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Diurnal Changes in Maize Leaf Photosynthesis[1]

I. CARBON EXCHANGE RATE, ASSIMILATE EXPORT RATE, AND ENZYME ACTIVITIES

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ABSTRACT

Diurnal changes in photosynthetic parameters and enzyme activities were characterized in greenhouse grown maize plants (*Zea mays* L. cv Pioneer 3184). Rates of net photosynthesis and assimilate export were highest at midday, coincident with maximum irradiance. During the day, assimilate export accounted for about 80% of net carbon fixation, and the maximum export rate (35 milligrams CH_2O per square decimeter per hour) was substantially higher than the relatively constant rate maintained through the night (5 milligrams CH_2O per square decimeter per hour). Activities of sucrose phosphate synthase and NADP-malate dehydrogenase showed pronounced diurnal fluctuations; maximum enzyme activities were generally coincident with highest light intensity. Reciprocal light/dark transfers of plants throughout the diurnal cycle revealed that both enzymes were deactivated by 30 minutes of darkness during the day, and they could both be substantially activated by 30 minutes of illumination at night. During 24 hours of extended darkness, sucrose phosphate synthase activity declined progressively to an almost undetectable level, but was activated after 1.5 hours of illumination. Thus, the diurnal fluctuation in maize sucrose phosphate synthase can be explained by some form of light modulation of enzyme activity and is not due to an endogenous rhythm in activity. No diurnal fluctuations were observed in the activities of NADP-malic enzyme or fructose 6-phosphate-2-kinase. Phosphoenolpyruvate carboxylase was activated by light to some extent (about 50%) when activity was measured under suboptimal conditions *in vitro*. The results suggested that the rates of sucrose formation and assimilate export were closely aligned with the rate of carbon fixation and the activation state of sucrose phosphate synthase.

Regulation of carbon fixation and partitioning, as well as the export of photoassimilates is of primary importance to plant growth and productivity, and may differ among C_4 and C_3 species. Under optimal conditions, C_4 photosynthesis is characterized by higher rates of CO_2 fixation, sucrose formation and assimilate export compared with C_3 plants (2). Carbon assimilation by the C_4 pathway is not completely light saturated at full sunlight and therefore the rate of CO_2 uptake is subject to large changes with natural fluctuations in light intensity during the day. In contrast, carbon assimilation in C_3 plants is usually saturated at a lower irradiance and therefore does not fluctuate with changes in natural illumination to the same extent as C_4 plants.

Recently, photosynthesis by the C_3 plant soybean was characterized diurnally. In this study (10), the activity of SPS[4] was shown to oscillate with an endogenous rhythm. SPS activity was correlated positively with assimilate export rate during the day and maximum SPS activity was similar to the measured assimilate export rate. Maize (11) and barley (12) leaf SPS have recently been shown to be light activated; however, it is not known whether SPS from these sources may be controlled by an endogenous rhythm in activity as well as by short-term light modulation.

Our goal in conducting the present study was to characterize diurnal changes in maize leaf carbon assimilation, partitioning and export, in relation to changes in the activities of SPS and key enzymes of the C_4 pathway. To determine whether diurnal fluctuations in enzyme activities were caused by an endogenous rhythm, enzyme activities were also measured in plants after reciprocal light/dark transfers at different times during the diurnal cycle.

MATERIALS AND METHODS

Plant Culture. Maize (*Zea mays* L. cv Pioneer 3184) plants were grown in 15 liter pots (two plants per pot), in the greenhouse during June and July, in a medium containing Terra Lite,[5] sterile top soil and perlite (1:1:1). A commercial slow release fertilizer (Sta-Green Pro Start, 13-6-6, Brawley Seed Co., Mooresville, NC) was incorporated at the recommended rate into the soil at planting. Plants received aged tap water (to reduce chlorine content of city water) daily and at 10 d intervals the soil was supplemented with alternating treatments of 10 ml of 3.2 M KNO_3 and 1.6 M NH_4NO_3. Plants were grown under supplemental lights (Sylvania 1000 W metalarc lamps) which provided a

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[4] Abbreviations: SPS, sucrose phosphate synthase; CER, carbon exchange rate; F26BP, fructose 2,6-bisphosphate; F6P,2K, fructose 6-phosphate-2-kinase; PEP, phosphoenolpyruvate; PEPCase, phosphoenolpyruvate carboxylase; Pi, inorganic phosphate; NADP-MDH, NADP-malate dehydrogenase; NADP-ME, NADP-malic enzyme.

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fluence rate of 400 μmol/m^2·s at plant level, in the absence of sunlight. Lights were on from 0.5 h after sunrise until 0.5 h before sunset. The plants used in this study were 22 d old; at this stage of development, leaf position 8 (acropetally numbered) was beginning to emerge from the whorl. Experiments were conducted on consecutive sunny days.

Experimental Protocol. The studies were conducted over a 27 h period (0900 h on d 1 to 1200 h on d 2), divided into 3 h intervals. For each interval, six different plants were used. All measurements were made on a 13 cm long segment of leaf 6, starting 10 cm from the leaf tip. At the beginning and end of each interval, three leaf punches (2 cm^2 total area) were collected separately from each plant to provide six paired samples for measurements of carbohydrates. The leaf punches were stored at −20°C prior to lyophilization. During the interval, CER was measured. After taking the leaf punches at the end of the interval, the remaining tissue was harvested for enzyme and metabolite analyses. The midrib was quickly removed from the 13 cm segment and one-half of the leaf blade was taken for measurement of enzyme activities. The other half of the blade was divided; half was taken for measurement of F26BP and the other half for measurement of all other metabolites. Results of metabolite measurements are presented in the companion study (15). Tissue was immediately frozen in liquid N_2 and stored at −80°C prior to analyses.

The study actually spanned a period of 2 consecutive days; however, the results were combined so that data were plotted starting from 0600 h, until 0300 h. This was done for ease of discussion and was possible because the overlapping points from d 1 and 2 were very close in all measurements. Thus, most data points are the means of 6 replicate samples, while those taken between 0900 h and 1200 h are means of 12 replicates.

To examine the effects of light/dark transfer on enzyme activities and metabolite levels, three plants were transferred to a darkened growth chamber during the light period or conversely to an illuminated growth chamber (about 600 μE/m^2·s at the level of the plants) during the normal dark period. After 0.5 h, tissue from leaf 6 was harvested as described above. Growth chamber temperature approximated temperature in the greenhouse.

CER and Assimilate Export Rate. Net carbon exchange was measured on leaf 6 at the middle of each sampling interval. Uptake or release of CO_2 was measured in a closed system using a LiCor Model 6000 Portable Photosynthesis Meter (LiCor, Lincoln, NE). Photosynthetic rate, measured as mg CO_2/dm^2·h, was converted to mg CH_2O/dm^2·h by multiplying by 0.68 (molar ratio of the two carbon forms). Mass carbon export rate was calculated as the difference between net carbon assimilation during the 3 h interval and the change in leaf dry weight (from lyophilized leaf punches) as described by Terry and Mortimer (14).

Enzyme Extraction and Assay. Frozen leaf samples (0.2–0.3 g) were ground in a chilled mortar with about 0.2 g sea sand and 2.5 ml extraction buffer (50 mM Hepes-NaOH [pH 7.5], 10 mM $MgCl_2$, 1 mM EDTA, 5 mM DTT, 2% (w/v) PEG-20, 0.5% [w/v] BSA and 0.02% [v/v] Triton X-100). The extract was filtered through Miracloth (Behring Diagnostics, LaJolla, CA) and an aliquot was collected for Chl determination prior to centrifugation at 1100*g* for 2 min. Unless specified otherwise, extracts were desalted by centrifugal gel filtration (3) into a buffer containing 50 mM Hepes-NaOH (pH 7.5), 10 mM $MgCl_2$, 1 mM EDTA, 2.5 mM DTT and 0.5% (w/v) BSA. NADP-MDH was assayed as described by Nakamota and Edwards (9). Extracts were assayed before desalting because inactivation of NADP-MDH is very rapid. After tissue extraction, 25 μl of the Miracloth filtered extract was added to 1.0 ml of 50 mM Hepes-NaOH (pH 8.0), 1.0 mM EDTA and 0.2 mM NADPH and centrifuged in a Beckman Microfuge (approximately 10,000*g*) for 10 s. Absorbance at 340 nm of 900 μl of this mixture was monitored prior to initiating the reaction with 100 μl of 20 mM oxaloacetate.

NADP-ME: Assay mixtures (1.0 ml) contained 50 mM Hepes-NaOH (pH 8.0), 10 mM EDTA, 5 mM DTT, 0.5 mM NADP, and 5 mM malate. After addition of 25 μl desalted extract, the change A_{340} was monitored prior to initiating the reaction by adding 25 mM $MgCl_2$. Activity of NADP-ME was also measured in nondesalted extracts to assess dilution during desalting. Dilution was minimal (≤10%); however, enzyme activities in each sample were corrected for the calculation dilution.

PEPCase: Desalted extracts were assayed under suboptimal and optimal conditions for PEPCase activity. Suboptimal assays (1.0 ml) contained 50 mM Hepes-NaOH (pH 7.2), 5 mM $MgCl_2$, 0.15 mM NADH, 1.0 mM $NaHCO_3$, 2 units malate dehydrogenase, and 50 μl desalted extract. After measurement of the background rate of NADH oxidation, reactions were initiated by addition of 0.3 mM PEP. Optimal conditions were similar to the above assay, except that activity was measured at pH 8.0 and assays contained 3.0 mM PEP. SPS was measured in a mixture containing 25 μl of 50 mM Hepes-NaOH (pH 7.5), 15 mM $MgCl_2$, 70 mM UDP-glucose and 14 mM fructose 6-P plus 45 μl desalted extract (70 μl final volume). Reactions were incubated for 5 min and then terminated by addition of 70 μl of 1 N NaOH. After a 10 min incubation in boiling water, product (sucrose plus sucrose phosphate) was visualized by addition of 250 μl of 0.1% (w/v) resorcinol in 95% ethanol and 750 μl of 30% HCl, followed by incubation at 80°C for 8 min. After cooling, the A_{520} was measured. Sucrose phosphate synthase activity was measured against a zero time blank, obtained by adding enzyme extract to a reaction mixture containing 70 μl of 1 N NaOH. The sucrose formed was quantitated by comparison to a sucrose standard curve.

F6P,2K: Reaction mixtures contained 90 μl of a mixture of 0.1 M Tris-HCl (pH 7.4), 5 mM K-phosphate, 5 mM fructose 6-P, 15 mM glucose 6-P, 4 units/ml creatine phosphokinase, 5 mM phosphocreatine, 2 mM ATP, and 5 mM $MgCl_2$, plus 60 μl of desalted leaf extract. The reaction was initiated by adding the desalted enzyme extract. Aliquots (20 μl) were removed at 5 min intervals, placed in 80 μl of 0.1 N NaOH and frozen at −20°C prior to F26BP measurement. F26BP formed during the incubation was quantitated by a modified procedure originally described by Van Schaftingen *et al.* (18). Reaction mixtures (1.0 ml) contained partially purified potato tuber pyrophosphate:fructose 6-phosphate phosphotransferase (PPi-PFK) (18), 4 units aldolase, 1 unit trioseP isomerase, 10 units glycerol 3-P dehydrogenase in 50 mM Tris-HCl (pH 8.0), and 5 mM $MgCl_2$. The amount of F26BP formed after 5 min due to F6P,2K activity was quantitated by measuring activation of the PPi-PFK reaction.

All enzyme assays were conducted at 33°C, which represents the average leaf temperature during the experimental period.

Nonstructural Carbohydrate Analysis. Measurements of starch and soluble sugars (sucrose and hexose sugars) in lyophilized leaf discs were carried out at described by Huber *et al.* (6).

Chlorophyll Measurement. Chl concentration was measured in Miracloth filtered extracts according to the procedure of Wintermans and De Mots (19).

Statistical Analysis. Diurnal changes in the measured parameters were statistically analyzed by one-way analysis of variance. Values are means of six determinations ± SE.

RESULTS

Diurnal Changes in Photosynthetic Parameters. Figure 1 shows the diurnal profiles of carbon fixation and assimilate export in greenhouse grown maize plants. Both CER and export increased in the morning, reached a maximum at about noon, and declined

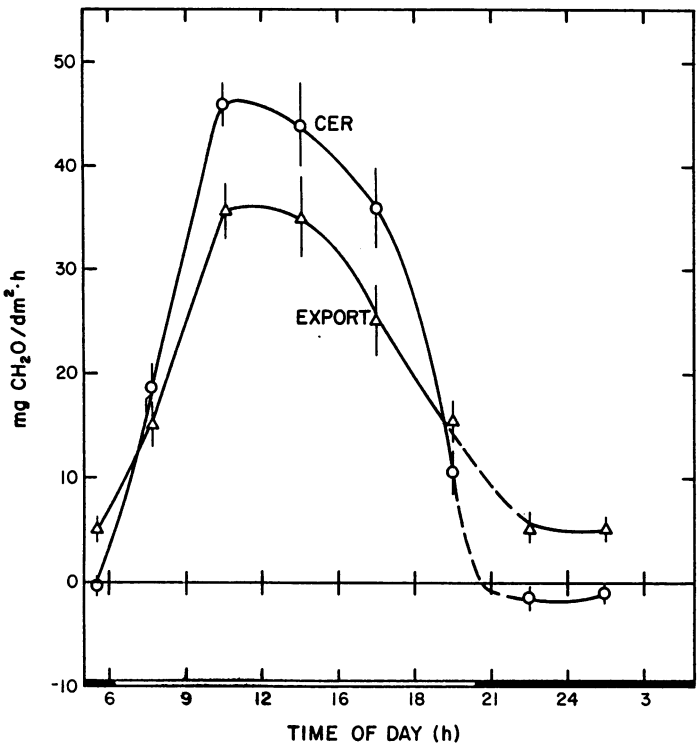

FIG. 1. Diurnal changes in net CER (O) and assimilate export rate (Δ) of a maize leaf during a normal day/night cycle. The solid bar at the bottom of the figure indicates darkenss and the open bar, light.

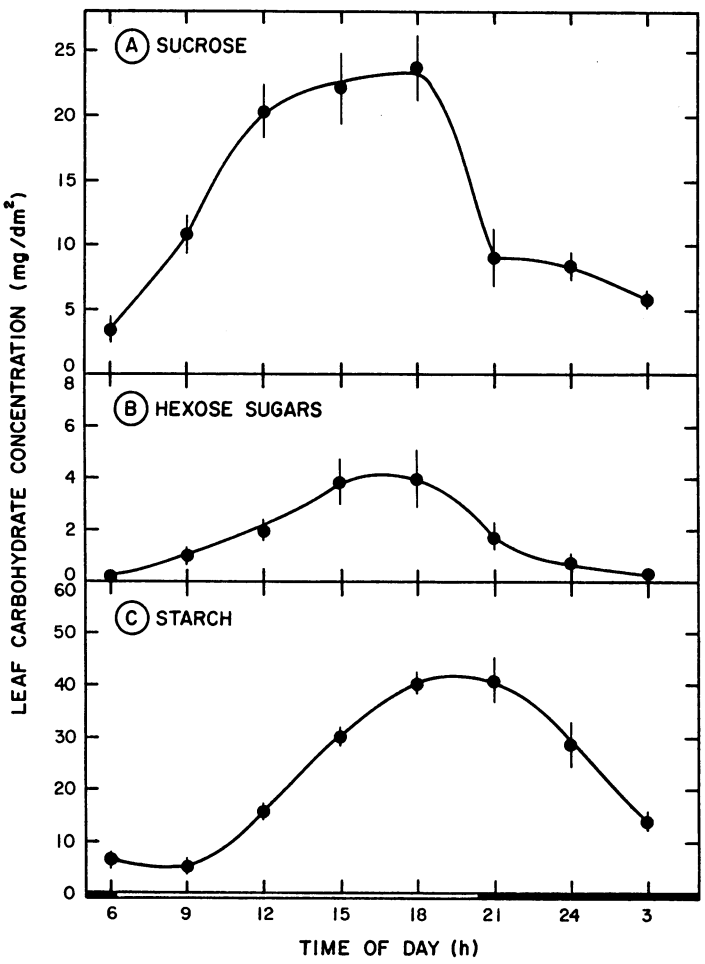

FIG. 2. Diurnal changes in maize leaf carbohydrate concentration. A, Sucrose; B, hexose sugars (glucose, fructose, glucose 6-P, fructose 6-P); C, starch. Note differences in ordinate scales of each panel.

in the afternoon. This pattern generally paralleled changes in light intensity during the photoperiod. Maximum CER was 47 mg $CH_2O/dm^2 \cdot h$ (67 mg $CO_2/dm^2 \cdot h$). Of the net carbon fixed at midday, about 80% was exported immediately. Assimilate export rate toward the end of the light period exceeded the rate of carbon assimilation and was coincident with a depletion of leaf sucrose (Fig. 2A). Respiratory loss of carbon at night was low and relatively constant (Fig. 1). Assimilate export remained fairly constant throughout the night at about 5 mg $CH_2O/dm^2 \cdot h$ (Fig. 1) and was apparently supported by mobilization of leaf starch (Fig. 2C).

Total carbon fixed during the day amounted to 455 mg CH_2O/dm^2. Of the total fixed, 81% was exported during the day (369 mg CH_2/dm^2) and 19% was stored in the leaf (86 mg CH_2O/dm^2). Maize leaves accumulated a substantial pool of sucrose during the day, but the soluble sugar pool was largely depleted within 0.5 h of the onset of darkness (Fig. 2A). Thus, at the end of the day, starch was the predominant form of carbon retained in the leaf (40 mg/dm²) (Fig. 2), representing 76% of the total nonstructural carbohydrate pool. Assimilate export at night accounted for 83% of the decrease in carbohydrate reserves, and the remaining 17% could be accounted for by respiration. Starch was the predominant source of carbon for these processes, and essentially all of the starch that accumulated in the leaf during the day had been mobilized by the end of the night (Fig. 2C).

Diurnal Changes in Enzyme Activities. A pronounced diurnal fluctuation in SPS activity was observed (Fig. 3, curve A); SPS activity was highest at midday, then decreased and was relatively constant at night. During the light period, transfer of plants to a darkened growth chamber for 0.5 h reduced SPS activity by about 65%. Sucrose phosphate synthase activity after transfer to darkness was about the same as the activity observed at night (approximately 60 μmol/mg Chl·h). Conversely, illumination (0.5 h) of plants during the normal dark period resulted in a substantial activation of SPS activity (Fig. 3, curve A). However, SPS activity after illumination at night was not as high as that observed during the day. We attribute this to the fact that natural irradiance (midday about 1200 $\mu E/m^2 \cdot s$) was about twofold higher than that provided experimentally (about 600 $\mu E/m^2 \cdot s$). In a second experiment, maize plants were subjected to 24 h of darkness after the end of the normal dark period (*i.e.*, extended darkness) to determine whether oscillations in SPS activity would occur in a constant environmental condition. As shown in Figure 3, curve B, SPS activity declined progressively in extended darkness so that after 24 h of extended darkness, SPS activity was almost undetectable. However, after a subsequent 90 min exposure to sunlight, SPS activity increased significantly. At that point, SPS activity was about half of the activity observed when plants were not subjected to 24 h of extended darkness. Because fluctuations in SPS activity did not occur under constant conditions, it appears that maize SPS activity is not controlled by an endogenous rhythm, as is the case for soybean SPS (10). The rapid activity changes in SPS after light/dark transfers indicated that the diurnal fluctuation in maize leaf SPS activity was the result of some form of light modulation. Because the highest SPS activity was observed when irradiance was greatest, it appears that light activation of SPS may require a high light intensity for saturation.

The fluctuations in SPS activity during the photoperiod were closely aligned with the diurnal pattern of assimilate export (Figs. 1 and 3A). This relationship suggests that SPS activity may be correlated with the flux of carbon into sucrose, and that light modulation of SPS activity is of physiological significance.

NADP-malate dehydrogenase is light modulated in both C_3

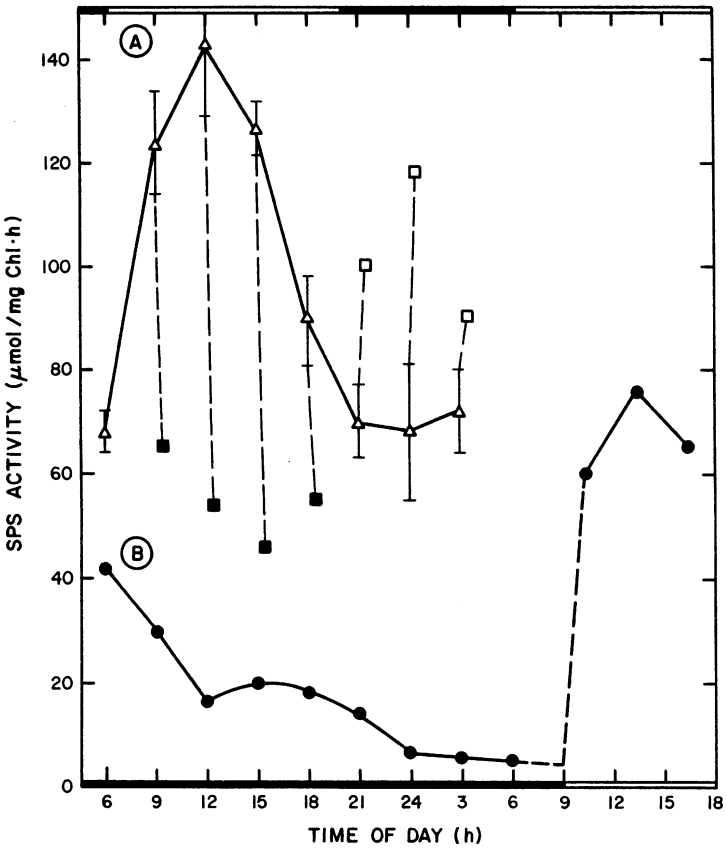

FIG. 3. Changes in maize leaf SPS activity during (A) a normal day/night cycle and (B) extended darkenss. As indicated by the dashed line in (A), plants were transferred during the day to darkness (■), and at night to an illuminated growth chamber (□). In (B), plants were exposed to 27 h of extended darkness followed by exposure to natural light. The bars at the top and bottom of the figure correspond to photoperiod conditions in (A) and (B), respectively.

and C_4 plants (1), and results consistent with these findings were obtained in the present study. The activity of NADP-MDH was undetectable in the dark, but increased greatly in the light. Maximum activity of NADP-MDH was observed at midday (Fig. 4A), which indicated a close relationship between enzyme activity and light intensity (16).

PEPCase activity, measured over the 24 h period, is shown in Figure 4B. When assayed under optimal conditions (pH 8.0, saturating substrates), PEPCase activity was high and no diurnal changes were observed. When activity was measured under suboptimal conditions (pH 7.2, low PEP concentration), activity was reduced by over 90%, and there was some indication of light activation. The activity of PEPCase in the light (30 μmol/mg Chl·h) was slightly higher than in the dark (20 μmol/mg Chl·h). However, the light/dark difference in PEPCase activity was not statistically significant.

NADP-malic enzyme, the principle C_4 acid decarboxylating enzyme in maize, was assayed throughout the diurnal cycle. No fluctuation in activity was observed (data not shown). In the present study, NADP-ME remained at a constant activity of about 340 μmol/mg Chl·h, and served as a useful control for monitoring plant-to-plant variability.

As shown in the companion report (15), there were pronounced diurnal fluctuations in the concentration of F26BP in maize leaves. Accordingly, we were interested in determining whether the activity of F6P,2K fluctuated diurnally (Fig. 5). F6P,2K activity, when measured in the presence of Pi, an activator, was quite high (about 85 μmol/mg Chl·h), and relatively constant throughout the experimental period. Similarly, no diurnal fluctuation in F6P,2K activity was observed when assays were conducted in the presence of the kinase inhibitor, 3-P-glycerate, which reduced activity by about 30% (Fig. 5).

DISCUSSION

To our knowledge, the results obtained in the present study provided the most complete diurnal characterization of changes in CER, assimilate export rate, enzyme activities and carbohydrate concentration of maize source leaves. Results from this study confirm earlier reports that maize leaves have a high capacity to assimilate and export carbon. This is apparent when these results are compared with data from similar studies using the C_3 plants, soybean and spinach (5). For comparison, the results with maize, which are expressed on a leaf area basis, have been converted to a leaf fresh weight basis. Cumulative net carbon fixation during the photoperiod was higher in maize (288 mg CH_2O/g fresh weight·photoperiod) compared to soybean and spinach (160 and 48 mg CH_2O/g fresh weight·photoperiod, respectively). Of the carbon assimilated during the photoperiod, assimilate export accounted for 80, 68, and 46% in maize, soybean, and spinach, respectively. Starch accumulation accounted for 14% of the carbon fixed in maize and 27% and 15%, respectively, in soybean and spinach. Since maize exports comparatively more of the carbon fixed during the day, it follows that relatively less carbon will be available for export at night. These results suggest that the partitioning of carbon may be different in C_4 compared to C_3 species, and this may have important implications for diurnal growth and metabolism in these different plant types. Although a substantial pool of sucrose accumulated in maize leaves during the day, it was rapidly depleted close to the end of the photoperiod. At this time the rate of assimilate export exceeded CER, but starch reserves were not mobilized. Therefore, stored sucrose was the primary carbon source for export at the end of the day.

Plants photosynthesizing via the C_4 pathway require high light intensities to saturate CO_2 fixation and Moss *et al.* (8) have shown that 90% of the fluctuations in carbon assimilation in maize can be explained by changes in light intensity alone. Light modulation of Calvin cycle enzymes is common to both C_3 and C_4 species. Certain enzymes of the C_4 assimilatory pathway are also light modulated, namely NADP-MDH and pyruvate Pi dikinase. Both of these enzymes are located in the mesophyll

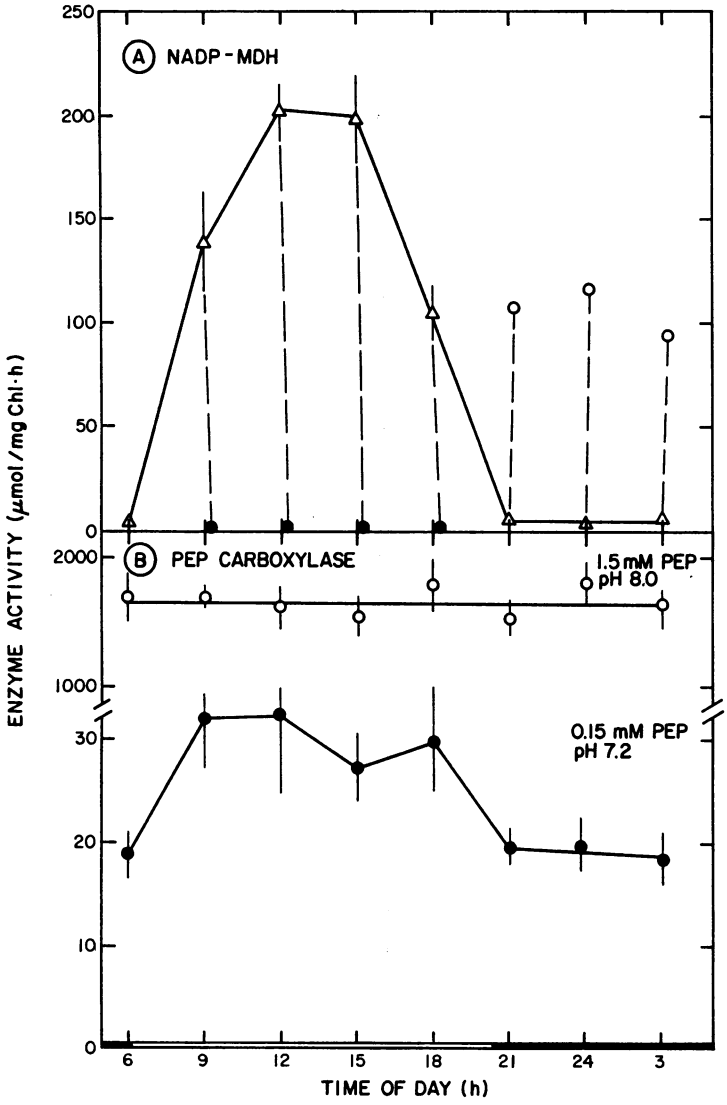

FIG. 4. Diurnal changes in the activities of (A) NADP-MDH and (B) PEPCase in maize leaves. As indicated by the dashed lines in (A) plants were transferred during the day to a darkened growth chamber (●) and at night to an illuminated growth chamber (○). In (B), PEPCase activity was assayed under optimal (○) (3.0 mM PEP, pH 8.0) or suboptimal (●) (0.15 mM PEP, pH 7.2) conditions. Note the break in the ordinate axis in (B).

chloroplast, and required high irradiance for maximal activation (1). This study confirms the occurrence of light activation of maize SPS (11), and provides new information about the diurnal changes of SPS activity in a natural environment. Maize SPS activity was highest at midday, coincident with the period of highest irradiance. This result implies that SPS light activation may require a high light intensity for saturation. If this were the case, then activation of SPS, NADP-MDH, and pyruvate Pi dikinase would be similar. However, because SPS is localized in the maize mesophyll cytosol (17), the mechanism of light activation cannot be readily explained by fluctuations in chloroplastic metabolites (including reduced thioredoxin) that are thought to be involved in the modulation of pyruvate Pi dikinase and NADP-MDH (1).

Diurnal fluctuations in soybean SPS activity have also been documented; however oscillations in soybean SPS activity persisted under constant light or dark conditions and therefore can be attributed to an endogenous rhythm in activity (10). Thus, the diurnal fluctuation in SPS activity in maize and soybean leaves have a fundamentally different basis.

Surcrose phosphate synthase activity was almost undetectable after 24 h of extended darkness. Exposure to light for 1.5 h after extended darkness resulted in a substantial increase in SPS activity, but only to the level normally observed for the dark enzyme. Transfer of plants to the light for 0.5 h during the normal dark period, resulted in higher SPS activities than those observed after extended darkness. These data suggest that light activation of SPS may involve multiple mechanisms with different time dependencies.

Light modulation of PEPCase has been reported in *Salsola soda* (7) and other C_4 species. In the study with *S. soda*, light activation of PEPCase was more pronounced when activity was measured under suboptimal conditions of low pH and low PEP concentration. When similar conditions were used to assay maize PEPCase, some diurnal fluctuation was apparent, but the effect was not as substantial as that observed with *S. soda*. When assayed under suboptimal conditions, PEPcase activity was reduced by more than 90% of the maximum activity observed under optimal conditions. Thus, cytosolic pH and PEP concentration *in vivo* are factors that may determine the physiological significance of the apparent light effect on PEPCase in maize.

The cytosolic concentration of F26BP is important in the regulation of sucrose formation since it is a potent inhibitor of cytosolic fructose 1,6-biphosphatase (for a recent review see Ref. 4). The level of F26BP in the cell is mediated by the activities of F6P,2K and fructose 2,6-bisphophatase, which are both localized primarily in the leaf mesophyll of maize, and are regulated by metabolic effectors (13). In the present study, F6P,2K activity was measured diurnally in the presence of Pi, a kinase activator, and separately with 3-P-glycerate, a kinase inhibitor, plus Pi. No

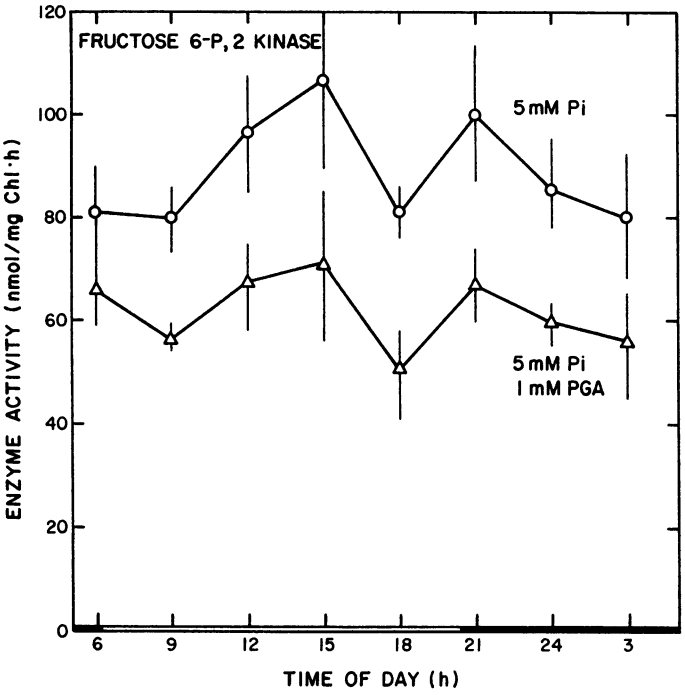

FIG. 5. Activity of F6P,2K in maize leaves harvested at different times of the diurnal cycle. Enzyme activity was assayed in the presence of 5 mM Pi (O), or with 5 mM Pi plus 1 mM 3-P-glycerate (Δ).

significant diurnal fluctuations in F6P,2K activity were observed under the conditions tested. There is some evidence in spinach however, that reciprocal changes in F6P,2K and F26BPase activities occur during the photoperiod (M. Stitt, personal communication). Thus, the possibility remains that in maize, alterations in F26BP synthesis:degradation are mediated by changes in fructose 2,6-bisphosphate activity. Alternatively, the maximum activities of both enzymes may be constant, but changes in metabolite levels *in vivo* may regulate the kinase and phosphatase activities to bring about changes in the F26BP concentration.

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