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Temperature Dependence of Photosynthesis in *Agropyron smithii* Rydb.[1]

I. FACTORS AFFECTING NET CO_2 UPTAKE IN INTACT LEAVES AND CONTRIBUTION FROM RIBULOSE-1,5-BISPHOSPHATE CARBOXYLASE MEASURED *IN VIVO* AND *IN VITRO*

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ABSTRACT

As part of an extensive analysis of the factors regulating photosynthesis in *Agropyron smithii* Rydb., a C_3 grass, we have examined the response of leaf gas exchange and ribulose-1,5-bisphosphate (RuBP) carboxylase activity to temperature. Emphasis was placed on elucidating the specific processes which regulate the temperature response pattern. The inhibitory effects of above-optimal temperatures on net CO_2 uptake were fully reversible up to 40°C. Below 40°C, temperature inhibition was primarily due to O_2 inhibition of photosynthesis, which reached a maximum of 65% at 45°C. The response of stomatal conductance to temperature did not appear to have a significant role in determining the overall temperature response of photosynthesis. The intracellular conductance to CO_2 increased over the entire experimental temperature range, having a Q_{10} of 1.2 to 1.4. Increases in the apparent Michaelis constant (K_c) for RuBP carboxylase were observed in both *in vitro* and *in vivo* assays. The Q_{10} values for the maximum velocity (V_{max}) of CO_2 fixation by RuBP carboxylase *in vivo* was lower (1.3-1.6) than those calculated from *in vitro* assays (1.8-2.2). The results suggest that temperature-dependent changes in enzyme capacity may have a role in above-optimum temperature limitations below 40°C. At leaf temperatures above 40°C, decreases in photosynthetic capacity were partially dependent on temperature-induced irreversible reductions in the quantum yield for CO_2 uptake.

In most C_3 plants which have been examined, photosynthetic temperature inhibition is fully reversible up to a high temperature threshold, beyond which irreversible effects predominate (for review, see Ref. 3). Among the factors which are known to regulate the degree of reversible temperature inhibition are stomatal conductance limitations (11, 31), and the O_2 inhibition of photosynthesis (photorespiratory reactions), which includes the differential liquid phase solubilities of O_2 and CO_2 as temperature increases (20). Recent discussions by Farquhar *et al.* (9, 10) and Collatz (5) suggest that the steady state concentration of RuBP[2] may regulate the activity of RuBP carboxylase, a potentially rate-limiting step in the reductive pentose phosphate cycle. It has been further suggested that temperature limitations to the rate of regeneration of RuBP may be partially responsible for the reversible inhibition of photosynthesis observed at above-optimum temperatures (3).

Temperature has also been shown to affect both the K_m (CO_2) and V_{max} of RuBP carboxylase *in vitro* (2, 24). Weis (35) has suggested that a temperature-dependent inactivation of RuBP carboxylase occurs at above-optimal temperatures in intact spinach chloroplasts. Such temperature effects may be partly responsible for the reversible temperature limitations on net photosynthesis observed for intact leaves. However, difficulties arise in interpreting these data due to the uncertainties encountered in using *in vitro* studies to describe processes *in vivo*.

Photosynthetic inhibition at analysis temperatures beyond the point of reversibility is due in part to irreversible inhibition of the quantum yield for CO_2 fixation, and decreased activity of certain enzyme reactions (4). An irreversible reduction in the quantum yield has been used as an indication of heat damage to the photosynthetic apparatus (29), and is presumably related to the integrity of the thylakoid membranes (1).

Agropyron smithii is a C_3 perennial grass which constitutes a major biomass producer of the short- and mixed-grass prairie ecosystems. Plants of this species initiate growth during the early spring months when seasonal temperatures are relatively cool (6). Flowering occurs in June; thus, this species completes a major portion of its life cycle before the commencement of high air temperatures which characterize the remainder of the growing season. Laboratory studies have indicated that net CO_2 uptake is severely limited when this species is grown or analyzed at temperatures above 25 to 30°C (19, 36). In light of this apparent intolerance of high temperature, we have chosen to fully characterize the mechanisms underlying temperature inhibition in *A. smithii*. The present study was conducted with two goals in mind: to describe the factors which regulate the temperature dependence of gas exchange of intact leaves, and to further characterize the role of RuBP carboxylase in the temperature response of intact leaf photosynthesis. The experimental approach emphasized comparisons between RuBP carboxylase activities measured *in vitro* and *in vivo*.

MATERIALS AND METHODS

Plant Material. Sod blocks containing *Agropyron smithii* Rydb. were collected from the Pawnee grassland in northeastern Colorado. The sods were divided, transplanted into a peat-sand mixture, and maintained in a growth chamber (model E 15; Conviron) at 20/15°C day/night air temperatures (12 h at each temperature). The photoperiod during growth was 14 h with the light being provided by a bank of fluorescent and incandescent lamps at an irradiance of 100 nE cm^{-2} s^{-1} at plant height. Leaf temperatures during the light period averaged 20.5°C. Plants were watered

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[2] Abbreviations: RuBP, ribulose 1,5-bisphosphate; VPD, vapor pressure difference.

daily, and nutrient solution was applied once each week. All plants were grown under these conditions for 4 months prior to experimental analysis. Plants were clipped periodically such that leaf age for the experimental plants was standardized at 35 ± 5 d.

Gas Exchange. Measurements of CO_2 and water vapor exchange were conducted with an open IR gas analysis system. A single attached leaf was sealed into a temperature-controlled cuvette constructed from polycarbonate which has the properties of being relatively impermeable to CO_2 and water vapor. The cuvette was enclosed in a modified growth chamber for additional temperature control. Leaf boundary layer conductance to gas exchange was maximized within the cuvette through the use of a small fan. Measurements of water loss from blotter paper leaf replicas indicated that the boundary layer conductance was 45 mm s^{-1}. Temperatures of the enclosed leaf were measured with 0.10 mm diameter copper-constantan thermocouples appressed to the lower leaf surface. Leaf temperatures were manually adjusted by means of a peltier-cooled heat exchanger (model 809-3040-01; Cambion) which formed the base of the cuvette. Using this apparatus, leaf temperatures were controlled within ±0.5°C of the desired temperature. A 1-kw multivapor lamp (Sylvania), supplemented with three 300-w medium flood lamps (Sylvania), filtered through 20 cm of H_2O, provided light intensities of 200 nE cm^{-2} s^{-1} (PAR) within the leaf cuvette, as measured with a quantum sensor (model 1776; LiCor Instruments). This combination of lamps provided a relatively even spectral distribution (~100 μw cm^{-2} nm^{-1}) between 400 and 700 nm as measured with a spectroradiometer (model SR; ISCO Co.). Exceptions to the spectral balance were noted between 575 and 630 nm, as rather high intensities (200–300 μw cm^{-2} nm^{-1}) were emitted. Light intensities for the quantum yield determinations were controlled with neutral density filters (cheesecloth) and measured continuously with the same quantum sensor mounted within the cuvette adjacent to the enclosed leaf. The air stream used in the gas exchange measurements was produced by mixing gas from cylinders containing 21% O_2 in N_2, or 2% O_2 in N_2, and a second containing pure CO_2 using a pair of Wösthoff mixing pumps (model 1 SA 27/3F). The resulting gas stream was humidified by bubbling through a 2-L flask filled with distilled H_2O slightly acidified with H_2SO_4, and submerged in a temperature-controlled water bath. Excess water vapor was removed by passing the air stream through a second 2-L flask which was half-filled with glass beads and submerged in the water bath. A portion of the resulting gas stream was passed at a constant flow rate through a drying column containing $MgClO_4$ and into the reference cell of a differential CO_2 analyzer (model 315B; Beckman Instruments). The remainder of the gas stream was passed through the leaf cuvette, a dew-point hygrometer (model 880; EG and G Instruments), a $MgClO_4$ drying column, and the sample cell of the CO_2 analyzer. Gas flow rates were measured before and after the leaf chamber, as well as before entering the reference cell of the CO_2 analyzer, with three flow meters (model 1370-01 A2AAA; Brooks Co.). Flow rates were adjusted such that the CO_2 differential within the leaf cuvette was maintained at less than 30 μl l^{-1}. In order to assess the magnitude of measurement error which is inherent in the components of the gas analysis system, the degree of alteration in known concentrations of CO_2 and water vapor was measured over the entire experimental temperature range. The results indicated that less than 1% alteration existed at all temperatures. The zero point of the differential CO_2 analyzer was adjusted before each photosynthesis measurement. Additionally, the CO_2 analyzer was calibrated with 4 to 5 differential gas concentrations at the beginning of each experimental day. Leaf areas were measured with a leaf area meter (model LI-3000; Lambda Instruments).

In the photosynthetic temperature response experiments, measurements were initially conducted at a leaf temperature of 20°C. After steady-state photosynthesis and transpiration rates were achieved, the temperature was adjusted in steps down to 10°C. The temperature was then increased in 2 or 5°C intervals with steady-state rates being recorded up to 48°C. For the photosynthetic irradiance response, leaves were first exposed to a light intensity of 40 nE cm^{-2} s^{-1} (400–700 nm) until a steady-state photosynthesis rate was achieved. Subsequently, the light intensity was lowered in five steps, ending in total darkness. Upon reestablishment of the photosynthesis rate at 40 nE cm^{-2} s^{-1}, the intensity was increased in several steps to full irradiance (200 nE cm^{-2} s^{-1}). For the experiments in which the CO_2 concentration was varied, a steady-state photosynthesis rate was established at 340 μl l^{-1} CO_2 (external to the leaf), whereupon the CO_2 concentration was reduced in appropriate steps to the CO_2 compensation point. Upon re-establishment of the photosynthesis rate at 340 μl l^{-1} CO_2, the CO_2 concentration was increased in several steps to a maximum of 800 μl l^{-1} CO_2. In this series of experiments, the data are based on soluble CO_2 concentrations at each experimental temperature and barometric pressure (34). Values for the intracellular conductance to CO_2 (C_i) were obtained as the initial slope of the photosynthetic response to CO_2 concentration (generally less than 4–6 μM). The absolute level of O_2 inhibition of photosynthesis is calculated as the difference between the photosynthesis rates with 2% O_2 and with 21% O_2. The percentage inhibition of photosynthesis by 21% O_2 is calculated as:

$$(P_{2\%_{O_2}} - P_{21\%_{O_2}}/P_{2\%_{O_2}}) \cdot 100 \quad (1)$$

Leaf transmittance and reflectance measurements were conducted on freshly cut leaf discs, at 5-nm wavelength intervals between 400 and 700 nm, with the integrating sphere described by Robberecht and Caldwell (28). Absorbance was calculated by subtraction. Since the absolute amount of energy incident upon the leaf and leaf absorbance were known for each 5-nm wavelength interval, the amount of energy absorbed by the leaf was accurately determined over the entire PAR range.

Measurement of Kinetic Constants for RuBP Carboxylase *in Vivo*. The measurement of RuBP carboxylase *in vivo* was conducted by assessing the response of intact leaf net photosynthesis to rate-limiting CO_2 concentrations. This approach requires several assumptions, and consideration of possible errors, especially at high leaf temperatures. However, the approach which we have used should allow us to make estimates of kinetic constants for RuBP carboxylase *in vivo*. The major assumptions inherent in the measurement of kinetic constants (K_c and V_{max}) for RuBP carboxylase in intact leaves are: (a) the principal resistance is biochemical and RuBP carboxylase is the rate-limiting step for CO_2 fixation at low CO_2 concentrations; and (b) net CO_2 uptake at low CO_2 concentrations obeys Michaelis-Menten kinetics (21). The analysis was conducted by constructing double-reciprocal plots with 1/P as the ordinate and $1/[CO_2] - \Gamma$ as the abscissa. This treatment neglects the effects of dark respiration which may be significant at high leaf temperatures. Thus, the K_c and V_{max} as determined in this study may be underestimated relative to the true *in vivo* values. An additional source of error may be present at the low CO_2 concentrations in which K_c and V_{max} were determined inasmuch as decreased activation of RuBP carboxylase may occur. Further studies on the magnitude of these effects *in vivo* are required. Kinetic parameters were obtained from double-reciprocal plots, in which all linear relationships were evaluated using least-squares regression analysis. In this study, the apparent K_c is defined as the CO_2 concentration which resulted in half V_{max} in either the *in vivo* or *in vitro* assays.

Measurement of RuBP Carboxylase Activity *in Vitro*. Activities of RuBP carboxylase were determined in crude leaf extracts derived from the same plants used in the gas exchange studies. In the interest of obtaining maximum activity of the enzyme *in vitro*, preilluminated leaves were killed in liquid N_2 and extracted in a medium containing HCO_3^- and Mg^{2+} (M. Ku, personal commu-

nication). Approximately 1 g fresh weight of the leaf material was clipped, weighed, and immediately frozen with liquid N_2 in a mortar. The leaves were ground into a fine powder in the presence of 15 ml of grinding medium. The grinding medium consisted of 100 mM Tricine buffer (pH 8.0), 5 mM DTT, 20 mM $MgCl_2$, 10 mM $NaHCO_3^-$, and 5% PVP. The extract was kept frozen (−80°C) until each assay by periodic additions of liquid N_2. Approximately 5 to 10 min prior to initiating each assay, a 2- to 3-ml aliquot of the frozen extract was removed from the mortar and allowed to thaw at room temperature. The thawed extract was pressed through a 40-μm nylon net attached to a 20-ml syringe and immediately assayed.

The assays were conducted in small glass vials which were half immersed in a temperature-controlled water bath. The assay buffer (100 mM CO_2-free Tricine, pH 8.0), which contained 5 mM DTT and 20 mM $MgCl_2$, was added to each reaction vial approximately 2 min prior to each assay in order to insure temperature equilibration. N_2 was bubbled through the assay medium for the entire 2-min equilibration period. Carbonic anhydrase (100 units) and RuBP (0.4 mM) were added to the assay medium at 60 and 45 s prior to initiating each assay, respectively. The appropriate concentration of $NaH^{14}CO_3^-$ (7.0 mCi mM^{-1}) was added 5 s before initiating the reaction, thus minimizing losses of $^{14}CO_2$ to the atmosphere above the reaction medium. The reaction was initiated by the addition of 20 μl of crude extract. The final volume of the reaction mixture was 1 ml. The reaction was terminated after 30 s (1 min at 10 and 15°C) by the addition of 200 μl of 6 N HCl. The contents of each reaction vial were transferred to a scintillation vial and dried for 24 h at 60°C. The quantity of $^{14}CO_2$ incorporated was counted with a liquid scintillation spectrometer (model LS 7000; Beckman Instruments).

The quantity of soluble protein in aliquots of each extract was determined according to the method of Lowry *et al.* (26). The average amount of protein present in each extract was 1.8 mg ml^{-1}. Specific activities were expressed on a leaf area basis after determining the amount of soluble protein per unit leaf area (~0.57 mg cm^{-2}).

RESULTS

The Temperature Dependence of Net Photosynthesis. The temperature dependence of net CO_2 uptake in *A. smithii* was similar to that reported for other C_3 species, when analyzed in the presence of 21% O_2 and 340 μl l^{-1} CO_2 (hereafter referred to as normal air; Fig. 1). A photosynthetic maximum was observed at 25°C, beyond which temperature inhibition increased progressively, resulting in zero net CO_2 uptake at 48°C. The inhibitory effects of high temperature on net CO_2 uptake were fully reversible up to 40°C (data not shown). When leaves were exposed to temperatures of 45°C for 20 min, and subsequently allowed to recover at 25°C for 1 h, a 20% reduction in the rate of light-saturated net photosynthesis was observed (measured at 25°C).

In an atmosphere of low O_2 and normal CO_2 (2% O_2, 340 μl l^{-1} CO_2), the photosynthetic temperature response was characterized by increased rates of net CO_2 uptake at all temperatures, and a shift in the temperature optimum to between 30 and 35°C. Further increases in the light-saturated rate of net photosynthesis at all analysis temperatures were observed when the CO_2 concentration external to the leaf was increased to 800 μl l^{-1} CO_2 (Fig. 1). The temperature optimum for net CO_2 uptake was observed to shift to 35 to 40°C at this higher CO_2 concentration.

The Temperature Dependence of O_2 Inhibition of Photosynthesis. The difference between the net photosynthesis rates determined with 21% and 2% O_2 (both with 340 μl l^{-1} CO_2) represents the amount of inhibition imposed by 21% O_2. This difference is expressed as a percentage of the photosynthesis rate in 2% O_2 in Figure 2. The temperature response of O_2 inhibition reached a maximum of 65% at 45°C. When calculated on an absolute basis,

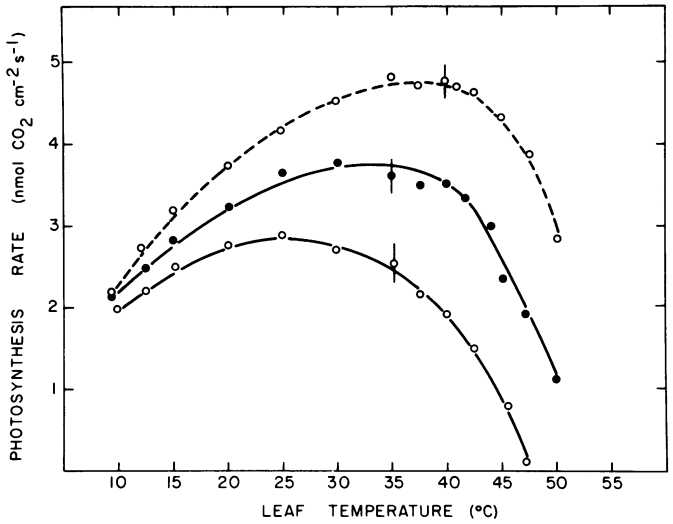


FIG. 1. The temperature dependence of net photosynthesis in the presence of 21% O_2, 340 μl l^{-1} CO_2 (O——O); 2% O_2, 340 μl l^{-1} CO_2 (●); 2% O_2, 800 μl l^{-1} CO_2 (O---O). Points represent the mean of three to four replicates. Vertical bars represent the maximum ± 1 SE in any treatment in this and all other figures.

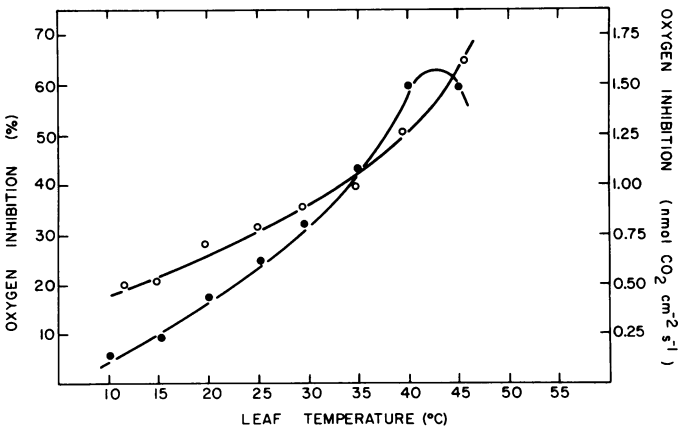


FIG. 2. The temperature dependence of O_2 inhibition of photosynthesis, expressed as a percentage of the photosynthesis rate observed in 2% O_2, 340 μl l^{-1} CO_2 (O); and on an absolute basis (●). Points represent the mean of five replicates.

the degree of O_2 inhibition exhibited a similar temperature response pattern (Fig. 2). One exception to the similarity was observed above 40°C, whereby the absolute amount of O_2 inhibition did not increase.

The Temperature Dependence of Stomatal and Intracellular Conductances. The stomatal conductance to CO_2 uptake (C_s) increased only slightly over the entire experimental temperature range, when assayed at a low and relatively constant VPD between the leaf and air (Fig. 3A). When the dewpoint temperature of the airstream entering the leaf cuvette was adjusted so that a slight increase in the VPD occurred with increasing temperature, then decreases in stomatal conductance were observed at the higher leaf temperatures (Fig. 3B). Stomatal responses to the VPD, such as those presented in Figure 3B, could have a marked effect on the temperature response of net photosynthesis in C_3 plants. This occurs because in most C_3 plants net photosynthesis rates are not CO_2-saturated at ambient air concentrations. Thus, any decrease in the availability of this substrate, in this case by diffusion limitations, would decrease the rate of CO_2 fixation. In order to prevent the stomatal response to VPD from interfering with the

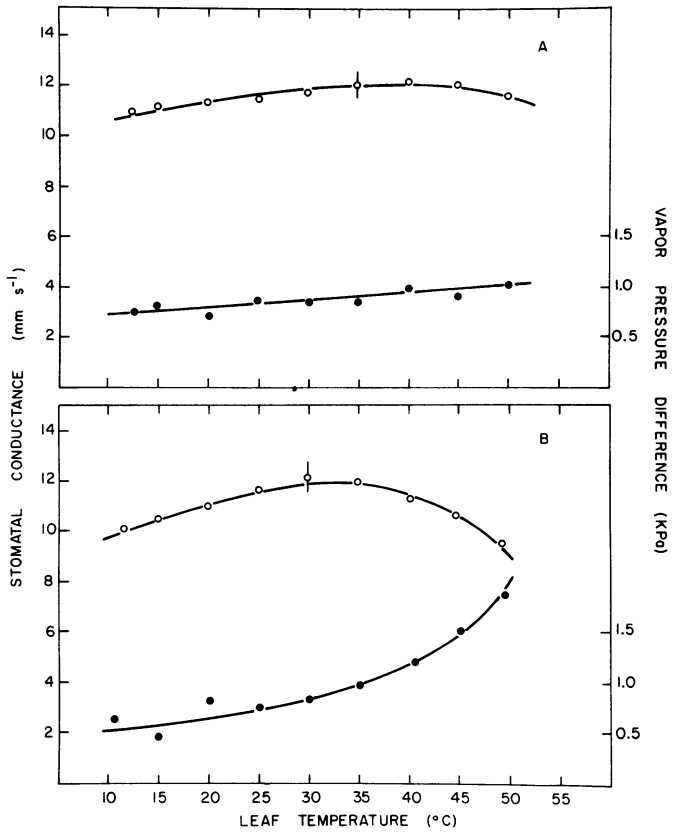


FIG. 3. A, The temperature dependence of stomatal conductance to CO_2 (○) in the presence of a relatively constant and low VPD (●); B, the temperature dependence of stomatal conductance to CO_2 in the presence of a slightly increasing VPD (●). Points represent the mean of three replicates.

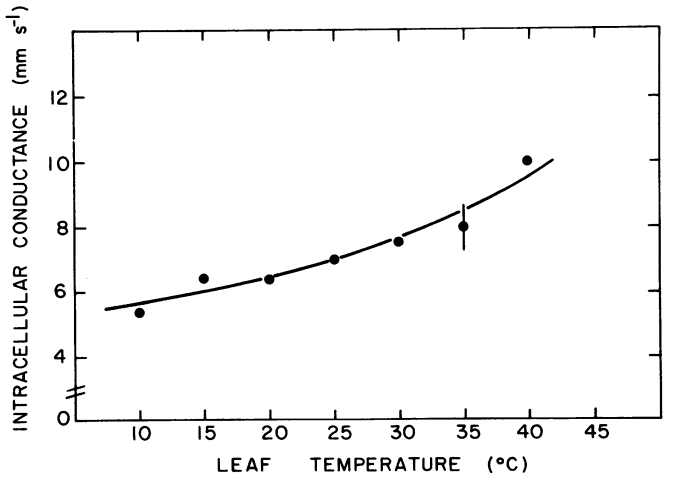


FIG. 4. The temperature dependence of the intracellular conductance to CO_2. Points represent the mean of three replicates.

temperature response of photosynthesis, all experiments were conducted with a relatively low VPD (<1.0 kPa).

The intracellular conductance to CO_2 (C_i) increased as a function of increasing leaf temperature (Fig. 4). Values of C_i were less than those for C_s over the entire experimental temperature range (10–40°C). Thus, C_i would represent the more limiting conductance to CO_2 diffusion when considered in series with C_s. The Q_{10} of C_i ranged from 1.2 to 1.4 over the experimental temperature range.

The Temperature Response of RuBP Carboxylase Measured *in Vivo* and *in Vitro*. Analysis of leaf photosynthesis at various temperatures and CO_2 concentrations (under 2% O_2) were made in order to assess the *in vivo* properties of RuBP carboxylase (see "Materials and Methods"). Leaf temperature had a marked affect on the CO_2 dependence of intact leaf net photosynthesis (Fig. 5, A and B). Although the response of net photosynthesis to CO_2 concentration was assayed at 5°C intervals between 10 and 40°C, we have only presented data from the 10 and 35°C assays for brevity. We chose to present data from the 35°C analysis temperature, rather than 40°C, because irreversible high temperature damage to chloroplast components other than RuBP carboxylase conceivably could have influenced the CO_2 response of net photosynthesis at 40°C (Fig. 10).

The net photosynthesis rate of intact leaves approached substrate saturation at progressively higher CO_2 concentrations as leaf temperatures were increased. Deviations from Michaelis-Menten kinetics were observed at high CO_2 concentrations (greater than 8 μM) *in vivo*, at all experimental leaf temperatures (Fig. 5C). The deviations were of least magnitude at the lowest temperature (10°C) and became progressively more pronounced as leaf temperatures were increased.

A series of experiments were conducted to characterize the time course of CO_2 fixation through RuBP carboxylase in crude leaf extracts (data not shown). The presence of an initial lag in CO_2 fixation was not observed within the shortest time interval (10 s). These results indicate that the state of activation of RuBP carboxylase did not change during the assay period. The quantity of $^{14}CO_2$ fixed was linear over the entire time range (10–90 s).

Activities of RuBP carboxylase measured *in vitro* increased at any given CO_2 concentration as assay temperatures were increased (Fig. 6). Data from two representative temperatures are presented, although the response of RuBP carboxylase activity to CO_2 concentration was measured at 5°C intervals from 10 to 45°C. Deviations from Michaelis-Menten kinetics were not observed at any experimental temperature when the data from the *in vitro* assays were expressed in double-reciprocal plots (Fig. 6C).

Values for the apparent K_c which were determined from the double-reciprocal plots at each temperature *in vitro* are presented in Figure 7. The values were very similar at any given temperature, and exhibited approximately equal increases as temperature increased, whether determined from the *in vivo* or *in vitro* assays.

The temperature response of the calculated V_{max} of RuBP carboxylase *in vivo* was characterized by a Q_{10} of 1.3 to 1.6 between 10 and 40°C (Fig. 8). In contrast, the Q_{10} of RuBP carboxylase assayed *in vitro* was 1.8 to 2.2 between 20 and 40°C. Relatively high Q_{10} values (4.0–5.3) were observed at the lower assay temperatures (10–20°C) *in vitro*. When expressed on an absolute basis, the V_{max} values determined from RuBP carboxylase assayed *in vitro* were lower than those for the *in vivo* assays at all experimental temperatures.

The Photosynthetic Response to Irradiance as Affected by Leaf Temperature. The results reported in Figure 9 indicate that light saturation of net photosynthesis occurred between 100 and 125 nE cm^{-2} s^{-1} absorbed PAR (125–150 nE cm^{-2} s^{-1} incident PAR), at saturating CO_2 concentrations, between 25 and 35°C. Increasing experimental leaf temperatures from 25 to 35°C had a very slight effect on the light dependence of net photosynthesis within the range of low light intensities (Figs. 9 and 10). The initial slope of the light response curve (quantum yield for CO_2 uptake; ϕ) was not reduced as leaf temperatures increased from 20 to 35°C; however, the absolute magnitude of net CO_2 uptake at each light intensity was reduced slightly (Fig. 10).

The quantum yield was measured as 0.081 to 0.083 mol CO_2 fixed per mol quanta (PAR) absorbed at leaf temperatures below 35°C. These values are similar to those reported for other C_3 plants in the presence of saturating CO_2 (8, 22). At leaf tempera-

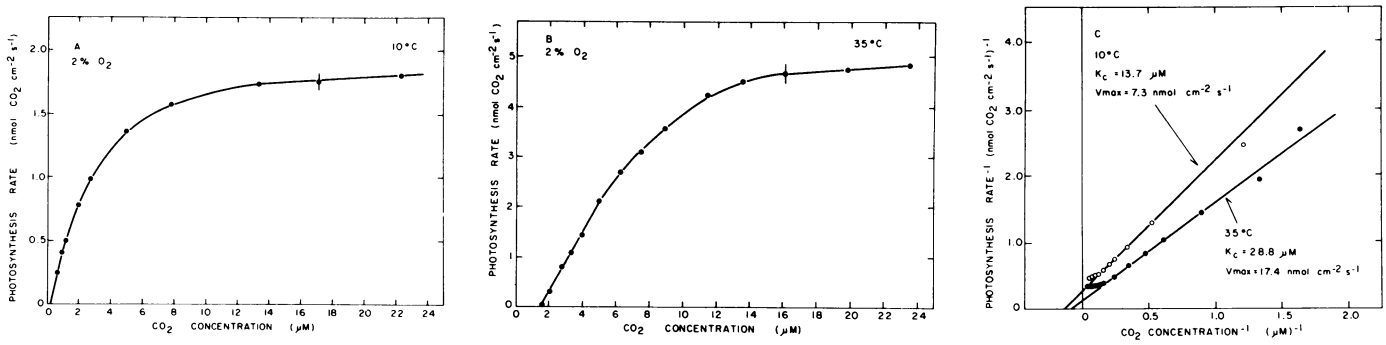


FIG. 5. The response of net photosynthesis to CO_2 concentration *in vivo* at 10°C (A) and 35°C (B). Data are also presented in a double-reciprocal plot (C). The CO_2 compensation point (Γ) has been subtracted from each CO_2 concentration in the double-reciprocal plot. Points which deviated from the linear relationship at high CO_2 concentrations (greater than 8 μM) were not used in calculating the kinetic constants (V_{max} and K_c). Note different scales for ordinates of A and B. Points represent the mean of three replicates in A and B. The data presented in C are from one leaf.

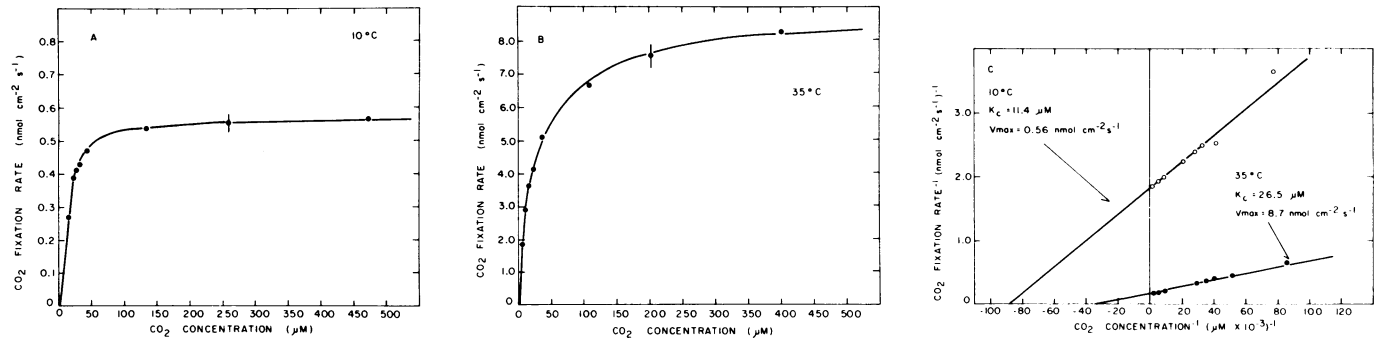


FIG. 6. The response of CO_2 fixation to CO_2 concentration by RuBP carboxylase assayed *in vitro* at 10°C (A) and 35°C (B). Data are also presented in a double-reciprocal plot (C). Note different scales for ordinates of A and B. Points represent the mean of three to four replicates.

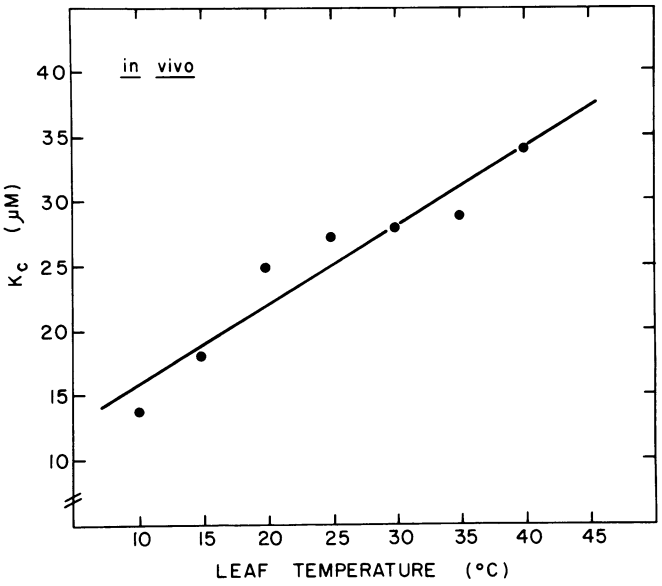
tures above 35°C, marked reductions in the quantum yield for CO_2 uptake were observed (Fig. 10). Reversibility of the temperature-dependent reduction in quantum yield was assessed by comparing the quantum yield before and after a 30-min high temperature pretreatment (Fig. 11). At 40°C, the reduction in quantum yield was completely reversible, while irreversible reductions occurred above 41°C.

DISCUSSION

The shape of the temperature response curve for photosynthesis in normal air for *A. smithii*, is similar to previously reported temperature responses for C_3 plants grown in cool-temperature regimes (for review, see Ref. 3). The cumulative results of these studies suggest a common pattern of habitat temperature adaptation among C_3 plants native to cool-temperature environments. In most of these cool-adapted plants temperature optima for photosynthesis occur between 20 and 30°C, with fairly distinct upper temperature thresholds between 40 and 44°C, beyond which net photosynthesis is irreversibly inhibited (4, 7, 29, 30). Recent studies in our laboratory (data not shown), as well as those by Kemp and Williams (19) and Williams (36), indicate that *A. smithii* possesses very little potential for photosynthetic acclimation to temperature. In fact, in these latter studies, net photosynthesis was reduced at all analysis temperatures when grown in a 35°C day/15°C night temperature regime, relative to a 20°C day/15°C night temperature regime. Together these studies demonstrate a high degree of correlation between the temperature response of photosynthesis for *A. smithii* and seasonal phenology patterns *in situ* (described in the "Introduction").

The inhibition of net photosynthesis at temperatures above the optimum does not appear to be due to decreasing stomatal conductance (Fig. 3A). It should be noted, however, that the response of stomatal conductance to leaf temperature, in this case, was determined in the presence of a low and relatively constant leaf to air vapor pressure difference (VPD). Previous studies have indicated that many plant species exhibit a marked decrease in stomatal conductance in response to increases in the leaf to air VPD (for review, see Ref. 13). In the current study, we also observed decreases in stomatal conductance for *A. smithii* if the leaf to air VPD increased with temperature (Fig. 3B). Stomatal responses of this type could significantly modify the temperature dependence of net photosynthesis for *A. smithii in situ*, as the leaf to air VPD often increases diurnally as a function of increasing leaf temperatures. The magnitude of this potential modification is observed by comparing the temperature response of photosynthesis for *A. smithii* in the current study, with that reported by Kemp and Williams (19). In the latter study, the VPD was allowed to increase over the entire experimental temperature range, resulting in marked reductions in net photosynthesis between 25 and 35°C with concomitant decreases in stomatal conductance. In the current study, the VPD was maintained at a relatively constant value, and only slight reductions in net photosynthesis occurred between 25 and 35°C with virtually no decrease in stomatal conductance.

From the results presented in Figure 2, it is evident that the reactions involving the O_2 inhibition of photosynthesis comprise a substantial proportion of the reversible high temperature inhibition of photosynthesis in normal air. Maximal O_2 inhibition occurred at 40°C, approximately 15°C higher than the temperature optimum for net photosynthesis. These results are consistent with several previous studies, in which maximal O_2 inhibition was observed to occur 10 to 15°C higher than the photosynthetic temperature optimum (15, 17, 27). In other studies, however, O_2 inhibition is reported to be greatest at the photosynthetic temperature optimum (16, 23). Temperature-induced limitations to the



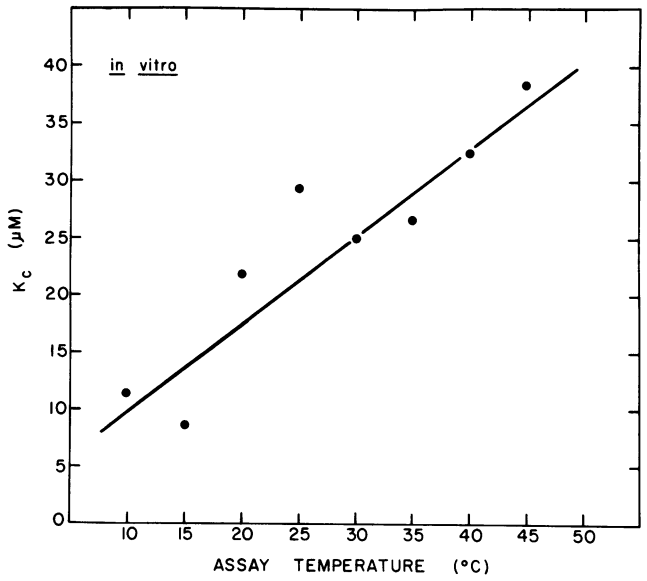


FIG. 7. The response of apparent K_c to temperature determined from *in vivo* and *in vitro* assays. Points represent the mean of three replicates.

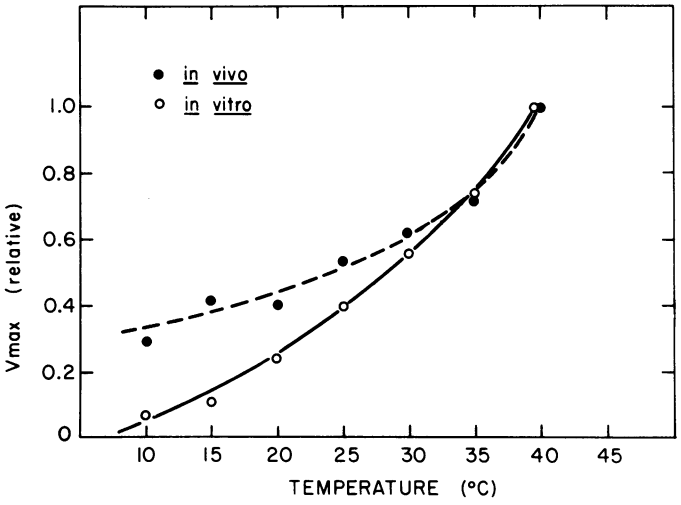


FIG. 8. The temperature dependence of the V_{max} for CO_2 fixation determined from *in vivo* and *in vitro* assays. The V_{max} values for 40°C were 24.5 and 11.9 nmol CO_2 cm^{-2} s^{-1} (2.58 and 1.25 μmol CO_2 mg^{-1} protein min^{-1}) for the *in vivo* and *in vitro* assays, respectively. Points represent the mean of three replicates.

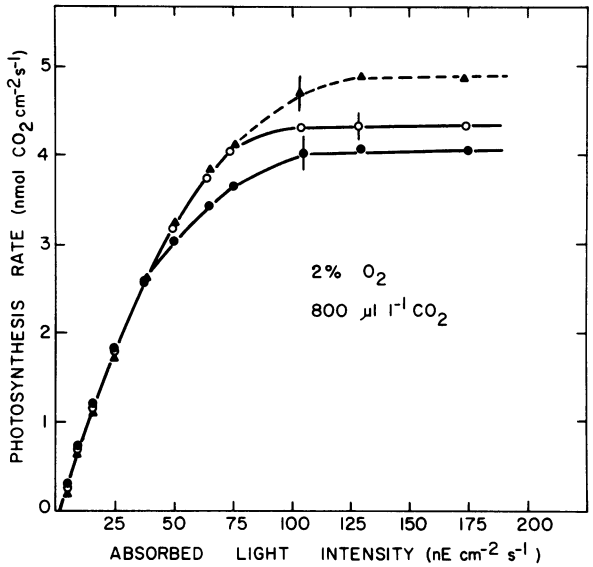


FIG. 9. The response of net photosynthesis to absorbed light intensity determined at 25°C (●); 30°C (○); and 35°C (▲). Points represent the mean of three to five replicates.

absolute amount of O_2 inhibition occurred above 40°C in the present study. Concomitant reductions in CO_2 uptake which are independent of O_2 inhibition (reflected in reduced photosynthesis rates above 40°C in 2% O_2; Fig. 1), may result in reductions in the rate of regeneration of RuBP. Since the oxygenase activity of RuBP carboxylase/oxygenase is dependent upon the steady-state concentration of RuBP, reduced rates of RuBP regeneration could contribute to reduced levels of O_2 inhibition. The percentage inhibition of net photosynthesis by 21% O_2 increased continuously over the entire experimental temperature range (Fig. 2). The range of the per cent inhibition expressed by *A. smithii* (20–65%) is similar to that reported for other C_3 species (8, 23).

Further limitations to net photosynthesis at above-optimal temperatures in *A. smithii* may have been due to the dependence of CO_2 solubility on temperature. The liquid phase solubility of CO_2 decreases markedly as leaf temperatures increase (20). In normal air, wherein the photosynthesis rates of C_3 plants are typically not CO_2 saturated, this decreased availability of substrate may impose significant restrictions on CO_2 uptake at high temperatures. Evidence of this effect in *A. smithii* was observed in the results presented in Figure 1. The temperature optimum for photosynthesis increased by approximately 5 to 7°C when the CO_2 concentration external to the leaf was increased from 340 to 800 μl l^{-1}.

There has been some debate in the literature as to whether the CO_2 response of intact leaf photosynthesis is characterized by Michaelis-Menten kinetics (14, 18, 21). Recent studies by Lilley and Walker (25) have shown that at saturating CO_2 concentrations, rates of CO_2 fixation in spinach chloroplasts are lower than rates in extracted RuBP carboxylase preparations. They suggested the presence of a photochemical limitation to the rate of RuBP regeneration. The absence of deviations from Michaelis-Menten kinetics for RuBP carboxylase assayed *in vitro* (Fig. 6) indicates that the catalytic capacity of RuBP carboxylase is not affected by high CO_2 concentrations. Ku and Edwards (21) have further demonstrated a deviation from Michaelis-Menten kinetics at sat-

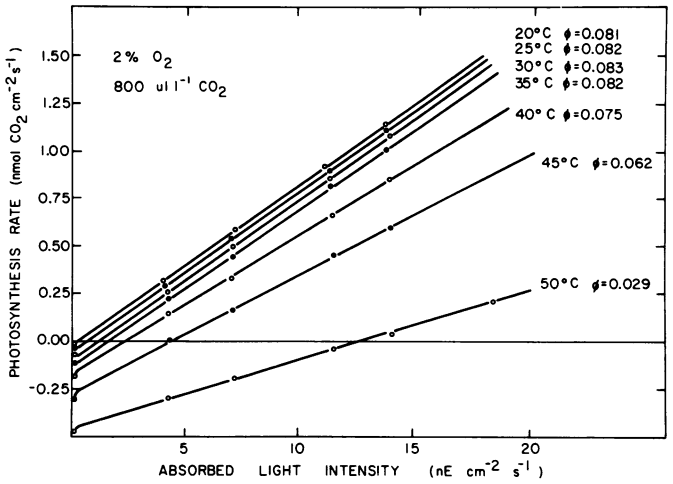


FIG. 10. The response of net photosynthesis to rate-limiting levels of absorbed light intensity at different leaf temperatures. Analysis temperatures are indicated in the same order as the response curves. The quantum yield for CO_2 uptake (ϕ) is also reported for each analysis temperature. Points represent data from one leaf which were typical of three replicates.

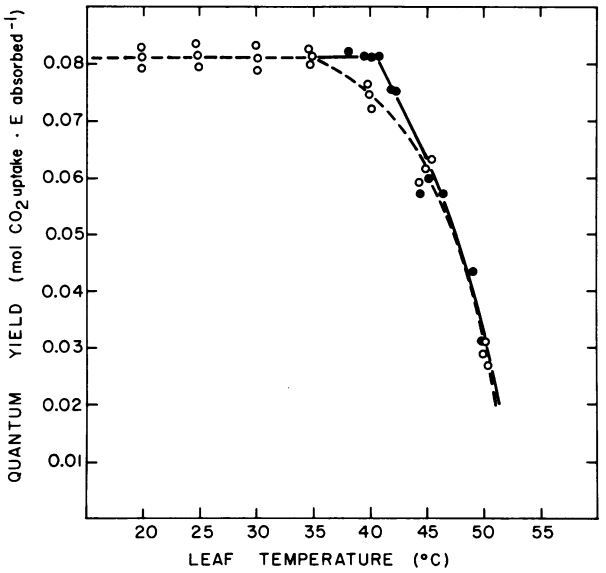


FIG. 11. The quantum yield of CO_2 uptake as a function of leaf temperature. The quantum yield was either determined at each temperature (○; data from Fig. 6) or at 20°C after a 30-min pretreatment at each temperature (●).

urating CO_2 concentrations in intact leaves of wheat. In the current study, we observed similar deviations at CO_2 concentrations greater than 8 μM *in vivo*, at all temperatures. The decreased magnitude of the deviations at 10°C, relative to 35°C, suggests that the photosynthetic (photochemical) limitations at high CO_2 concentrations are temperature dependent. Thus, the temperature dependence of CO_2 uptake in the presence of saturating CO_2 (Fig. 1), may be a reflection of the temperature dependence of the photochemical and/or biochemical limitations of the rate of RuBP regeneration.

It is reasonable to assume that the CO_2 dependence of intact leaf photosynthesis follows Michaelis-Menten kinetics at low CO_2 concentrations (measured under low O_2 to reduce photorespiration), as the concentration of RuBP would not be rate-limiting (21). In that case, the initial slope of the CO_2 response curve (intracellular conductance, C_i) would be equivalent to the ratio V_{max}/K_c. The latter relationship occurs because at very low CO_2 concentrations the contribution of substrate concentration to the denominator of the Michaelis-Menten equation

$$P = \frac{V_{max}[CO_2]}{K_c + [CO_2]} \quad (2)$$

becomes negligible, and equation 2 can be rewritten as:

$$P = \frac{V_{max}[CO_2]}{K_c} \quad (3)$$

Thus, by analyzing the linear response of net photosynthesis to rate-limiting CO_2 concentrations, we can assess the ratio V_{max}/K_c as the slope (C_i). In the current study, when V_{max} and K_c were calculated from only the linear portion of the CO_2 response curve, C_i and V_{max}/K_c did not deviate by more than 6% (data not shown). Ideally, the temperature response of C_i (V_{max}/K_c) should exhibit a Q_{10} of approximately 2 (reflecting the Q_{10} of RuBP carboxylase), if there were no temperature induced changes in K_c, and CO_2 uptake at low CO_2 concentrations is limited by RuBP carboxylase. The fact that C_i does not exhibit a Q_{10} of 2 (Fig. 4) suggests that temperature-dependent changes in K_c and/or V_{max} are occurring *in vivo*. Evidence of temperature-dependent changes in the V_{max} of RuBP carboxylase *in vivo* is provided in Figure 8. The decreased Q_{10} of V_{max} *in vivo*, relative to *in vitro*, may be due to temperature-dependent changes in the chloroplast stromal environment, such that the V_{max} of RuBP carboxylase becomes increasingly limited as temperature increases. In a recent study, Weis (35) suggested that a progressive, reversible inactivation of RuBP carboxylase occurs at above-optimum temperatures in intact spinach chloroplasts. Further studies are needed to characterize the biochemical environment of the chloroplast as a function of temperature, and thus, establish the precise relationship between the low Q_{10} of RuBP carboxylase *in vivo* (Fig. 8) and the low Q_{10} of intact leaf photosynthesis between 20 and 40°C (Fig. 1).

Increases in the apparent K_c for RuBP carboxylase as temperature increases may also have a role in intact leaf photosynthetic limitations at above-optimum temperatures (Fig. 7). In the current study, the temperature dependence of the apparent K_c was less than previously reported (2, 24); however, increases in the apparent K_m (CO_2) with increased temperature were observed in those studies. Values for the apparent K_c were similar in both the *in vivo* and *in vitro* assays (Fig. 7). These values are similar to the range of apparent K_m (CO_2) which have recently been determined for a number of C_3 grasses (13–26 μM) (37). In the current study, we have simplified the kinetics of the CO_2 response curve *in vivo* by assuming that the physical transfer component of CO_2 diffusion from the cell wall to the site of carboxylation, is a minor component relative to the biochemical conductance of the carboxylation step. The validity of this assumption is supported by the similar values for apparent K_c from both the *in vitro* and *in vivo* assays. Indeed, in those studies which have assumed the transfer component to be a major limitation to CO_2 uptake, the apparent K_c has been determined as an unreasonably low value (18, 33). The presence of deviations from Michaelis-Menten kinetics *in vivo* required us to estimate the kinetic constants for intact leaves at CO_2 concentrations below the apparent K_c. At substrate concentrations below K_c, it is more difficult to determine kinetic constants. This problem was especially obvious at high leaf temperatures. However, the similarities among the values of K_c *in vivo* and *in vitro* for several experimental leaves (Fig. 7) suggest that valid estimates of kinetic constants for intact leaves can be derived.

The V_{max} of RuBP carboxylase measured *in vitro* at various temperatures is greater than the CO_2-saturated rate of photosynthesis in intact leaves. However, the *in vitro* V_{max} of the enzyme is lower than expected when compared with theoretical *in vivo* V_{max} (Fig. 8). While it is probably difficult to obtain maximum activation and specific activity with RuBP carboxylase *in vitro*, the *in*

vitro K_c of the enzyme in the present study may be similar to that *in vivo* (Fig. 7). A major assumption we have made in the current study is that although the *in vitro* assay environment may have reduced the absolute value for V_{max} of RuBP carboxylase, it has not affected the relative response to assay temperature. The complexity of RuBP carboxylase only allows tentative conclusions and further studies are required to evaluate how temperature influences activation and catalysis *in vivo*.

Net photosynthesis in *A. smithii* was saturated with respect to light intensity at approximately 70% of full sunlight, when measured in the presence of saturating CO_2 (Fig. 9). At leaf temperatures above 35°C, decreases in photosynthetic capacity were partially dependent on temperature-induced limitations to the light-dependent reactions of photosynthesis (Fig. 10). Decreases in the absolute magnitude of net CO_2 uptake at each of these limiting light intensities as leaf temperatures increased were likely due to the presence of a progressively larger amount of mitochondrial respiration contributing to CO_2 efflux from the leaves (12). The temperature-dependent reductions in quantum yield were irreversible above 41°C (Fig. 11). These results are consistent with previous observations of the temperature (40–49°C) at which irreversible reductions of the quantum yield for CO_2 uptake, and/or large increases in temperature-dependent Chl fluorescence, occur for a number of C_3 and C_4 species (7, 29, 30). It has been suggested that these reductions in efficiency are primarily due to heat damage to the thylakoid membranes (1, 29). In order to further characterize high temperature damage to the photosynthetic apparatus in *A. smithii* at the subcellular level of organization, a separate series of studies with isolated chloroplast grana have been conducted. The results from these studies are reported in an accompanying report (32).

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