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Changes in Levels of Intermediates of the C_4 Cycle and Reductive Pentose Phosphate Pathway under Various Light Intensities in Maize Leaves[1]

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ABSTRACT

The rate of CO_2 assimilation and levels of metabolites of the C_4 cycle and reductive pentose phosphate pathway in attached leaves of maize (*Zea mays* L.) were measured over a range of light intensity from 0 to 1,900 microEinsteins per square meter per second under a saturated CO_2 concentration of 350 microliters per liter and a limiting CO_2 concentration of 133 microliters per liter. The level of ribulose 1,5-bisphosphate (RuBP) stayed almost constant (around 60 nanomoles per milligram chlorophyll [Chl]) from low to high light intensities under 350 microliters per liter. Levels of 3-phosphoglycerate (PGA) increased from 100 to 650 nanomoles per milligram Chl under 350 microliters per liter CO_2 with increasing light intensity. The calculated RuBP concentration of 6 millimolar (corresponded to 60 nanomoles per milligram Chl) was about two times above the estimated RuBP binding-site concentration on ribulose bisphosphate carboxylase-oxygenase (Rubisco) of ~2.6 millimolar in maize bundle sheath chloroplasts in the light. The ratio of RuBP/PGA increased with decreasing light intensity under 350 microliters per liter CO_2. These results suggest that RuBP carboxylation is under control of light intensity possibly due to a limited supply of CO_2 to Rubisco through the C_4 cycle whose activity is highly dependent on light intensity. Pyruvate level increased with increasing light intensity as long as photosynthesis rate increased. A positive relationship between levels of PGA and those of pyruvate during steady-state photosynthesis under various conditions suggests that an elevated concentration of PGA increases the carbon input into the C_4 cycle through the conversion of PGA to PEP and consequently the level of total intermediates of the C_4 cycle can be raised to mediate higher photosynthesis rate.

Recently, studies have been conducted on changes in photosynthetic metabolite levels during the induction period (9, 20, 28), and under steady-state photosynthesis with normal atmospheric conditions (19, 26) and with varying CO_2 concentration (31) in maize leaves. These studies have increased our understanding of C_4 photosynthesis and they provide a new approach to research in C_4 plant. In part they have shown that there are sufficient concentration gradients between the mesophyll and bundle sheath to facilitate intercellular transport of certain C_4 cycle intermediates by diffusion during C_4 photosynthesis (19, 26). The C_4 cycle and RPP[2] pathway are linked (7) and there is increasing evidence that complex interactions occur under different conditions in order to develop and maintain a balance in photosynthetic metabolites. The results of a transient peak of RuBP during the initial phase of induction in maize leaves led us to conclude that there is a non-autocatalytic buildup of RuBP well before CO_2 assimilation reaches a maximum (30, 32). This non-autocatalytic buildup of RuBP early in induction in maize leaves is suggested to be important in the initiation of C_4 photosynthesis (32). Further studies demonstrated a transient peak of PEP during the initial phase of induction in maize leaves, suggesting that there is also a limitation on PEP carboxylase during this period (9, 28). This phenomenon coincides well with recent findings of a light modulation of PEP carboxylase (5, 13). Modulation of PEP carboxylase by light has been initially reported in several C_4 species with a 2- to 3-fold increase following a dark to light transition (17, 18, 25). However, recent reports revealed that there were up to 30-fold differences in PEP carboxylase activity during dark to light transitions in maize when assayed in simulated conditions of dark or light or in the presence of effectors (5, 13). Studies of metabolite levels during induction in maize leaves (9, 20, 28) provided a basis for *in vitro* simulation of conditions *in vivo*. The total level of the C_4 cycle intermediates (malate, aspartate, pyruvate, PEP, and alanine) increased during induction (28) and also with increasing CO_2 concentration (31, 32) as the photosynthesis rate increased. These findings suggested that input of carbon into the C_4 cycle and an increase in the total level of the C_4 cycle intermediates is required to increase the photosynthetic rate in maize leaves (9, 28, 31, 32).

Although the influence of light intensity on metabolite levels in C_4 plants has not been reported, this is an important environmental factor controlling C_4 photosynthesis. Thus, the purpose of this study was to measure changes in photosynthetic metabolite levels in maize leaves under steady-state photosynthesis with varying light intensities.

MATERIALS AND METHODS

Plant Material. Seeds of *Zea mays* L. (variety Chuseishu-B) were obtained from Nihonsogyo, Tokyo, Japan. Plants were grown as described previously (27). The largest fully expanded leaves of plants 5 to 6 weeks old were used for the experiments.

Leaf Gas Exchange Measurements and Killing Procedure.

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[2] Abbreviations: RPP, reductive pentose phosphate; DHAP, dihydroxyacetonephosphate; FBP, fructose-1,6-bisphosphate; F6P, fructose 6-phosphate; G6P, glucose 6-phosphate; PEP, phosphoenolpyruvate; PGA, 3-phosphoglycerate; Rubisco, ribulose 1,5-bisphosphate carboxylase-oxygenase; RuBP, ribulose-1,5-bisphosphate.

Measurements were made on attached maize leaves using high speed freeze clamp equipment which was essentially the same as described by Badger *et al.* (2). The leaf temperature was kept constant at 30.5 ± 1°C. After obtaining steady-state photosynthesis (20–50 min), a leaf was very rapidly punched out. The procedures used in this study were similar to those described elsewhere (31).

Measurements of Metabolites. Frozen samples were extracted with 3% $HClO_4$ and neutralized samples were used for the enzymatic determination of metabolites as previously described (28, 31). In determining the metabolite contents of leaf tissue, corrections were made based on the average recoveries (72–97%) previously reported (28). The values obtained for FBP are maximum estimates, since the assay used also measures half the sedoheptulose-bisP pool, due to the action of aldolase.

Chl Determination. Pheophytin was extracted from $HClO_4$ residues into 80% acetone. Pheophytin was measured by the method of Vernon (36) and converted to Chl units.

RESULTS AND DISCUSSION

The rate of photosynthesis and photosynthetic metabolite levels were measured under a saturated CO_2 concentration of 350 $\mu l \cdot L^{-1}$ and a limiting concentration of CO_2 of 133 $\mu l \cdot L^{-1}$ with increasing light intensity (Figs. 1 and 2). The photosynthetic CO_2 assimilation rate in maize leaves is saturated around an atmospheric CO_2 concentration of 350 $\mu l \cdot L^{-1}$ (31). Stomatal conductance is under control of light intensity (24). However, intercellular CO_2 concentration under low light intensity (50 $W \cdot m^{-2}$) was approximately the same as that found under high light intensity (620 $W \cdot m^{-2}$) in maize leaves (24). Thus, an atmospheric CO_2 concentration of 350 $\mu l \cdot L^{-1}$ is saturating concentration of CO_2 under low light intensity in maize leaves. Photosynthetic CO_2 assimilation rates increased up to light intensities of 1900 and 300 $\mu E \cdot m^{-2}\ s^{-1}$ under CO_2 concentration of 350 $\mu l \cdot L^{-1}$ and 133 $\mu l \cdot L^{-1}$, respectively (Figs. 1 and 2). The maximum photosynthetic CO_2 assimilation rates under a saturated CO_2 concentration of 350 $\mu l \cdot L^{-1}$ and a limiting CO_2 concentration of 133 $\mu l \cdot L^{-1}$ were about 40 and about 17 $\mu mol\ CO_2 \cdot m^{-2}\ s^{-1}$, respectively. The initial slopes of the light response curves of photosynthesis under the two different concentrations of CO_2 were the same (Figs. 1 and 2). These results are consistent with earlier reports (1, 12). Under a concentration of 133 $\mu l \cdot L^{-1}$, the limiting supply of CO_2 limits photosynthesis above a light intensity of 300 $\mu E \cdot m^{-2}\ s^{-1}$.

Changes in the levels of RuBP in C_3 plants with increasing light intensity under atmospheric level of CO_2 have been studied extensively (2–4, 21–23). The levels of RuBP are about 35 to 70 $nmol \cdot mg^{-1}$ Chl under low light intensities in C_3 plants (5–15 $W \cdot m^{-2}$) (3, 23). The RuBP levels increase to 150 to 300 $nmol \cdot mg^{-1}$ Chl with increasing light intensity up to about 10% of full sunlight (200 $\mu E \cdot m^{-2}\ s^{-1}$ or 50 $W \cdot m^{-2}$) in C_3 plants (2–4, 21, 22). Above 10% of full sunlight the RuBP level stays almost constant or slightly decreases up to 1650 $\mu E \cdot m^{-2}\ s^{-1}$ or 350 $W \cdot m^{-2}$ (70–80% of full sunlight) as the photosynthesis rate increases in C_3 plants (3, 4, 21, 22). However, in the study by Badger *et al.* (2) the RuBP levels continued to increase up to more than 500 $nmol \cdot mg^{-1}$ Chl above a light intensity of 400 $\mu E \cdot m^{-2}\ s^{-1}$ as the photosynthesis rate increased in C_3 plants. The reason for the above discrepancy in the changes in RuBP levels in C_3 plants at higher light intensities is not clear at the moment. The RuBP binding-site concentrations on Rubisco in chloroplasts of C_3 plants are between 3.5 and 4.5 mM (15). Levels of RuBP of 35 to 70 $nmol \cdot mg^{-1}$ Chl under low light intensities corresponds to concentrations of 1.4 to 2.8 mM based on an assumed stromal volume of 25 $\mu l \cdot mg^{-1}$ Chl. Under low light intensity (5–15 $W \cdot m^{-2}$), the RuBP levels dropped below the concentration of RuBP binding-site on Rubisco in C_3 plants. Thus, in C_3 plants, under low light intensity the regeneration of RuBP may be the major limitation on photosynthetic carbon assimilation. On the other hand, above light intensities of 200 $\mu E \cdot m^{-2}\ s^{-1}$ or 50 $W \cdot m^{-2}$ (about 10% of full sunlight) the RuBP levels were above the concentration of RuBP binding-site on Rubisco. Thus, under these conditions not only the supply of ATP and NADPH but also the capacity of RuBP carboxylation was limiting. In contrast to C_3 plants, in the present study with the C_4 plant maize RuBP levels stayed almost constant around 60 $nmol \cdot mg^{-1}$ Chl with increasing light intensity from 50 $\mu E \cdot m^{-2}\ s^{-1}$ (where the photosynthesis rate was only 4% of the maximum rate) to 1900 $\mu E \cdot m^{-2}\ s^{-1}$ under 350 $\mu l \cdot l^{-1}$ CO_2 or to 300 $\mu E \cdot m^{-2}\ s^{-1}$ under 133 $\mu l \cdot l^{-1}$ CO_2 as long as the photosynthesis rate increased (Figs. 1 and 2). Under a limiting concentration of CO_2 of 133 $\mu l \cdot L^{-1}$, the RuBP level increased up to 150 $nmol \cdot mg^{-1}$ Chl above a light intensity of 300 $\mu E \cdot m^{-2}\ s^{-1}$ although the photosynthesis rate did not increase above this light intensity (Fig. 2). RuBP levels of 60 to 150 $nmol \cdot mg^{-1}$ Chl correspond to 6 to 15 mM RuBP in bundle sheath chloroplasts assuming that RuBP is retained in the bundle sheath chloroplasts and 40% of the Chl in maize leaves is in bundle sheath chloroplasts (16) with a stromal volume of 25 $\mu l \cdot mg^{-1}$ Chl. These RuBP levels of 6 to 15 mM are far above the calculated RuBP binding-site concentration of about 2.6 mM in maize bundle sheath chloroplasts (28). These results indicate that even under a low light intensity of 50 $\mu E \cdot m^{-2}\ s^{-1}$ (only 2.5% of full sunlight) regeneration of RuBP does not limit RuBP carboxylation in C_4 plant. J. Perchorowicz and R. G. Jensen (unpublished data) also measured RuBP level in maize seedlings with increasing light intensity and their results corresponded to RuBP of 2.7 mM, 8.7 mM, and 6.4 to 5.7 mM in maize bundle sheath chloroplasts at a light intensity of 50, 100, and 230 to 1500 $\mu E \cdot m^{-2} \cdot s^{-1}$, respectively (based on the same assumption mentioned above). Even though, there were small discrepancies between our results and theirs, but their results essentially support the suggestion mentioned above.

The level of PGA increased rapidly and then gradually with increasing light intensity under 350 $\mu l \cdot L^{-1}$ CO_2 (Fig. 1). Under 133 $\mu l \cdot L^{-1}$ CO_2 the PGA level increased up to 300 $\mu E \cdot m^{-2}\ s^{-1}$ and decreased slightly thereafter with increasing light intensity (Fig. 2). The levels of DHAP continued to increase from almost nil in the dark to 500 to 600 $nmol \cdot mg^{-1}$ Chl with increasing light intensity under CO_2 concentration of 350 $\mu l \cdot L^{-1}$ and 133 $\mu l \cdot L^{-1}$ (Figs. 1 and 2). The ratio of PGA/DHAP increased from about 1 (>1,300 $\mu E \cdot m^{-2}\ s^{-1}$) to approximately 3 (<200 $\mu E \cdot m^{-2}\ s^{-1}$) with decreasing light intensity under CO_2 concentration of 350 $\mu l \cdot L^{-1}$ (Fig. 1). This could be due to the limiting supply of NADPH and ATP under low light intensity.

The ratio of RuBP/PGA increased with decreasing light intensity below 200 $\mu E \cdot m^{-2}\ s^{-1}$ at 350 and 133 $\mu l \cdot L^{-1}$ CO_2 (Fig. 3a). These results also suggest that RuBP carboxylation is limited under low light intensity.RuBP is believed to be confined to bundle sheath chloroplasts in C_4 plants, while PGA is considered to exist in the cytoplasm and chloroplasts of both mesophyll and bundle sheath (19, 26). For a more complete assessment of the regulation of RuBP carboxylation from considering the ratio of RuBP/PGA in the bundle sheath chloroplasts, we would need to know the PGA level in the bundle sheath chloroplasts. In C_3 plant 90% of the PGA was inside the chloroplast as PGA^{3-} due to the pH gradient between stroma and cytoplasm (10) and in maize leaves 75% of the PGA was in the bundle sheath in the light (26). Thus, a large proportion of PGA may be in the bundle sheath chloroplasts in the light in maize leaves, and the differences in the ratio of RuBP/PGA in maize leaves under different light intensities may be regarded as reflecting the differences in the ratio of RuBP/PGA in the bundle sheath chloroplasts under these conditions. Under a limiting concentration of CO_2 with a high light intensity, it is reasonable that RuBP carboxylation but

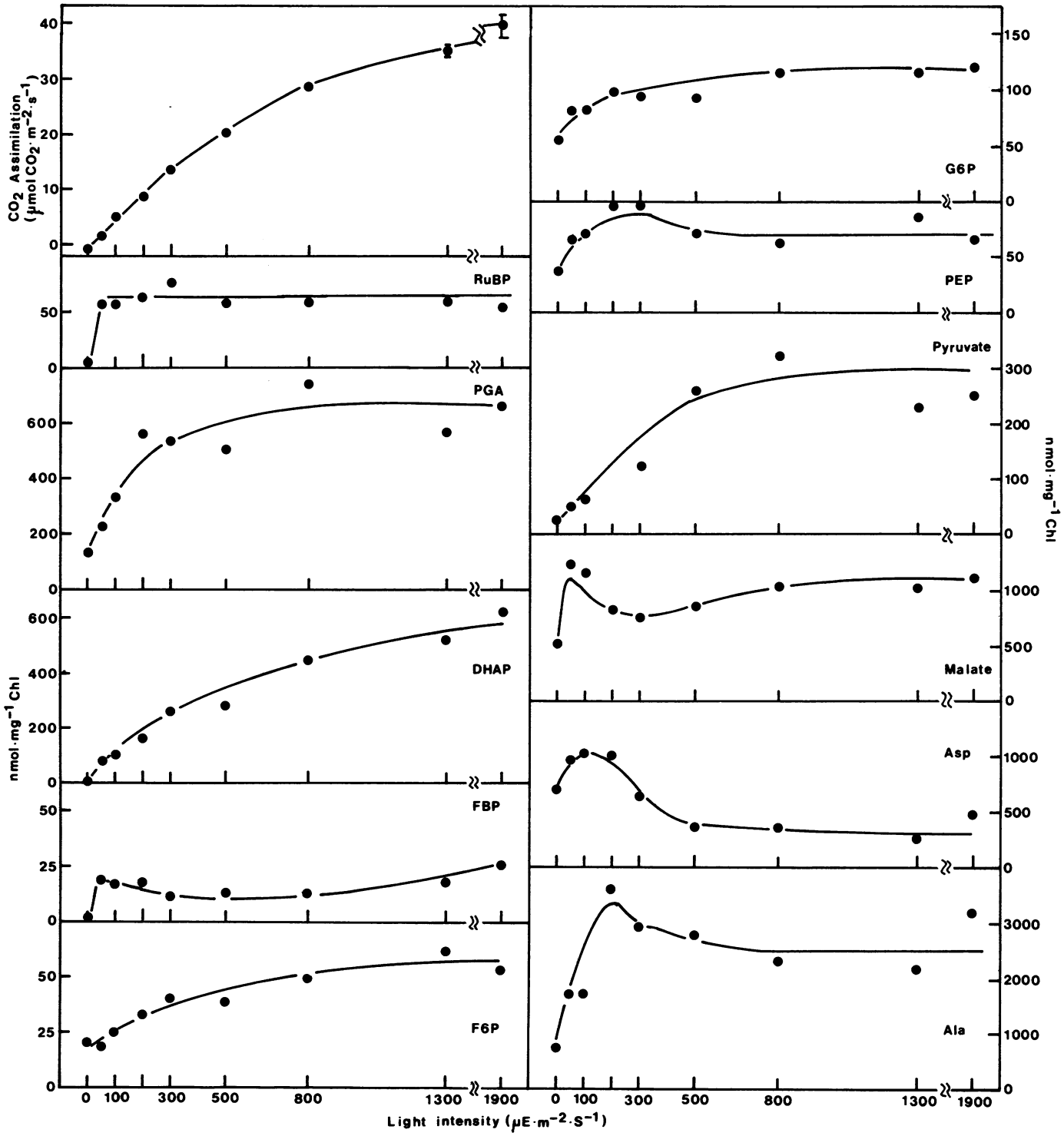

FIG. 1. Changes in the rate of CO_2 assimilation and levels of metabolites of the C_4 cycle and RPP pathway in an attached maize leaf as a function of light intensity under 350 $\mu l \cdot L^{-1}$ CO_2. Three separate measurements were made on the rate of CO_2 assimilation at each light intensity utilized. Three samples were taken at each light intensity and combined for metabolite assays. Mean values ± SE are shown for the rate of CO_2 assimilation.

not RuBP regeneration is limited in maize leaves due to a limiting supply of CO_2 (31). Under these conditions, the ratios of RuBP/PGA were extremely high (Fig. 3b) (data were taken from Usuda [31]). These facts indicate that a high RuBP/PGA ratio could be a good circumstantial evidence of limitation on RuBP carboxylation. It may further be inferred that the high ratio of RuBP/PGA under low light intensity (<200 $\mu E \cdot m^{-2}$ s^{-1}) is due to a limitation of RuBP carboxylation but not RuBP regeneration.

In C_4 photosynthesis the supply of CO_2 to Rubisco is mediated by decarboxylation of malate. The rate of CO_2 assimilation and malate synthesis, and consequently the rate of malate decarboxylation, is highly dependent on light intensity. Previously, we found that activities of Rubisco and pyruvate,Pi dikinase were similar to the maximum photosynthetic CO_2 assimilation rate with different C_4 species and in maize leaves of various ages (27, 34). We also showed that maximum activities of pyruvate,Pi

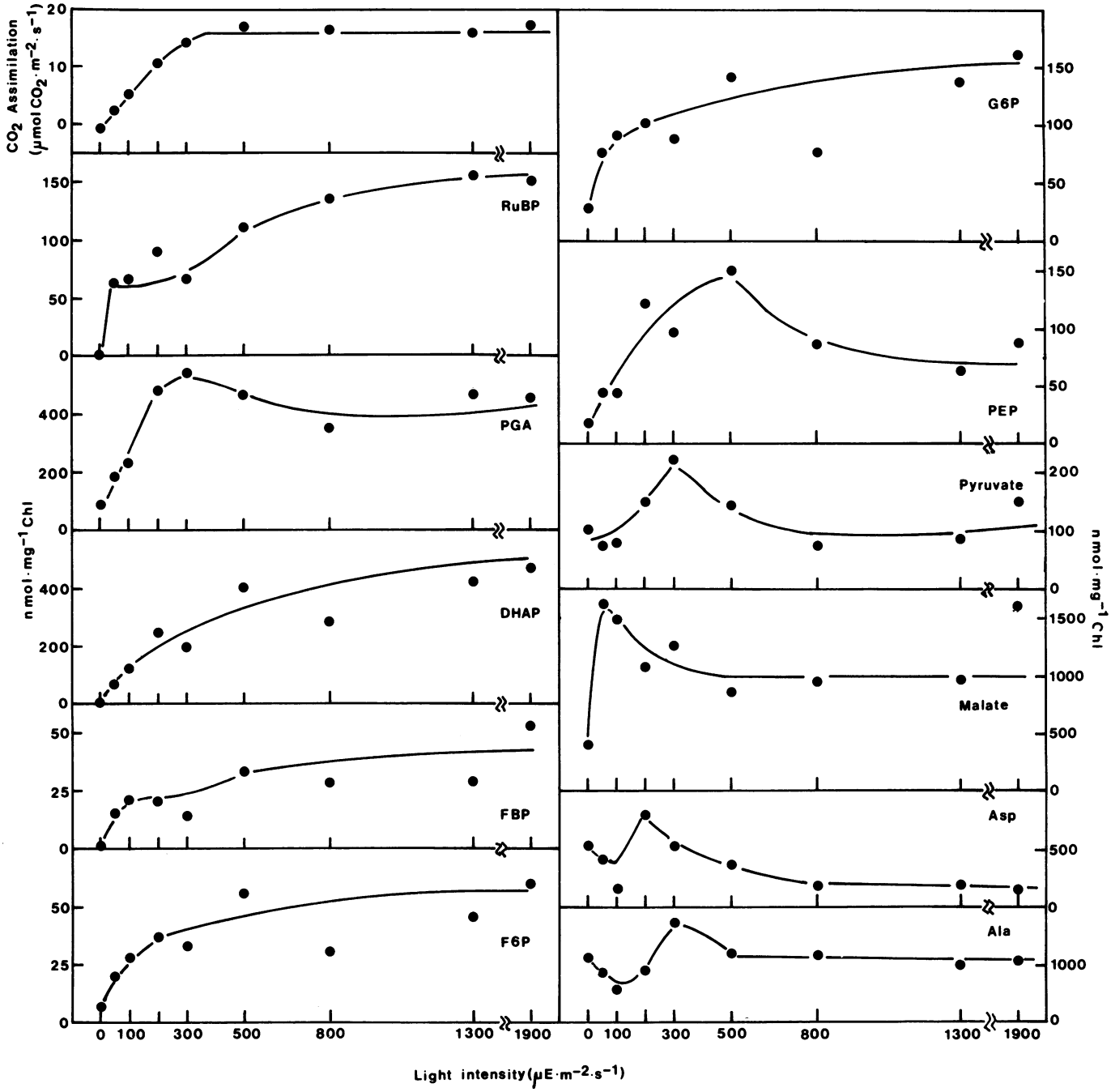

FIG. 2. Changes in the rate of CO_2 assimilation and levels of metabolites of the C_4 cycle and RPP pathway in an attached maize leaf as a function of light intensity under 133 $\mu l \cdot L^{-1}$ CO_2. Three separate samples were taken as described in the legend of Figure 1.

dikinase were generally just sufficient to accommodate photosynthesis in maize leaf from low (160 $\mu E \cdot m^{-2}\ s^{-1}$) to high (1800 $\mu E \cdot m^{-2}\ s^{-1}$) light intensity (33, 35). The pyruvate level increased from 50 to 300 nmol·mg^{-1} Chl with increasing light intensity (Fig. 1). About 50% of pyruvate in maize leaves was in mesophyll cells under high light intensity (26). If we assume that 60% of the Chl in maize leaves is in mesophyll chloroplasts (16) with a stromal volume of 25 $\mu l \cdot L^{-1}$ and tentatively that 40 to 80% of pyruvate in mesophyll cells is in the mesophyll chloroplasts under high light intensity, then 300 nmol pyruvate·mg^{-1} Chl corresponded to 5 to 10 mM of pyruvate in mesophyll chloroplasts which is far above the K_m for pyruvate of pyruvate,Pi dikinase (0.25 mM) (6). Thus, the level of pyruvate does not limit the activity of pyruvate,Pi dikinase under high light intensity. Although we have to know the pyruvate level in the mesophyll chloroplasts especially under low light intensity in order to assess whether pyruvate level limits the activity of pyruvate,Pi dikinase or not, the results mentioned above lead to the following conclusions: (a) Under saturating CO_2 and saturating light intensity, activities of pyruvate,Pi dikinase and Rubisco could play major roles in determining the maximum photosynthesis rate. (b) Under moderate to high light intensities (200 to 1900 $\mu E \cdot m^{-2} s^{-1}$) the C_4 cycle (primarily pyruvate,Pi dikinase activity which is highly dependent on the light intensity [33, 35]) may limit the photosynthesis rate. Consequently the rate of CO_2 supply to Rubisco could be dependent on light intensity. (c) Under low light intensities (<200 $\mu E \cdot m^{-2}\ s^{-1}$) the rate of CO_2 supply to Rubisco through the C_4 cycle and/or activity of Rubisco is

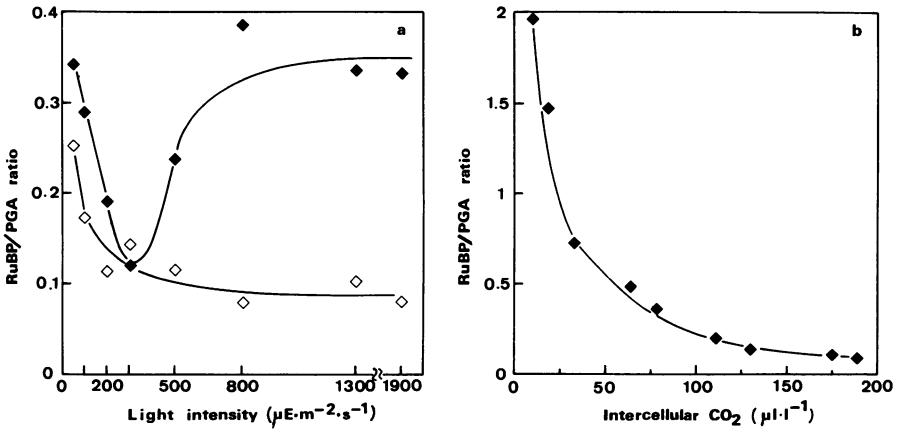

FIG. 3. Ratios of RuBP/PGA in an attached maize leaf under steady-state photosynthesis under various conditions. Data were taken from Figure 1 (◇) and Figure 2 (◆) in a panel of (a) and previous results (31) (◆) in a panel of (b). For further details, see the legend of Figure 1.

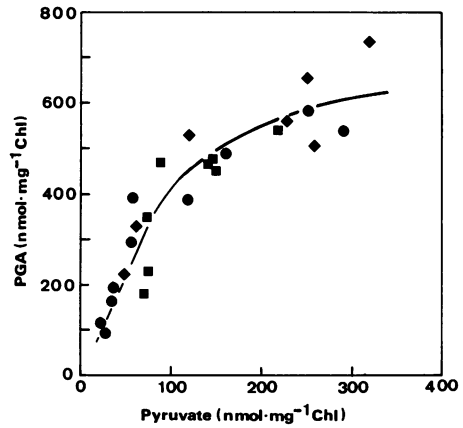

FIG. 4. Relationship between the levels of PGA and those of pyruvate in an attached maize leaf under various conditions. Data were taken from Figure 1 (under a constant CO_2 concentration of 350 $\mu l \cdot L^{-1}$ with increasing light intensity, ♦), from Figure 2 (under a constant CO_2 concentration of 133 $\mu l \cdot L^{-1}$ with increasing light intensity, ■), and from the previous results of (31) (under a constant light intensity of 1300 $\mu E \cdot m^{-2} s^{-1}$ with increasing intercellular CO_2 concentration, ●).

severely reduced relative to RuBP regeneration resulting in high RuBP/PGA ratio. Recently, Furbank and Hatch (8) found that leaf CO_2 (total inorganic carbon) pool in *Urochloa panicoides* (PEP carboxykinase type) decreased almost linearly together with photosynthesis rate with decreasing light intensity. Thus, under low light intensity CO_2 supply to Rubisco seems to be reduced, however, the actual CO_2 concentration in maize bundle sheath chloroplasts under low light intensity is not clear at the moment. If CO_2 supply is reduced and its reduced CO_2 concentration is limiting RuBP carboxylation under low light intensity, then some basis is needed to explain the low apparent photorespiration in C_4 plant at low light intensity. (In C_4 plant the CO_2 compensation point is low throughout all light intensities above the light compensation point [14] and there is no Kok effect [14].) If CO_2 supply is reduced but still its reduced CO_2 concentration is above the saturating concentration of CO_2 for Rubisco under low light intensity, then there are enough CO_2 and RuBP for maximum activity of Rubisco. But actually the rate of carboxylation was quite low in a steady state photosynthesis under low light intensity. Previously we found that more than 70% of Rubisco was detected as an active form from darkened maize leaves without activation treatment (29). There may be additional factors controlling Rubisco activity other than the activation state of Rubisco under low light intensity, if CO_2 supply is enough for Rubisco under these conditions.

With increasing light intensity (>300 $\mu E \cdot m^{-2} s^{-1}$) at 133 $\mu l \cdot L^{-1}$ CO_2, the ratio of RuBP/PGA increased (Fig. 3a). This could be also due to a limitation of CO_2 supply to Rubisco relative to regeneration of RuBP under these conditions.

Previously, we found in maize leaves that the level of total intermediates in the C_4 cycle (malate, aspartate, pyruvate, PEP, and alanine) increased during the latter stage of the photosynthetic induction period (28) and also under steady-state photosynthesis with increasing intercellular CO_2 concentration (31). We suggested that there was carbon input into the C_4 cycle from the RPP pathway to increase the level of the C_4 cycle intermediates which is required to enhance the photosynthesis rate. Furbank and Leegood also suggested that there was a connection between the C_4 cycle and the RPP pathway in maize leaves, possibly through the conversion of PGA to PEP during photosynthetic induction (9). With increasing light intensity the total level of the C_4 cycle intermediates increased under 350 $\mu l \cdot L^{-1}$ CO_2 but fluctuated under 133 $\mu l \cdot L^{-1}$ CO_2 (Figs.1 and 2). This inconsistency could be due to a photosynthetically inactive pool of malate (11), aspartate, and alanine which could mask real changes in levels of the photosynthetically active pools of these compounds. In contrast to these metabolites, the level of pyruvate may reflect the total level of photosynthetically active intermediates of the C_4 cycle under steady-state photosynthesis, because a large proportion of pyruvate seemed to be photosynthetically active (28). Pyruvate levels increased with increasing light intensity (Figs. 1 and 2) and with increasing intercellular CO_2 concentration (31) as long as the photosynthesis rate increased. Under 133 $\mu l \cdot L^{-1}$ CO_2 the pyruvate levels began to decrease and PGA levels also slightly decreased above light intensity of 300 $\mu E \cdot m^{-2} s^{-1}$ while the photosynthetic CO_2 assimilation rate did not change above this light intensity (Fig. 2). The positive relationship between the levels of PGA and those of pyruvate under steady-state photosynthesis under various conditions (Fig. 4) suggest that an elevated concentration of PGA could increase the carbon input into the C_4 cycle through the conversion of PGA to PEP and consequently, the level of total intermediates of the C_4 cycle could be raised. Further studies including measurements of metabolite levels in specific compartments are needed to elucidate the regulation of partitioning of PGA to PEP and PGA to DHAP for the regeneration of RuBP and the synthesis of sucrose.

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