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# Changes in Photosynthetic Capacity in Rice Leaves from Emergence through Senescence. Analysis from Ribulose-1,5-bisphosphate Carboxylase and Leaf Conductance

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Changes in the rates of gas exchange and the amount of ribulose 1,5-bisphosphate (RuBP) carboxylase protein were determined in the 12th leaf blades of rice during the reproductive stages. RuBP carboxylase exhibited a large change similar to that in the assimilation rate at 2% $O_2$ throughout the leaf's life, but its decrease during senescence was barely faster than the decrease in the assimilation rate. Consequently, the overall relationship was slightly curvilinear. By contrast, leaf conductance decreased more slowly than the assimilation rate which resulted in the intercellular $CO_2$ concentration increasing during senescence.

In order to determine the maximum activity of RuBP carboxylase at the intercellular $CO_2$ concentration, the kinetic parameters and their pH response were determined using purified, and completely activated, rice RuBP carboxylase. The maximum carboxylase activity at the intercellular $CO_2$ concentration was linearly correlated with the assimilation rate at 2% $O_2$ ($r$=0.989), and was very close to that needed to account for the assimilation rate.

We conclude that changes in both the amount of RuBP carboxylase protein and leaf conductance reflect the change in photosynthesis during the life span of the leaf.

**Key words**: Activation (RuBP carboxylase) — Conductance — Leaf senescence — *Oryza sativa* — Photosynthesis — Ribulose-1,5-bisphosphate carboxylase.

Leaf photosynthesis in cereal crops during the reproductive stages sustains seed development [see Murata and Matsushima (1975) for a review]. Therefore, changes in the photosynthetic activity during these periods are directly related to crop productivity. It is important to maintain a high photosynthetic capacity in the leaves for as long as possible during seed development. In order to establish the improved breeding or cultural methods for enhancing crop productivity, we first of all need to clarify the factors responsible for the change in photosynthesis with leaf age.

Leaf photosynthesis generally exhibits large changes with leaf age. As the leaf develops and chloroplasts are assembled, the photosynthetic activity increases to a maximum rate just after full expansion. Thereafter, the leaf steadily loses its photosynthetic capacity during senescence. These changes are affected by a variety of factors, such as nitrogen nutrition, water stress, the light interception, and other physiological parameters.

The photosynthetic rate in the leaves is controlled by both stomatal conductance and

Abbreviations: DTT, dithiothreitol; E, einstein(s); RuBP, ribulose 1,5-bisphosphate.

mesophyll capacity for photosynthesis [see Farquhar and Sharkey (1982) for a review]. Mesophyll capacity is considered to depend, in particular, upon the in vivo regulation of RuBP carboxylase [EC 4.1.1.39] activity in mature leaves. A biochemical model of photosynthesis in leaves of $C_3$ species developed by Farquhar et al. (1980) suggests that the $CO_2$ assimilation rate is limited by the RuBP saturated rate of the RuBP carboxylase activity at low $CO_2$ concentrations and by the rate allowed by RuBP regeneration capacity at high $CO_2$ concentrations. In addition, a number of studies on the enzymic properties of RuBP carboxylase suggest that the amount of the enzyme as well as the degree to which the enzyme is activated under given field conditions should be considered as regulating photosynthesis [see Jensen and Bahr (1977) for a review]. However, the role of RuBP carboxylase responsible for the change in photosynthesis with leaf age had remained uncertain.

We recently indicated that the amount of RuBP carboxylase protein can be a limiting factor in photosynthesis throughout the life span of the leaf based on the following three findings (Makino et al. 1983b). First, the in vitro RuBP carboxylase activity is highly correlated with photosynthesis. Second, the specific activity of the carboxylase protein remains constant. Third, the in vitro carboxylase activity calculated at the atmospheric $CO_2$ concentration is comparable to the in situ photosynthetic $CO_2$-assimilation rate per mg of carboxylase protein during the life span of the leaf. This study examines whether the change in the photosynthetic $CO_2$-assimilation rate can be accounted for by the changes in both the amount of RuBP carboxylase protein and leaf conductance throughout the life of rice leaves during the reproductive stages.

## Materials and Methods

*Plant materials*—Rice (*Oryza sativa* L., cv Sasanishiki) plants were grown to the ripening stage in a greenhouse by water culture method as reported previously (Mae and Ohira 1981). The basal nutrient solution used contained 1 mM $NH_4NO_3$, 0.6 mM $Na_2HPO_4$, 0.3 mM $K_2SO_4$, 0.4 mM $MgCl_2$, and 0.2 mM $CaCl_2$, and other minor nutrients. The solution was renewed once a week and the pH was adjusted to 5.0 with 2 M HCl. The strength of the nutrient solution was varied depending on the age of the plants as described before (Makino et al. 1983b). The age of all the plants was checked by marking the leaves on the main stems with ink every 5 to 7 days. Heading time, defined as the time when half of the ears on the main stems became visible, was 107 days after germination (August 21, 1983).

The 12th leaf blades on the main stems were used as samples throughout the experiments. The tips of the leaves emerged from the 11th leaf sheaths on the 75th day after germination. Samples were collected 26 times for about 80 days from leaf emergence through senescence. All photosynthetic measurements and collections were made between 10 a.m. and 12 p.m. (noon).

*Photosynthetic measurements*—The rates of $CO_2$ exchange and transpiration were determined using an open gas exchange system. The 12th leaf blade attached to the plant was inserted into an acrylic chamber and illuminated through a flowing water filter by nine 500-W flood lamps. Light intensity was adjusted to 1,700 $\mu E \cdot m^{-2} \cdot s^{-1}$ (85 klux) at the upper surface of the leaf. Leaf temperature was measured with a fine copper-constantan thermocouple attached to the lower surface of the leaf and adjusted to 25°C by controlling the temperature of the chamber water jackets. Air was passed through the chamber at a rate of 63 $mmol \cdot min^{-1}$ using a precision flow meter. It was obtained from a high pressure gas cylinder containing 350 $\mu l \cdot liter^{-1}$ $CO_2$ and 2% (v/v) $O_2$ in $N_2$ (Tanuma-Sanso) or the atmospheric air containing 340 to 360 $\mu l \cdot liter^{-1}$ $CO_2$, and saturated at 16.7°C with water vapor, corresponding to 19 mbar vapor pressure. $CO_2$ concentration and water vapor pressure in the air entering and leaving the chamber were measured with an infrared gas analyzer (Hitachi-Horiba ASSA-2) and a dew point hygrometer

(Ace model AKD), respectively. The measurements were performed until a steady-rate of $CO_2$ exchange was obtained. Upon termination of the experiment, the leaf blade was quickly cut off, and its fresh weight and leaf area were measured, then the leaf was used for the immunochemical assay described below.

Gas exchange parameters were calculated using the equations reported by Wong et al. (1978).

*Determinations of soluble protein and RuBP carboxylase*—A fresh leaf was immediately homogenized in 50 mM phosphate buffer (pH 7.5) containing 5 mM DTT and 12.5% (v/v) glycerol at a ratio of leaves to buffer of 0.125 g·ml$^{-1}$ in a chilled mortar with a pestle and acid-washed quartz sand to ensure complete maceration. The soluble fraction was obtained by centrifuging the homogenate at $39{,}000 \times g$ for 20 min at 0 to 4°C. Soluble protein was measured as the N content with Nessler's reagent after Kjeldahl digestion, using trichloroacetic acid precipitate, which had been washed once with ethanol. The amount of the protein was calculated by multiplying its N content by 6.25.

RuBP carboxylase content in the soluble fraction was determined by rocket immunoelectrophoresis (Laurell 1966, LKB 1978). Specific antibodies against RuBP carboxylase were raised in white rabbits (Makino et al. 1983b). Gels of $9.0 \times 10.5 \times 0.1$ cm contained 1% (w/v) agarose, 74 mM Tris, 25 mM barbital, 0.34 mM Ca-lactate (pH 8.6), and sufficient antibodies to allow detection of 1 to 5 μg of carboxylase. Serial dilutions of RuBP carboxylase purified from rice leaves (Makino et al. 1983a) were used as a standard protein. Wells 3 mm in diameter were punched and 4 μl of the diluted soluble fraction or the standard protein was placed in each well. The electrophoresis was conducted at 10 mA for about 5 h using the Tris/barbital/Ca-lactate buffer. A standard curve was plotted and unknown values were calculated from the regression line.

*Kinetic studies of carboxylation reaction*—The kinetic parameters related to carboxylation and their pH response were determined using the RuBP carboxylase purified from rice leaves (Makino et al. 1983a). The enzyme (5 to 10 mg·ml$^{-1}$) was stored at 0 to 4°C in 50 mM Na-phosphate (pH 7.5), 1 mM DTT, 0.1 mM EDTA, and 12.5% (v/v) glycerol. Prior to assay, it was pretreated at 37°C for 1 h, and passed through a Sephadex G-25 column equilibrated with 100 mM Bicine/NaOH (pH 8.3), 25 mM $MgCl_2$, and 5 mM $Na_2HPO_4$. It was stored at room temperature in a 1.0-ml screw-capped vial and activated by injecting $NaHCO_3$ solution to a final concentration of 10 mM.[1] The maximum specific activity of this enzyme preparation was 3.1 μmol $CO_2$·min$^{-1}$·mg carboxylase protein$^{-1}$ when assayed at 30°C and pH 7.8.

RuBP carboxylase activity was measured at 25°C by $^{14}CO_2$ fixation into acid-stable products. Enzyme reactions were conducted in 1.0-ml screw-capped Micro V vials equipped with septa of Teflon rubbers (Wheaton). The vials were first flushed with $N_2$, and injections into the vials were made with precision microliter syringes. Reaction buffers were prepared as $CO_2$- and $O_2$-free as possible by pH adjustment with carbonate-free NaOH and flushing with $N_2$ for 2 h prior to use. The $CO_2$ concentrations in the assay buffers were calculated from the $NaH^{14}CO_3$

[1] Under this condition, the rice leaf carboxylase was completely activated. This was checked by performing the activation kinetics as described by Lorimer et al. (1976). Although RuBP carboxylase is generally considered to be fully activated with a preincubation at 20 mM $MgCl_2$, 10 mM $NaHCO_3$, and pH 8.0 to 8.6 [see Jensen and Bahr (1977) for a review], the rice enzyme was not necessarily completely activated under this condition (the amount of the deactivated enzyme was 10 to 15%). Therefore, $Na_2HPO_4$ (5 mM) was added to the activation mixture. It is a positive effector for promoting enzyme activation which is a weak competitive inhibitor of catalysis with respect to RuBP ($Ki = 1$ mM, McCurry et al. 1981). Since it was diluted 50-fold to a concentration of 100 μM in the catalytic reaction mixture and the concentration of RuBP used during catalysis was 600 μM (30 times the $K_m$ for RuBP), the inhibitory effect could be ignored (for details, see the legend to Fig. 6).

solution added and the carryover of $HCO_3^-$ from the enzyme preactivation solution, using the Henderson-Hasselbach equation at pK' of 6.37 (Lorimer et al. 1976).

Tetrasodium RuBP and purified carbonic anhydrase were obtained from Sigma Chemical Co. and $NaH^{14}CO_3$ from New England Nuclear. All other reagents were from Wako Pure Chemical Industries, Ltd.

## Results

The 12th leaf blades emerged from the 11th leaf sheaths on the main stems 75 days after germination, and became fully expanded 8 days later. After 80 days from leaf emergence, the leaves senesced completely and all of their parts dried up. The leaf area and the fresh weight increased during leaf expansion, and remained almost constant from full expansion until late senescence, but then declined with drying up (Fig. 1).

*Changes in gas exchange parameters*—Rates of photosynthesis were measured at 2% and 21% $O_2$ in this study. The former assimilation rate is generally considered to correspond to the true photosynthetic capacity, and the latter is apparent photosynthesis observed under the natural conditions. Therefore, the difference between them can be regarded as photorespiration.

Fig. 2A shows the changes in the rates of $CO_2$ assimilation at 2% and 21% $O_2$ in the 12th leaf blade from emergence through senescence. Both assimilation rates increased rapidly during leaf expansion, and reached maximum about 10 days after full expansion, then declined steadily during senescence. The ratio of the assimilation rates at 2% to 21% $O_2$ remained constant at $1.37 \pm 0.07$ throughout the experimental period (Fig. 2B). These results indicate that the leaf consumes about 35% of its photosynthetic capacity by photorespiration throughout its life.

Fig. 3 shows the changes in conductance to vapor diffusion and the intercellular $CO_2$ concentration in the 12th leaf blade from emergence through senescence. Only data obtained at 2% $O_2$ are shown. Leaf conductance exhibited a large change similar to that in the $CO_2$-assimilation rate with age. However, leaf conductance declined more slowly than the $CO_2$-assimilation rate during senescence (see Fig. 2A), and consequently the intercellular $CO_2$ concentration increased from 200 to 330 $\mu l \cdot liter^{-1}$ during this period (Fig. 3B). These results indicate that stomatal resistance is not a limiting factor responsible for a change in photosynthesis with leaf age although the positive correlation between the $CO_2$-assimilation rate and conductance was observed (Fig. 5B). Similar results were recently obtained with wheat (Evans 1983).

*Change in RuBP carboxylase protein*—The amounts of RuBP carboxylase and other soluble

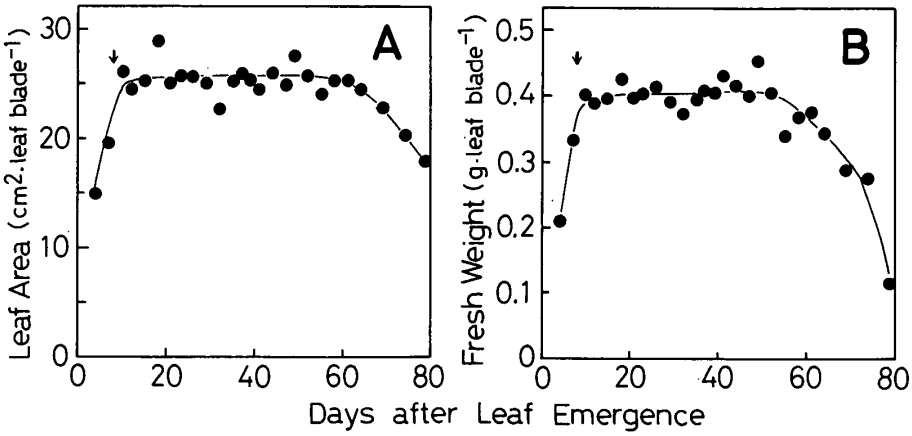


**Fig. 1** Changes in the leaf area (A) and the fresh weight (B) of the 12th leaf blade on the main stem of rice from leaf emergence through senescence. The arrow indicates the time when the 12th leaf blade was fully expanded.

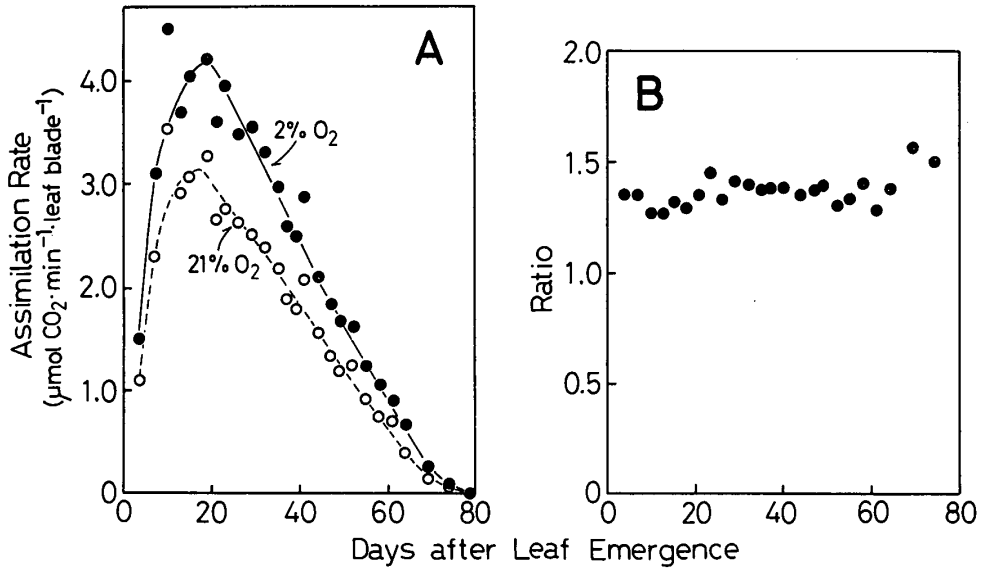


**Fig. 2** A, Changes in the rates of $CO_2$ assimilation at 2% (v/v) $O_2$ (●) and 21% (v/v) $O_2$ (○) in the 12th lea blade on the main stem of rice from leaf emergence through senescence. Measurements were made at a light intensity of 1,700 $\mu E \cdot m^{-2} \cdot s^{-1}$, a leaf temperature of 25°C, and air conditions of 350 $\mu l \cdot liter^{-1}$ $CO_2$ and 19 mbar vapor pressure, using an open gas exchange system. B, Change in the ratio of $CO_2$-assimilation rates at 2% (v/v) $O_2$ to 21% (v/v) $O_2$ shown in this figure A.

proteins were examined in the 12th leaf blades, which had been used for the photosynthetic measurements. The RuBP carboxylase content was determined by rocket immunoelectrophoresis. By SDS-polyacrylamide slab gel electrophoresis of the pellet fraction obtained by centrifugation, we verified that the carboxylase was completely extracted.

Change in RuBP carboxylase protein approximately paralleled that in the assimilation rate

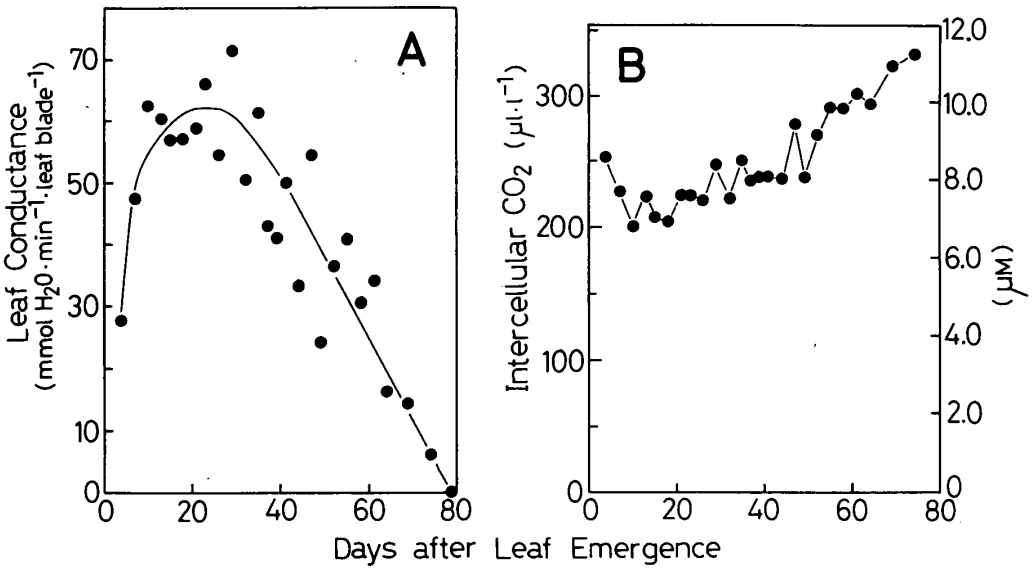


**Fig. 3** Changes in conductance to vapor diffusion (A) and the intercellular $CO_2$ concentration (B) in the 12th leaf blade on the main stem of rice from leaf emergence through senescence. Measurements were made at a light intensity of 1,700 $\mu E \cdot m^{-2} \cdot s^{-1}$, a leaf temperature of 25°C, and air conditions of 350 $\mu l \cdot liter^{-1}$ $CO_2$, 2% (v/v) $O_2$ and 19 mbar vapor pressure, using an open gas exchange system. The right vertical axis (B) shows the concentration of $CO_2$ ($\mu M$) in solution which is equilibrated with that in the intercellular air spaces.

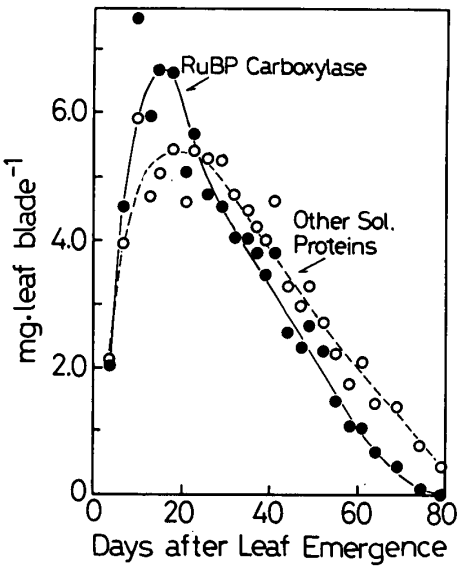


**Fig. 4** Changes in the amounts of RuBP carboxylase (●) and other soluble proteins (○) in the 12th leaf blade on the main stem of rice from leaf emergence through senescence. The leaf was homogenized in 50 mM phosphate buffer (pH 7.5) containing 5 mM DTT and 12.5% (v/v) glycerol. After centrifugation, the amounts of RuBP carboxylase in the soluble fraction were determined by rocket immunoelectrophoresis (Laurell 1966, LKB 1978).

during the life span of the leaf (Fig. 4). However, the relationship between the $CO_2$-assimilation rate and the carboxylase protein content was slightly curvilinear (Fig. 5A). Its slope declined when the carboxylase content exceeded about 20 mg·dm$^{-2}$. This was because the decline in RuBP carboxylase content was slightly faster than that of assimilation rate during senescence.

*Kinetic properties of purified RuBP carboxylase (pH response of the kinetic parameters)*—In order to determine the maximum activity of the rice leaf carboxylase at the intercellular $CO_2$ concentration under the optimal pH condition, the pH response of the kinetic parameters were examined using the purified carboxylase. These studies were conducted using completely activated enzyme (Fig. 6). Although RuBP carboxylase is generally assayed at pH 8.0 to 8.3, the optimal pH for *Vmax* of the rice enzyme was 7.8. The measured $K_m(CO_2)$ decreased with increasing pH. This variation is a similar response to that obtained with other higher plant carboxylases (Servaites et al. 1977, Badger 1980). The pH response to the maximum activity at the physiological $CO_2$ concentration (a value of 8 μM was used, see Fig. 3B) was calculated from the kinetic

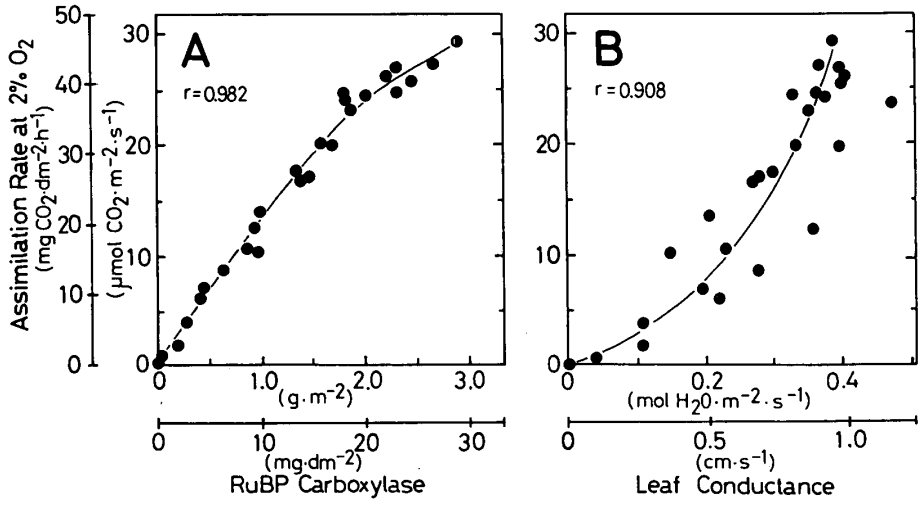


**Fig. 5** Relationship between the rate of $CO_2$ assimilation at 2% (v/v) $O_2$ and the content of RuBP carboxylase (A), and conductance to vapor diffusion (B) on a leaf area basis. Data are obtained from Fig. 1, 2, 3 and 4. Correlation coefficient was determined using first-order kinetics.

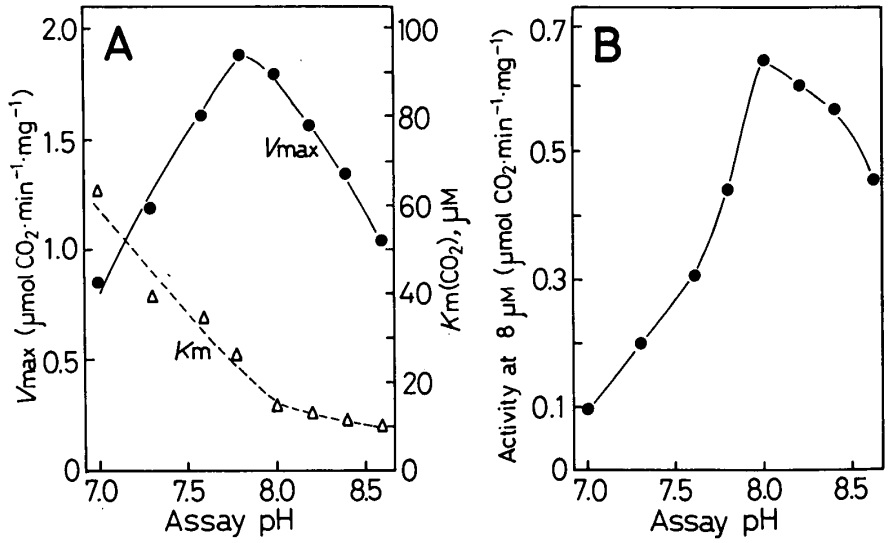


**Fig. 6** A, The response to pH of $K_m$ (△) and *Vmax* (●) for RuBP carboxylase purified from rice leaves. Assays (500 μl, final volume) were carried out at 25°C in 100 mM Hepes/NaOH (pH 7.0 to 7.8) and Bicine/NaOH (pH 7.8 to 8.6) containing 25 mM $MgCl_2$, 0.6 mM RuBP, different $NaH^{14}CO_3$ (1.0 mCi·mmol$^{-1}$) concentrations (0.2, 0.5, 1.0, 2.0, 3.0, 5.0 and 10.0 mM), and 5 Wilbur-Anderson units carbonic anhydrase in 1.0-ml screw-capped vials flushed with $N_2$. Enzyme reactions were initiated by injecting 10 μl of enzyme (8.5 μg and 9.3 μg), which had been activated with 100 mM Bicine/NaOH (pH 8.3), 25 mM $MgCl_2$, 10 mM $NaHCO_3$ and 5 mM $Na_2HPO_4$. At the same time, 1-μl solutions of carbonic anhydrase were added in order to completely equilibrate between $CO_2$ and $HCO_3^-$ during the reaction. After 1 min, the reactions were stopped by adding 100 μl 2 M formic acid. The slopes and intercepts of the plots of $[CO_2]$/velocity versus $[CO_2]$ were determined by calculating the first-order regression lines from the obtained data. All assays were performed in duplicate. B, The response to pH of RuBP carboxylase activity at the physiological $CO_2$ concentration. Enzyme activity at the physiological $CO_2$ concentration (8 μM ⇆ 235 μl·liter$^{-1}$, see Fig. 3B) was calculated from the $K_m$ and *Vmax* shown in this figure A using the Michaelis-Menten equation.

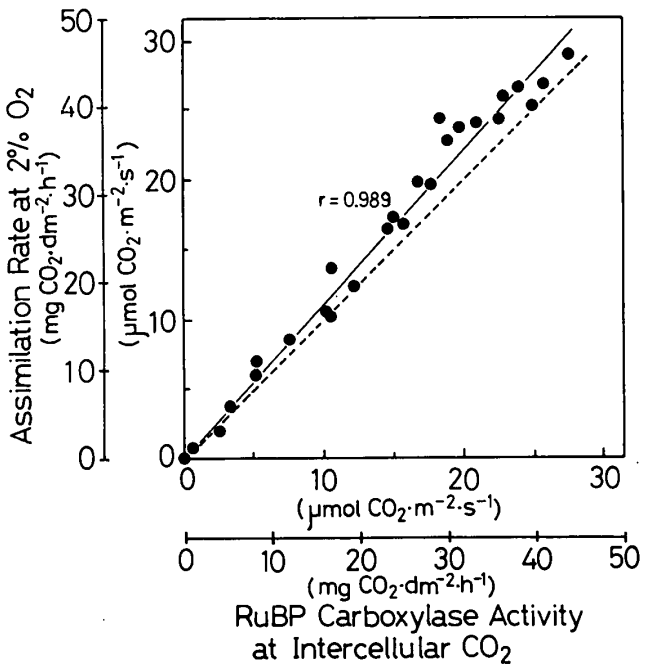


**Fig. 7** In situ rate of $CO_2$ assimilation at 2% (v/v) $O_2$ versus the in vitro RuBP carboxylase activity at the intercellular $CO_2$ concentration. Data of the $CO_2$-assimilation rate at 2% (v/v) $O_2$ are obtained from Fig. 2. RuBP carboxylase activity was calculated from the amount of the enzyme protein (Fig. 4), the intercellular $CO_2$ concentration (Fig. 3B), and the kinetic parameters at the optimal pH [Fig. 6, $K_m$ ($CO_2$) = 14.4 μM and *Vmax* = 1.79 units·mg carboxylase protein$^{-1}$ at pH 8.0 and 25°C], using the Michaelis-Menten equation. The solid line indicates the regression line obtained from the experimental data ($y = 1.09x + 0.38$). Regression analysis was performed using first-order kinetics. The dashed line represents the relationship when both values are completely consistent with each other.

parameters shown in Fig. 6A (Fig. 6B). The response of this activity shows an optimum at 8.0 with a sharp decline below pH 7.8. This optimum is similar to the pH that observed in the chloroplast stroma under illumination (Werdan et al. 1975).

*The $CO_2$-assimilation rate and RuBP carboxylase activity at the intercellular $CO_2$*—The maximum activity of RuBP carboxylase at the intercellular $CO_2$ concentration throughout the leaf's life was calculated from the amount of the enzyme protein (Fig. 4) and its kinetic parameters at the optimal pH (Fig. 6). It is important to note that the enzymic properties of RuBP carboxylase are unchanged during the life span of the leaf. Previously, we have found that the specific activity of RuBP carboxylase ($\mu$mol $CO_2 \cdot min^{-1} \cdot$mg carboxylase protein$^{-1}$) remains constant in rice leaves from emergence through senescence (Makino et al. 1983b) and comparable to the activity of the purified carboxylase (Makino et al. 1983a). In addition, RuBP carboxylase is synthesized en masse during leaf expansion and persists, with little or no turnover, until senescence (Mae et al. 1983, Makino et al. 1984). Thus, it is reasonable to conclude that its enzymic properties are unchanged throughout the leaf's life.

Fig. 7 shows the relationship between the $CO_2$-assimilation rate at 2% $O_2$ and the maximum activity of RuBP carboxylase at the intercellular $CO_2$ concentration. The calculated carboxylase activity was linearly correlated with the $CO_2$-assimilation rate measured in situ ($r$=0.989), and was very close to that needed to account for the assimilation rate.

## Discussion

Our previous work suggested that the amount of RuBP carboxylase protein can be a limiting factor in photosynthesis throughout the life span of the leaf (Makino et al. 1983b). As shown in Fig. 5A, however, the relationship between the $CO_2$-assimilation rate at 2% $O_2$ and the RuBP carboxylase content was slightly curvilinear. The slope of this relationship declined significantly when the carboxylase content per unit leaf area exceeded 20 mg$\cdot$dm$^{-2}$. Similar trends are found by other investigators (Friedrich and Huffaker 1980, Uchida et al. 1980, 1982). While the importance of the carboxylase is recognized, these results indicate that RuBP carboxylase protein is not the sole determinant responsible for the change in photosynthesis with leaf age. Here, it is important to note the change in stomatal aperture with age since the $K_m(CO_2)$ value of RuBP carboxylase is close to the atmospheric $CO_2$ concentration. Although the change in leaf conductance per se was not closely related to the change in the assimilation rate (Fig. 2 and 3), the RuBP carboxylase activity calculated at the intercellular $CO_2$ concentration was *linearly* correlated to the $CO_2$-assimilation rate at 2% $O_2$ (Fig. 7).

A biochemical model of photosynthesis in $C_3$ species developed by Farquhar et al. (1980) suggests that the $CO_2$-assimilation rate is limited by the RuBP saturated rate of the RuBP carboxylase activity at low $CO_2$ concentrations and by the rate allowed by RuBP regeneration at high $CO_2$ concentrations. In addition, von Caemmerer and Farquhar (1981) suggested that the latter limitation depends upon electron transport capacity. It is important to note the transition from limitation due to RuBP carboxylation capacity to one due to electron transport capacity to regenerate RuBP. The $CO_2$ concentration at which the transition occurs, depends upon the ratio of electron transport capacity to RuBP carboxylation capacity (Farquhar and von Caemmerer 1982). It seems to vary with irradiance, temperature, and to a lesser extent $O_2$ concentration (von Caemmerer and Farquhar 1981). von Caemmerer and Farquhar (1981) reported that the transition at 2% $O_2$ occurred near 200 $\mu$l$\cdot$liter$^{-1}$ $CO_2$ at 20°C and near 320 $\mu$l$\cdot$liter$^{-1}$ $CO_2$ at 28°C in *mature* kidney bean leaves under saturating light. In our case with rice leaves (at 25°C), the intercellular $CO_2$ concentration increased from 200 to 330 $\mu$l$\cdot$liter$^{-1}$ during senescence (Fig. 3B). As the change in the transition with leaf age was not examined in this study, we cannot conclude whether the limitation due to RuBP regeneration capacity came

into play during senescence. However, since the decrease in RuBP carboxylase activity during senescence is much faster than the decreases in electron transport activity (Camp et al. 1982), Chl and membrane protein contents (Makino et al. 1983b), the ratio of potential electron transport capacity to RuBP carboxylation capacity may increase during senescence. Therefore, RuBP regeneration probably cannot be a limiting factor responsible for the change in photosynthesis with leaf age. If this limitation had occurred significantly, the results in Fig. 7 should have been curvilinear and the RuBP carboxylase activity calculated at the intercellular $CO_2$ concentration should have been well in excess of the assimilation rate.

Recently, Evans (1983) reported that the initial slope of the $CO_2$-assimilation rate against the intercellular $CO_2$ concentration varied nonlinearly with RuBP carboxylase activity. He discussed that this was due to a finite wall and liquid-phase conductance to $CO_2$ diffusion which causes a drop in $CO_2$ concentration between the intercellular spaces and the chloroplast stroma. Considering this $CO_2$-diffusion model, however, RuBP carboxylase activity at the stromal $CO_2$ concentration is insufficient to account for the measured $CO_2$-assimilation rate. The finite wall and liquid-phase conductance to $CO_2$ diffusion is complicated by the fact that carbonic anhydrase exists in both chloroplast and cytoplasm (Graham et al. 1974). Since carbonic anhydrase promotes the equilibrium between $CO_2$ and $HCO_3^-$ under given pH conditions, this enzyme would function to reduce the drop in $CO_2$ concentration between the intercellular spaces and the chloroplast stroma. Farquhar et al. (1982) concluded from considerations of observed carbon isotope fractionations that the $CO_2$ concentration in the chloroplast stroma is only marginally less than that in the intercellular spaces.

Friedrich and Huffaker (1980) observed that the mesophyll conductance decreased rapidly during leaf senescence. However, since its decrease during senescence is accompanied by decreased $CO_2$-assimilation capacity, it would not cause a significant $CO_2$ drop in the chloroplast stroma.

The in vitro RuBP carboxylase activity calculated from the kinetic parameters at the optimal pH was never sufficient to account for the in situ $CO_2$-assimilation rate at 2% $O_2$ (Fig. 7), although the kinetic studies were conducted using completely activated enzyme (Fig. 6). This was not due to incomplete extraction of RuBP carboxylase as no carboxylase was detected in the pellet fraction following SDS-polyacrylamide slab gel electrophoresis (data not shown). Presumably, this was mainly attributed to slight overestimation of the true catalytic $K_m(CO_2)$ of the carboxylase. Despite stringent precautions, it is difficult to completely eliminate contaminating $CO_2$ from buffers for the enzyme assay. We estimate this inevitable $CO_2$ to be at most 2 $\mu$M, using the kinetic equilibrium constants for activation of the enzyme (data not shown). Therefore, the maximum activity at the intercellular $CO_2$ concentration would be only just sufficient to account for the in situ assimilation rate.

A number of studies on the enzymic properties of RuBP carboxylase suggest that the amount of the enzyme protein as well as the degree to which the enzyme is activated under given field conditions should be considered as regulating photosynthesis [see Jensen and Bahr (1977) for a review]. The in vivo activation state of the carboxylase responds to limiting light (Sicher and Jensen 1979, Robinson et al. 1979, Perchorowicz et al. 1981, 1983). However, whether all of the enzyme in the leaf can be maintained in a completely activated state under saturating light has yet to be elucidated. Therefore, the important point to emphasize here is that we used *completely activated enzyme* in order to determine the kinetic parameters. Although the molecular mechanism of activation of the enzyme is largely understood, the in vivo regulation of it remains less clear. If one subjects the in vitro enzyme to the physiological conditions (7 to 10 $\mu$M $CO_2$, 5 to 10 mM $Mg^{2+}$, pH about 8.0), the amount of enzyme in the activated state is only 20 to 30% (Lorimer et al. 1976, Hatch and Jensen 1980). Several chloroplast metabolites, such as NADPH, 6-phosphogluconate, and fructose 1,6-bisphosphate, stimulate enzyme acti-

vation, and seem to fully activate the enzyme under the above physiological conditions (Hatch and Jensen 1980). Considering here the physiological aspects, however, the following three contradictions must be recognized.

First, since these metabolites interact with enzyme at a catalytic site for RuBP (Badger and Lorimer 1981, McCurry et al. 1981, Jordan et al. 1983), an enzyme molecule cannot be simultaneously catalytically competent and activated by one of the metabolites. Second, the amounts of these metabolites within stroma are not necessarily sufficient to account for that of RuBP carboxylase active sites (3 to 4 mM, Jensen and Bahr 1977). Third, the substrate RuBP decreases the steady state level of the active ternary enzyme form by binding to the deactivated enzyme (McCurry et al. 1981). The RuBP bound to the deactivated enzyme is not released during a 1-h gel filtration (Paech and Tolbert 1978). However, mechanisms that preclude the binding of RuBP to the deactivated enzyme have yet to be found. Nevertheless, the results in Fig. 7 suggested that none of the enzyme remain in the deactivated state in vivo. The linear correlation between the assimilation rate and the maximum activity of RuBP carboxylase calculated at the intercellular $CO_2$ concentration (Fig. 7) suggests that activation of the enzyme cannot be a limiting factor responsible for the change in photosynthesis with leaf age and that the in vivo enzyme can be in a completely activated state throughout the leaf's life.

We conclude that for rice leaves during the reproductive stages, changes in both the amount of RuBP carboxylase protein and leaf conductance reflect the change in the photosynthetic assimilation rate. Activation of RuBP carboxylase is not a limiting factor responsible for the change in photosynthesis and the in vivo enzyme probably can be in a completely activated state throughout the leaf's life.

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