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Influence of Temperature and O_2 Concentration on Photosynthesis and Light Activation of Ribulosebisphosphate Carboxylase Oxygenase in Intact Leaves of White Clover (*Trifolium repens* L.)

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ABSTRACT

Detached leaves of white clover (*Trifolium repens* L.) were kept for 1 h under various conditions of temperature, oxygen concentration and light intensity. Rates of photosynthesis were measured where appropriate and then ribulosebisphosphate carboxylase oxygenase (RuBPCO) was extracted rapidly and its initial activity measured immediately.

The extracted activity increased with increased intensity of illumination of the leaves. Where leaves were pretreated at low light intensity, the lower the temperature of the leaves the higher the extracted activity of RuBPCO. At high light intensity temperature did not affect the activity of subsequently extracted RuBPCO but the light intensity which was necessary for maximum activity increased with temperature. Activity of RuBPCO from leaves pretreated in the dark was least when CO_2 was low and temperature high. Leaves, pretreated at low temperatures and high light intensity in 20% O_2, yielded higher activity in extracts than leaves pretreated under similar conditions but in 2% O_2. A relatively weak temperature response of photosynthesis at low irradiances was associated with a decrease in extractable RuBPCO activity with increasing temperature. A strong temperature dependence of the oxygen inhibition of photosynthesis was associated with lower extractable RuBPCO activity in leaves pretreated at low oxygen concentration at low temperatures. With leaves from plants grown at low temperatures prior to treatment of leaves, oxygen inhibition of photosynthesis was less temperature dependent and activity of RuBPCO in extracts was not decreased by low O_2 at low temperatures. Differences in the activation of RuBPCO appear to influence photosynthesis and account for an absence of oxygen inhibition of photosynthesis at low temperatures in plants grown in warm conditions.

Key words: Ribulosebisphosphate carboxylase oxygenase activation; Photosynthesis; Temperature; O_2 effect; White clover.

INTRODUCTION

Ribulosebisphosphate carboxylase oxygenase (RuBPCO) catalyses the fixation of CO_2 in the photosynthetic pentose-phosphate cycle and of O_2 in the photorespiratory glycolate pathway in C_3 plants. The catalytic properties of RuBPCO influence the response of photosynthesis and photorespiration to temperature and O_2 concentration in soybean leaves (Laing, Ogren, and Hageman, 1974) and cells (Servaites and Ogren, 1978). Thus, Berry and Farquhar (1978) based their model of C_3 photosynthesis on the catalytic properties of RuBPCO.

However, some discrepancies have been found between measured photosynthesis and rates of photosynthesis calculated on the basis of the catalytic properties of RuBPCO. Thus, the temperature responses of photosynthesis in *Agropyron smithii* Rydb. (Monson, Stidham, Williams, Edwards, and Uribe, 1982), and *Nerium oleander* L. (Berry and Björkman, 1980) are weaker and the temperature response of oxygen inhibition of photosynthesis in wheat leaf segments is stronger than would be expected from calculations (Arrabaca, Keys, and Whittingham, 1981).

The present study investigates the possibility that changes in enzyme activity are, to some extent, responsible for these discrepancies. Activation of purified RuBPCO involves the ordered binding of activating CO_2 to the enzyme in a rate determining step followed by the rapid addition of Mg^{++} to form the activated ternary complex at increased pH (Lorimer, Badger, and Andrews, 1976). A number of chloroplast metabolites are known to influence the activation equilibrium and to inhibit competitively the catalytic reaction of RuBP (Hatch and Jensen, 1980; Curry, Pierce, Tolbert, and Orme-Johnson, 1981). Badger and Lorimer (1981) showed that such effectors of activation interact at the RuBP binding site and proposed the following model for effector-mediated activation of RuBPCO:

$$\begin{array}{ccccc} E... & \rightleftharpoons & E.C.. & \rightleftharpoons & E.C.M. \longrightarrow \text{catalysis} \\ \uparrow\downarrow & & \uparrow\downarrow & & \uparrow\downarrow \\ E...F & \rightleftharpoons & E.C..F & \rightleftharpoons & E.C.M.F \end{array}$$

(E = enzyme, C = activating CO_2, M = Mg^{++}, F = effector)

The activation state of RuBPCO in intact leaves can be estimated by measuring the initial activity of fresh extracts made at around 0 °C in CO_2-free buffer (Mächler and Nösberger, 1980). This measurement indicates the amount of potentially active enzyme sites (all E.C.. forms of the enzyme: E.C.., E.C.M., E.C..F_r, E.C.M.F_r, where F_r means effectors which dissociate rapidly from the enzyme).

The activation state of RuBPCO *in vivo* is low in the dark and increases as light intensity is increased (Bahr and Jensen, 1978; Mächler and Nösberger, 1980; Perchorowicz, Raynes, and Jensen, 1981). Chloroplasts, when illuminated, show an increase in pH and Mg^{++} concentration in the stroma (Werdan, Heldt, and Milovancev, 1975; Portis and Heldt, 1976). This change in stromal conditions may be the cause of light activation of RuBPCO since the affinity of the enzyme for activating CO_2 increases as pH and Mg^{++} concentration are increased. The present study reports the influence of temperature and oxygen concentration on the kinetics of light activation in intact leaves of white clover and relates the results to photosynthesis.

MATERIALS AND METHODS

Plant material

White clover plants (*Trifolium repens* L. ecotype Chur) were propagated vegetatively and grown in pots (15 cm diameter) filled with perlite. The pots were placed in growth chambers (PGV-36, Conviron) with day/night temperatures of either 20/16 °C or 10/7 °C. The photoperiod was 16 h with light being provided by a bank of fluorescent tubes and incandescent bulbs giving an irradiance of 380–400 μmol m^{-2} s^{-1} (400–700 nm) at plant height. Plants were irrigated daily with nutrient solution (Hammer, Tibbits, Langhans, and McFarlane, 1978). The leaves used in the experiments had reached full expansion 6–12 d before sampling.

Gas exchange

CO_2 exchange was measured with an open infrared gas analysis system. Two leaves were detached during the photoperiod and enclosed in a temperature controlled cuvette of copper and glass. In some experiments, leaf temperature was measured with a thermocouple attached to the lower leaf surface.

Leaf boundary layer resistance was minimized by the use of a small fan placed in the cuvette. The air stream needed for gas exchange measurements was produced by mixing gas from cylinders containing pure O_2, N_2 or CO_2 using Wösthoff mixing pumps. The light source was a 400 W sodium vapour lamp, separated from the leaves by a 8·0 cm water layer. Light intensity was varied by changing the distance between light source and cuvette.

Initial activity of RuBPCO

Leaves were pre-adapted in the gas exchange cuvette for 60 min. At this time a constant activation state of RuBPCO was attained. RuBPCO was extracted rapidly at 2 °C in a CO_2-free buffer with 20 mM $MgCl_2$ and its initial activity measured as described earlier (Mächler and Nösberger, 1980). The temperature during assay was 10 °C (Figs 1–4) or 17·5 °C (Fig. 5).

Additional experiments were performed to examine the influence of pretreating the leaves under various conditions for 60 min on the amount of RuBPCO that can be activated in the presence of 10 mM $NaHCO_3$ and 20 mM $MgCl_2$. Leaves were ground rapidly in an ice cold mortar in the extraction buffer to which $NaHCO_3$ had been added to give a final concentration of 10 mM. 2·0 ml of the homogenate were centrifuged for 10 min at 40 000 × *g* at 2 °C. The supernatant liquid was then heated at 30 °C for 5 min and assayed at 20 °C. RuBPCO activities were expressed as a percentage based on the activity of control leaves which had not been pretreated before extraction of RuBPCO.

RESULTS AND DISCUSSION

Influence of temperature and CO_2 concentration on light activation of RuBPCO

Initial activity tests of RuBPCO, rapidly extracted in CO_2-free buffer, are assumed to give an estimate of the activation state of RuBPCO *in vivo*, although inactive E.C.. complexes (E.C.., E.C..F_r, and E.C.M.F_r) also appear as active enzyme in the assay and, thus, may cause overestimation. Differences in initial activity were not due to enzyme synthesis or degradation during the pretreatments of the leaves. Pretreatments of leaves for 60 min had no significant effect on RuBPCO activity when extraction occurred in the presence of 10 mM $NaHCO_3$ and 20 mm $MgCl_2$. RuBPCO in extracts from leaves which had been pre-incubated at different temperatures rapidly reached a similar activity when $NaHCO_3$ was added (Fig. 1). Similar results had been obtained for RuBPCO rapidly extracted from wheat leaves which had been pre-incubated at different CO_2 levels (Mächler and Nösberger, 1980). The relatively low activities in extracts with 10 mM $NaHCO_3$ may be attributed to the inhibitory effect of HCO_3^- on enzyme activation (Mächler and Nösberger, 1980).

Initial activity of RuBPCO, rapidly extracted from leaves, was increased as intensity of prior illumination of the leaves was increased (Fig. 2). The amount of activity extracted following exposure to bright light was relatively independent of temperature during illumination. However, light saturation was attained at a lower light intensity when temperature was decreased. Thus, the decreased activation state of RuBPCO with increasing temperature that was observed earlier (Mächler, 1981), is attributed to non-saturating light. The decreased requirement for light with decreased temperature may be due to a change in the properties of the enzyme or to a change in the chemical environment of RuBPCO in the chloroplast stroma; Mg^{++} concentration, pH or level of effectors may change with temperature and affect the amount of E.C..– complexes (E.C.., E.C.M., E.C..F_r and E.C.M.F_r). The decrease in enzyme activity with increasing temperature does not seem to be due to decreased solubility of CO_2. Increasing CO_2 concentrations inhibit the light activation of RuBPCO (Mächler, 1981). The highest initial activities were observed when the light intensity was high and when the CO_2 concentration was low, indicating a high affinity of the enzyme for the activating CO_2 in the light (Table 1).

The activation state of RuBPCO was also affected by temperature and CO_2 concentration during pre-incubation in the dark: RuBPCO was almost completely inactivated when leaves of white clover were incubated in the dark at 20 °C and in the absence of CO_2 (Table 1).

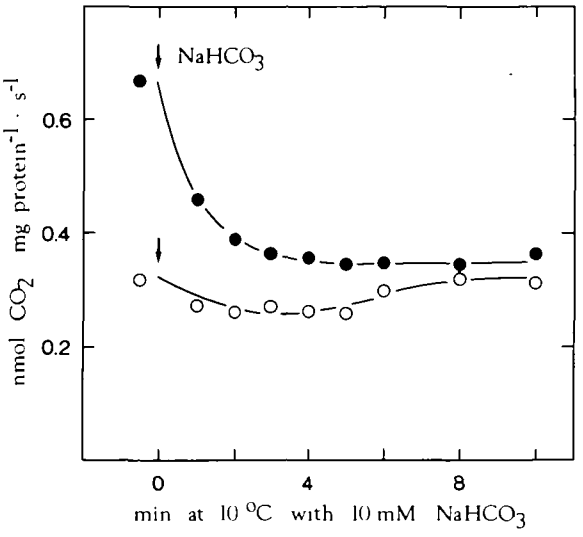

FIG. 1. Influence of $NaHCO_3$ on the activity of RuBPCO rapidly extracted from leaves of white clover which had been exposed previously to high and low temperatures for 60 min. Leaves from plants grown at 20/16 °C were exposed to an atmosphere with 310 cm^3 m^{-3} CO_2 and 20% O_2 at a photon flux density of 320 μmol m^{-2} s^{-1} at 25 °C (○) or 5 °C (●) for 60 min. Initial activity of RuBPCO in extracts was measured at 10 °C. Thereafter, $NaHCO_3$ was added to a concentration of 10 mM and extracts were transferred to a waterbath of 10 °C (↓). Initial activity of the enzyme was measured at 10 °C at intervals.

Initial activity after 60 min in the dark increased as temperature was decreased or CO_2 concentration was increased (Fig. 2 and Table 1). Lack of dark inactivation has been reported for spinach chloroplasts (Bahr and Jensen, 1978) and for protoplasts of wheat and barley (Robinson, McNeil, and Walker, 1979). In barley protoplasts, dark inactivation occurred when HCO_3^- concentration was low (Sicher, 1982).

At increased CO_2 concentrations, when initial activity in the dark was already high, low light intensities caused inactivation of the enzyme (Table 1). This light inhibition of RuBPCO at high CO_2 concentrations may be due to the inhibitory effect of HCO_3^- (Mächler and Nösberger, 1980) which increases in proportion due to the pH shift in the stroma when leaves are illuminated.

Response of photosynthesis and activity of RuBPCO to temperature in the presence of 2% and 20% O_2

The temperature response of photosynthesis was relatively weak in the presence of 20% O_2 at a photon flux density of 600 μmol m^{-2} s^{-1}, whereas there was a sharp increase in photosynthesis with increasing temperature in the presence of 2% O_2 (Fig. 3A). Temperature during pre-incubation of leaves influenced initial activity of rapidly extracted RuBPCO. In the presence of 20% O_2, RuBPCO activity decreased with increasing pre-incubation temperature; in the presence of 2% O_2, activity was less at low temperature than in the presence of 20% O_2 but increased with increasing temperature so that there was no difference in activity between leaves kept in 2% O_2 and 20% O_2 at 20 °C and 25 °C (Fig. 3B).

Activity of extracted RuBPCO and photosynthesis seem to be related: the decrease in enzyme activity with increasing temperature, in the presence of 20% O_2, was associated with a weak temperature response of photosynthesis. The decreasing effect of oxygen on activity

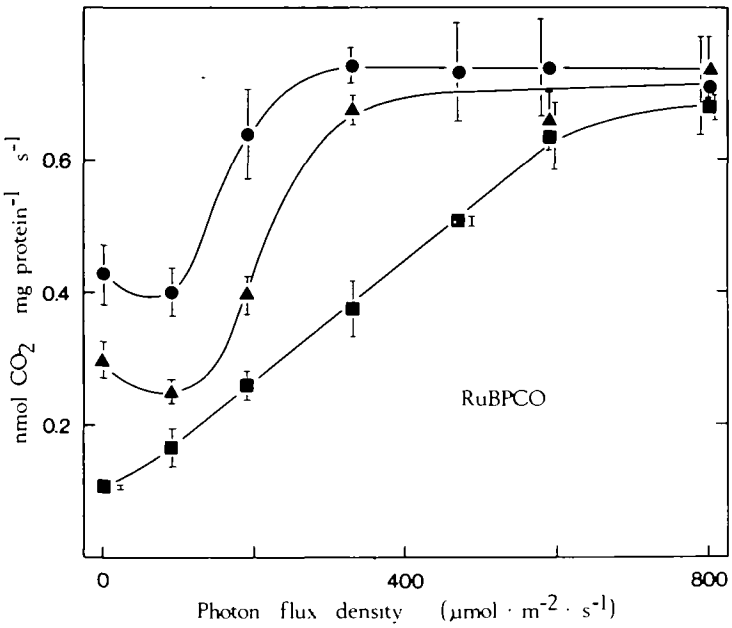

FIG. 2. Influence of temperature and light on the activity of RuBPCO in leaves. Leaves from plants grown at 20/16 °C were exposed for 60 min to an atmosphere with 20% O_2, 340 cm^3 m^{-3} CO_2, at temperatures of 7 °C (●) 16 °C (▲) or 25 °C (■) at various light intensities. Initial activity of RuBPCO in extracts was measured at 10 °C. Curves were fitted by eye. Vertical bars represent 2× standard errors of the mean.

TABLE 1. *The activity of RuBPCO extracted from leaves after periods in darkness or light with various concentrations of CO_2*

Leaves were kept for 60 min at 20 °C in the dark, or in low or high irradiance, and at either the CO_2 compensation point of photosynthesis or CO_2 concentrations as indicated. Thereafter RuBPCO was rapidly extracted and its initial activity tested at 10 °C. Values in parentheses ( )[a] show the activities obtained when RuBPCO from pretreated leaves was extracted in a buffer containing $NaHCO_3$ at a concentration of 10 mM and heated for 5 min at 30 °C. Values are expressed as the percentage activity as compared with untreated leaves.

CO_2 concentration (cm^3 m^{-3})	Light intensity (μmol m^{-2} s^{-1})		
	Dark	100	500
	Initial activity (nmol CO_2 mg^{-1} protein s^{-1})		
0/40/41·5	0·077 (92)[a]	0·392	0·867 (94)[a]
300	0·167	0·208	0·967
1000	0·242	0·217	0·783
5000	0·693 (96)[a]	0·285	0·433 (122)[a]

of RuBPCO with increasing temperature seems to be related to the strong temperature dependence of oxygen inhibition of photosynthesis (Figs 3A, B).

The catalytic properties of RuBPCO seem to be mainly responsible for the influence of temperature on oxygen inhibition of photosynthesis and photorespiration since the affinity of RuBPCO for CO_2 decreases as temperature is increased, more so than does the affinity for

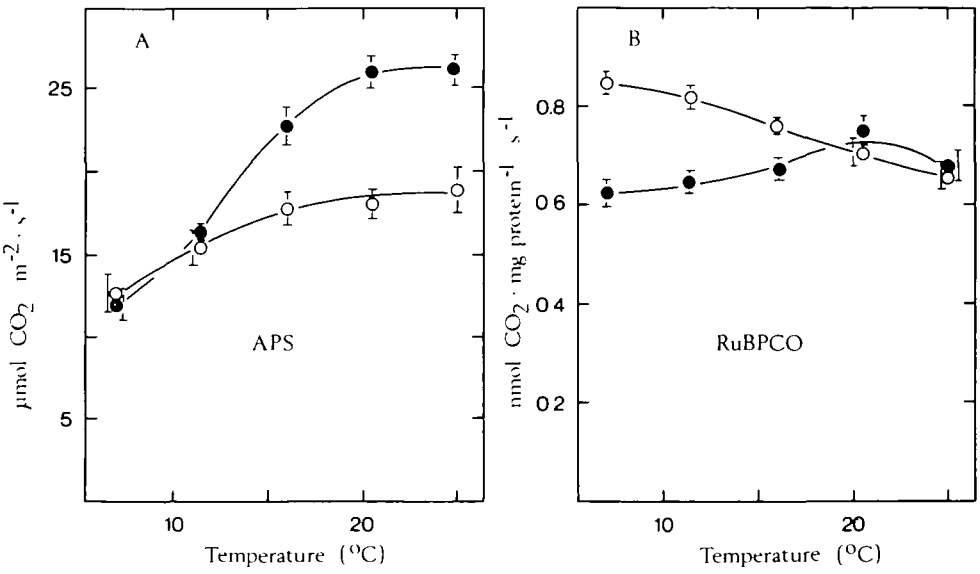

FIG. 3. Activity of RuBPCO and the rate of photosynthesis in leaves exposed to 2% O_2 and 20% O_2 at various temperatures. Leaves from plants grown at 20/16 °C were exposed for 60 min to an atmosphere with 2% O_2 (●) or 20% O_2 (○), 340 cm^3 m^{-3} CO_2, at a photon flux density of 600 μmol m^{-2} s^{-1} and at various temperatures. Apparent photosynthesis in the conditions of treatment (APS in A) was determined and thereafter RuBPCO rapidly extracted. Initial activity of the enzyme (B) was measured at 10 °C. Curves were fitted by eye. Vertical bars represent 2× standard errors of the mean.

O_2 (Laing *et al.*, 1974; Badger and Collatz, 1977). Moreover, the ratio of the solubilities of O_2 to CO_2 increases with temperature (Ku and Edwards, 1977*a*, *b*). However, temperature dependence of oxygen inhibition of photosynthesis in wheat leaf segments at normal or slightly increased CO_2 concentrations is stronger than would be expected from calculations based on the catalytic properties of RuBPCO (Arrabaca *et al.*, 1981). Oxygen enhancement of photosynthesis at low temperatures (Jolliffe and Tregunna, 1968; Mächler and Nösberger, 1978; Cornic and Louason, 1979) and at increased CO_2 concentrations (McVetty and Canvin, 1980) cannot be assigned to the kinetic properties of RuBPCO.

The present data suggest that the discrepancy between the temperature dependence of O_2 inhibition of photosynthesis and of CO_2 fixation rates, calculated from kinetic data of RuBPCO, can be explained, at least partly, by the temperature dependent effect of O_2 on the activity of RuBPCO.

The influence of 2% O_2 and 20% O_2 on activity of RuBPCO extractable from illuminated leaves in relation to the oxygen inhibition of photosynthesis at low temperature (9·5°C)

Initial activity of RuBPCO in leaves grown at 20/16 °C and pre-incubated for 60 min at a leaf temperature of 9·5 °C under high light intensity was lower when 2% O_2 instead of 20% O_2 was present during pre-incubation. On the other hand activities of extracts activated in the presence of 10 mM $NaHCO_3$ and 20 mM $MgCl_2$ did not differ significantly from each other (Fig. 4A—legend). Following exposure to 2% O_2, maximum activity was attained at a photon flux density of 200 μmol m^{-2} s^{-1} during exposure, whereas in the presence of 20% O_2, 350 μmol m^{-2} s^{-1} were needed to produce extracts with maximum activity of RuBPCO. Oxygen inhibition of photosynthesis at the same temperature was 20% at low light and declined when light intensity was increased from 150–200 μmol m^{-2} s^{-1} (Fig. 4B). This phenomenon is in

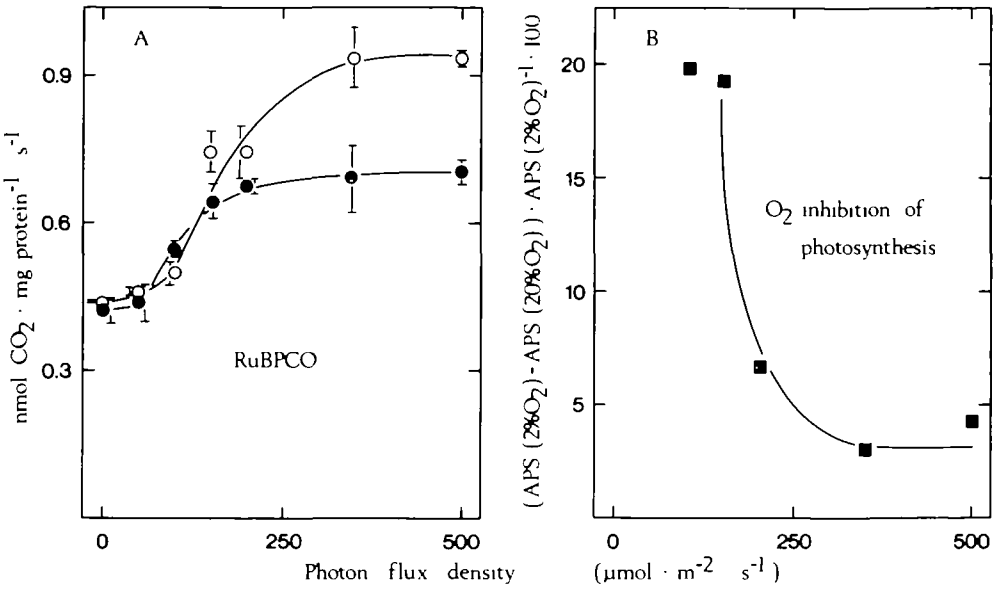

FIG. 4. Activity of RuBPCO and oxygen inhibition of photosynthesis in leaves exposed to 2% O_2 and 20% O_2 at low temperature and various light intensities. Leaves from plants grown at 20/16 °C were exposed for 60 min to an atmosphere with 310 cm^3 m^{-3} CO_2 and 2% O_2 (●) or 20% O_2 (○) at various light intensities. Leaf temperature was held constant at 9·5 °C. Apparent photosynthesis in the conditions of treatment (APS) was determined and thereafter RuBPCO rapidly extracted. Initial activity of the enzyme (A) was measured at 10 °C. Oxygen inhibition of photosynthesis (B) was calculated as follows: $|(APS(2\%\ O_2) - APS(20\%\ O_2))/APS(2\%\ O_2)| \times 100$. When RuBPCO was extracted in a buffer containing $NaHCO_3$ at a concentration of 10 mM, no significant difference was found between extracts of leaves pretreated in 2% O_2 or 20% O_2 for 60 min (activities were 113% and 117% respectively as compared with untreated leaves). Curves were fitted by eye. Vertical bars represent 2× standard errors of the mean.

contrast to the general conception of the Warburg effect, which is supposed to be relatively independent of light intensity (Jolliffe and Tregunna, 1968; Servaites and Ogren, 1978; Osmond and Björkman, 1972).

The decline in oxygen inhibition of photosynthesis (Fig. 4B) and the divergence of the activity at 2% O_2 and 20% O_2 (Fig. 4A) with increased illumination of leaves occurred at about the same light intensity and seem to be related.

Inactivation of RuBPCO, associated with photo-inhibition of photosynthesis at low temperature in 1% O_2 and in the absence of CO_2, was observed in detached wheat leaves (Boyle and Keys, 1982). In our experiments, oxygen inhibition of photosynthesis was decreased only when CO_2 concentration was ambient or high (see also Cornic and Louason, 1979), a condition which does not induce photo-inhibition.

Influence of growth temperature on oxygen dependence of photosynthesis and activity of RuBPCO in leaves

The effect of O_2 on photosynthesis and activity of RuBPCO was influenced by temperature during growth. White clover leaves, grown at 10/7 °C and measured at either 10 °C or 20 °C, showed a continuous decrease in photosynthesis as oxygen was increased from 2% to 60% O_2 (Fig. 5A). Activity of RuBPCO was not markedly affected by oxygen under the same conditions (Fig. 5B). Photosynthesis of leaves grown at 20/16 °C also showed a continuous

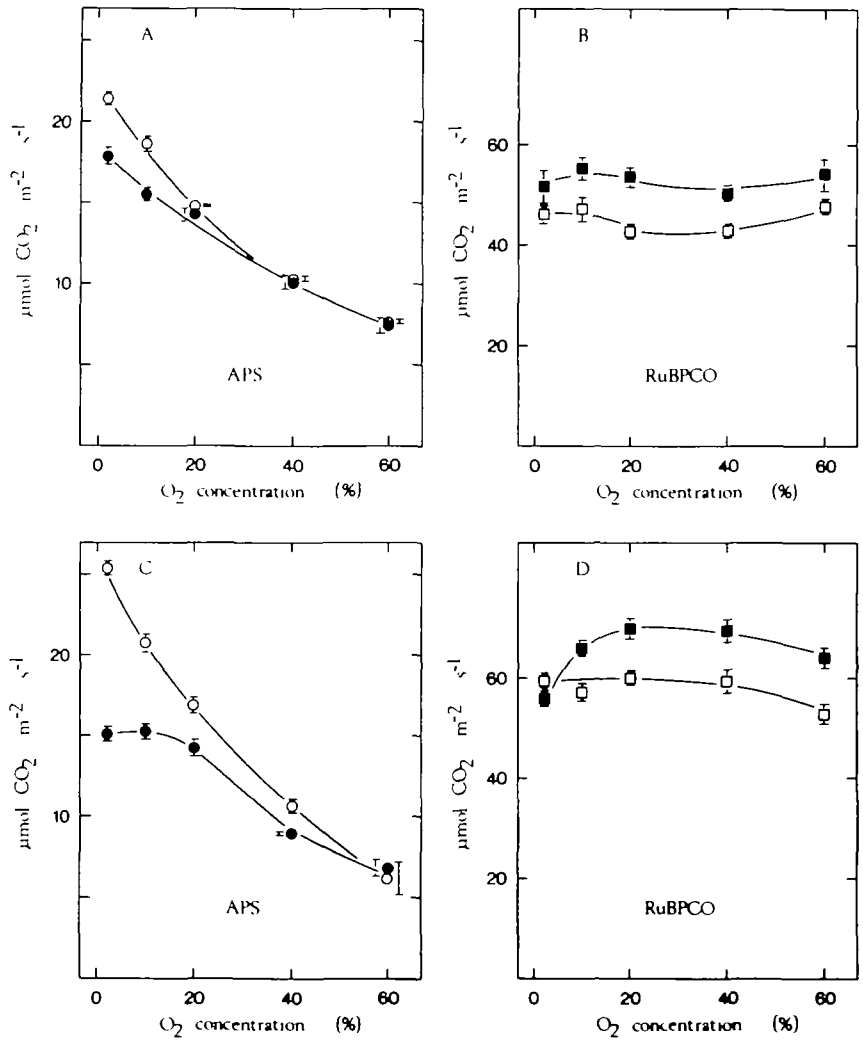

FIG. 5. Activity of RuBPCO and the influence of growth temperature on oxygen dependence of apparent photosynthesis. Leaves from plants grown at 10/7 °C (A and B) and 20/16 °C (C and D) were exposed for 60 min to atmospheres with 310 cm^3 m^{-3} CO_2 with various O_2 concentrations at 10 °C (●, ■) or 20 °C (○, □) at a photon flux density of 500 μmol m^{-2} s^{-1}. Apparent photosynthesis in the conditions of treatment (APS) was determined (A and C) before extracting RuBPCO. Initial activity (B and D) was measured at 17·5 °C. Vertical bars represent 2× standard errors of the mean.

decrease from 2% O_2 to 60% O_2 when measured at 20 °C but at 10 °C, photosynthesis did not decrease from 2% O_2 to 20% O_2 although it did decrease above 20% O_2 (Fig. 5C). Activity of RuBPCO increased from 2% O_2 to 20% O_2 at 10 °C in these leaves (Fig. 5D). The results suggest a relation between oxygen inhibition of photosynthesis and oxygen effect on activation state of RuBPCO. This has been discussed already.

Temperature during growth affects properties of RuBPCO (Huner and Macdowall, 1978). RuBPCO, purified from unhardened winter rye, was more susceptible to cold inactivation than RuBPCO from hardened winter rye. RuBPCO from unhardened plants had a lower

apparent affinity for CO_2 at low pH and low temperatures (Huner and Macdowall, 1978, 1979). It is possible that the difference in the effect of oxygen on warm and cold adapted plants, at low temperature, is related to these enzyme properties. On the other hand, differences in the properties of chloroplast membranes could affect the stromal conditions and also influence RuBPCO activation.

CONCLUSIONS

The RuBPCO reaction may limit photosynthesis mainly at rate saturating light intensities. Therefore, parameters describing the capacity of RuBPCO to fix CO_2 *in vivo* have been introduced into biochemical models of C_3 photosynthesis, namely the kinetic parameters of the enzyme (Berry and Farquhar, 1978) and a constant describing the total concentration of catalytic enzyme sites (Farquhar, von Caemmerer, and Berry, 1980). Calculated rates of photosynthesis are indeed close to measured rates when RuBPCO is fully activated. However, the model increasingly overestimates measured photosynthesis at higher temperatures (Berry and Björkman, 1980). This may be due, at least in part, to a reduction of the amount of active enzyme sites at higher temperatures (see also Weis, 1981). In addition, full activation is neither attained when CO_2 concentration is high nor when, at low temperatures, O_2 concentration is low. Under these conditions, the model is also expected to overestimate photosynthesis.

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