Plant Physiol. (1983) 72, 297–302
0032-0889/83/72/297/06/$00.50/0

Nitrogen and Photosynthesis in the Flag Leaf of Wheat (*Triticum aestivum* L.)

Received for publication November 1, 1982

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ABSTRACT

Wheat (*Triticum aestivum* L. cv Yecora 70) plants were grown with various concentrations of nitrate nitrogen available to the roots. Sampling of flag leaves began after they had reached full expansion and continued throughout senescence. Rates of gas exchange, ribulose-1,5-bisphosphate (RuP_2) carboxylase activity, and the amounts of chlorophyll, soluble protein, nitrogen, and phosphorus were determined for each flag leaf. Rate of CO_2 assimilation was uniquely related to total leaf nitrogen irrespective of nutrient treatment, season, and leaf age. Assimilation rate increased with leaf nitrogen, but the slope of the relationship declined markedly when leaf nitrogen exceeded 125 millimoles nitrogen per square meter. Chlorophyll content and RuP_2 carboxylase activity were approximately proportional to leaf nitrogen content. As leaves aged, RuP_2 carboxylase activity and calculated Hill activity declined in parallel. With normal ambient partial pressure of CO_2, the intercellular partial pressure of CO_2 was always such that rate of assimilation appeared colimited by RuP_2 carboxylation and RuP_2 regeneration capacity.

The initial slope of rate of CO_2 assimilation against intercellular partial pressure of CO_2 varied nonlinearly with carboxylase activity. It is suggested that this was due to a finite conductance to CO_2 diffusion in the wall and liquid phase which causes a drop in CO_2 partial pressure between the intercellular spaces and the site of carboxylation. A double reciprocal plot was used to obtain an estimate of the transfer conductance.

Many studies have related increase in CO_2 assimilation rate to increase in leaf nitrogen (*Nicotiana tabacum*, 22; *Oryza sativa*, see 32; *Triticum aestivum*, 17). Whenever a sufficiently broad range of leaf nitrogen contents has been examined, it has been consistently found that the relationship is nonlinear, the slope declining as nitrogen content increases (*Beta vulgaris*, 16; *Glycine maxima*, 13; *Gossypium hirsutum*, 31, *Oryza sativa*, 25). Thomas and Thorne (27) found that the addition of 200 kg $N \cdot ha^{-1}$ to a wheat crop increased protein and Chl contents per unit leaf area by 27% and 15%, respectively, without measurable increases in assimilation rate per unit leaf area. Similarly, Migus and Hunt (14) and Gregory *et al.* (5) found no response of assimilation rate per unit leaf area to the amount of nitrogen applied to field-grown wheat.

The aims of the present work were to: first, determine the relationship between CO_2 assimilation rate and nitrogen content in the flag leaf of wheat; second, see if different nitrogen treatments or senescence altered the relative proportions of leaf proteins; and third, examine the relationship between the CO_2 assimilation rate measured under CO_2 limiting conditions and the *in vitro* RuP_2[1] carboxylase activity.

[1] Abbreviation: RuP_2, ribulose 1,5-bisphosphate.

MATERIALS AND METHODS

Plant Material. Three experiments were done: two during summer and one during winter. Plants of *Triticum aestivum* L. cv Yecora 70 were grown in a glasshouse in well-spaced 5-L pots of sandy loam. There were two plants per pot and eight replicate pots per treatment. The plants were watered daily and given 500 ml of Hewitt's nitrate nitrogen nutrient solution (6) three times a week. The summer crop had two nitrate treatments, 12 and 2 mM nitrate, and the winter crop had five treatments, 12, 2, 0.5, 0.1, and 0 mM nitrate. Nitrate concentrations in the nutrient solution were varied by substituting chloride for nitrate. Plants were induced to flower by subjecting them 7 d after emergence to continuous light for 7 d. Thereafter, the photoperiod was extended to 16 h with incandescent lights. The glasshouse average daily maxima and minima were 28/18°C and 20/13°C for the summer and winter crops, respectively. Plants were sprayed twice before flag leaf emergence with Metacystox to control mites.

Gas Exchange. A system having two double-sided leaf chambers was used to measure gas exchange from each side (2.25 cm^2) of each of two leaves concurrently. The boundary layer conductances were 0.8 and 1.25 mol m^{-2} s^{-1} for the upper and lower sides, respectively. CO_2-free air with known humidity was mixed with 10% CO_2 in air to generate different partial pressures of CO_2, which were measured with a Hartmann and Braun absolute infrared gas analyzer (IRGA). Air flow rates into the chambers were controlled and measured with Brooks (R-2-25-A) rotameters with sapphire floats. Differences in partial pressures of CO_2 and H_2O entering and leaving the chambers were measured with a Beckman 865 CO_2 IRGA and an ADC series 225 H_2O IRGA. Leaf temperature was controlled by varying the temperature of water flowing through water jackets on the chamber and was measured with a fine copper-constantan thermocouple (No. SCPSS-020E-6 Omega; Stamford, CT) pressed against the lower surface of the leaf. The leaves were illuminated with a water-cooled 2,500-w Xenon arc (XB 2500, Osram) lamp. Plants were placed in the gas exchange system for 1 h before measurements were begun. All measurements were made with PAR flux at the leaf surface being 1,800 μmol quanta m^{-2} s^{-1}, leaf temperature 23°C, and leaf to air vapor pressure difference 13 mbar. The partial pressure of CO_2 was 340 μbar except when assimilation rate (A) as a function of intercellular CO_2 partial pressure (p_i) was being determined. Rates of gas exchange, and associated parameters, were calculated using the equations of von Caemmerer and Farquhar (28). The calculated p_i has recently been confirmed by direct measurement (24). The initial slope of each A:p_i curve was taken from a linear regression of 6 points on the curve below a p_i of 100 μbar. The maximum Hill activity was estimated from each A:p_i curve, with p_i between 300 and 600 μbar, using equation A9 of von Caemmerer and Farquhar (28):

$$H = J_a/4 = 4.5(p_i + 7\Gamma_*/3)(A + R_d)/(p_i - \Gamma_*)/4$$

where H is Hill activity (μmol O_2 m^{-2} s^{-1}), J_a is the electron transport rate (4e/O_2), p_i is partial pressure of CO_2 inside the leaf (μbar), Γ_* is CO_2 compensation point without R_d (30 μbar), A is rate of CO_2 assimilation (μmol CO_2 m^{-2} s^{-1}), and R_d is dark respiration rate (μmol CO_2 m^{-2} s^{-1}).

Flag Leaf Measurements. From each nitrogen treatment, rates of gas exchange were measured repeatedly on three flag leaves at approximately weekly intervals. RuP_2 carboxylase (EC 4.1.1.39) activity, Chl, soluble protein, total nitrogen, and phosphorus contents were all determined on a fourth flag leaf the morning following gas exchange measurement. The assay for RuP_2 carboxylase activity was a modification of the method of Lorimer, Badger, and Andrews (12). About 10 cm^2 of leaf was ground in a Ten Broeck homogenizer in 5 ml of extraction buffer (50 mM Hepes-KOH [pH 7.0], 1 mM Na_2EDTA, 2 mM $MgSO_4$, 10 mM $NaHCO_3$, 10 mM DTT, 1% Polyclar) and centrifuged at 2,500g for 5 min. The supernatant liquid (25 μl) was used for the assay, which was done in triplicate. The assay medium consisted of 100 mM Tricine-OH (pH 8.1), 20 mM $MgCl_2$, 20 mM $NaH^{14}CO_3$ (18.5 GBq/mol). The final assay volume was 0.5 ml and the assay was done at 30°C in scintillation vials. After preincubation of the extract for 2 min, the reaction was started by the addition of 25 μl of 20 mM RuP_2. The assay was stopped after 60 s with 250 μl 5 N HCOOH and the vials were blown dry and counted in a scintillation counter. The assay was completed within 10 min of collecting the leaf. Chl was determined in 80% acetone. The MacKinney-Arnon equations (1) were transformed to give molar concentrations: 22.22 D_{645} + 9.057 D_{663} = x μmol Chl l^{-1}.

The supernatant liquid (1 ml) was also used to determine soluble-protein nitrogen. An equal volume of 10% (w/v) TCA was added to the supernatant liquid and centrifuged. The pellet was resuspended in 5% (w/v) TCA and recentrifuged. The supernatant liquid was discarded and the quantity of soluble-protein nitrogen in the pellet was found from a microKjeldahl digestion (11) and colorimetric ammonia determination with a Technicon autoanalyzer. Total leaf nitrogen and phosphorus were also measured, using the microKjeldahl method on another portion of the leaf.

Statistics. The lines shown in Figures 3, 5, and 6 were fitted by least squares regression. The least significant differences in Figure 1 were calculated from a one-way analysis of variance. Student's t test with $P < 0.05$ was used to determine significant differences.

RESULTS

Several characteristics of the winter crop are presented in Table I. The means of the first 4 weeks of measurements after full leaf expansion are presented, except for the ratio of Hill activity to initial slope which is the mean of all determinations. Greater nitrogen availability enhanced tillering and resulted in increased dry weight of the plants, which is reflected in their increased leaf area. The change in nitrogen contents per unit leaf area was relatively small and still smaller was the change in rate of CO_2 assimilation per unit leaf area between nitrogen treatments. RuP_2 carboxylase activity and Chl content reflect the leaf nitrogen content.

The rate of CO_2 assimilation per unit leaf aea was monitored from soon after full expansion until leaf death (Fig. 1a). Because of higher growth temperatures, the summer crop matured earlier than the winter crop. For clarity, only data for the highest and lowest nitrate treatments are shown. With the winter crop at anthesis, the assimilation rate of leaves from the 12 and 2 mM treatments were not significantly different, but both rates were significantly higher than those from the three lowest nitrate treatments (0, 0.1, 0.5 mM). Leaf conductance (g) and intercellular partial pressure of CO_2 (p_i) were similar between treatments until senescence began. Assimilation rate then declined faster than conductance which resulted in the p_i increasing (Fig. 1b).

Several A:p_i curves from flag leaves of the winter crop are given in Figure 2. The same leaves were measured at weekly intervals, but only three curves from the 12 and 0 mM treatments are shown. Initially, flag leaves from the two treatments behaved similarly. The photosynthetic capacity after anthesis was stable for 5 weeks in the high-nitrate plants, whereas it declined progressively in the low-nitrate plants. There was no significant change in the ratio of maximum Hill activity to initial slope from full expansion through senescence in any treatment, so the mean of all determinations is presented in Table I. The only significant difference among treatments, is between the ratio for the 12 mM nitrate leaf and the 0 mM nitrate leaf.

The proportions of Chl content, RuP_2 carboxylase activity, and soluble protein to total leaf nitrogen were all independent of the nitrate treatment. The proportion of Chl to total nitrogen was slightly lower in the summer than the winter (3.7 ± 0.1 *versus* 4.7 ± 0.1 mmol Chl/mol N; Fig. 3a). There was no seasonal effect on RuP_2 carboxylase activty (1.25 ± 0.05 mmol CO_2 s^{-1}/mol N; Fig. 3b) or soluble protein (0.51 ± 0.01 mol soluble-protein N/mol N) as a proportion of total leaf nitrogen. Despite the continuous increase of Chl content, RuP_2 carboxylase activity, and phosphorus (Fig. 4) with increase in leaf nitrogen content, the rate of CO_2 assimilation did not increase much with increase in nitrogen content above approximately 125 mmol m^{-2} (Fig. 5). Leaves containing nitrogen in excess of 150 mmol m^{-2} occurred only in the summer crop when there was a higher specific leaf weight (49.8 ± 0.7 *versus* 36.8 ± 0.5 g m^{-2} in winter).

The relationship between RuP_2 carboxylase activity assayed *in vitro* and the initial slope of the A:p_i curve is presented in Figure 6a. Initial slope was proportional to activity in winter, but inclu-

Table I. *Total Leaf Area and Characteristics of Young, Fully Expanded Flag Leaves Grown in Winter (Means ± SE)*

	Nitrate Treatment (mM)				
	0	0.1	0.5	2	12
Leaf area/plant (cm^2)	57 ± 2	56 ± 5	130 ± 12	378 ± 26	880 ± 128
Flag leaf area (cm^2)	19 ± 1	18 ± 1	25 ± 2	35 ± 1	44 ± 1
Leaf nitrogen (mmol m^{-2})	81 ± 4	86 ± 6	82 ± 6	122 ± 3	135 ± 9
CO_2 assimilation rate (μmol m^{-2} s^{-1})	24.4 ± 0.7	23.3 ± 0.8	23.4 ± 0.9	29.1 ± 1.0	28.2 ± 0.6
RuP_2 carboxylase activity (μmol CO_2 m^{-2} s^{-1})	123 ± 17	114 ± 10	99 ± 15	148 ± 3	183 ± 15
Chl (mmol m^{-2})	0.36 ± 0.01	0.42 ± 0.03	0.42 ± 0.03	0.52 ± 0.01	0.62 ± 0.02
Hill activity/initial slope (mol O_2 μbar mol CO_2^{-1})	312 ± 7	293 ± 16		300 ± 13	265 ± 10

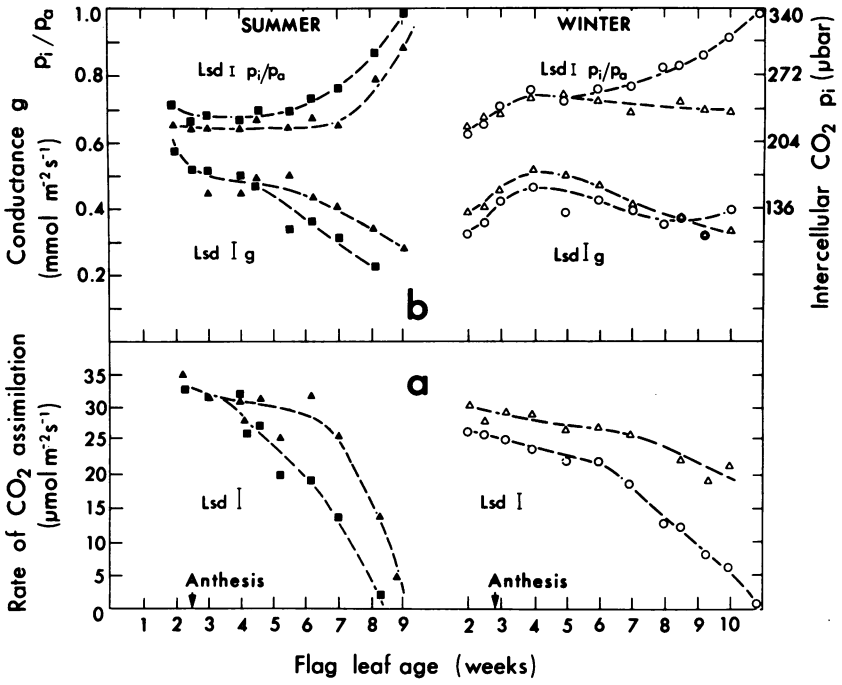

FIG. 1. a, CO_2 assimilation rate through the life of the flag leaf. (▲, ■), data from the summer crop (means of six replicates); (△, ○), the winter crop (means of four replicates). (▲, △), 12 mM; (■), 2 mM; (○), 0 mM nitrate treatment; (↓), start of anthesis. b, Conductance and intercellular CO_2 pressure through the life of the flag leaf. p_i/p_a is the ratio of the intercellular CO_2 pressure to the external ambient CO_2 pressure.

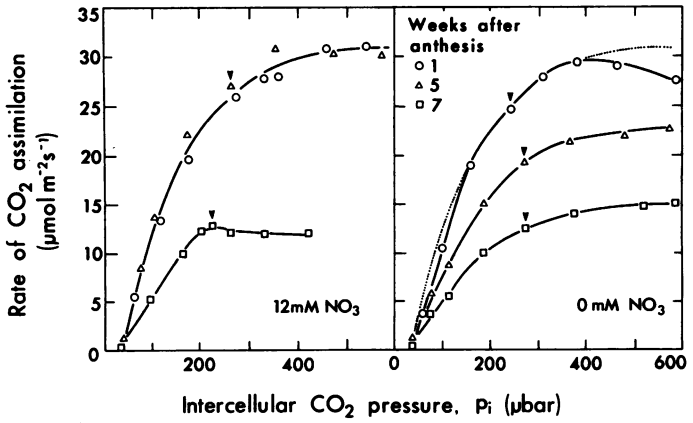

FIG. 2. Assimilation rate *versus* intercellular CO_2 pressure for 12 and 0 mM nitrate treatments. The curves are shown for three different times: (○), 1 week; (△), 5 weeks; (□), 7 weeks after anthesis. The arrow indicates the operating position at standard ambient conditions. (·····), line from the 12 mM treatment repeated for ease of comparison.

sion of the summer data, with generally higher activities, suggests that the overall relationship is curvilinear.

DISCUSSION

CO_2 Assimilation Rate and Leaf Nitrogen Content. The relationship between CO_2 assimilation rate per unit leaf area and total leaf nitrogen per unit leaf area (Fig. 5) appears to be general in that, whether the change in nitrogen content was achieved through nutrition, seasonal conditions, or senescence, the effect on assimilation was the same. Gregory, Marshall, and Biscoe (5) found that the relationship between assimilation rate and nitrogen was different between fertilized and unfertilized plots. Although leaves from the 12 mM nitrate winter treatment had not died by the end of the experiment, the other four treatments were indistinguishable from one another. Also, three different crops behaved similarly. Therefore, a single relationship appears to be justified.

The response of wheat to nitrogen fertilizer is in marked contrast to other plants such as *G. hirsutum* (31) or *Phaseolus vulgaris* (28) in which different amounts of nitrogen fertilizer resulted in plants with widely varying assimilation rates per unit leaf area and $A:p_i$ curves. Leaves from wheat plants grown in winter with 12 or 0 mM nitrate initially had similar $A:p_i$ curves. Nitrogen deficient plants senesced earlier than those grown with high nitrogen, and it was only then that differences between nitrogen treatments became apparent. That CO_2 assimilation rate per unit leaf area did not respond to addition of nitrogen fertilizer to field-grown wheat (27) is due to several factors. Even with very little nitrogen available to the roots, young fully expanded leaves never contained less than 80 mmol N m^{-2}. A 100-fold reduction in nitrate concentration resulted in only a 50% reduction in nitrogen content per unit leaf area which in turn only reduced assimilation rate by 20%. In a field-grown crop, nitrogen fertilization would result in a greater leaf area index which would reduce the light received by each leaf. The small increases in assimilation rate could then be offset by greater shading. As Gregory *et al.* (5) point out, most agricultural soils have nitrate concentrations in the mM range, which means only small increases in assimilation rate can be expected from increases in leaf nitrogen content.

Partitioning of Nitrogen between Protein Fractions. The relative proportion of soluble protein, RuP_2 carboxylase activity, and Chl were unaltered by the plant's nutrient treatment. Also, the ratios of Chl and soluble protein to total nitrogen were both constant throughout senescence. The increase in the ratio of Chl to nitrogen in the winter-grown leaves is perhaps a result of less light during winter. It suggests that more of the insoluble protein is associated with Chl-protein complexes because the proportion of insoluble nitrogen to total nitrogen was independent of the season.

Natr (15) recognized the importance of RuP_2 carboxylase activity in the response to nitrogen nutrition, but it does not appear to increase to a greater extent than other nitrogenous components. Wittenbach (30) and Peoples *et al.* (19) have both demonstrated that the specific activity of RuP_2 carboxylase is constant until late in senescence in the wheat leaf. Consequently, the activities meas-

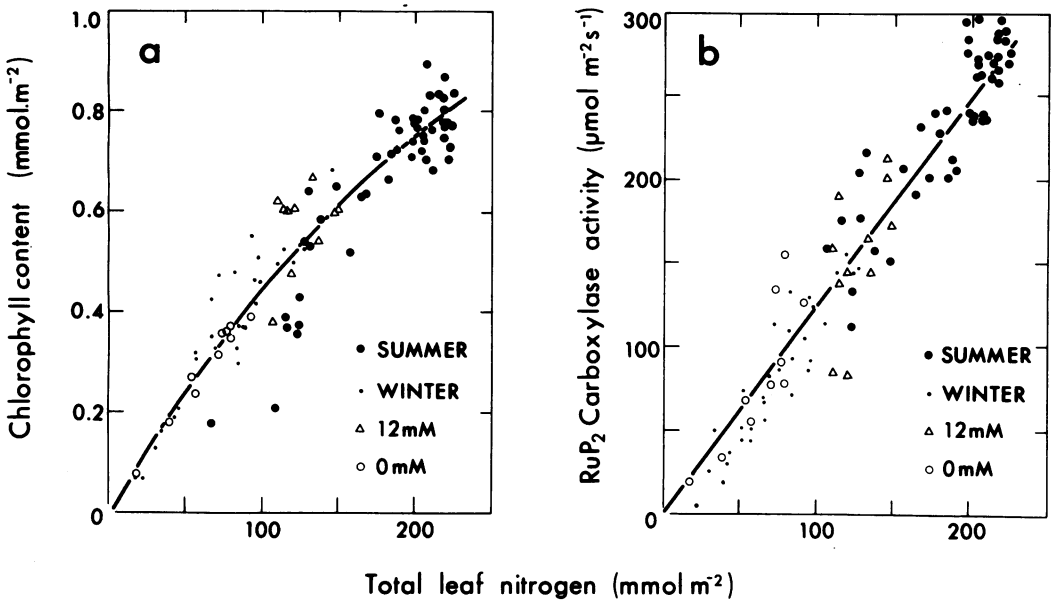

FIG. 3. a, Chl content *versus* total leaf nitrogen. $r^2 = 0.99$; $n = 102$. b, RuP_2 carboxylase activity *versus* total leaf nitrogen. $y = 1.25\ x$; $r^2 = 0.98$; $n = 102$. (●), summer; (△, ·, ○), winter; (△), 12 mM; (·), 2, 0.5, 0.1 mM; (○), 0 mM nitrate.

ured here should reflect the amount of RuP_2 carboxylase protein. The ratio of RuP_2 carboxylase activity to soluble-protein nitrogen or total nitrogen declined between 2 and 4 weeks of leaf age but then remained constant throughout senescence. Wittenbach (30) and Peoples *et al.* (19) also found that the ratio of RuP_2 carboxylase to soluble protein remained constant until late senescence but then declined. With barley, Friedrich and Huffaker (4) found a relatively constant RuP_2 carboxylase specific activity, but found that the ratio of RuP_2 carboxylase to soluble protein declined throughout senescence. It would seem that, in some situations, RuP_2 carboxylase can be remobilized more rapidly than other soluble proteins.

Senescence and the Loss of Photosynthetic Capacity. During senescence of *P. vulgaris* leaves, there appears to be some alteration in the balance between the Chl-protein complexes (8) as well as a large decrease in coupled non-cyclic electron transport rate (9). The latter appeared to be due to impaired electron flow between PSII and PSI (10). It was suggested that impaired electron flow was the cause of reduced assimilation rate during senescence. On the other hand, Friedrich and Huffaker (4) suggest that the loss of RuP_2 carboxylase is the primary event responsible for the decline in assimilation rate with senescence. The initial slope of the $A:p_i$ curve is believed to be proportional to the *in vivo* RuP_2 carboxylase activity (Fig. 6a) while the CO_2-saturated portion reflects the Hill activity (3). The ratio of Hill activity and initial slope thus gives a measure of the relative activities of RuP_2 regeneration and carboxylation (28). Inasmuch as no change in the ratio of Hill activity to initial slope could be discerned during senescence, it seems that photosynthetic metabolism is dismantled in a coordinated way such that an efficient balance between the various processes is maintained. The ambient operating point on the $A:p_i$ curve from leaves of all ages and all nitrate treatments was in the transition zone where assimilation rate is colimited by RuP_2 carboxylation and regeneration (28). Neither RuP_2 carboxylase activity nor the rate of electron flow can therefore be said to be the cause of the decline in the rate of assimilation during senescence.

Thimann and Satler (26) proposed that stomatal closure is the cause of senescence in barley leaves kept in the dark. During senescence of barley leaves, Friedrich and Huffaker (4), using a dual isotope porometer, observed a small decline in p_i and suggested this was due to a stomatal limitation. However, only the lower surface was measured. In the experiment reported here, stomatal limitation certainly cannot be said to have occurred.

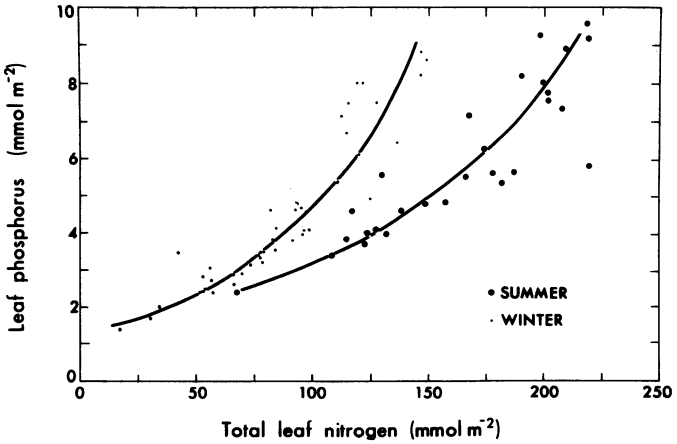

FIG. 4. Relationship between phosphorus and nitrogen contents of the flag leaf. (●), summer; (·), winter.

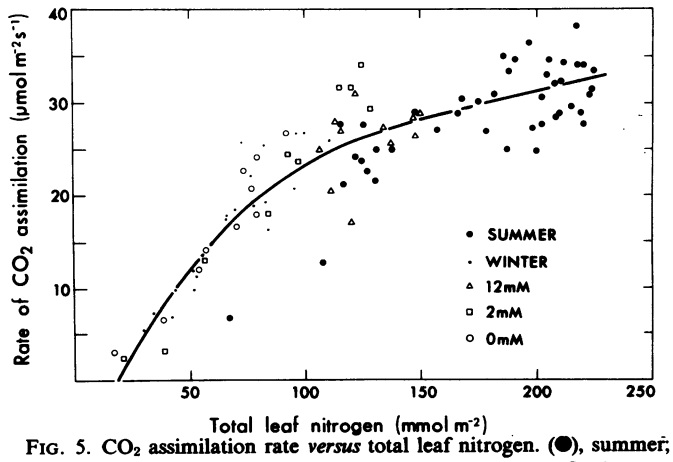

FIG. 5. CO_2 assimilation rate *versus* total leaf nitrogen. (●), summer; (△, □, ·, ○), winter; (△), 12 mM; (□): 2 mM; (·), 0.5, 0.1 mM; (○), 0 mM.

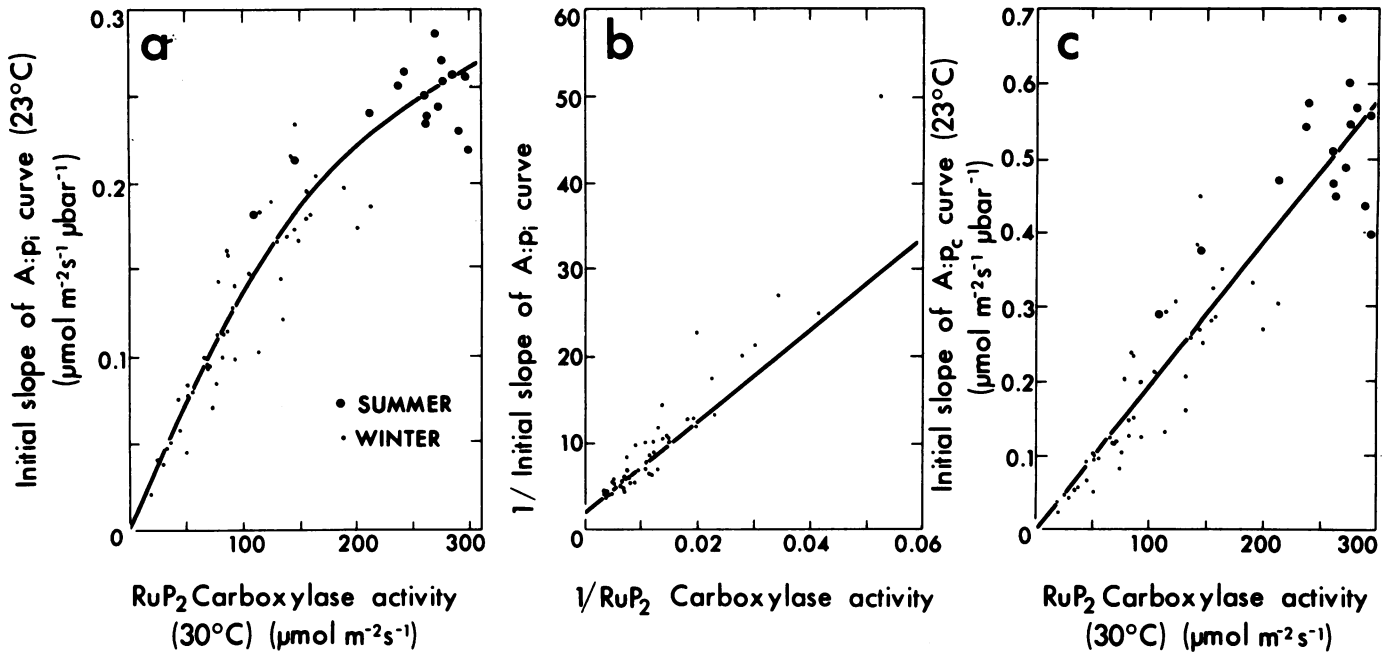

FIG. 6. a, Initial slope of $A:p_i$ curve *versus* RuP_2 carboxylase activity. (●), summer, (·), winter. b, Double reciprocal plot of Figure 6a. Maximal initial slope, $g_w = 0.49 \pm 0.03$ mol m^{-2} s^{-1} bar^{-1}, RuP_2 carboxylase activity at 0.5 $g_w = 245 \pm 25$ μmol m^{-2} s^{-1}. c, Initial slope of assimilation rate against partial pressure of CO_2 at the carboxylase enzyme (p_c) *versus* RuP_2 carboxylase activity.

First, p_i increased by 100 μbar during senescence. Second, the ambient operating point was always at the transition zone of the $A:p_i$ curve which could not be the case if stomatal conductance was a limitation.

Assimilation Rate and RuP_2 Carboxylase Activity. A biochemical model of photosynthesis developed by Farquhar *et al.* (3), using the current estimates of RuP_2 carboxylase kinetic constants, suggests that isolated RuP_2 carboxylase activity is only just sufficient to account for *in vivo* rates of assimilation. Both von Caemmerer and Farquhar (28) and Seemann and Berry (23) show the close relationship between CO_2-limited assimilation rate and RuP_2 carboxylase activity. In both cases, the assimilation rate was slightly greater than would be predicted from the *in vitro* enzyme kinetic constants. For the winter-grown leaves of wheat, there was a close relationship between CO_2-limited assimilation and RuP_2 carboxylase activity, but for leaves developing in summer there was a departure from linearity. Some curvature is also apparent in von Caemmerer and Farquhar's (28) data. There are two possible causes of the curvature. Either some of the enzyme is inactive in the leaf but functional when assayed *in vitro*, or there is a significant drop in CO_2 pressure between the intercellular spaces and the site of carboxylation due to a finite wall and liquid-phase conductance to CO_2 diffusion (g_w). An estimate of g_w can be obtained from a double reciprocal plot of Figure 6a. Using the method of Wilkinson (29), g_w was found to be 0.49 ± 0.03 mol m^{-2} s^{-1} bar^{-1} or ~0.47 mol m^{-2} s^{-1}, and half the maximum conductance occurred with a carboxylase activity of 245 ± 25 μmol m^{-2} s^{-1} (Fig. 6b). Raven and Glidewell (21) have attempted to derive the wall and liquid-phase resistances, and they estimate that they range from 10 to 15 s cm^{-1} on a mesophyll cell area basis. They assume a mesophyll cell area to leaf area of 20. Parker and Ford (18) have measured the mesophyll cell area to leaf area for *T. aestivum* as 10.5, although their method underestimates the ratio. If a value of 15 is used, the estimate of g_w by Raven and Glidewell becomes 0.4 to 0.6 mol m^{-2} s^{-1} which is in good agreement with g_w derived in Figure 6b. The initial slope of assimilation rate *versus* the partial pressure of CO_2 at the site of carboxylation, p_c, can be calculated by the equation $k = \alpha/(1 - \alpha/g_w)$, where k is the initial slope of the $A:p_c$ curve or carboxylation efficiency (2), α is the initial slope of the $A:p_i$ curve, and g_w is the wall and liquid-phase conductance (Fig. 6c).

It has been suggested by several workers (7, 20) that some RuP_2 carboxylase is inactive in the chloroplast and acts as a storage protein. If there is no decline in the pressure of CO_2 between the intercellular spaces and the site of carboxylation, the results in Figure 6 suggest that 65% of RuP_2 carboxylase activity in excess of 155 μmol m^{-2} s^{-1} would have to be inactive in the leaf, representing one third of the RuP_2 carboxylase in the summer-grown wheat leaf. One would expect this to form a discrete and visible structure within the chloroplast which was not found when sections of the leaf were examined by electron microscopy. With the g_w obtained from Figure 6b, none of the enzyme would be inactive *in vivo*, and indeed, with the current kinetic parameters for the enzyme, there is insufficient *in vitro* activity to account for the observed assimilation rates.

Acknowledgments—I thank Drs. G. D. Farquhar, L. T. Evans, I. R. Cowan, and T. D. Sharkey for critical reading of the manuscript.

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