1978). This means that the small subunit is subject to the laws of Mendelian inheritance and the large subunit is inherited maternally. Studies of the variability of RuBP Case among primitive and advanced photosynthesizing organisms (Takabe and Akazawa, 1975) have revealed a strong uniformity of the structure and properties of the large subunit from the various plant and bacteral sources, whereas there is a great deal of variability in the primary structure and properties of the small subunit. Jordan and Ogren (1981) reported in another survey of primitive and advanced plants that there seemed to be a trend in the evolution of RuBP Case to a form that is capable of a more efficient utilization of CO_2. These changes would therefore most likely involve the small subunit.

CATALYTIC PROPERTIES OF RUBP CASE

RuBP Case was first recognized by Weissbach et al (1956) as the catalyst for the CO_2-fixation step in the "dark" reactions of photosynthesis. In this reaction, a molecule of CO_2 is added to the second carbon on a molecule of ribulose bisphosphate forming a 2-carboxy,3-keto,pentose bisphosphate intermediate which subsequently is cleaved to form two molecules of phosphoglycerate,or PGA (Calvin, 1956). Later studies (Ogren and Bowes, 1971) have shown that RuBP Case also catalyzes an oxygenase reaction, in which O_2 is added to RuBP which cleaves to form a molecule of PGA and a molecule of phosphoglycolate, the latter of which is converted to glycolic acid, the substrate for photorespiration. In addition to the substrates RuBP and CO_2 (or O_2), RuBP Case activity also requires Mg^{2+}, presumed earlier (Kawashima and Wildman, 1970) to function as a cofactor to catalysis. In addition, the enzyme shows a pH optimum in the alkaline range, between 7.8-8.0 in the presence of 10 mM Mg^{2+}. In kinetic studies of RuBP Case it was observed that the enzyme had a high affinity for RuBP, with a K_m (RuBP) estimated at around 25 µM (Laing and Christseller, 1980). The substrates CO_2 and O_2 have been shown to compete for the same catalytic

site (Ogren and Bowes, 1971). Several kinetic studies (Lorimer and Andrews, 1973; Laing et al., 1974; Badger and Andrews, 1974) have demonstrated that the kinetic constants for CO_2 and O_2 are identical, where $Ki(O_2)$ for carboxylation = $K_m(O_2)$ for oxygenation and $Ki(CO_2)$ for Oxygenation = K_m (CO_2) for carboxylation.

ACTIVATION OF RUBP CASE IN VITRO

The question of the affinity of RuBP Case for CO_2 was a puzzling enigma before the mechanism of RuBP Case activation was fully understood. Early estimations of the $K_m(CO_2)$ were very large (10-30 mM), greatly exceeding atmospheric CO_2 levels, and could not allow for compatable enzyme activity in vivo (Akazawa, 1979; Kawashima, 1970). To explain this difference between the low affinity for CO_2 fixation by purified enzyme and the high affinity of CO_2 fixation which the enzyme exhibits in the leaf, it was proposed that the enzyme exists in a different kinetic state in the leaf. In a study (Jensen and Bahr, 1974) of the kinetic properties of the enzyme from lysed spi-

nach chloroplasts, a labile form of the enzyme having a very low $K_m(CO_2)$ (11-18 μM) was observed immediately following lysis of chloroplasts into assay medium. This form was rapidly converted to a high K_m form, comparable to purified enzyme (K_m = 20-25 mM CO_2).

Pon et al (1963) first reported that the linearity of the RuBP Case reaction in vitro depends on the order in which Mg^{2+}, CO_2, and RuBP were added. A lag in activity was observed when the reaction was initiated with Mg^{2+} and CO_2, but a rapid and linear initial activity was observed upon addition of RuBP to enzyme preincubated in Mg^{2+} and CO_2. Later studies with purified enzyme (Lorimer, 1976, 1977, 1978; Laing and Christseller, 1976) have shown that purified RuBP Case was activated by CO_2 and Mg^{2+} in a process distinct from catalysis. Lorimer et al (1976) demonstrated that the activation of RuBP Carboxylase most likely occurred by a two-step process. First CO_2 binds to the inactive enzyme (E) in a slow, rate-limiting step followed by the rapid binding of Mg^{2+}, resulting in the fully activated enzyme complex ($E\text{-}CO_2\text{-}Mg^{2+}$). Removal of CO_2 and Mg^{2+} by gel filtration deactivated the enzyme. The extent of activation was dependent on the preincubation pH (optimum 8.6) and the CO_2 and Mg^{2+} concentrations in the activation

medium (optima at 20 and 10 mM, respectively). The effector CO_2 was shown to be distinct from the substrate CO_2, and bound at a site near the catalytic site (Lorimer et al, 1977). Activation of the carboxylase function resulted in a comparable activation of the oxygenase function as well and the $K_m(CO_2)/K_m(O_2)$ ratio was not changed by activation. Failure to fully activate RuBP Case in CO_2 and Mg^{2+} prior to assay would result in overestimations of the $K_m(CO_2)$. It was observed in kinetics studies with the purified enzyme (Laing and Christseller, 1980) that a Lineweaver-Burke plot of the $K_m(CO_2)$ of purified spinach RuBP Case that had not been preactivated, deviated from a linear relationship expected for Michaelis-Menten kinetics. A linear relationship was observed only when enzyme had been fully activated by CO_2 and Mg^{2+}, and assayed for a short time interval (30 s). Deviations from simple Michaelis-Menten kinetics are understandable if the enzyme is activated during the assay, since in a determination for $K_m(S)$ it is assumed that the amount of catalytically active enzyme is constant during the assay. It was also shown that the reaction rate of fully active enzyme is virtually independent of the Mg^{2+} concentration, indicating that Mg^{2+} may only be required for activation. This places the requirement of Mg^{2+} for catalysis in doubt, although other investigators (Sugiyama

et al., 1968; Nishimura and Akazawa, 1973; Takabe and Akazawa, 1973) have reported a Mg^{2+}-dependent enhancement of the RuBP Case reaction. They also observed that Mg^{2+} shifted the pH optimum from alkaline nearer to neutral. The necessity of an alkaline pH for total activation provided an indication as to the nature of the activator binding site. It was proposed that activator CO_2 was binding to a group on the enzyme with an alkaline pK, probably an amine (Lorimer et al, 1976). Lorimer (1981) has identified this site to be the ε-amino group of Lysine #254.

Variations in the amount of CO_2, H+ or Mg^{2+} in the environment of the enzyme or a shortened preincubation period would contribute to lower amounts of active enzyme. Therefore , the different K_m (CO_2) forms of the enzyme reported by Jensen and Bahr (1974) were explained by Lorimer et al. (1976) to be the result of low Mg^{2+} and CO_2 in the assay medium, causing deactivation of the in vivo state of the enzyme. For enzyme that has been fully activated by CO_2 and Mg^{2+}, the measured $K_m(CO_2)$ was low enough (20 μM) to account for observed rates of CO_2 fixation in the leaf under normal atmospheric conditions.

IN VIVO REGULATION OF RUBP CASE ACTIVATION

RuBP Carboxylase is located in the stroma of the chloroplast, a site where its activity could be conceivably controlled *in vivo* by light-dependent changes in H^+ and Mg^{2+} levels. Upon illumination, protons move from the stromal compartment into the thylakoid space in exchange for Mg^{2+}, raising the stromal pH and Mg^{2+} concentrations, thus favoring enzyme activation. In a study by Lin and Nobel (1971) with isolated *Pisum sativum* chloroplasts, it was observed that CO_2-fixation in chloroplasts rapidly isolated from darkened leaves was lower than in chloroplasts from illuminated leaves, but was enhanced by the addition of 5 mM Mg^{2+}. This indicated a possible role of Mg^{2+} in regulating photosynthesis. The dependance of *in vivo* RuBP Case activity on illumination and the necessity for intact chloroplast membranes has been demonstrated (Jensen and Bahr, 1974; Lorimer et al., 1978). Whether changes in stromal pH and Mg^{2+} influence RuBP Carboxylase activation in the chloroplast depends on the magnitude of the differences between stromal pH and Mg^{2+} before and after illumination. The shift in stromal pH prior to and following illumination has been generally estimated to be from approximately 7 to 8 (Heldt et al., 1974; Jen-

sen and Bahr, 1974; Robinson and Walker, 1981). Heldt et al. (1974) observed a shift of RuBP Case activity from near 0 to maximal with a pH shift from 7 to 8. Lorimer et al. (1978) also observed an effect of similar magnitude on the enzyme when stromal pH was reduced with the addition of nitrite to the chloroplast suspension medium. Total Mg^{2+} levels in spinach chloroplast stroma have been estimated (Grimmler et al., 1974; Portis and Heldt, 1976) to be 20 mM. However, it has been proposed that most of this Mg^{2+} exists in a bound state, and that the stromal free Mg^{2+} change following illumination is only about 3 mM (Portis and Heldt, 1976). This value differs from an earlier estimation of the magnitude of the change in Mg^{2+} levels in Pisum chloroplasts upon illumination, which was approximately 10 mM (Lin and Nobel, 1971). If the later estimation is more accurate, RuBP Carboxylase activation in vivo would have to be significantly effected by relatively small changes in stromal Mg^{2+} levels. A number of studies have implicated RuBP and other Calvin cycle intermediates as having significant roles in regulating RuBP Case activation in vivo. Pyridine nucleotides and sugar phosphates were among the compounds considered as possible effector molecules. These compounds often showed significant stimulatory or inhibitory effects on RuBP Case activity (Lorimer et al., 1978), but effects varied with

concentration and pH. For example, fructose bisphosphate was observed by Buchanan and Schurmann (1973) to inhibit RuBP Case activity, and shown to stimulate RuBP Case activity in another study (McCurry et al., 1981). Substrate inhibition of activity by RuBP was observed by Dailey and Criddle (1980) and Sicher and Jensen (1979), whereas Paech et al. (1978) suggested that byproducts of RuBP breakdown, and not RuBP itself, may inhibit RuBP Case activity. However, the significance of the role of sugar phosphates in regulating RuBP Case activation is questionable. The effects of these different compounds on RuBP Case activity in vitro are observed in high concentration, much higher than their actual concentration in the intact chloroplast. Considering the abundance of RuBP Case in the leaf (0.4-0.5 mM RuBP Case, 3-4 mM RuBP binding sites) (Heldt and Sauer, 1971), it is unlikely that many of these compounds would be present in significant concentration to have an effect on RuBP Case activation in vivo (Jensen and Bahr, 1977; Lorimer et al., 1978). Furthermore, since many of these possible effector molecules, i.e. NADPH, 6-phosphogluconate, were shown to interact at the catalytic site, thereby inhibiting binding of the substrate RuBP (Badger and Lorimer, 1981), it is unlikely that a molecule that occupies the RuBP binding site while maintaining the enzyme in an active state could be considered an

effector of considerable importance *in vivo*.

Is RuBP Case in a partially active state?

The extent to which RuBP Case is activated *in vivo* is as yet undetermined. Electron micrographs of *Avena* chloroplasts have revealed the presence of Fraction 1 protein in a crystalline state (Gunning *et al.*, 1968). This has led to the suggestion (Kawashima and Wildman, 1971) that RuBP Case exists part of the time in a "quasi-crystalline" state, which may be removed from involvement in catalytic activity. Furthermore, Lorimer (1981) states that purified RuBP Case remains essentially inactive *in vitro* when subjected to conditions of CO_2 and Mg^{2+} and pH believed to apply to its environment in the stroma (10 μM CO_2, 1-2 mM Mg^{2+}, pH 8.0). The $K_{act}(CO_2)$ of the enzyme was estimated by Laing and Christseller (1976) to be about 100 μM, which is well above atmospheric CO_2 concentration. Therefore the percent of the RuBP Case that is active *in vivo* could be an important factor limiting photosynthesis.

Recent studies in which RuBP Case activity was assayed immediately after homogenization of leaf tissue have suggested that RuBP Case may exist in a state of only partial activation *in vivo*. Perchorowicz *et al.* (1981) measured RuBP Case activity in an extract of wheat leaf tissue, assayed directly without prior activation, and observed a lower rate compared to activity observed in extract preincubated prior to assay in HCO_3^-, Mg^{2+}, and a pH of 7.7. This lower rate was believed to represent the *in vivo* state of RuBP Case activation in the leaf, termed "initial" activity, and the higher rate represented the total amount of activatable RuBP Case. The initial activation state is preserved in harvested leaf tissue by storage of the leaf tissue at 0 C (Perchorowicz *et al.*, 1982; McDermitt *et al.*, 1982). At 25°C, initial activity in the leaf homogenate changed rapidly to to a new active state that was dependent on Mg^{2+} and CO_2 levels in the homogenate (McDermitt et al., 1982). Maximal percent activation of RuBP Case (initial/total activity) was estimated to be 60% for wheat, 70% for spinach (Perchorowicz *et al.*, 1981, 1982, 1983) and 83% for soybean (Perchorowicz *et al.*, 1982; McDermitt *et al.*, 1982).

RuBP Case initial activity and control of photosynthesis rate

Leaf photosynthesis rate and RuBP Case initial activity was observed to increase with increasing irradiance, presumably as a result of increased stromal Mg^{2+} and pH (Perchorowicz et al., 1981, 1983; Sicher, 1982). This would indicate that RuBP Case in vivo activity was regulating photosynthesis rate in the light. However, photosynthesis in low light may not necessarily be reduced by dark-deactivation of RuBP Case, which has been proposed by some (Robinson et al, 1979) not to occur in vivo. Perchorowicz et al. (1981, 1983) observed that RuBP Case percent activation does not decrease as rapidly in a transition from light to dark as it does in a transition from high to low irradiance. Since the change in photosynthesis rate with the transition from light to dark more closely ressembled the observed pattern for the change in RuBP levels (Perchorowicz, 1981) it was proposed that RuBP levels (controlled by fructose bisphosphatase and sedcheptulose bisphosphatase activity), and not RuBP Case activity, was limiting photosynthesis rate in the dark.

RuBP Case may exist partly in a form that cannot be activated even by CO_2 and Mg^{2+}. In a study where RuBP Case initial and total activities were measured in soybean over a diurnal time-period (McDermitt et al., 1982), it was reported that total as well as initial RuBP Case activity increased significantly with increasing irradiance. It was suggested that there was a mechanism other than pH and Mg^{2+} and CO_2 by which RuBP Case activation was regulated by light.

EFFECT OF IRRADIANCE ON LEAF DEVELOPMENT

It is well-documented that the growth of sun-adapted plants in irradiance levels which are suboptimal for maximal growth has a significant effect on their physiology and anatomy (Boardman, 1977). Modifications in leaf anatomy during growth under reduced irradiance include a reduction in stomatal frequency and leaf thickness, the result of reduced cell size and number, but an increase in cell chlorophyll content, due to increased granal development in the chloroplasts (Bjorkman, 1971; Crookston, 1975). The size of the individual light-harvesting com-

plexes in the chloroplasts of Atriplex patula was shown in one study (Bjorkman, 1971) to be unaffected by irradiance during growth, but the relative quantity of electron carriers (acceptor P, cyt b559, cyt b6 and cyt F) decreased with lower irradiance.

Maximal leaf photosynthetic activity as well as the saturating irradiance level for photosynthesis, were also reduced (Bjorkman, 1971; Bowes et al., 1972; Singh et al., 1974).

Effect of Irradiance on RuBP Case Activity

A reduction in the levels of Fraction 1 protein was also observed during growth in low irradiance. The amounts present could be reversed upon transfer from low to high irradiance (Blenkinsop and Dale, 1974; Gauhl, 1975). The activity of RuBP Case was likewise reduced during growth in low irradiance (Gauhl, 1975; Blenkinsop and Dale, 1974, Bowes et al., 1972; Singh et al., 1974). The reduction in RuBP Case activity in response to decreased irradiance levels was sometimes similar

(Gauhl, 1975), greater (Blenkinsop and Dale, 1974), or lesser (Bowes et al., 1972) than the reduction in maximal photosynthesis rate. In these earlier studies the in vivo state of the enzyme was not measured. It would therefore seem likely that some of the inconsistensies reported in correlating photosynthesis with RuBP Case activity as effected by irradiance are caused by differences in the methods for measuring RuBP Case. Very few studies have directly investigated whether RuBP Case percent activation may be strongly affected by the irradiance in which the plant is grown. One recent study with field-grown soybeans (McDermitt et al., 1982) has shown that RuBP Case percent activity varied in leaves depending on their position in the canopy. Leaves at the top of the canopy, those exposed to direct sunlight, had 85% of the RuBP Case in an in vivo active state. Leaves lower in the canopy, and presumably shaded, had only 30% activation of RuBP Case.

CHAPTER 1: ACTIVATION STATE OF RIBULOSE BISPHOSPHATE CARBOXYLASE IN SOYBEAN LEAVES

ABSTRACT

Conditions for extraction and assay of ribulose 1,5 bisphosphate carboxylase present in an *in vivo* active form (initial activity) and an inactive form able to be activated by Mg^{2+} and CO_2 (total activity) were examined in leaves of soybean, *Glycine max* (L.) Merr. cv Will. Total activity was highest after extracts had preincubated in $NaHCO_3$ (5 mM saturating) and Mg^{2+} (5 mM optimal) for 5 minutes at 25°C or 30 minutes at 0°C before assay. Initial activity was about 70% of total activity. $K_{act}(Mg^{2+})$ and $K_{act}(CO_2)$ were approximately 0.3 mM and 36 µM, respectively. The carry-over of endogenous Mg^{2+} in the leaf extract was sufficient to support considerable catalytic activity. While Mg^{2+} was essential for both activation and catalysis, Mg^{2+} levels greater than 5 mM were increasingly inhibitory to catalysis. Similar inhibition by high Mg^{2+} was also observed in filtered, centrifuged, or desalted extracts and partially purified enzyme. Activities were also stable for

up to 3 hours in leaf extracts stored at 0°C. Initial activity quickly deactivated at 25°C in the absence of high CO_2. Total activity slowly declined irreversibly upon storage of leaf homogenate at 25°C.

INTRODUCTION

Purified RuBP Case[1] requires preincubation in Mg^{2+} and CO_2 before assay to attain maximal activity [for details, see reviews by Jensen and Bahr (6) and Lorimer (12)]. The model for this activation [12] involves the stepwise addition of CO_2 and Mg^{2+} to the enzyme in the following sequence:

Enzyme + CO2 <--> Enzyme-CO2 + Mg2+ <--> Enzyme-CO2-Mg2+
(E form, inactive) (EC form, inactive) (ECM form, active)

The K_{act} for Mg^{2+} and CO_2 measured with purified enzyme are

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[1] Abbreviations: RuBP Case: ribulose 1,5-bisphosphate carboxylase; LN_2: liquid nitrogen; Chl: chlorophyll.

approx 1 and 0.1 mM, respectively [4, 8]. However, the concentrations of Mg^{2+} and CO_2 which are present in the chloroplast may be insufficient for complete activation [8, 12]. A number of workers have estimated the amount of RuBP Case which is in an active state in leaves. Perchorowicz et al. [17, 18, 19] measured percent activation, the quotient of initial and total activity, to estimate the fraction of the total RuBP Case which is in the active state. Initial activity was measured by the addition of a filtered leaf-extract, homogenized in a buffered medium without added Mg^{2+} and CO_2, to an assay medium containing saturating Mg^{2+} and CO_2 concentrations. Total activity was measured in a similar manner, but after homogenate had preincubated for 10 min in saturating Mg^{2+} and CO_2 to attain full activation. Other workers [2, 14] have measured percent activation of RuBP Case in wheat leaves but have included Mg^{2+} in the homogenizing medium as a necessary precaution against deactivation of the active enzyme during extraction. The percent activation of RuBP Case in wheat leaves is significantly influenced by irradiance level [15, 17, 18], CO_2 [2, 15, 17], temperature [14], and has been found to increase with increases in leaf photosynthesis rate [2, 15, 17, 18]. For example, increasing the illumination of wheat leaves increased percent activation from 20 to 60% and photosynthesis rate from 0 to 60 μmol CO_2 (mg

Chl h)-1 [17]. Hence the amount of active RuBP Case in the leaf could be an important factor in regulating the rate of photosynthetic CO_2 fixation and its physiological significance and control needs to be examined. Percent activation varies considerably among different species of plants as well as the optimal conditions for measuring it [19]. Before examining the influence of environmental factors on the percent activation of RuBP Case in soybean leaves, we have established procedures for optimizing extraction and assay of initial and total enzyme activity in soybean leaves.

MATERIALS AND METHODS

Plant Material Soybeans [Glycine max (L.) Merr. cv Will] were grown in vermiculite in chambers under a 12 h photoperiod (25°C day and 20°C night) and illuminated with 540 μE m-2 s-1 provided by a combination of fluorescent and incandescent lamps. Plants were watered daily with a balanced nutrient solution.

LEAF HOMOGENIZATION

Fully-mature leaves, generally from the third to fifth node, were sampled in the middle of the light period by either removing leaf discs (0.5 Cm^2) with a cork borer or removing and quickly submerging the entire trifoliolate leaf into LN_2 or an ice water bath. Leaves stored in LN_2 were ground to a fine powder in a LN_2-chilled mortar. Samples (0.1 g) of LN_2-frozen leaf powder or leaf discs were homogenized by hand for 30 s with a Tenbrock glass homogenizer in 5 ml of ice cold homogenizing medium [50 mM Tris-Cl, 5 mM DTT, 10 mM isoascorbic acid, and 0.1% (w/v) BSA, pH 8.0 at 0°C] and stored in an ice bath or immediately assayed. In some cases, either 5 mM $MgCl_2$ and/or $NaHCO_3$ was added to the homogenization medium as indicated in the text.

RUBP CASE ASSAYS

RuBP Case activity was measured in a medium containing (final

concentration): homogenate (approx 5 µg Chl), Tris-Cl (100 mM), pH 8.0 at 25°C, isoascorbic acid (10 mM), DTT (5 mM), BSA (0.1% w/v), Mg^{2+} (concentration as indicated in text), $NaH^{14}CO_3$ (20 mM, 0.1 Ci mol^{-1}), and RuBP (0.4 mM) (total volume, 0.51 ml) in a stoppered 4 ml plastic vial. Vials were shaken reciprocally (3 s^{-1}) in a 25°C water bath. Initial activity was measured by the addition of 50 µl of extract by means of a positive displacement micropipette (Sigma) to the assay medium and terminated after 1 min by the addition of 0.2 ml of 6 N formic acid. Total activity was measured in a similar manner except that the extract was added to the assay medium and incubated at 25°C with shaking for 5 min before initiating the assay by the addition of RuBP. After assay, vials were purged with air and dried overnight at 70°C. Acid-stable ^{14}C was determined by scintillation counting and corrected for quenching by external standard method. Ba_2RuBP (Sigma) was solubilized with excess Dowex-50-H+ and after filtration the pH was taken to 6.5 with KOH. This solution was stored at 0°C and added immediately before assay. RuBP concentration was routinely assayed using crystalline tobacco RuBP Case and $NaH^{14}CO_3$. Chlorophyll was determined by the procedure of Wintermans and DeMot [23] and soluble protein using a dye-binding reagent (Bio-Rad) and a BSA (fraction V) standard. Carbon

dioxide concentration was calculated from bicarbonate concentration using a pK' value of 6.38 [8]. Corrections were made in the specific activity of assay $H^{14}CO_3^-$, due to carry-over of $H^{12}CO_3^-$ if added to the homogenizing medium. No corrections were made for the presence of atmospheric CO_2 in assay medium.

In this study, initial activity refers to activity measured immediately upon addition of the extract at 0°C to an assay medium containing RuBP. Final activity refers to activity measured after the extract had been preincubated for a period of time, usually 5 min at 25°C, before assay. Total activity was measured after extract had preincubated in 20 mM $NaHCO_3$ and 5 mM Mg^{2+} for 5 min before assay. All assays were linear for at least 3 min.

RESULTS

Optimum pH for Activation and Assay Both catalysis and activation in 5 mM Mg^{2+} and 20 mM $NaHCO_3$ were optimal at pH 8.0. Initial and total activities were reduced about 30% at the

extremes of pH range of 7.4 to 8.8 (data not shown).

Magnesium Concentration and Time Course of Activation RuBP Case activity increased with time upon preincubation of extract in an assay medium containing added Mg^{2+} (2 to 5 mM optimal) and 20 mM $NaHCO_3$ before addition of RuBP (Fig. 1). Activity remained constant between 5 and 20 min of preincubation. Incubation of extract in an an assay medium without added Mg^{2+} resulted in no substantial loss of activity with time. The addition of Mg^{2+} (5 mM optimal) to the assay medium slightly increased the initial activity (measured without added Mg^{2+}) has been reported earlier [19]. In some of the experiments which follow we continued to measure this "endogenous" activity in the event that it might have significance. However, subsequent work [22] has shown that this activity is the result of carry-over of endogenous Mg^{2+} present in the leaf extract. Soybean leaf extracts were found to contain approximately 15 to 20 umol Mg^{2+} mg Chl^{-1} (1.5 to 2.0 mM Mg^{2+}) as measured by atomic absorption spectroscopy. After a a ten-fold dilution into assay medium, the final assay concentration of endogenous Mg^{2+} would be 0.15 to 0.2 mM Mg^{2+}. This endogenous Mg^{2+} is sufficient for maintaining considerable activity (Fig. 2). While the conversion of E to EC is considered to occur

slowly (minutes) at assay temperature, the conversion of EC to ECM occurs very rapidly [13]. We also observed that preincubation of extract in 20 mM $NaHCO_3$ followed by assay in 5 mM Mg^{2+} and 20 mM $NaHCO_3$ increased total activity, but the change in activity resulting from either increasing or decreasing assay Mg^{2+} concentration was so rapid that it could not be measured inan interval as short as 5 s.

Assay of initial and final activities at added Mg^{2+} levels greater than 5 mM was increasingly inhibitory (Figs. 1 and 2). This inhibition was unexpected in that 20 mM Mg^{2+} is optimal for activation and assay of purified spinach carboxylase [13] and 30 mM for filtered soybean leaf extracts [19]. We then determined if this inhibition also occured in filtered, centrifuged, and desalted extracts or partially purified enzyme, because Mg^{2+} has also been reported to bind RuBP Case to chloroplast membranes [16]. High Mg^{2+} levels also inhibited final RuBP Case activities in homogenates which had been either filtered through a nylon net (10 µm opening), centrifuged for 1 min at 12,000 X g, or centrifuged and then passed through a 5 ml syringe filled with Sephadex G-25 (equilibrated in homogenizing medium without BSA) by centrifugation at 300 X g for 1 min (Fig. 2). About 20% of the final activity was lost by filtra-

tion, centrifugation, or desalting, based on the amount of Chl present in the original homogenate. Enzyme which was partially purified by precipitation by 20 mM $MgCl_2$ in the presence of 18% (w/v) PEG-4000 [3] and then passed through a small column of Sephadex G-25 equilibrated in homogenizing medium without BSA also showed a similar pattern of inhibition by Mg^{2+} concentrations greater than 5 mM. The $K_{act}(Mg^{2+})$ for partially purified enzyme was approximately 0.3 mM (Fig. 2). Of interest is the observation that the ratio of the activity in the presence of endogenous Mg^{2+} only (zero added Mg^{2+}) to maximal activity (5 mM added Mg^{2+}) was not reduced by filtration or centrifugation but was reduced by partial purification of the enzyme. Partial purification might be expected to remove all endogenous Mg^{2+} from the extract.

To determine whether Mg^{2+} was inhibiting activation, catalysis or both, LN_2-frozen leaf powder was extracted at 25°C in homogenizing medium containing increasing Mg^{2+} concentrations, 0 to 50 mM, and 20 mM $NaHCO_3$ and incubated for 5 min before assay. Similar activities were observed for all extracts if the assay medium were adjusted such that the final concentration added Mg^{2+} in the assay was 5 mM (data not shown). However, when the homogenates were assayed in the same concentration of added

Mg^{2+} as was present in the homogenizing medium, a similar inhibition of final activity was observed (Figs. 1 and 2). These data indicate that added Mg^{2+} levels above 5 mM inhibit catalysis directly and not activation, in that the inhibition is rapidly reversible upon lowering Mg^{2+} concentration to 5 mM.

Carbon Dioxide Concentration Carbon dioxide is required for the conversion of E to EC and also as substrate for the enzyme. In order to determine its effect on activation only, we extracted leaf powder in homogenizing medium containing increasing concentrations of HCO_3^- or HCO_3^- and 5 mM Mg^{2+} and assayed initial and final (after incubation in homogenizing medium for 5 min at 25°C) activities (Fig. 3). Increasing HCO_3^- concentration had little effect on initial activity measured immediately after extraction, except at very high concentrations. Initial activities assayed with 5 mM Mg^{2+} were much higher than activities assayed in the absence of added Mg^{2+}, as shown previously (Figs. 1 and 2). Final activities assayed in the presence of Mg^{2+} were reduced after incubation of extract at low HCO_3^- concentration and increased with increasing HCO_3^- concentration, saturating at approx 5 mM. The enzyme did not completely deactivate in the absence of added HCO_3^- even though an effort was made to eliminate atmospheric

CO_2 from both homogenization and assay media before conducting the experiment [14]. This inability to deactivate the enzyme completely by eliminating CO_2 has been observed previously with purified enzyme [13] and is probably the result of endogenous Mg^{2+} stabilizing the EC complex. The amount of added HCO_3^- required to promote one-half maximal activation [$K_{act}(HCO_3^-)$] is approx 2.2 mM (53 µM CO_2) in the presence of endogenous Mg^{2+}. If 5 mM Mg^{2+} were added to the homogenizing medium, initial activities were not different, but final activities were slightly higher and the $K_{act}(HCO_3^-)$ was reduced to 1.5 mM (36 µM CO_2). When leaf powder was homogenized and assayed without added Mg^{2+}, final activities were lower and did not substantially increase above initial levels at higher HCO_3^-.

RuBP Concentration Initial and total activities increased with increasing RuBP concentration, but did not saturate even at 1 mM RuBP. Between 0 and 0.12 mM RuBP, both activities followed Michaelis-Menten kinetics [K_m(RuBP) = 31 µM] but deviated from these kinetics at higher RuBP levels (Fig. 4). The ratio of initial to total activity or percent activation decreased about 20% with increasing RuBP concentration from 0-1 mM.

Other Compounds The addition of 0.1% (w/v) BSA, 5 mM DTT, and 10 mM Na isoascorbate had little effect on both initial and total activities (data not shown), but these were added as a precaution against denaturing substances and proteases in the homogenate.

Enzyme Stability in the Leaf Initial and final activities did not increase significantly with while leaves were stored in ice or LN_2 for periods of up to 4 h (Fig. 5). Leaves stored in ice had activities similar to those measured in a homogenate prepareed from leaf discs removed from those leaves immediately before being added to ice water. However, both initial and final activities were about 30% higher when leaves were frozen and ground in LN_2 before extraction. In a following experiment, leaf discs were removed from the same leaflet and half were extracted immediately in a glass homogenizer. The other half was ground to a powder in LN_2 and then extracted. Total activities were measured before and after passage of ech extract through a French pressure cell at 5000 psi to completely lyse any unbroken cells. Activities were highest in the extract of LN_2-frozen leaf powder and about 40% lower in the extract of fresh leaf tissue, but similar in both extracts after pressure-cell treatment (about 8% lower). Evidently

extraction of leaves directly in a glass homogenizer did not completely rupture all leaf cells. Microscopic examination of fresh leaf homogenates revealed many cells with intact chloroplasts. Homogenates of LN_2-frozen leaf powder were observed to contain fewer cells, but chloroplasts in these cells were all ruptured.

Stability in the Homogenate Initial activities of extracts prepared and stored at ice temperatures remained constant with time (Fig. 6A). Initial activities were higher if 5 mM Mg^{2+} was included in the assay medium; however initial activities were no different if Mg^{2+} was included in the homogenizing medium as well as the assay. Extracts prepared in medium containing HCO_3^- or Mg^{2+} and HCO_3^- and assayed in Mg^{2+} both increased in activity similarly with time and attained optimal activity after 30 min. The addition of HCO_3^- to the homogenizing medium had no effect on initial activity assayed without Mg^{2+} indicating that Mg^{2+} concentration was limiting for total activity. Total activities decreased about 20% over the 2 h period. Extracts prepared in medium without HCO_3^- and stored at 25°C quickly lost activity before reaching a stable but lower final activity (Fig. 6b). Activities were about 30% higher if 5 mM Mg^{2+} was included in the homogenizing and assay mediums but

deactivation occurred nonetheless. Extracts prepared at 25°C in HCO_3^- and Mg^{2+} and assayed in Mg^{2+} increased in activity over the first 5 min before gradually decreasing with time. Activities of extracts prepared in HCO_3^- and assayed without Mg^{2+} remained constant for 15 min before also gradually losing activity. This loss of total activity at 25°C is apparently different from deactivation and is irreversible. Total activities, measured after 5 min incubation in saturating Mg^{2+} and CO_2, were similar in all extraction buffers and decreased about 38% after 2 h at 25°C. The irreversible loss of final activity appeared to be more rapid if the extracts were exposed to high HCO_3^- concentrations during preincubation (Fig. 6B).

DISCUSSION

RuBP Case activity in soybean leaf extracts showed many similarities to activity in purified enzyme from leaves of soybean and other species. Preincubation of homogenate in high CO_2 and Mg^{2+} increased enzyme activity above initial levels presumably by stabilizing enzyme. The K_{act} (Mg^{2+}) and K_{act}(CO_2) are simi-

lar to those reported for purified enzyme measured under the same conditions [4, 7]. The presence of endogenous Mg^{2+} in leaf extract complicates the accurate measurement of the kinetic constants for activation. Following a ten-fold dilution of extract into the assay medium, the endogenous Mg^{2+} concentration is sufficiently high to support appreciable catalytic activity because $K_{act}(Mg^{2+})$ is comparatively low, approx 0.3 mM measured with with partially purified enzyme (Fig. 2).

Magnesium concentrations above 5 mM were inhibitory to catalysis but not activation. This inhibition was similar in partially purified RuBP Case (Fig. 2), essentially free of all Chl, thus eliminating the possibility that inhibition may have occurred because of binding of RuBP Case to chloroplast membranes [15]. This inhibition of soybean RuBP Case by high Mg^{2+} is insignificant as regards assay because the enzyme is approximately 94% activated by 5 mM Mg^{2+} in the presence of 20 mM $NaHCO_3$ (Fig. 2). Laing et al [9] found purified soybean RuBP Case to be inhibited about 85% by 46 mM Mg^{2+} compared to 6 mM Mg^{2+}. Recently, Bahr et al. [1] also reported that purified tobacco enzyme was increasingly inhibited by increasing Mg^{2+} concentrations above 2.5 mM, while the spinach enzyme showed

maximal activity with 25 mM Mg^{2+}. Such differences among species reaffirms the necessity for optimizing activation and assay conditions for a particular species [19].

The K_m(RuBP) measured for soybean leaf homogenates is identical to that reported for purified soybean enzyme [8]. We also observed a deviation from Michaelis-Menten kinetics with RuBP concentration above 0.12 mM. Laing and Christseller [8] explained the increased activity with higher levels of RuBP as substrate activation of enzyme which was already fully activated by CO_2 and Mg^{2+}. Total activities (measured after incubation in optimal CO_2 and Mg^{2+} for activation) increased at a greater rate than initial activities resulting in a slight decrease in percent activation with increasing RuBP concentration (Fig. 4).

Both initial and final activities were stable in leaves stored in an ice water bath (19 and Fig. 5) or stored frozen in LN_2 (Fig. 5). Activities of leaf tissue previously frozen and ground in LN_2 are higher than activities measured in leaves extracted directly without freezing because of a complete rupturing of the leaf cells. Evidently not all of the RuBP Case is released from the cells by the LN_2 treatment because filtering

or centrifuging removed about 20% of the total activity (Fig. 2). An attempt to increase total activity by passing an extract of LN_2-frozen leaf powder through a French pressure cell did not increase activity indicating that all of the enzyme in an extract of LN_2-frozen leaf tissue is accessible to RuBP.

Both initial and total activity were very stable for at least 2 h if extracts were stored at ice temperature (Fig. 6A). The inclusion of 5 mM Mg^{2+} in the extraction medium did not increase initial activity, but the inclusion of 20 mM bicarbonate either alone or with Mg^{2+} resulted in enzyme activation both at 0°C (Fig. 6A) and 25°C (Fig. 6B). At the latter temperature, activation was much faster. The observed stability of initial activity in leaf extracts prepared in the absence of added Mg^{2+} and CO_2 is in contrast to the rapid deactivation which occurs in purified spinach enzyme upon removing Mg^{2+} by dilution [7] or gel filtration [22] or the addition of excess Na_2EDTA (Servaites, unpublished) results in a rapid loss of initial activity at 0°C. While removing Mg^{2+} deactivates the enzyme, the addition of Mg^{2+}, above 2 mM, does not appear to further activate the enzyme, but hold the active enzyme in a non-equilibrium state. Homogenizing leaf tissue at 25°C (Fig.

6B) in the absence of added Mg^{2+} and CO_2 or warming a cold extract (data not shown) results in a rapid loss of initial activity to a new equilibrium dependent upon the Mg^{2+} and CO_2 level.

Upon incubation of extract at 25°C, total activity is slowly and irreversibly lost (Fig. 6B). However, the loss of total activity was essentially eliminated upon a ten-fold dilution of extract into assay medium (Fig. 1). Removal of low molecular weight compounds from the extract by gel filtration had no effect upon final activities (Fig. 2). This irreversible loss of activity with time is probably the result of protease action rather than progressive inhibition by small molecular weight compounds present in the leaf extract. Extracts of soybean leaves are known to contain a number of endoproteases which can rapidly degrade the large subunit RuBP Case [21] which presumably results in a loss of activity. For an unknown reason loss of activity was more rapid when leaves were extracted and stored at 25°C in medium containing HCO_3^-. Machler and Nosberger [14] have also reported a higher loss of initial activity of wheat leaf homogenates stored in the presence of HCO_3^-. They attributed this loss of activity as a direct inhibition of the enzyme by HCO_3^-. This loss of total activity upon warming

the homogenate is unfortunate in that the activation by CO_2 is more rapid at higher temperatures and is usually conducted immediately before assay of total activity. It may be advisable if critical measurements of total activity (E, EC, and ECM forms) are being made to allow diluted extract to activate at ice temperatures for 30 to 60 min in a medium containing bicarbonate and Mg^{2+} before measuring total activity.

Much evidence indicates that photosynthesis rate at low CO_2 concentration is correlated with RuBP Case activity [5, 6, 18]. However, when measurements of RuBP Case activity and photosynthesis rate are made on the same leaf, RuBP Case activity substantially exceeds photosynthesis rate [5, 18]. There are at least two reasons for this apparent inconsistency. First, photosynthesis rate is measured at air level of CO_2 (10 μM CO_2), while RuBP Case activity is measured at saturating CO_2 concentration (i.e., greater than 100 μM CO_2). Second, RuBP Case activity is measured after conversion of E and EC to ECM form by activation with CO_2 and Mg^{2+}, while the true rate of *in vivo* carboxylation is probably a function of only the ECM form. If our measurements of initial activity are a true reflection of *in vivo* carboxylation rates, then only 70% of the total carboxylase (E, EC, ECM) is in the active form (ECM) in the soybean

leaves we examined and this activity may better correlate to rates of photosynthesis than total RuBP Case activity.

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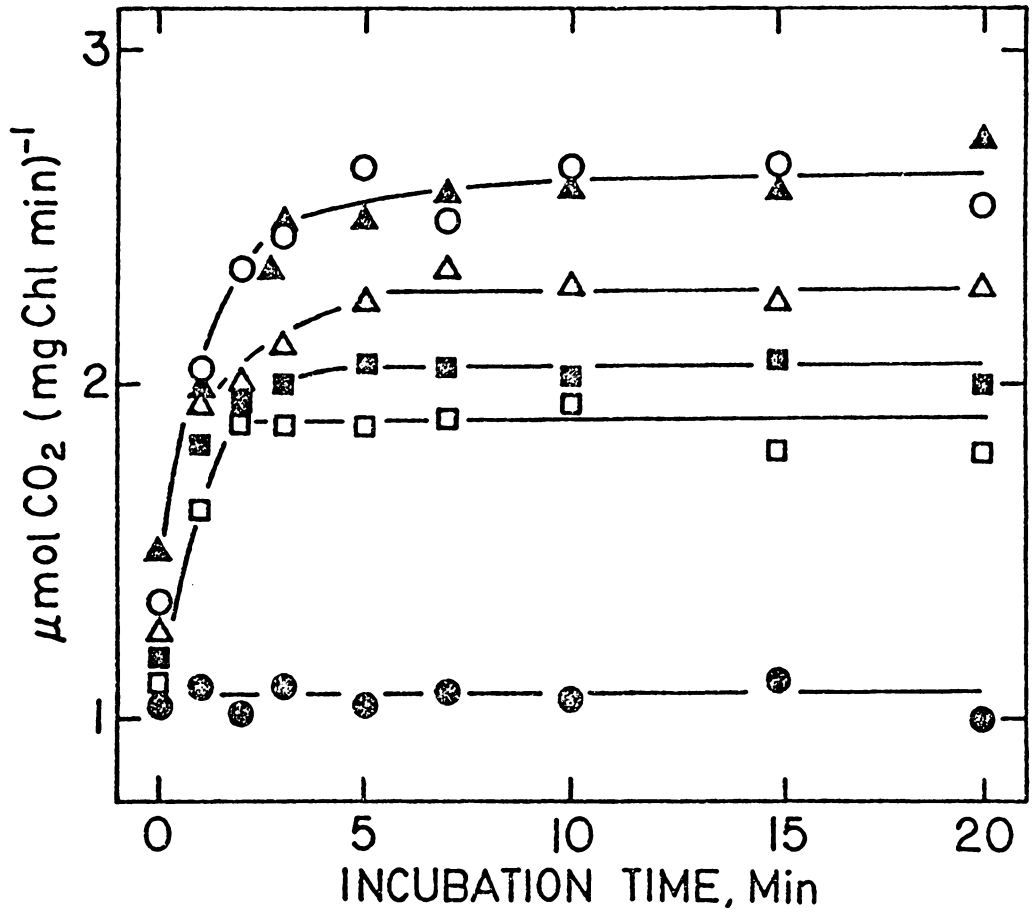

Figure 1. Effect of Mg^{2+}, incubation time on RuBP Case activity: Time course of activation of RuBP Case activity in 20 mM $NaHCO_3$ and increasing concentrations of Mg^{2+}. Leaf extracts were prepared from the same LN_2-frozen leaf powder at 0°C and added to assay medium containing 20 mM $NaHCO_3$ and either 0 (●), 2 (○), 5 (▲), 10 (△), 20 (■), or 30 (□) mM Mg^{2+} at 25°C. After the indicated time, RuBP was added to initiate the assay and terminated after 1 min.

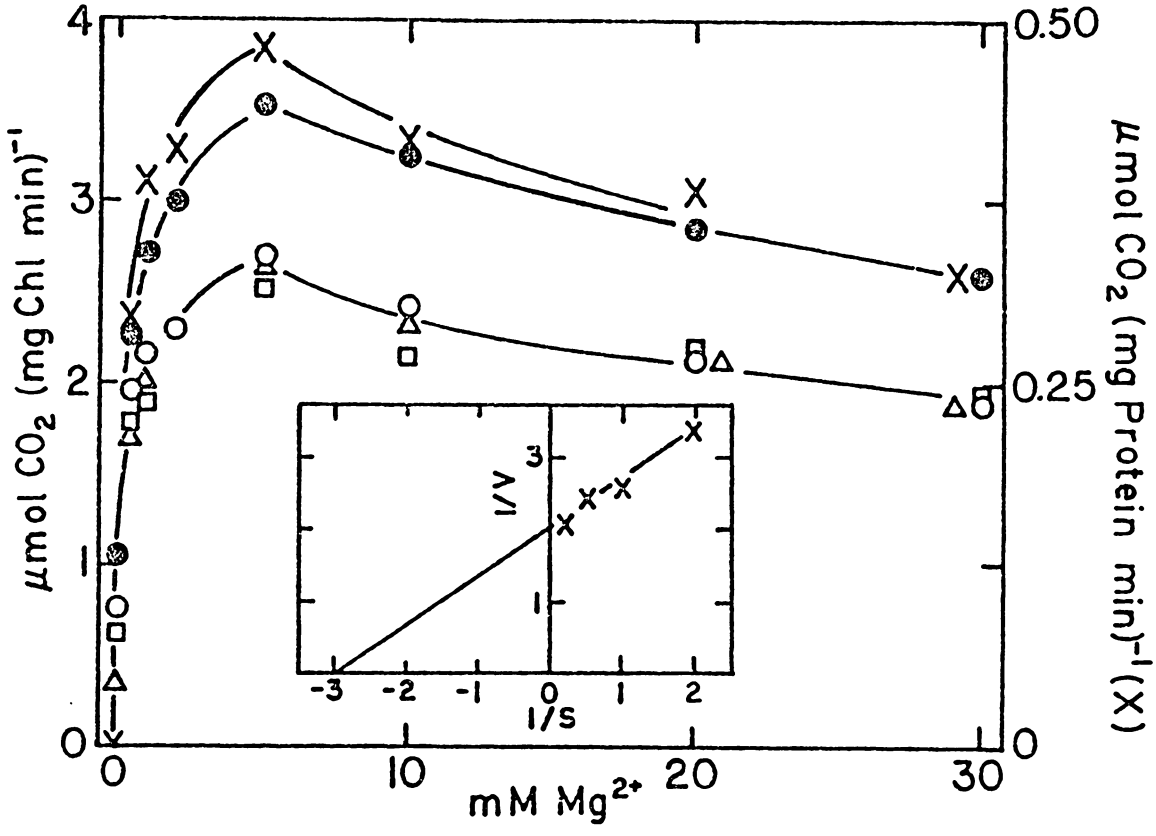

Figure 2. Effect of Mg^{2+} concentration on final RuBP Case activity: Leaf extracts were prepared at 0°C from the same LN_2-frozen leaf powder and aliquots either added directly to assay medium (●) or added after either filtration (O), centrifugation (□), centrifugation and desalting (Δ), or partial purification (X) of the extract. After incubation for 5 min in assay medium containing Mg^{2+} (as indicated) and 20 mM $NaHCO_3$, assays were initiated by the addition of RuBP and terminated after 1 min. Means of triplicate assays are shown (avg SD = ± 4.4%). Inset: double reciprocal plot of Mg^{2+} concentration vs. final activity of partially purified soybean RuBP Case.

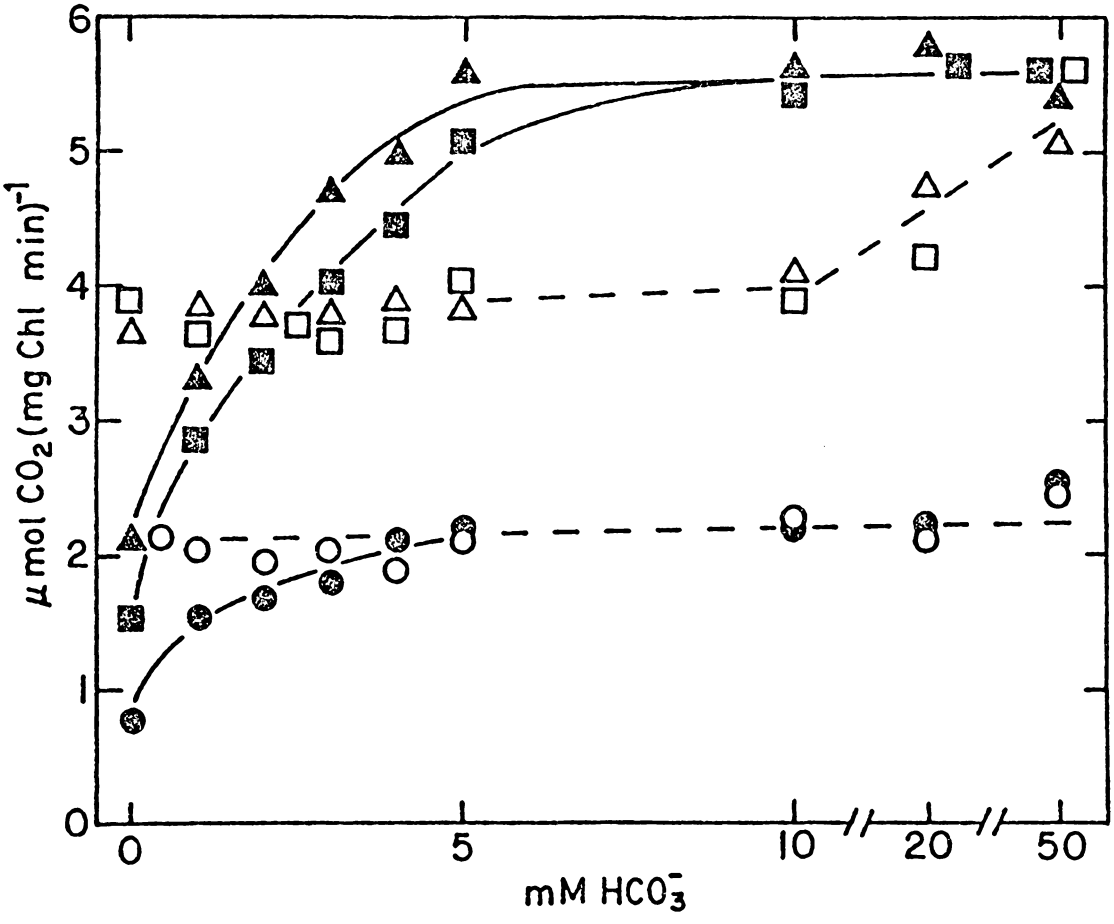

Figure 3. Effect of HCO_3^- concentration during preincubation on initial and final RuBP Case activity: Leaf extracts were prepared from the same LN_2-frozen leaf powder at 0°C and varying concentrations of HCO_3^- plus 0 mM Mg^{2+} (●,○,■,□) or 5 mM Mg^{2+} (▲,△) and assayed (single measurements) immediately (○,□,△, initial activity) or assayed after extracts had preincubated for 5 min at 25°C (●,■,▲, final activity). The assay medium contained 20 mM $NaHCO_3$ and either 0 mM Mg^{2+} (●,○) or 5 mM Mg^{2+} (■,□,▲,△). Means of triplicate assays are shown (avg. SD = ± 5%).

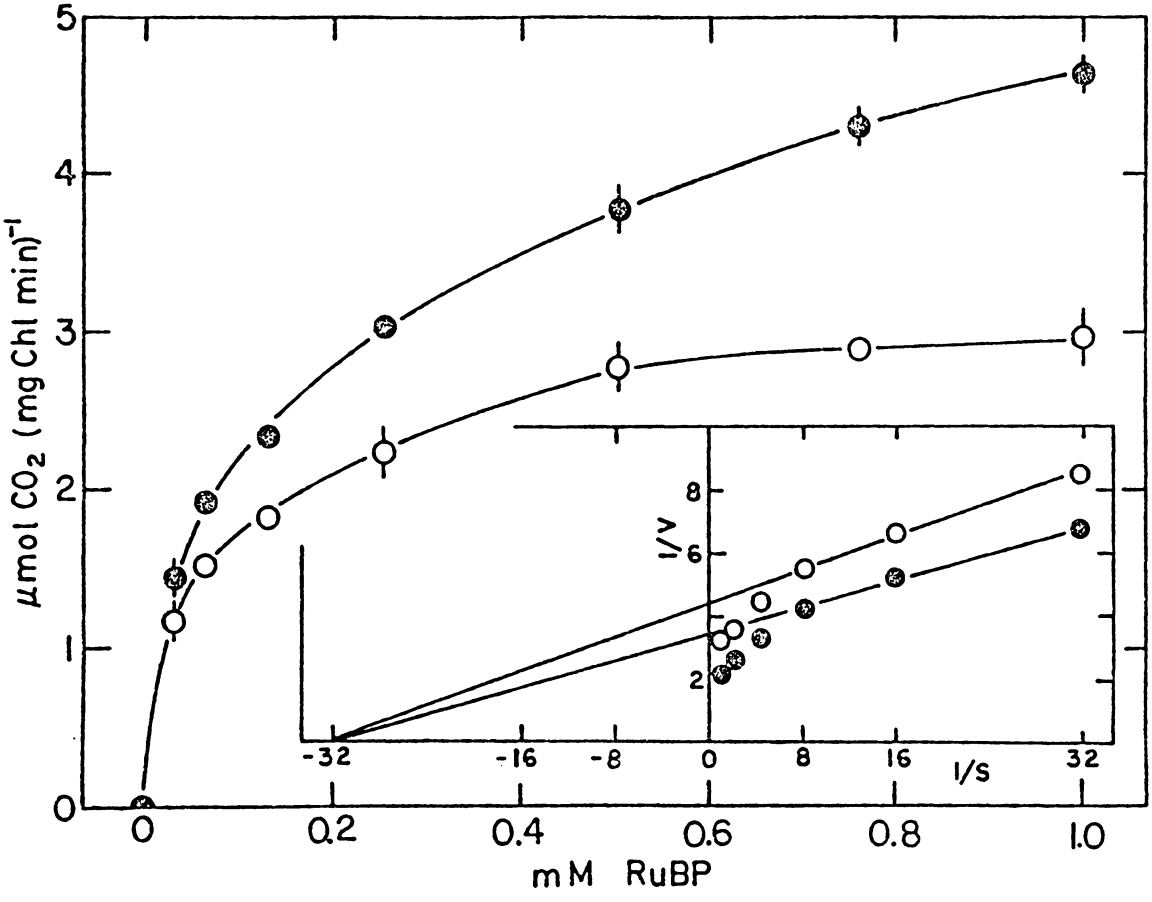

Figure 4. RuBP response curve for initial and total RuBP Case activity: Extracts of LN_2-frozen leaf powder were assayed immediately in assay medium containing 5 mM Mg^{2+}, 20 mM $NaHCO_3$, and RuBP concentration as indicated (O, initial activity) or after preincubation in assay medium for 5 min before the addition of RuBP (●, total activity). Triplicate measurements were made from the same extract and vertical bars represent SD of the mean. Inset: Double reciprocal plot of RuBP concentration vs. initial and final activities.

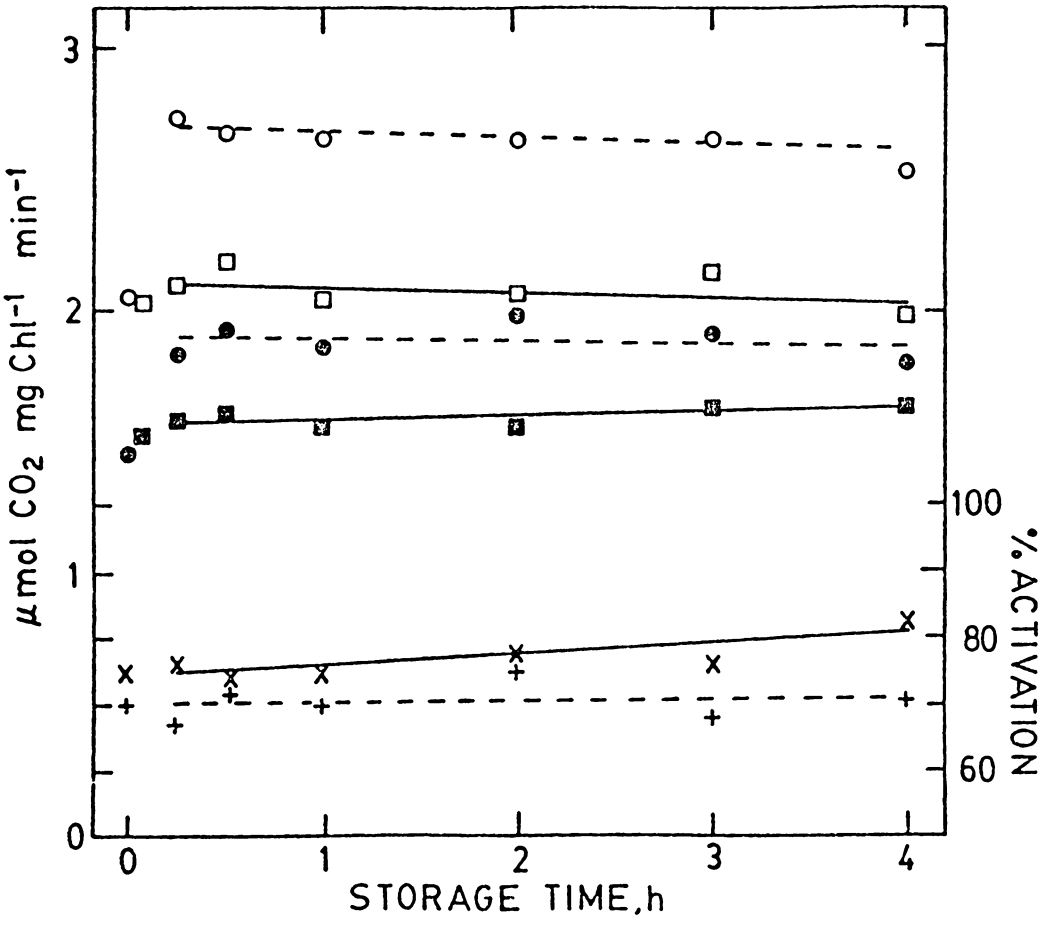

Figure 5. Stability of RuBP Case activity in soybean leaves stored in ice water or LN_2. Leaf discs were removed from leaves and immediately homogenized at 0°C and aliquots added to assay medium at 25°C. These leaves were then submerged into LN_2 (●,○,-) or ice-water (■,□,X). At the times indicated leaf tissue was removed and extracts prepared and assayed. Different leaves were used for the two treatments. Initial activities (●,■) and final activities (○,□) were assayed in medium containing 5 mM Mg^{2+} and 20 mM $NaHCO_3$. Percent activation (+,X) was determined as (initial activity) (total activity)$^{-1}$.

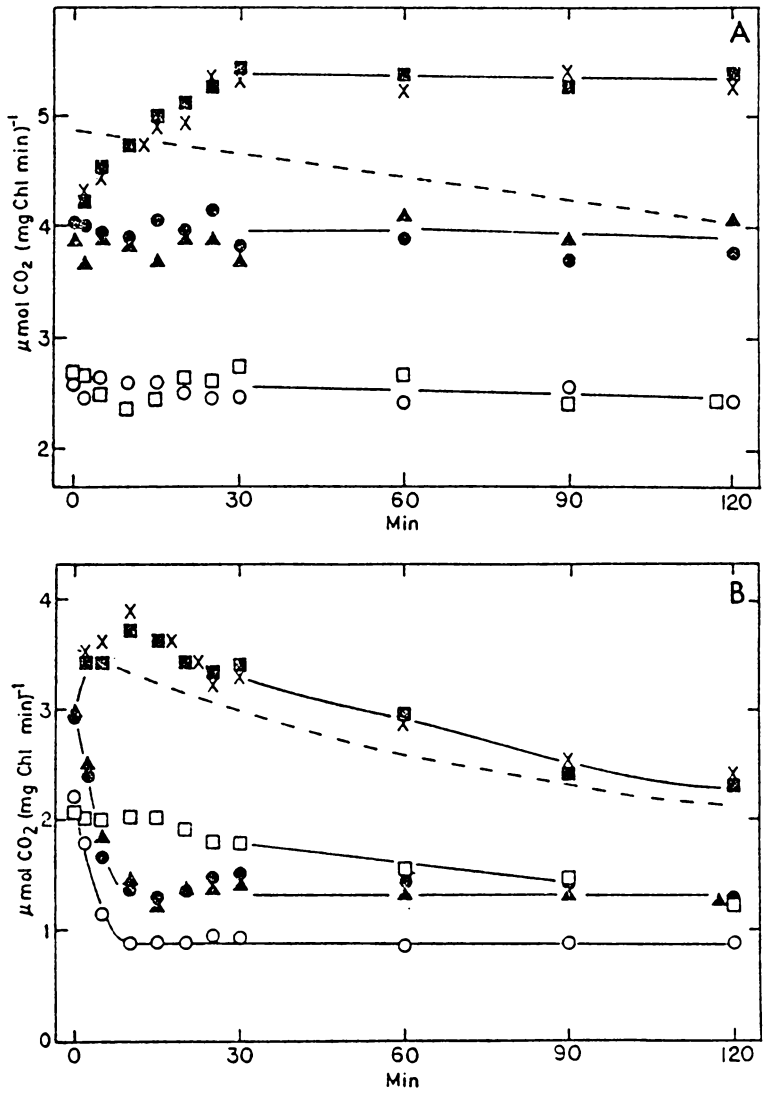

Figure 6. Stability of RuBP Case activity in soybean leaf extracts stored at 0°C (A) or at 25°C (B): Extracts were prepared from the same LN_2-frozen leaf powder in homogenizing medium (○,●), medium plus 20 mM $NaHCO_3$ (■,□), medium plus 5 mM $MgCl_2$ (▲), or medium plux 20 mM $NaHCO_3$ and 5 mM $MgCl_2$ (X) and assayed (single measurements) at the times indicated in medium plus 0 mM $MgCl_2$ (○,□) or 5 mM $MgCl_2$ (●,■,▲,X). Different leaves were used in A and B. Dashed line (----) indicates mean of total activity for all extracts.

CHAPTER 2: EFFECT OF IRRADIANCE DURING GROWTH OF GLYCINE MAX ON PHOTOSYNTHETIC CAPACITY AND PERCENT ACTIVATION OF RUBP CASE

ABSTRACT

Ribulose bisphosphate carboxylase (RuBP Case) initial activity (in vivo activity) and total activity (activity present after complete activation by CO_2 and Mg^{2+}) were assayed simultaneously in extracts of leaves of soybean (Glycine max cv. Will) plants which were grown under 4 different irradiance levels, 100%, 70%, 53% and 20% full sun (FS), in a greenhouse. Total RuBP Case carboxylase activity on a leaf area basis decreased with decreasing irradiance during growth, but was not different on a dry weight basis. The percentage of total RuBP Case which was active in vivo (initial activity/ total activity) was found to be similar for both high and intermediate light treatments (approx. 94%), but significantly lower (74%) for the low light treatment. However, when low-irradiance-grown plants were exposed to 100% FS, percent activation increased to a level comparable to that of the higher irradiance-grown plants (92%), indicating that the inactive portion of RuBP Case in the 20% FS treatment was further activatable in vivo. Maximal leaf photosynthesis rate on an area basis was significantly lower and saturated at a lower irradiance for the lower

irradiance-grown plants. Initial RuBP Case activity also decreased with decreasing irradiance and was found to correlate (r^2=0.84) with leaf photosynthesis rate. On a dry weight basis, leaf photosynthesis rate and initial RuBP Case activity were identical among leaves of the 4 light treatments. Thus, decreases in leaf photosynthesis rate observed upon growing plants in reduced irradiance are proportional to similar decreases in the in vivo RuBP Case activity.

INTRODUCTION

The growth of sun-adapted plants in irradiance levels which are suboptimal for maximal growth has a significant effect on their physiology and anatomy [for review see ref. 3]. Modifications in leaf anatomy during growth under reduced irradiance include a reduction in stomatal frequency and leaf thickness, the result of reduced cell size and number, but an increase in leaf chlorophyll content, due to increased granal development in the chloroplasts, and a reduction in stromal volume and photochemical; capacity [1, 5]. Maximal leaf photosynthetic activ-

ity at saturating irradiance is also reduced [1, 4, 5, 19]. The reduction in photosynthetic capacity in leaves grown under low irradiance is accompanied by a reduction in amount and activity of RuBP Case, the enzyme which catalyzes the CO_2-fixation step of the pentose phosphate reduction pathway, and in total leaf protein [1, 2, 7] However, the change in RuBP Case activity in response to changing irradiance levels is sometimes similar [4, 7], greater [2] or lesser [5] than the observed change in maximal photosynthesis rate. These previous studies were conducted before it was known that the enzyme requires preincubation in high CO_2 and Mg^{2+} for maximal activity [9]. Furthermore, not all of the RuBP Case enzyme, which can be activated *in vitro*, may be active *in vivo* [13, 14, 15, 17, 18]. Perchorowicz *et al.* [13, 14, 15] found that RuBP Case activity present in a leaf extract immediately after homogenization (initial, or *in vivo* activity) was considerably lower than the RuBP Case activity present after the extract had preincubated for some time in CO_2 and Mg^{2+} before assay (total activity). Therefore, it would seem likely that some of the inconsistensies reported in correlating photosynthesis with RuBP Case activity [2, 5, 7] may be the result of combined differences in the methods for measuring RuBP Case, as well as differences in the irradiance level at the time the leaves were assayed.

The purpose of this study was to determine if permanently altering a number of chloroplast morphological and physiological characteristics (e. g., chlorophyll and protein content, stromal volume, photochemical activity) by reducing irradiance during growth would, to some degree, permanently alter the amount of RuBP Case active *in vivo*. Since initial RuBP Case activity has been shown to undergo a reversible light activation-dark deactivation in wheat leaves [13, 15] and barley protoplasts [18], initial and total RuBP Case activities were measured in leaves of soybeans at the irradiance in which they were grown (100% to 20% FS) and after being subjected to a brief exposure to different irradiance levels. In addition, leaf thickness, specific leaf weight (SLW), soluble protein, chlorophyll, and the irradiance-response of photosynthesis were also measured to serve as a basis of comparison with previous studies and to observe the relationship that these may have with changes in initial RuBP Case activity.

MATERIALS AND METHODS

Plant Culture: Soybeans (Glycine Max (L.)Merr., cv. Will) were grown in 1-l pots containing equal amounts of perlite, vermiculite, Turface and peat moss to which was added 1 g of $CaCO_3$ per pot. After germination, pots were placed inside wooden frame boxes (approx. 0.7 m on each side) supporting black polypropylene shade cloth (Propex, Amoco Fabrics) of shade densities of 30%, 47%, and 80%. A fourth set of pots was unshaded to serve as a control. Six pots were used for each shade treatment. Plants were grown in a temperature-controlled greenhouse (22/29°C, day/night,) and irrigated daily, alternating between water and a modified Hoagland's nutrient solution.

RuBP Carboxylase Assays: Fully-expanded trifoliate leaves on the uppermost part of the plant (not shaded) were selected and either the center leaflets were excised or leaf discs (0.7 cm^2) were removed with a cork borer. Leaf tissue was then immediately frozen in liquid N_2 (LN_2). Care was taken to prevent shading the leaf tissue during the few seconds between excision and freezing in LN_2. Leaf tisue was stored in LN_2 until extraction. Irradiance was measured with a quantum sensor (Li-Cor model Q-5550) within each shade box at the time of leaf harvest. The remaining leaflets of the trifoliate leaf were

excised and used for leaf chlorophyll, SLW, and leaf protein measurements.

Liquid N_2-frozen leaf tissue was placed in a LN_2-chilled mortar and ground into a fine powder. Approximately 0.1 g of this powder was transferred to an ice-chilled glass homogenizer and homogenized with 1 ml of extraction medium [100 mM Tris-Cl, pH 8.0 at 0°C, 0.1% (w/v) Casein, 5 mM $MgCl_2$] and stored on ice. When leaf discs were used as opposed to an entire leaflet, 5 discs were ground to a powder inside a glass homogenizer chilled with LN_2. Afterwards, the homogenizer was transferred to an ice bath and warmed to ice temperature, then the tissue was homogenized in 1 ml of extraction medium. The time from extraction to assay was approximately 1 min.

Assays were conducted in stoppered 4-ml polypropylene scintillation vials in a 25°C water bath (Gilson, model SGRP-14) and were shaken reciprocally at 3 s^{-1}. Leaf extract (50 µl) was added to 0.45 ml of an assay medium: (final concentration) 100 mM Tris-Cl, pH 8.0 at 25°C, 5 mM $MgCl_2$, 20 mM $NaH^{14}CO_3$ (0.1 Ci mol^{-1}), 0.4 mM RuBP, and 0.1% (w/v) Casein. Initial activity was measured by the addition of extract to the complete assay mixture. Final activity was attained after extract was allowed

to incubate for 5 min in an assay medium without RuBP, with shaking at 25°C, and the assay initiated by the addition of RuBP. After 1 min, the assays were terminated by the addition of 0.2 ml of 6 N formic acid. The vials were flushed with air for 5 min and then dried at 70°C overnight. After drying, vial contents were resuspended in 0.2 ml of H_2O and 3 ml of Liquiscint (National Diagnostics, Somerville, NJ) was added. Radioactivity was determined by scintillation spectroscopy.

Magnesium was included in both the extraction and assay mediums as a necessary precaution to maintain the in vivo amount of enzyme in the active (enzyme-CO_2-Mg^{2+} or ECM) form during extraction and assay [16]. Inactive enzyme (E form) is only activated by preincubation in high CO_2 and Mg^{2+} [16]. Mg^{2+} also converts the EC (enzyme-CO_2) form to the active ECM form. Therefore, initial activity is a measure of both the EC and ECM forms, and total activiy is a measure of all 3 forms: E, EC, and ECM [14].

Specific leaf weight, leaf protein, and chlorophyll measurements Twenty-five leaf discs (0.7 cm^2) were removed from leaflets. Ten of these discs were weighed, frozen in LN_2 and extracted in a glass homogenizer with sufficient 50 mM Tris-Cl

buffer to make a final concentration of 0.05 g fr.wt. ml^{-1}. The extract was allowed to sit on ice for approximately 30 min, after which insoluble material was removed by centrifugation at 12000xg for 1 min and the supernatant was stored at -20°C. Protein concentration was later determined using a dye-binding assay [6].

Another 5 discs were weighed and extracted in an ice- chilled mortar with 10 ml of methanol. Insoluble material was removed by centrifugation at 1000xg for 5 min and the chlorophyll concentration was determined spectrophotometrically [11].

SLW (g dry cm^{-2}) was determined by weighing the remaining 10 leaf discs after drying overnight at 70°C.

Photosynthesis measurements: Photosynthesis was measured in a closed system consisting of (in series): a peristaltic pump (Cole-Parmer), a flow-meter, a CO_2 generator, a plexiglass leaf chamber (12.5 x 4.0 x 13.5 cm), a condensor (-20°C) and an infra-red gas analyzer (Mine Safety, model 303). Air was circulated through the system at a rate of 1 l/min.

A single trifoliolate leaflet was excised under water and

inserted into the leaf chamber, with the petiole remaining in a small reservoir. The leaf was supported by wires, stretched diagonally across the width of the chamber. The chamber was sealed and immersed in a water bath (25°C), and illuminated with a tugsten light source. Photosynthesis rate was measured as the change in CO_2 concentration between 350-250 µl l^{-1} CO_2.

Leaf thickness measurement Leaf thickness was determined from photomicrographs of thin sections, cut by hand with a razor blade, from representative leaves of each of the 4 treatments.

RESULTS AND DISCUSSION

Chlorophyll, SLW, soluble protein, and initial and total RuBP Case activities were measured in leaves of all 4 light treatments at 3 different times (20, 40, and 60 days after planting). Plants were all of the same developmental age on each of the sampling dates for the 4 light treatments. Variation in measurements among the sampling dates was reduced by

normalizing the means of the three lower irradiance treatments to the mean of the 100% FS control (Table 1). Lower irradiance during growth was accompanied by a significant decrease in SLW (Table 1), and a smaller decrease in leaf thickness (Table 1, Fig. 7). Two distinct palisade layers were observed in leaves of 100% FS-grown plants. In the leaf sections from 70% and 53% FS, the cells in the lower palisade layer were shorter and in appearance seemed to resemble spongy mesophyll cells. In the leaf sections from 20% FS, there was only 1 distinct palisade layer. Leaf chlorophyll levels on an area basis were the same for all 4 light treatments, but on a dry weight basis chlorophyll increased with lower irradiance during growth, being 2.3 times higher in the lowest irradiance treatment. Examination of the leaf thin-sections showed that the number of chloroplasts per cell was similar for all 4 leaf types, about 15 chloroplasts per mesophyll cell. Therefore, the chlorophyll concentration per chloroplast is probably higher in the leaves grown in lower light. Previous studies [1, 5] have reported that the higher leaf chlorophyll concentration per chloroplast was related to more extensive chloroplast granal development. In short, the effects of altering irradiance during growth on observed changes in leaf anatomy, SLW and chlorophyll concentration are consistent with those reported in previous studies

[1, 3, 4, 5].

Both initial and total RuBP Case activities on an area basis decreased with decreasing irradiance during growth, but on a dry weight basis total activity was not significantly different (Table 2). Leaf protein on a dry weight basis increased significantly (Table 1), resulting in a lower specific activity of RuBP Case with decreasing irradiance, but this decrease was not large enough to be significant. The specific activity of RuBP Case was found to decrease about one-half in Atriplex [1] and about one-third in barley leaves [2] following growth in low irradiance. Bjorkman et al. [1] attributed reduction in RuBP Case activity, and presumably a comparable reduction in Fraction 1 protein [2], as part of the plant's "economizing" scheme, whereby with shading, light-harvesting capacity is increased by diverting more protein into increased chloroplast granal development at the expense of RuBP Case protein. Percent activation was similar for leaves grown in 100%, 70% and 50% FS, being about 93% between 30 and 60 days after planting, whereas percent activation in the leaves from 20% FS was consistently lower, about 74%. This means that for the high and intermediate irradiance treatments almost all of the leaf RuBP Case was in an active (EC or ECM) form in vivo. Only in the low-

est irradiance treatment was there a significant amount of RuBP Case (26%) maintained in an inactive (E) form.

We next determined whether this partial state of activation of RuBP Case in the shaded (20% FS) treatment was permanent or if the inactive RuBP Case could be further activated in the leaf by increasing irradiance. Plants grown in 100% FS were switched with plants grown in 20% FS, and RuBP Case initial and total activities were measured throughout one photoperiod. Activities were also measured in control plants which were grown and maintained in either 100% or 20% FS. The results of this experiment (Table 3) show that percent activation of RuBP Case in the leaves of the shade adapted plant, when exposed to 100% FS, was increased to the level of the 100% FS control and percent activation of RuBP Case in the 100% FS leaves when shaded was reduced to the level of the 20% FS control maintained in its original low-light conditions. Changing the irradiance level for only 1 day was probably not sufficient time to alter total RuBP Case activity, which is probably dependent upon the amount of RuBP Case protein present in the leaves as a result of the growth conditions before switching. Therefore, the inactive portion of RuBP Case in the 20% FS is indeed activatable under high light. The significantly lower

initial activity on a dry weight basis observed in the 20% FS-grown leaves (Table 1) is the result of the irradiance level to which the leaves were exposed immediately before harvest. If percent activity of RuBP Case in vivo is regulated by stromal Mg^{2+} and H+ concentration as a result of photosynthetic electron transport, then it would appear that even chloroplasts in leaves grown in very low irradiance (20% FS) have sufficient photochemical capacity to completely activate almost all of their RuBP Case.

In that an exposure to a different irradiance level during one day altered the percent activation of RuBP Case (Table 3), we then examined if a relationship existed between initial RuBP Case activity and leaf photosynthesis rate as a function of altering irradiance. Upon increasing irradiance, photosynthesis rate and initial RuBP Case activity increased rapidly and were both essentially constant after 5 min (data not shown). The irradiance response curves of photosynthesis rate, measured in leaves of plants grown under the 4 irradiance levels (Fig. 8), were typical of the photosynthesis response of sun-adapted plants grown in low irradiances [1, 4, 5, 7, 19]. The low-irradiance-grown plants had lower maximal leaf photosynthesis rates on an area basis. Photosynthesis rate satu-

rated at approximately the same irradiance level that the leaves recieved during growth as noted by the arrows in Figure 8. RuBP Case initial activity in leaves from the 4 treatments also increased with increasing irradiance in a manner similar to photosynthesis rate. To determine if a close relationship existed between RuPB Case initial activity and photosynthesis rate, we plotted RuBP Case initial activity vs. photosynthesis, in Fig. 9. A positive and significant correlation ($r^2 = 0.84$) existed between photosynthesis rate and initial RuBP Case activity as influenced by instantaneously increasing irradiance. The slopes and y-intercepts of the regression lines of the 4 light treatments were homogenious at the 5% level of significance as determined by an F-test. Apparently, while reduced irradiance during growth altered a number of leaf parameters (Table 1), the relationship between leaf photosynthesis rate and in vivo RuBP Case activity on a dry weight basis was not changed. At the lowest irradiance (7 $\mu E\ m^{-2}\ s^{-1}$), initial RuBP Case was only 50% of total activity. Photosynthesis under very low irradiance is probably limited more by the generation of RuBP than in vivo RuBP Case activity. Perchorowicz and Jensen [15] have shown that leaf RuBP concentration increased rapidly upon increasing illumination, saturating at about 250 $\mu E\ m^{-2}\ s^{-1}$. They attributed the further

increase in photosynthesis rate above this irradiance level to be due primarily to the further activation of RuBP Case *in vivo*.

From these data we conclude that irradiance level per se controlls *in vivo* RuBP Case activity in two ways. First, the level of irradiance during growth affects the total amount RuBP Case present on a leaf area basis because of a reduction in leaf volume. Secondly, irradiance level has a direct effect on the amount of RuBP Case functioning *in vivo* which is mediated by a light-activation mechanism, the exact nature of which is still unknown. Percent activation of RuBP Case was similar (94%) for the 53%, 70% and 100% FS-grown plants indicating that at least 53% FS was sufficient to activate most (94%) of the leaf RuBP Case to either the EC or ECM form, while 20% FS was sufficient for only partial (74%) activation, regardless of differences in irradiance during growth, the levels of RuBP Case protein, or the amount of chlorophyll in the leaf.

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Table 1. Leaf thickness, SLW, chlorophyll and protein concentrations as affected by irradiance during growth. Leaf thickness was measured on at 60 days after planting. The other parameters are the means of measurements from 3 sampling dates: 30, 40, and 60 days after planting. For analysis, measurements were normalized to the 100% FS control on each sampling date. Numbers in parentheses are the means of values derived by dividing the experimental by the 100% FS control for analysis. Means followed by the same letter are not significantly different at the 95% confidence level as determined by Duncan's multiple range test.

Irradiance	Leaf Thickness	SLW	Chlorophyll a+b		Protein	
%FS	μm	g dry wt dm^{-2}	mg dm^{-2}	mg g dry wt^{-1}	mg dm^{-2}	mg g dry wt^{-1}
100	231(1.00a)	0.327(1.00a)	2.84(1.00a)	8.68(1.00c)	24.8(1.00a)	76.4(1.00b)
70	183(0.79b)	0.224(0.67b)	2.80(0.99a)	13.3 (1.54b)	22.2(0.91a)	116 (1.39ab)
53	177(0.77b)	0.202(0.61bc)	2.96(1.05a)	15.2 (1.75b)	22.4(0.91a)	111 (1.47ab)
20	126(0.55c)	0.152(0.46d)	3.01(1.05a)	20.1 (2.31a)	17.1(0.70a)	115 (1.52a)

Table 2. Initial and total RuBP Case activities, percent activation, and specific total activity as affected by irradiance during growth. Means of triplicate measurements made on three sampling dates (30, 40, and 60 days after planting) are shown. For analysis, measurements were normalized to the 100% FS total activity for each sampling date. Numbers in pare theses are the means of values derived by dividing the experimental by the 100% FS total activity for analysis. Means followed by the same letter are not significantly different at the 95% confidence level as determined by Duncan's multiple range test.

Irradiance	Initial RuBP Case		Total RuBP Case		Percent Activation	Specific Total Activity
	mg CO_2 h^{-1}		mg CO_2 h^{-1}			mg CO_2 h^{-1}
	dm^{-2}	g dry wt^{-1}	dm^{-2}	g dry wt^{-1}		mg $protein^{-1}$
100	68.8(0.96a)	212(0.96ab)	71.8(1.00a)	222(1.00a)	0.96a	2.96(1.00a)
70	51.1(0.71b)	249(1.10a)	54.6(0.75b)	268(1.18a)	0.94a	2.50(0.87a)
53	47.5(0.66b)	244(1.10a)	50.4(0.70b)	262(1.17a)	0.94a	2.39(0.81a)
20	26.9(0.37c)	181(0.82b)	36.9(0.51c)	246(1.12a)	0.74b	2.15(0.74a)

Table 3. Initial and total RuBP Case activities measured at mid-day in 65 day-old soybean plants grown and maintained in either 100 or 20%FS and similar plants which were moved to the 20 and 100% FS conditions during the preceeding night period. Means followed by the same letter are not significantly different at the 95% confidence level as determined by Duncan's multiple range test.

Irradiance during growth (%FS)	Irradiance before measurement (%FS)	RuBP Case activity (mg CO_2 dm^{-2} h^{-1}) Initial	Total	Percent Activation
100	100	45.9a	51.4a	89a
20	100	30.5c	32.9b	93a
100	20	35.9b	53.0a	68b
20	20	22.0d	32.9b	67b

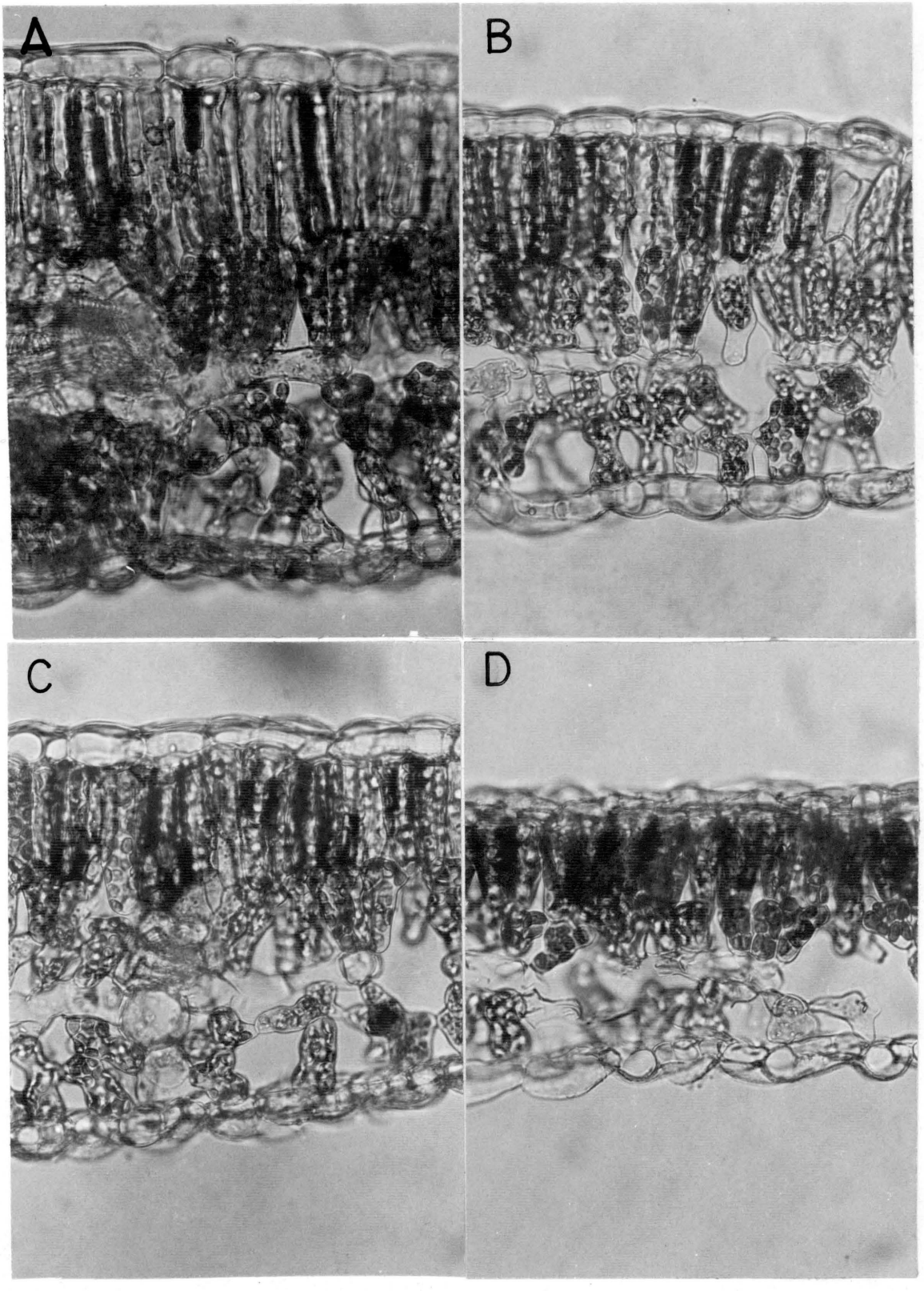

Figure 7. Effect of irradiance on leaf thickness and anatomy: Photomicrographs of thin sections of leaves from plants grown in (A) 100% FS, (B) 70% FS, (C) 53% FS, and (D) 20% FS, measured at 50 days after planting.

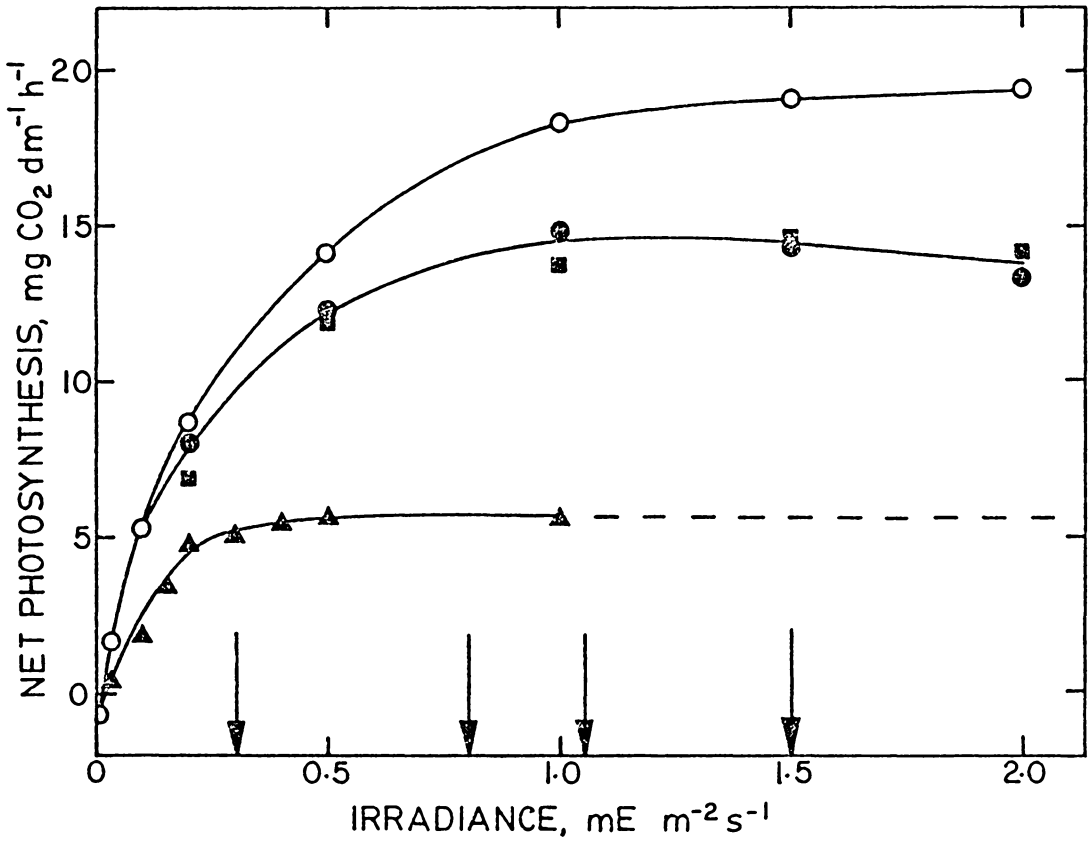

Figure 8. Photosynthesis response curves of soybean leaves grown in different irradiances: Photosynthesis rate of detached trifoliolate leaflets from 35-day old soybeans grown in 100% FS (O), 70% FS (●), 53% FS (□) and 20% FS (▲), as measured with increasing irradiance. Means of triplicate measurements are shown (avg. SD of mean for all measurements was 5%)

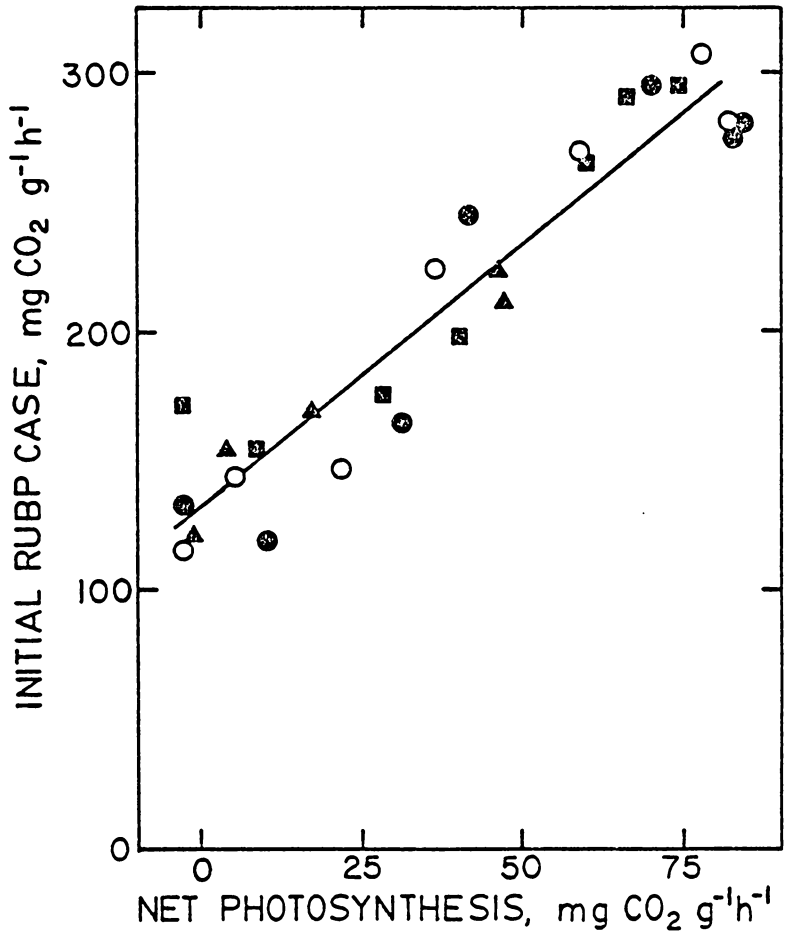

Figure 9. Comparison between initial RuBP Case activity and net photosynthesis rate: Plot of photosynthesis rates vs. initial RuBP Case activities measured at different irradiance levels, for plants grown in 100% FS (○), 70% FS (●), 53% FS (■), and 20% FS (▲). RuBP Case initial activity was measured in a leaflet similar to the one used for photosynthesis measurements. The leaflet was exposed to each irradiance for at least 10 min, and leaf discs were removed for RuBP Case assay. The solid line is the regression slope (1.94 ± 0.17) of all the data points and intercepts the y-axis at 131.4 ± 7.8. The total RuBP Case activity for all 4 treatments was 309.9 ± 32.2.

DISCUSSION

OPTIMIZATION OF RUBP CASE EXTRACTION AND ASSAY

The necessity for defining the optimal conditions for extracting and assaying RuBP Case activity in soybean leaves was prompted by a preliminary experiment (Appendix A). Our prior assumption, that RuBP Case initial activity was preserved upon rapidly freezing soybean leaves in LN_2 following removal from the plant, was correct according to the results of our study. Storing leaves in liquid N_2 or an ice bath were both effective in maintaining the activation state of the enzyme in leaf tissue for up to 4 hours (Fig. 5, Chapter 1). In addition, initial RuBP Case activity in extracts of leaf tissue was preserved by storage on ice for a period of up to 2 hr (Fig 6a, Chapter 1). Storage of homogenate at 25°C (Fig 6b, Chapter 1), in contrast, resulted in a rapid change in activity to a new level determined by the CO_2 and Mg^{2+} levels in the homogenate. These results are consistent with the hypothesis of McDermitt et al (1982), that RuBP Case activitation is kept displaced from equilibrium at 0°C, but not at 25°C. The inclusion of

dithiothreitol (DTT) or other added protectants to the extraction medium did not provide any noticeable benefit to stability of the enzyme, when assayed immediately after extraction. McDermitt et al. (1982), concluded that DDT was an essential protectant for long-term storage of the enzyme at 0°C. Dithiothreitol was excluded from the extraction medium in further studies (Chapter 2), because it was observed that DTT actually promoted loss of total activity during incubation at 25°C. Dithiothreitol may stimulate sulfhydryl-protease activity. The RuBP and CO_2 levels used in the earlier study (Appendix A) were sufficient for saturating RuBP Case activity. The possibility that differences in RuBP concentration could account for the observed variability from day to day (Fig. 10, Appendix A) cannot be ruled out. High RuBP concentrations were observed to further stimulate RuBP Case initial and total activity above activities predicted from Michaelis-Menten kinetics (Fig. 4, Chapter 1). Other sources of variability could have arisen from slight differences in the the pH of the extraction and assay, which was higher (8.2) than the optimum pH measured for RuBP Case activity (8.0; Chapter 1). In addition, the extract was allowed to preincubate at 25°C for 10 min. A time-course of RuBP Case activation (Fig. 1, Chapter 1) showed clearly that 5 min was adequate for maximal activation

at 25°C in the presence of 5 mM $MgCl_2$ and 20 mM $NaHCO_3$. Long incubation times may reduce activity in extracts from leaves of field- grown soybeans, particularly those of an older age, because protease activity is higher in older leaves (Wittenbach, 1980). This could have contributed total activities being lower than initial activities at the latter end of the growing season. The most surprising and troublesome effect was the level of Mg^{2+} on activity. Mg^{2+} was found to inhibit the rate of catalysis at concentrations greater than 5 mM (Figs. 1 and 3, Chapter 1 and Fig. 12, Appendix A). This was unexpected because 30 mM Mg^{2+} was found by Perchorowicz et al (1982) and McDermitt et al (1982) to be optimal for activation and assay of RuBP Case in leaf extracts of soybean. This effect would have caused further complications in our prior attempt to measure RuBP Case activity in field-grown soybeans. The unavoidable presence of endogenous Mg^{2+} carried over from the extract to the assay makes it difficult indeed to determine the in vivo the amount of enzyme present solely in the ECM form. Due to the extremely low $K_{act}(Mg^{2+})$ of 0.3 mM (Fig. 2, Chapter 1), and the rapidity with which Mg^{2+} binds to the EC form (Lorimer, 1976), it may be assumed that any RuBP Case present in the EC form would be converted to the ECM form by endogenous Mg^{2+} during extraction. However, the EC complex is not believed to repre-

sent a sizeable enzyme pool and most of the enzyme is believed to be in either the fully actvated (ECM) or inactive (EC) form (McCurry et al., 1981; Perchorowicz et al., 1983).

EFFECT OF IRRADIANCE DURING GROWTH ON RUBP CASE PERCENT ACTIVATION

After optimizing RuBP Case extraction and assay conditions, we investigated the effect of irradiance during growth on RuBP Case percent activity (Chapter 2). Altering irradiance during growth had a significant effect on total RuBP Case activity on a leaf area basis but percent activation was not affected by decreasing irradiance during growth from full sun to 50% full sun (Table 2, Chapter 2). A significant reduction in percent activation was observed, however, at 20% full sun, which is approximately 0.3 mE m^{-2} s^{-1}. This would imply that the observed reduction in maximal leaf photosynthesis rate on an area basis in plants grown in 70% and 53% full sun was not caused by a reduction in RuBP case percent activation. The principle reason the reduction in photosynthetic capacity on

an area basis for these plants can be attributed to a lower amount of total amount of RuBP Case activity present in the leaf. In the 20% full sun treatment, RuBP Case percent activation was reduced to 74%. However, the percent activation of RuBP Case in the 100% full sun plant was reduced to the same degree by exposure to 20% full sunlight (Table 3 and Fig. 9, Ch. 2; Fig 16, Appendix C). At the same time, RuBP Case percent activation in 20% full sun-grown plants was increased upon exposure to 100% full sunlight to the level observed in the 100% full sun plant. This would indicate that RuBP Case percent activation was not permanently altered by the irradiance level during growth. Hence, we conclude that percent activation is dependant on the irradiance level to which the leaf is exposed at the time of leaf removal and is not significantly affected by altered irradiance during growth.

Previous studies, in which RuBP Case initial activity and photosynthesis rate were measured in leaves or leaf protoplasts at different light intensities (Perchorowicz et al., 1981, 1983; Sicher, 1982), showed that RuBP Case initial activity and photosynthesis rate increased simultaneously with increasing irradiance. In this study, the irradiance response of photosynthesis and RuBP Case initial activity, measured in leaves

from the 4 different irradiance-growth treatments. The results (Fig. 9, Chapter 2) showed that initial RuBP Case activity also increased with increasing irradiance. In addition, RuBP Case initial activity was demonstrated in Fig. 9 to correlate with photosynthesis rate, when expressed on a leaf dry weight basis. Furthermore, RuBP Case percent activity in soybean leaves approached 100% in full sunlight (Tables 2 and 3, Fig. 9, Chapter 2). In other studies, percent activation in wheat seedlings saturated at only 60% and was not further activatable under strong light (Perchorowicz et al., 1981, 1983) This difference could be an actual species difference between wheat and soybean in the amount of RuBP Case which is able to be activated in vivo, or it could be explained as an age difference. In our study, we have observed that 20 day-old plants had a percent activation of only 70% for the 3 higher irradiance treatments, while percent activation for the 20% full sun treatment was around 50%. This could mean that in soybeans, RuBP Case is in a partial state of activation during early development, but increases with maturation to nearly 100% total activity under normal light conditions. The possibility that percent activation of RuBP Case may change with plant age was also suggested by our data from Appendix A, which shows percent activation increasing from 70% to over 100% during the

growing season. However, since there was much variability from day to day measurements, it is not possible to draw any conclusions of seasonal changes in percent RuBP Case activation in soybean based on these data alone; further experiments are required.

Another phenomenon we observed, which also merits further investigation, is the diurnal change in RUBP Case initial and total activity. Initial RuBP Case activity increased between morning and midday and decreased in the evening, presumably in response to changing irradiance (Figs. 16 and 17, Appendix C; Fig. 13, Appendix B) However, total RuBP Case activity also increased throughout the first half of the diurnal period to a maximum level by midday. This phenomenon has also been observed by other workers (McDermitt et al., 1982; Vu et al.., 1983). We also measured RuBP Case initial and total activity in chamber-grown soybeans throughout a diurnal period. Total as well as initial activity was observed to increase rapidly upon illumination (Fig. 17, Appendix C). RuBP Case protein levels were found to be relatively constant throughout the diurnal period (see Appendix B). Therefore the changes in total activity over a diurnal period may represent changes in the amount of RuBP Case that can be activated by Mg^{2+} and CO_2

alone and suggests that an additional mechanism exists to regulate the amount of RuBP Case which is able to be activated *in vivo*. A large portion of RuBP Case may be stored in an insoluble crystalline state in the dark (Gunning *et al*, 1968). The increase in total activity in the morning could then be caused by the release of the enzyme from this crystalline state. The mechanism by which this occurs may be connected to the Mg^{2+}/H+ changes in the stroma with light. Reformation of the crystalline state would be a slower process, to account for the slower decline in total activity at nightfall. Evidence that RuBP Case may exist in crystalline form *in vivo* can be seen in electron micrographs of *Avena* chloroplasts (Gunning *et al*, 1968), showing the presence of aggregates of chrystalline particles in the stroma. They were found to be proteinaceous, and closely ressembled purified crystalline Fraction 1 Protein.

APPENDIX A: SEASONAL CHANGES IN INITIAL AND FINAL ACTIVITIES OF RUBP CASE FROM LEAF HOMOGENATES OF FOUR SOYBEAN GENOTYPES

ABSTRACT

Initial activities (IA) and final activities (FA) of ribulose bisphosphate carboxylase (RuBP Case were measured in leaves of soybean [Glycine max (L.) Merr. genotypes ESSEX, WILLIAMS-79, WILL, V-78-1175] plants grown at the Virginia Tech Agronomy Farm. RuBP Case activities were measured eight times from four weeks after planting to senescence. Specific leaf weight and leaf chlorophyll dm^{-2} increased throughout the growing season and decreased abruptly before senescence. Soluble leaf protein dm^{-2} remained constant and also decreased at senescence. RuBP Case IA was determined by adding leaf homogenate directly to assay medium. RuBP Case FA was measured after incubation of leaf homogenate for 10 min in 10 mM $NaHCO_3$ and 20 mM $MgCl_2$. FA on an area basis slowly decreased throughout the growing season. IA did not vary significantly from FA except on the first sampling date when FA exceeded IA and twice in the middle

of the season when IA exceeded FA by about 100%. IA was optimal at 5 mM $MgCl_2$ and was inhibited at higher Mg^{2+} concentrations. Differences between IA and FA may reflect changes in the response of the enzyme to the level of Mg^{2+}, CO_2, and RuBP which are maximal for activation and assay.

INTRODUCTION

RuBP carboxylase catalyzes the first step in photosynthesis which is the incorporation of CO_2 with RuBP to form two molecules of P-glycerate. The enzyme also incorporates molecular oxygen with RuBP to form P-glycolate and P-glycerate. P-glycolate is the substrate for photorespiration. Hence, this one enzyme is believed to have an important role in regulating the rates of photosynthesis and photorespiration (3). Studies (1, 6, 8, 10, 11) using purified RuBP Case have shown that the enzyme must first be preincubated in Mg^{2+} and CO_2 to attain maximal activity. The Kact (concentration required for half maximal activation) have been measured to be approximately 100 µM CO_2 and 1 mM Mg^{2+} for the soybean enzyme (8) and 43 µM

CO_2 and 2 mM Mg^{2+} for the tobacco enzyme (6). Magnesium concentration in the chloroplast stroma has been measured to be about 2 mM (16) and CO_2 concentration is probably no greater than atmospheric, 10 μM (8). Hence, RuBP Case may be in a state of partial activation in the leaf. Perchorowicz et al (14) measured RuBP Case IA in wheat leaves immediately after homogenization and RuBP Case FA after incubation of the homogenate in saturating Mg^{2+} and CO_2. They found that the percent activation of RuBP Case increased with increasing light intensity but was never greater than 60%. It is not known if the percent activation of RuBP Case in the leaf varies with leaf age. Such changes could contribute to differences in photosynthesis which occur inplant canopies during the growing season (9). To examine this possibility, we measured IA and FA of RuBP Case in leaves of field-grown soybeans at weekly intervals between 4 weeks after planting to senescence. Four genotypes were examined of which two were genetically similar (WILLIAMS-79 and WILL), the third (ESSEX) quite different genetically from the first two, and the fourth (V-78-1175) was an experimental strain developed from a cross of WILLIAMS-79 and ESSEX.

MATERIALS AND METHODS

Plant Material

Soybeans [Glycine max (L.) Merr. cvs. ESSEX, WILL, WILLIAMS-79, and V-78-1175] were planted June 20, 1982, every 4 cm in 6 m rows at 0.9 m row widths. The growing site was situated one a Groseclose silt loam. The soil was fertilized with 34 kg P_2O_5 ha^{-1} and 83 kg K_2O ha^{-1} and treated with a mixture of the herbicides Basilin and Vernam applied at recommended rates pre-emergence. Leaves were collected between 1200 and 1400 h on clear days. For each cultivar, 5 mature trifoliolate leaves were selected at random from leaves at the top of the canopy. The central leaflets were removed, placed in a cheesecloth bag, and immediately plunged into a dewar of liquid N_2. The remaining two leaflets were placed in plastic bags and stored on ice for later specific leaf weight, leaf chlorophyll, and soluble leaf protein measurements. The stage of plant development was

recorded using the criteria of Fehr and Caviness (5).

RuBP carboxylase assays

Leaves, previously frozen in liquid N_2, were ground to a fine powder in liquid N_2 with a mortar and pestle. Approximately 0.1 g of leaf powder was removed and placed in an ice-chilled mortar and ground to a slurry with 5 ml of homogenization medium [50 mM tris-Cl, pH 8.2 at 25°C, 10 mM isoascorbic acid, 5 mM dithiothreitol (DTT) and 0.1% bovine serum albumen (BSA)]. Initial activity measurements were performed within 2 min after homogenization by adding 0.1 ml of homogenate to 0.9 ml of assay medium (100 mM tris-Cl, pH 8.2 at 25°C, 20 mM $MgCl_2$, 5 mM DTT, 0.4 mM dibarium RuBP, and 10 mM $NaH^{14}CO_3$ in a final volume of 1 ml) at 25°C. A 40 mM dibarium RuBP stock solution was made immediately before assay and stored on ice. Assays were performed in in 3 ml conical vials fitted with rubber stoppers. The assay mixture was stirred with a magnetic stir bar. At 30 sec time intervals over a 2 min periiod 50 µl aliquots were removed and injected into 4 ml plastic poly-scintillation

vials containing 0.1 ml of 5N acetic acid. Vials were purged with air and dried overnight at 70°C. To the vials was added 0.2 ml of H20 and, after mixing, 3 ml of Liquiscint (National Diagnostics, Inc., Somerville, New Jersey) was added Radioactivity was determined by scintillation spectroscopy. Final activity was measured in a similar manner to IA except that 0.1 ml of homogenate was added to assay medium without RuBP and after 10 min of incubation at 25°C, 10 µl of 40 mM dibarium RuBP was added to initiate the reaction. RuBP-dependent $^{14}CO_2$ fixation was linear for 2 min for both IA and FA. Activities were determined from linear regression analysis of $^{14}CO_2$ fixed per minute. Chlorophyll was measured in 0.1 ml aliquots of the homogenates. Initial activity and FA were each measured simultaneously from three different homogenates of the same leaf powder.

Leaf protein, leaf chlorophyll, and specific leaf weight measurements

From the leaflets stored on ice, 0.5 cm2 leaf discs were removed with a cork borer. Ten discs were dried overnight at

70°C to determine specific leaf weight. Chlorophyll content in 5 leaf discs was determined spectrophotometrically (17) after extraction in 25 ml of 95% ethanol. Protein was measured using the Bradford method (2). Ten leaf discs were homogenized at 0°C in sufficient 50 mM Tris-Cl buffer (pH 8.2 at 25°C) to make a 5% (w/v) homogenate. After incubation on ice for 20 min, the homogenate was centrifuged at 10,000 X g for 15 min and stored at -20°C until analysis. An aliquot of the homogenate was mixed with diluted dye reagent (Bio-Rad), and protein was calculated by comparing A_{595} against a BSA standard in the same buffer.

RESULTS AND DISCUSSION

Additional Mg^{2+} or CO_2 (above air levels) was not included in the homogenizing medium (14, 15) so as not to activate any inactive RuBP Case present in the homogenate. For convenience, activation and assay were performed in the same reaction vial (14, 15), using Mg^{2+} and HCO_3^- levels found sufficient to activate purified enzyme (8).

Preliminary experiments with leaves of chamber-grown soybean plants showed that storing leaves on ice for one hour before homogenization resulted in a substantial loss of RuBP Case IA compared with a homogenate made immediately after detaching the leaf. Homogenates from leaves stored in liquid N_2 for up to 5 hr showed little change in IA compared with that of an initial extract. IA was found to decrease slowly during storage of the homogenate on ice and therefore IA and FA were measured as rapidly as possible after homogenization. Incubation for 10 min at 25°C and assay of extract in buffer devoid of Mg^{2+} resulted in loss of activity as would be expected due to inactivation of the enzyme (8, 10). FA measurements were similar whether leaves were homogenized in homogenizing medium plus 20 mM $MgCl_2$ and 10 mM $NaHCO_3$ and then assayed or preincubated for 10 min in assay medium before adding RuBP.

Addition of compounds to the homogenization medium which protect against denaturing substances in the homogenate either had no effect (10 mM isoascorbate, 0.1% BSA, 0.1% casein, 2% PVP-40), slightly inhibited (1% BSA, 1% casein), significantly inhibited (20% Dowex-1-Cl), or slightly enhanced (5 mM DTT) both IA and FA to the wame extent. Similar results were found with leaves from plants at various stages of development. For

these reasons, 5 mM DTT, 10 mM isoascorbic acid, and 0.1% BSA were included in the homogenization medium, and 5 mM DTT in the assay. Filtration or centrifugation to remove particulates resulted in a loss of about 50% of IA and FA based on the amount of Chl present in the original homogenate. Studies are continuing on the optimization of procedures for measuring IA and FA in leaf homogenates and details will be presented in another publication [2].

RuBP Case FA when expressed on a leaf area basis (Figure 10) or leaf protein (not shown) slowly declined throughout the growing season. Final activity was highest on the first sampling date, probably because these leaves were not fully expanded, and declined rapidly before senescence. When expressed on a dry weight, or leaf chlorophyll basis, FA decreased considerably throughout the growing season, because of the large increase in specific leaf weight and chlorophyll (Figure 11). The earlier maturing cultivars (WILL, WILLIAMS, and V-78-1175) all of maturity group III were beginning to senesce 93 days

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[2] Servaites and Torisky (1984) Activation state of ribulose bisphosphate carboxylase in soybean leaves. Plant Physiol. In Press.

after planting as evidenced by decreases in specific leaf weight, leaf chlorophyll, and leaf protein levels (Figure 2), but ESSEX, a later-maturing cultivar of group V, did not show these changes. IA in field-grown soybean leaves was significantly (95% level) lower than FA on the first sampling date for all the genotypes WILL. In all the genotypes, the mean FA exceeded the mean IA until 60 days after planting when mean IA was greater than the mean FA. This difference was significant for all four genotypes (95% level) at 60 and 64 days after planting when IA was about twice FA. This difference appears to be due to an increase in IA, rather than a decrease in FA, in that FA was not lower at these times than at others. In contrast to previous reports in which IA was found to be considerably lower than FA (14, 15), these data would indicate that IA RuBP Case in soybean leaves at the top of the canopy throughout most of the growing season is either not different from or exceeds FA RuBP Case.

McDermitt et al (13), using a procedure similar to the one used here, found IA and FA of RuBP Case in leaf homogenates from field-grown soybeans to be identical during a diurnal time course, but homogenates from chamber-grown plants were found to have lower IA than FA. Further research needs to resolve the

differences observed between IA and FA which are controlled by light intensity (14), to determine if species differ in IA and FA RuBP CAse, and to optimize extraction and assay procedures for measuring FA and IA (15).

Higher IA than FA was unexpected in that this has not previously been reported. The difference could be attributed to a change in the state of activation of the enzyme during incubation because of sub-optimal levels of Mg^{2+} and CO_2 required for full activation, or a loss of activity during incubation at 25°C due to denaturing substances or proteases in the leaf which inhibited enzyme activity. To examine if Mg^{2+} was optimal for activation and subsequent assay we measured IA and FA in the presence of increasing concentrations of Mg^{2+} in homogenates of leaves collected 67 days after planting. Figure 12 shows only the data for ESSEX, but a similar response was measured for the other varieties. Final activity increased with Mg^{2+} concentration and saturated at 10 mM Mg^{2+}. This response is similar to that of purified enzyme (10). Initial activity was optimal at 5 mM Mg^{2+}. Magnesium concentrations (above 10 mM) necessary for activating purified RuBP Case were inhibitory to IA and in the absence of Mg^{2+}, IA was 60% of that at 5 mM. These data are quite different from those of Perchorowitz et al

(15) in which 30 mM Mg^{2+} was optimal for both activation and assay of RuBP Case from soybean leaves. Initial activity at 5 mM Mg^{2+} for the cultivars, ESSEX, V-78-1175, WILL, WILLIAMS-79, were 122, 188, 170, and 161 mg CO_2 dm^{-2} hr^{-1}, respectively. These rates are considerably higher than those reported by Hesketh et al. (7) for 29 different different field-grown soybean genotypes. Hence, it does not appear necessary and, in fact, could be inhibitory, to preincubate crude leaf homogenates from soybean in Mg^{2+} and CO_2 before assay of RuBP Case. Conditions for measuring optimal activity in leaf homogenates may be quite different from those of the purified enzyme and need to be determined for each species (15).

A number of workers (1, 4, 6, 8) have reported on the action of chloroplast intermediates such as RuBP, NADPH, 6-P-gluconate, and Pi, which act as effectors of activation of RuBP Case at suboptimal levels of Mg^{2+} and CO_2. These studies show that effectors bind loosely to the E-CO_2-Mg^{2+} complex and stabilize the complex in the absence of RuBP. Effectors apparently do not cause activation, but shift the equilibrium of the enzyme to the activated complex and have no effect on activation in the presence of levels of CO_2 and Mg^{2+} optimal for activation. Hence the presence of an effector(s) in the crude homogenate

cannot explain the higher IA over FA reported here, but may have a role in maintaining the state of activation of the carboxylase *in vivo* in the presence of physiological concentrations of CO_2 and Mg^{2+} or in a cold homogenate.

One min of incubation in saturating Mg^{2+} and CO_2 was sufficient time to attain maximal RuBP Case activity in a leaf homogenate from soybean (15). FA decreased about 20% in the following 4 min, presumably because of the action of phenolic compounds present in the leaf homogenate which denatured the enzyme (15). We found protectants, except DTT which increased both activities by 10%, to have little effect on preserving activity. Likewise, in the presence of RuBP final rates were linear for 3 min, but the possibility of alteration of RuBP Case during preincubation needs to be investigated.

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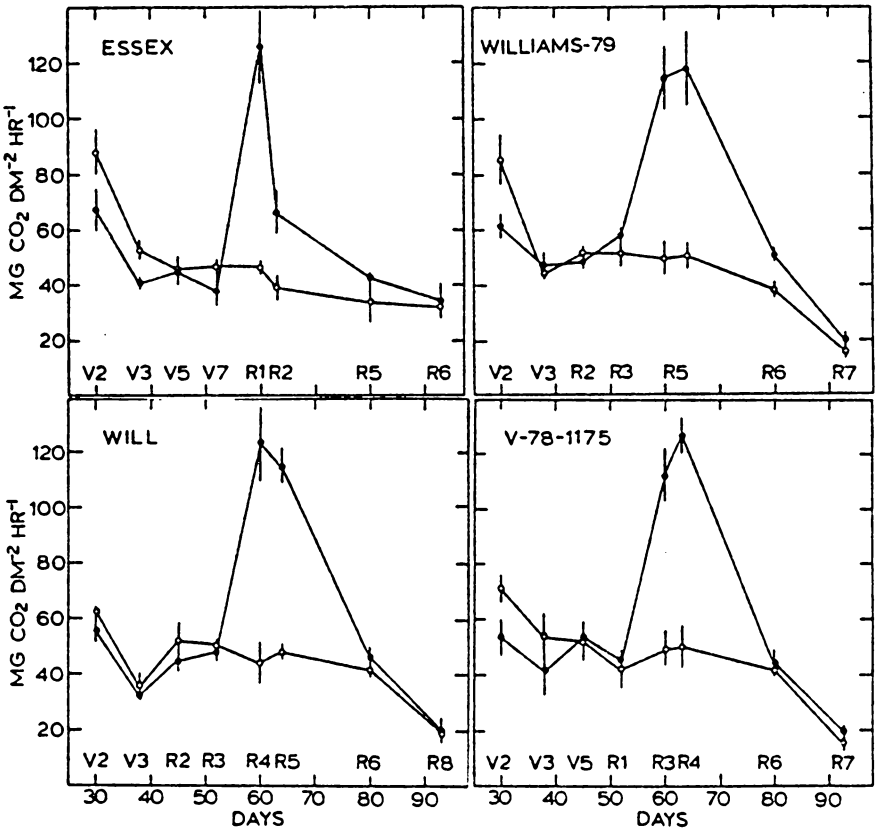

Figure 10. RuBP Case IA and FA in field-grown soybeans over the growing season: RuBP Case IA (●) and FA (○) measurements from homogenates of leaves of four soybean genotypes collected at various days after planting. Vertical lines indicate standard deviation of mean. Details of the assay procedures are stated in the text. Letters and numbers along the abscissa for each genotype indicate the developmental stage (5) of the plants at each sampling date.

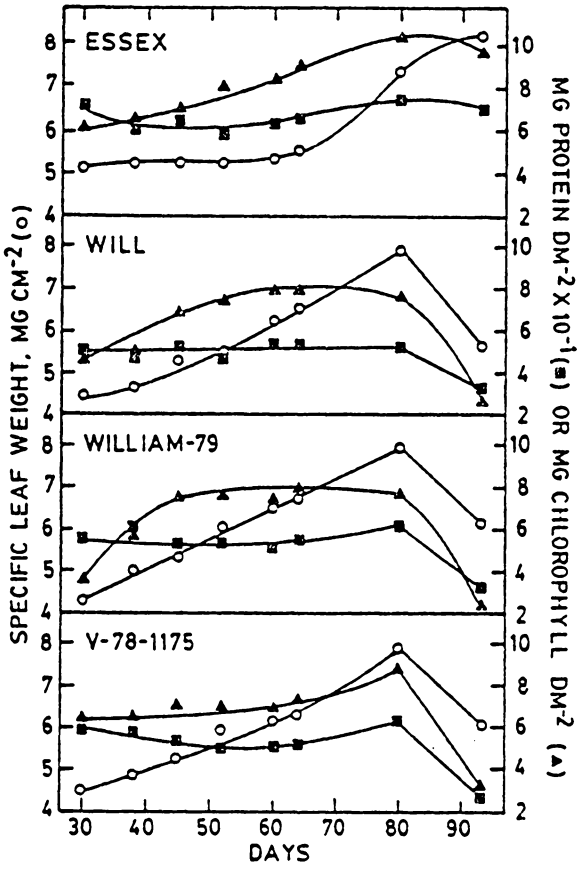

Figure 11. Seasonal changes in SLW, leaf chlorophyll and leaf protein: Leaf chlorophyll (▲) leaf protein (■) and specific leaf weight measurements (O) from leaves of four genotypes of soybean collected at various times after planting. Details of the assay procedures are stated in the text. Leaf protein levels are a factor 10X greater than legend.

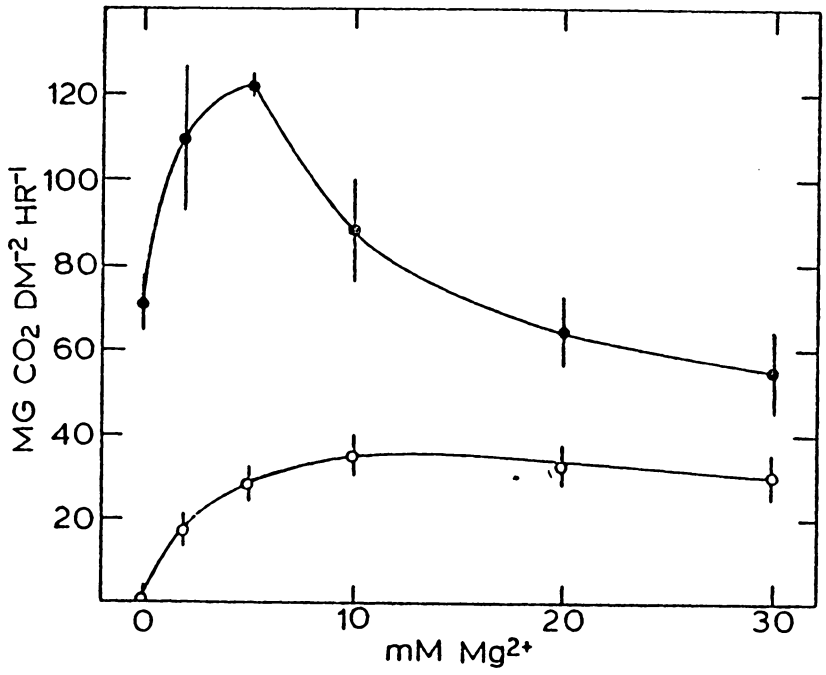

Figure 12. Magnesium response of RuBP Case initial, final activity: Effect of Mg^{2+} concentration in the assay medium on IA (●) or in the incubation and assay medium on FA (○). Assays were conducted as described in the text from homogenates of leaves of the genotype ESSEX collected 64 days after planting. FA and IA were measured simultaneously at each Mg^{2+} concentration from three separate homogenates. Vertical lines represent standard deviation of means.

APPENDIX B: DIURNAL CHANGES IN RUBP CARBOXYLASE ACTIVITY AND ACTIVATION STATE IN LEAVES OF FIELD-GROWN SOYBEANS

SUMMARY

Initial ribulose 1,5-bisphosphate carboxylase activity (that present in an in vivo state) and total activity (that which is made available after complete activation by CO_2 and Mg^{2+}) were measured in extracts of field-grown soybean (Glycine max cv. Will) leaves collected over a diurnal period. Initial activity increased with increasing irradiance, saturating at 1.2 millimoles $meter^{-2}$ $second^{-1}$, and decreased when irradiance level fell below this level. Total activity increased about three-fold during the first four hours and remained constant for the rest of the period. Percent activation (initial: total activity) was about 50% at the beginning and end of the diurnal period and remained near 100% for 6 hours in the middle of the day. Leaf photosynthesis and percent activation both increased similarly with increasing irradiance. The lower total activity observed in the morning hours could not be increased by gel-filtration of the extracts in the presence of

Mg^{2+} and CO_2 concentrations favoring complete activation. No marked changes were observed in the amount of total leaf protein on a gram dry weight basis in leaf samples collected throughout the diurnal period. We propose that light has two effects on ribulose 1,5-bisphosphate carboxylase activity: (1) light facilitates activation by CO_2 and Mg^{2+}, and (2) promotes the conversion of inactive enzyme present after a prolonged dark period to a form which is able to be activated.

INTRODUCTION

Ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBP Case) is an important photosynthetic enzyme directly involved in the regulation of the rates of both photosynthesis and photorespiration [1,2]. Purified RuBP Case requires activation with CO_2 and Mg^{2+} at high pH to attain maximal activity [2] and its activation in vivo may be regulated by light-induced changes in stromal Mg^{2+} and H+ concentrations [3]. Reversible light-activation and dark-deactivation of the enzyme has been demonstrated to occur in vivo in intact leaves [4,5] and iso-

lated leaf protoplasts [6,7] and chloroplasts [8]. The activation state of the enzyme has been shown to increase with irradiance and saturate at approximately the same irradiance as photosynthesis rate [4,6], indicating that activation of RuBP Case in vivo may be an important factor in controlling photosynthesis rate at most irradiance levels [4]. In this work, we have measured changes in the activation state of RuBP Case in field-grown soybeans as affected by changing natural irradiance levels during a daylight (diurnal) period to determine the extent of activation and its possible relevance to canopy photosynthesis.

MATERIALS AND METHODS

Soybeans [Glycine max (L.) Merr cv Will] were planted in Blacksburg, VA on June 20, 1983, every 4 cm in 6 m rows at 0.9-m row-widths. Before planting the soil was fertilized with 83 kg K_2O ha^{-1} and 34 kg P_2O_5 ha^{-1}. The soil was irrigated weekly when necessary. Three trifoliolate leaflets on different plants were selected at random from leaves in the uppermost

part of the canopy. Only leaves which were completely exposed (not shaded) were selected. At the indicated times, leaflets were removed, placed in a cheesecloth bag, and immediately immersed into a dewar flask containing LN_2. Leaves were stored in LN_2 until analysis was made on the following day. Immediately before assay the LN_2-frozen leaflets were ground to a fine powder in a LN_2-chilled mortar. Approximately 0.2 g of the leaf powder was extracted at 0°C in a ground-glass homogenizer with 5 ml of homogenizing buffer (50 mM Tris-HCl, 5 mM $MgCl_2$, and 10 mM dithiothreitol, pH 7.4 at 25°C). The addition of 5 mM Mg^{2+} to the extraction medium did not alter the in vivo percent activation of the enzyme [9], but was included as a precaution against possible deactivation of the enzyme during extraction [10]. Initial RuBP Case activity was measured by adding extract (50 µl) to an assay mixture containing (final concentrations): 50 mM Tris-HCl, 5 mM $MgCl_2$, 20 mM $NaH^{14}CO_3$ (0.1 Ci mol^{-1}), 0.5 mM RuBP, and 0.1% (w/v) casein, pH 8.0 at 25°C. After 1 min at 25°C, assays were terminated by the addition of 0.2 ml 6 N HCO_2H. After drying at 70°C, acid-stable ^{14}C was determined by liquid scintillation spectroscopy. Total RuBP Case activity was measured in a similar manner but the assay was initiated by the addition of RuBP after the extract had preincubated in the assay medium for 5 min to fully acti-

vate the enzyme. Further details of the assay procedure are provided elsewhere [9].

Chlorophyll was extracted with methanol and its concentration determined spectrophotometrically [11]. Total soluble protein was measured by addition of extract, from which particulate material was first removed by centrifugation at 12,000 X g for 10 min, to diluted dye reagent (Bio-Rad) and $A_{595\ nm}$ was compared to a BSA (fraction V) standard. The amount of RuBP Case protein present in leaf extracts was determined by spectrophotometric measurement of the amount of dye bound to protein after separation of protein by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and staining with Coomassie blue. The procedure was modified from that of Fenner et al. [12]. Leaf extract (0.1 g ml^{-1}) was added to an equal volume of extraction buffer [4% (w/v) SDS, 10% (w/v) 2-mercaptoethanol, 10% (w/v) glycerol, 50 mM Tris-HCl, pH 6.8 at 25°C] and boiled for 2 min. After centrifugation at 12,000 X g for 1 min, samples (approximately 20 ug protein) were subjected to SDS-PAGE (10% to 15% gradient) followed by staining with Coomassie Brilliant Blue R-250 (Serva) as described previously [13]. Gels were destained in isopropanol: acetic acid: water (25:10:65, v/v) for 12 h, followed by further destaining in repeated changes of

7% (v/v) acetic acid for an additional 24 h. Elution of dye from the gel was performed by cutting out stained bands, macerating them, and extracting the dye by incubation in 1% (v/v) SDS for 12 h at 25°C. After extraction, SDS solution was added to bring the sample solution containing the macerated gel to a defined volume in a graduated tube. The final volume was varied in order that the measured A_{600nm} was between 0.05 and 1.0. Absorbance values of similar gel areas not containing protein were subtracted from the sample absorbance values to reduce background absorbance. The percentage of RuBP Case protein in the leaf extract was determined by dividing the combined total absorbance values ($A_{600nm}ml^{-1}$ X ml $sample^{-1}$) of the large (55 kDa) and small (12.5 kDa) subunits by the total absorbance value of the entire gel (Fig. 13). In a preliminary experiment (Fig. 13), absorbance values of dye eluted from purified soybean RuBP Case and that present in a leaf extract were linear in the range of 0 to 30 µg of applied protein. The absorbance values of dye recovered from the entire gel corresponded to about 97% of the total applied protein for purified soybean RuBP Case and about 90% of the total applied leaf protein. Total SDS-extractable protein was determined in a similar manner, but from samples spotted and dried on filter paper [14]. Conversion of absorbance units to protein was based on a BSA

(fraction V) standard prepared in half-strength extraction buffer (A_{600nm} ml^{-1} µg $protein^{-1}$ = 0.15). For reference, crystalline tobacco RuBP Case had a measured value of 0.18.

Irradiance (400 to 700 nm) was recorded simultaneously throughout the diurnal period with a quantum sensor (Li-Cor model Q-5550) and a recorder at a 10 mV setting.

Photosynthetic CO_2 exchange was measured at 25°C in a closed system similar to the one described previously [15]. A leaflet was excised from the plant and sealed in a plexiglass cuvette with the petiole remaining in a small reservoir of water. The rate of change of CO_2 concentration between 350 and 250 µl l^{-1} was followed with an infrared gas analyzer (Mine Safety model 303) while the leaf was illuminated at varying irradiances (0-2 mmol m^{-2} s^{-1}) with a tungsten light source.

RESULTS

Initial (non-activated) and total (fully activated) RuBP Case

activities were measured at various times during a diurnal period in crude leaf extracts of field-grown soybeans at 45, 60, and 72 days after planting. Similar results were observed on all 3 dates. Figure 14 shows the response of the 72-day-old plants measured on a particularly clear day. Initial RuBP Case activity increased about 6-fold with increasing irradiance in the morning and approached a nearly constant level at an irradiance of approximately 1.2 mmol m^{-2} s^{-1}. Later in the day, initial activity decreased when irradiance fell below this level. Total activity increased about three-fold in the morning, but remained nearly constant for the remainder of the day. Percent activation [(initial activity) (total activity)$^{-1}$ X 100%] showed a parallel response to changing irradiance being about 50% at the beginning and end of the period and 100% for 6 h in the middle of the period.

In the field, increasing irradiance is probably accompanied by increasing leaf temperature, which may affect percent activation, although in the experiment shown in Figure 14, daytime air temperature was 20°C at both ends of the diurnal period and 25°C at midday. For this reason, leaves were taken into the laboratory and photosynthetic CO_2 exchange and percent activation measured as a function of increasing irradiance on simi-

lar leaflets (Fig. 15). Measurements were made after leaflets had equilibrated at the indicated irradiance for 30 min. As had been observed with wheat leaves [4] and barley leaf protoplasts [6], percent activation and photosynthesis rate increased simultaneously with increasing irradiance (Fig. 14).

While percent activation has previously been reported to increase with increasing irradiance, total activity did not change [4,6]. We therefore conducted further analysis of the samples collected 72 days after planting (Fig. 13) to determine the nature of the lower total activities in the morning hours. A number of chloroplast metabolites, e.g. ribulose 1,5-bisphosphate and ribose-5-phosphate, can bind to inactive enzyme and slow the rate of in vitro activation by CO_2 and Mg^{2+} [16]. RuBP binds more tightly than other intermediates, but we calculated that a 1-h gel filtration step removed almost all (97%) of the RuBP bound to inactive spinach enzyme [17]. We therefore extracted leaf samples in a homogenizing buffer to which was added 20 mM $NaHCO_3$ and 1 ml of this extract, after insoluble material was removed by centrifugation at 12,000 X g for 10 min, was applied to a 0.7 X 15 cm column of Sephadex G-25 equilibrated with homogenizing medium at a flow rate of 0.4 ml min^{-1}. One ml of the gel-filtered extract was collected after

the void volume and an aliquot was assayed for total activity. The gel-filtration treatment slightly increased activity about 10% in all samples compared to activity measured before gel filtration (data not shown), but had little effect on total activity compared to that measured in the original extract (Fig. 2). A second hypothesis we considered was that total RuBP Case activity was lower in the morning hours because of a reduced amount of RuBP Case protein in the leaf samples at that time. To examine this possibility, RuBP Case protein was determined in samples of leaf material collected during the diurnal period (Fig. 2) by separation using SDS-PAGE and quantified by measuring the intensity of staining with Coomassie blue. The large and small subunits of RuBP Case were well separated from other leaf polypeptides (Fig. 13). The average amount of RuBP Case protein in all leaf samples was 33 ± 3% of the total SDS-extractable leaf protein applied to the gels. Although the method employed for measuring RuBP Case protein is relative to a number of factors, e.g. staining procedures [12], staining intensity of specific proteins [14], the small relative differences observed in RuBP Case protein levels throughout the diurnal period cannot account for the 3-fold differences in total activity. Other leaf parameters measured on a g dry weight basis, such as mg Chl g^{-1} (7.0 ± 0.7), mg total

protein g^{-1} (96 ± 13), and mg soluble protein g^{-1} (88 ± 11), were not substantially different in the leaf samples collected over the diurnal period and there was no obvious pattern in the slight differences which were observed.

DISCUSSION

The degree of change in the activation state of RuBP Case in vivo and the biological significance of such a change is the subject of much recent speculation [18]. RuBP Case activity in field-grown soybeans was found to undergo a reversible change which occurred simultaneously with a change of incident irradiance from 0 to 1.2 mmol m^{-2} s^{-1}. In chamber-grown plants of wheat [4] and soybeans [9], maximal percent activation of RuBP Case in leaves was only 60 and 70% respectively, but in field-grown soybeans percent activation approached [19] and sometimes slightly exceeded 100% (Fig. 2, ref. 20). Percent activation changed slowly at the beginning and end of the diurnal period because of the relatively slow change in natural irradiance level. In the laboratory, however, increasing leaf

irradiance resulted in a rapid (within 5 min) increase in percent activation [4, 19]. In agreement with previous reports [4,6], percent activation and photosynthesis rate increased simultaneously with increasing irradiance (Fig. 15). Perchorowicz et al. [4] have suggested that the increase in photosynthesis with increasing irradiance is primarily the result of activation of RuBP Case because leaf RuBP concentration, another variable which could also control RuBP Case activity in vivo, saturated at a much lower irradiance, approximately 0.23 mmol m^{-2} s^{-1}. In the laboratory (Fig. 3), percent activation did not increase as rapidly with increasing irradiance as was observed in the field (Fig. 2). This difference might reflect differences in the light sources used (incandescent lamp vs. sunlight) and the angle of irradiance measurement. In the field, irradiance was measured perpendicular to the horizon while in the laboratory, irradiance was measured perpendicular to the leaf surface. Soybeans are able to alter leaf angle so to keep the leaf surface perpendicular to the incident solar radiation in the early morning and late afternoon [22]. This means that irradiance incident upon the leaf may have been higher in the morning and evening hours than irradiance measured directly overhead.

Total RuBP Case activity increased about three-fold during the first four hours of the diurnal period (Fig.2). Other recent reports have also described similar increases in total RuBP Case activity in leaves of soybean [19, 20] and peanut [23] collected between pre-dawn and mid-day. The observed change in total activity is large only after a prolonged dark period, which is perhaps the reason why it has not been observed by all workers [4,6]. In the laboratory, total activity, measured in leaves which had preincubated in the dark for only 1 h, was reduced only 20% (Fig. 15).

Others (20, 23). have also observed total activity to be low in the morning but remain high for a number of hours following dusk. Hence, while initial and total activity both increase simultaneously with increasing irradiance, total activity declines in the dark at a much slower rate than initial activity, indicating that the effect of light on the two activities may not be mechanistically similar.

The reason for the observed change in total activity is not known. The conditions we have employed for activating inactive enzyme in leaf extracts of soybean are optimal for complete activation and protection of the enzyme from denaturing sub-

stances in the leaf extract [9]. Mid-day total activities (Fig. 2) are similar or slightly higher than the total RuBP Case activities measured previously in 29 soybean genotypes [24]. It is unlikely that small molecular weight phosphorylated effectors, which can reduce the rate of activation of purified RuBP Case [16], had any affect on total RuBP Case activity. Gel filtration did not increase total activity in extracts of leaves collected in the early morning compared to those collected at mid-day (Fig. 13). However, the possibility exists that a very tight binding intermediate, which was not removed by gel filtration, could still be bound to the enzyme and slow the rate of activation in vitro. The lower total activity is not the result of a reduced amount of RuBP Case protein. RuBP Case protein did not undergo any significant breakdown and resynthesis during a 24-h period in barley leaves [25] and we observed no marked change in either soluble leaf protein, total leaf protein, or RuBP Case protein on a g dry weight basis during the diurnal period. Furthermore, exposure of soybean leaves, covered during the preceding night period, to full irradiance at mid-day resulted in a rapid increase in total activity to near control levels (uncovered leaves) in about 10 min [19]. Such rapid changes in total activity precludes the involvement of protein synthesis as the cause of the

effect [19].

We conclude that initial RuBP Case activity in field-grown soybean leaves increases about 6-fold during a diurnal period simultaneously with increases in natural irradiance. This increase may be the result of a light-facilitated activation of the enzyme similar to its activation by Mg^{2+} and CO_2 in vitro [2]. Total RuBP Case activity increases about 3-fold during the first 4 h of the diurnal period and remains high thereafter. In this case, light may play a role in rapidly converting the inactive enzyme to a form which is able to be activated by CO_2 and Mg^{2+} in vivo. In the dark, the enzyme slowly reverts to a form which is not able to be activated by CO_2 and Mg^{2+} alone.

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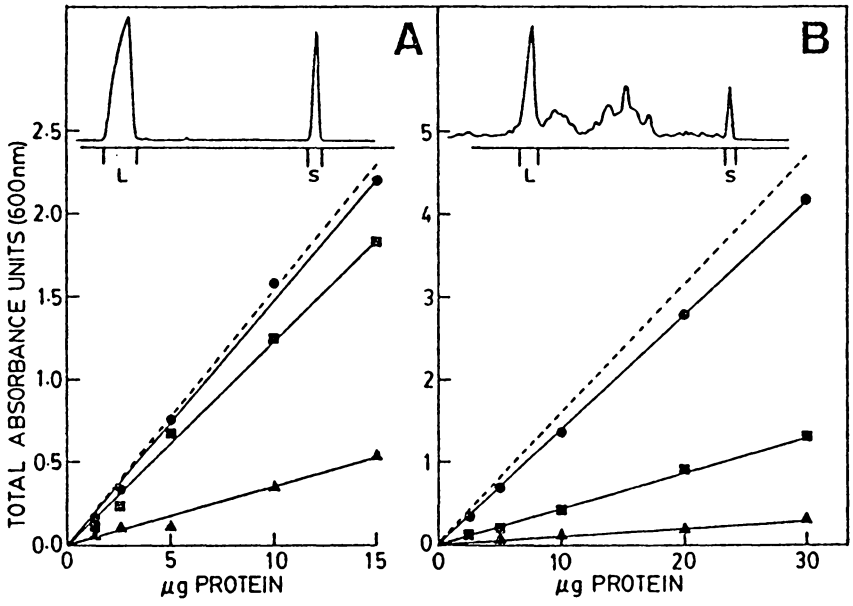

Figure 13. Standard curves derives from quantitative assay of RuBP Case protein: Varying amounts of purified soybean RuBP Case in half-strength extraction buffer (A) and SDS-extracted leaf sample (B) were subjected to SDS-PAGE as described in the text. Protein bands corresponding to the large (L,■) and small (S,▲) subunits of RuBP Case were cut out from the gel (as indicated by the brackets), eluted in 1% SDS, and A_{600nm} determined. Total (T,●) absorbance is the sum of the absorbance of the entire gel. The dashed line represents absorbance values calculated from direct measurement of protein extracts [14]. Inset: Scans (A_{520nm}) of SDS-plyacrylamide gels of purified soybean RuBP Case (A) and leaf extract (B).

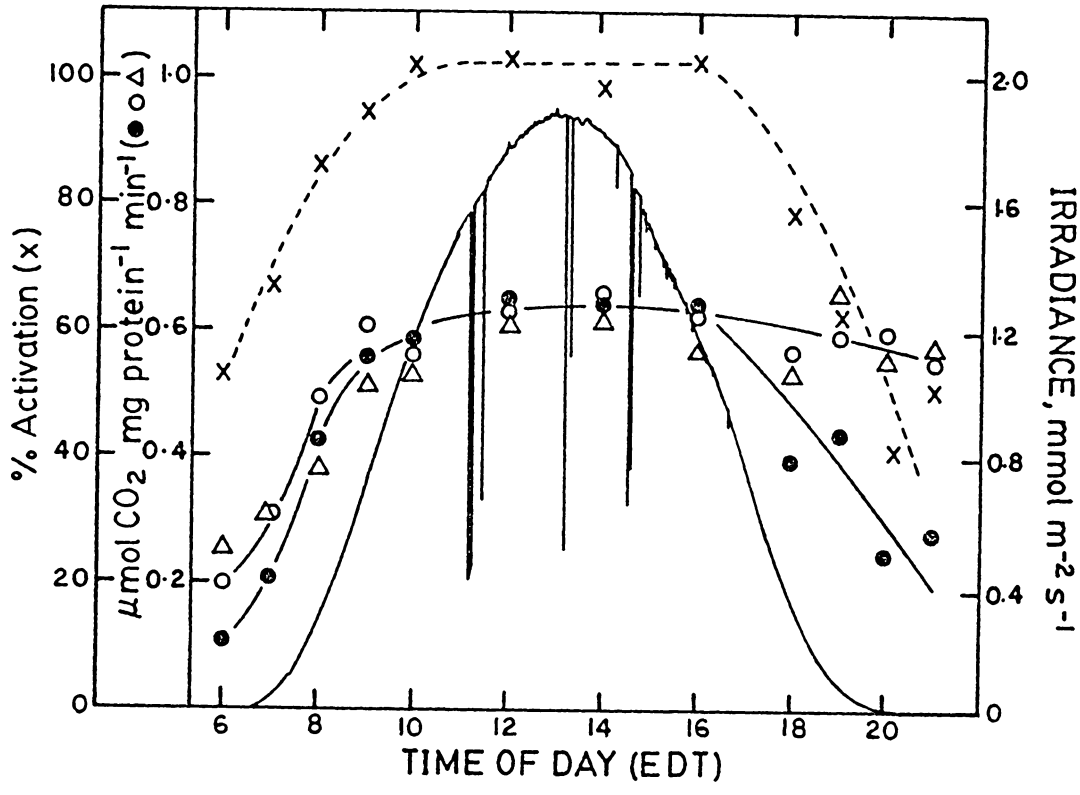

Figure 14. Diurnal time course of RuBP Case activity in field-grown soybeans at 72 days after planting: Initial (●, avg. SD = ± 8.1%) and total (O, avg. SD = ±7.7%) RuBP Case activities were measured in triplicate from leaf samples collected at the indicated times during the diurnal period (EDT = eastern daylight time). Percent activation (X) = (initial activity) (total activity)$^{-1}$ X 100%. Total RuBP Case activity (Δ, avg SD = ±2.2%) was measured after extracts were subjected to gel filtration on Sephadex G-25 as described in the text. Irradiance (continuous solid line) was recorded continuously throughout the diurnal period.

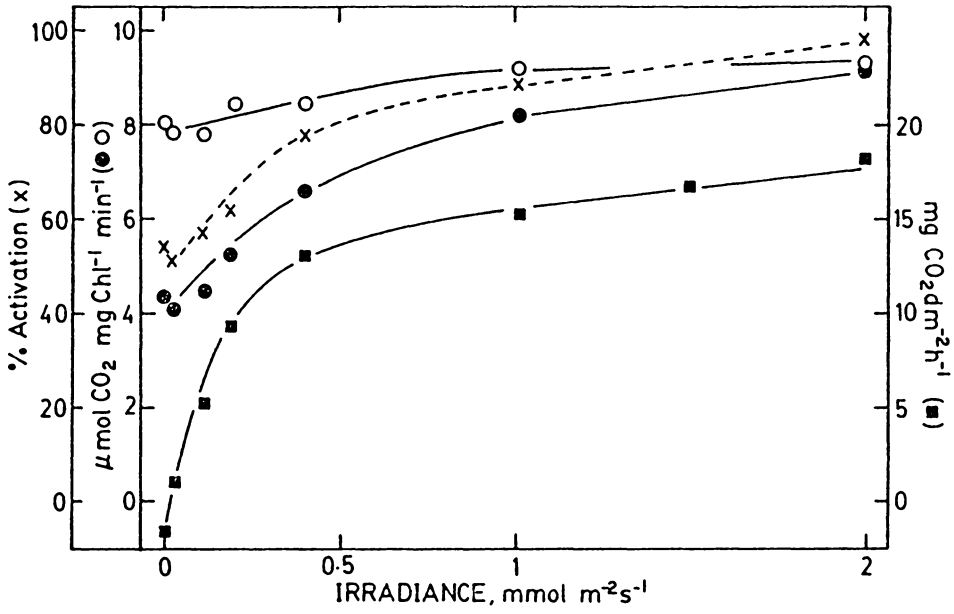

Figure 15. Irradiance response curve of leaf photosynthesis and RuBP Case activity in soybean leaves at 46 days after planting.: Soybean leaves were excised under water, brought into the laboratory and stored in the dark for 1 h. A single leaflet was placed in a sealed plexiglass cuvette which was maintained at 25°C and allowed to equilibrate at the indicated irradiance for 30 min before measurement of photosynthetic CO_2 exchange (■, avg. SD = ± 4.4%) and initial (◐, avg. SD = ± 1.9%) and total (O, avg SD = ± 2.8%) RuBP Case activities. For the RuBP Case measurements, 3 leaf discs (0.4 cm^2) were removed and immediately frozen in LN_2 and then analyzed for RuBP Case activity. Measurements were made in triplicate.

APPENDIX C: DIURNAL CHANGES IN RUBP CASE ACTIVITY IN GREENHOUSE-GROWN AND CHAMBER-GROWN SOYBEANS

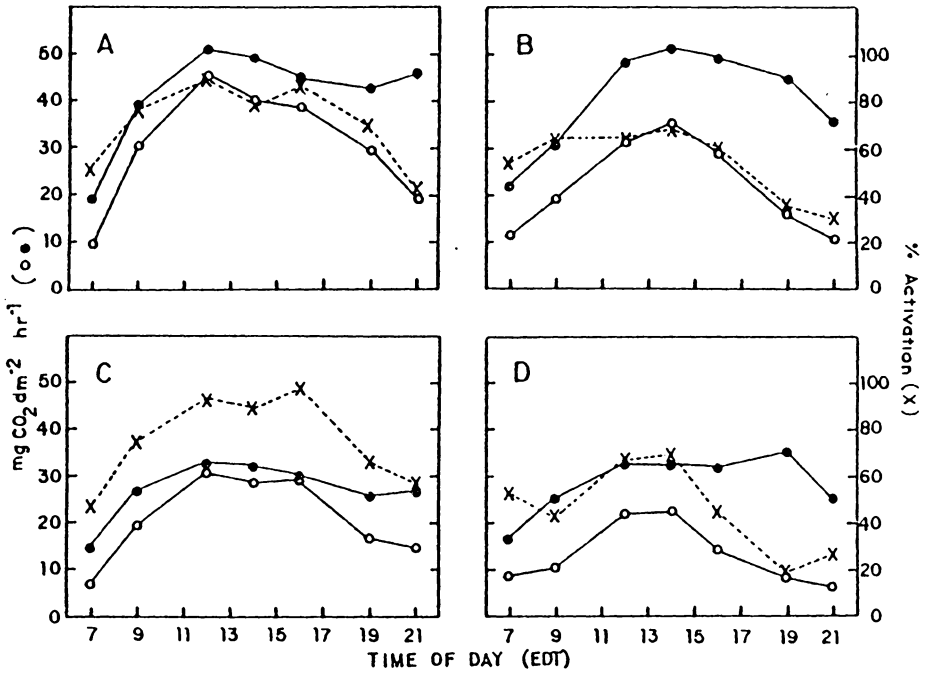

Figure 16. RuBP Case activity in greenhouse-grown soybeans over a diurnal period: Diurnal response of RuBP Case initial activity (O) and total activity (●) in 65 day-old soybeans, grown in á greenhouse under 100% full sun (A, B) or 20% full sun (C, D). Plants were exposed to either 100% full sun (A, C) or 20% full sun (B, D) throughout the diurnal period. RuBP Case activity was measured in leaf tissue samples collected at various intervals throughout the diurnal period, and frozen in liquid N_2. Percent activation (X) = (initial activity) (total activity)$^{-1}$ X 100%.

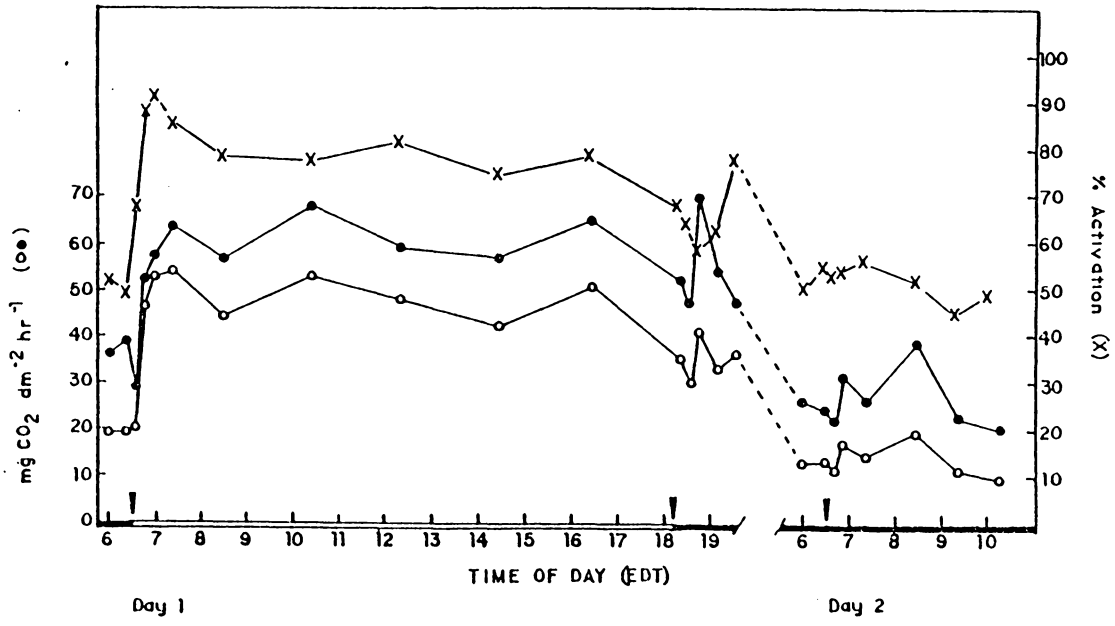

Figure 17. Diurnal RuBP Case activity in chamber-grown soybeans: Diurnal time course of RuBP Case initial activity (O), total activity (●) and percent activation (X) in 30 day-old soybean plants in a growth chamber. The total irradiance of the light source was 650 uE m-2 s-1. On DAY 1, leaf tissue samples for RuBP Case assay were collected throughout the plants' accustomed light cycle, which began at 6:30 and terminated at 18:30 (arrows). Following a 10-hr interval (break in abscissa), sampling was resumed and continued for 5 hr into the following day (DAY 2), during which the dark period was prolonged.

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