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Diurnal Changes in Maize Leaf Photosynthesis[1]

II. LEVELS OF METABOLIC INTERMEDIATES OF SUCROSE SYNTHESIS AND THE REGULATORY METABOLITE FRUCTOSE 2,6-BISPHOSPHATE

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HIDEAKI USUDA[2], WILLY KALT-TORRES, PHILLIP S. KERR[3], AND STEVEN C. HUBER*
United States Department of Agriculture, Agricultural Research Service, and Departments of Crop Science and Botany, North Carolina State University, Raleigh, North Carolina 27695-7631

ABSTRACT

Diurnal changes in the regulatory metabolite, fructose-2,6-bisphosphate (F26BP), and key metabolic intermediates of sucrose biosynthesis were studied in maize (*Zea mays* L. cv Pioneer 3184) during a day-night cycle. Whole leaf concentrations of dihydroxyacetonephosphate (DHAP) and fructose 1,6-bisphosphate changed markedly during the photoperiod. DHAP concentration was correlated positively with the rate of sucrose formation *in vivo* (assimilate export plus sucrose accumulation) and extractable activity of sucrose phosphate synthase (SPS). The changes closely followed net photosynthetic rate, which tracked irradiance. The other metabolic intermediates measured (glucose 6-phosphate, fructose 6-phosphate, and UDP-glucose) were either relatively constant over the 24 hour period or changed in a different pattern. Diurnal changes in leaf F26BP concentrations were pronounced, and fundamentally different than the pattern reported with other species. F26BP concentration decreased at the beginning of the day and remained low and constant; a 3- to 4-fold increase occurred with darkness, and slowly declined thereafter. In general, leaf F26BP concentration was negatively correlated with net photosynthetic rate, and also leaf DHAP concentration. Consequently, coordination of the regulation of cytosolic fructose 1,6-bisphosphatase and SPS was apparent. The results support the postulate that in maize leaves the activation state of SPS may be dependent on availability of DHAP and possibly other metabolites.

Sucrose plays an important role in plant metabolism as a major end product of photosynthesis, as the principal form of translocated carbon, and also as an important storage sugar (1). The pathway and regulation of photosynthetic sucrose synthesis in C_3 plants has been studied intensively. The rate of sucrose biosynthesis in C_3 plants could be controlled by the regulation of SPS[4] (7, 10) and cytoplasmic F16BPase (6, 8, 20). The regulatory metabolite F26BP is a potent inhibitor of the latter enzyme (8, 20), and may serve to coordinate the regulation of these two enzymes (8, 19).

In the companion paper (11), we show that the maximum rate of assimilate export (leaf area basis) in maize leaves is about 1.5 times higher than that in soybean leaves (12), and that there is a pronounced diurnal fluctuation in photosynthetic metabolism.

Several lines of evidence suggest that sucrose formation in maize leaves may be strictly compartmented within the mesophyll cell. SPS is mainly, if not exclusively, localized in the mesophyll cytoplasm in maize leaves (22). The proportion of sucrose synthesized in the mesophyll fraction in maize leaves after short-term $^{14}CO_2$-fixation was approximated to be 100% when its proportion was extrapolated back to time zero (5). In addition, activities of F6P,2K and F26BPase were localized mostly, if not solely, in the mesophyll tissue of maize leaves (14). Most of the leaf F26BP, a regulatory metabolite that inhibits the activity of cytoplasmic F16BPase, is also present in the mesophyll tissue of maize leaves (17).

The recent observation that the majority of chloroplastic F16BPase activity is in bundle sheath cells (23) is consistent with the previous findings that starch was strictly confined to the bundle sheath tissue in maize plants under normal conditions (4). Such spatial separation of sucrose and starch synthesis between mesophyll and bundle sheath cell may be unique to maize leaves. In C_3 plants, on the contrary, sucrose and starch synthesis occur within the same cell; sucrose synthesis occurs in the cytoplasm and starch synthesis in the chloroplast.

In the companion manuscript (11), we characterized diurnal changes in photosynthetic parameters of maize leaves in relation to changes in enzyme activities. Using the same experimental tissue in this study, we also measured changes in the levels of precursor metabolites of sucrose synthesis and the regulatory metabolite F26BP. Our objectives in undertaking this work were to relate changes in metabolites with photosynthetic processes, and activities of relevant enzymes.

MATERIALS AND METHODS

Plant Material. *Zea mays* L. (cv Pioneer 3184) was used in the experiments. For further details, see the companion study (11).

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[2] Permanent address: Laboratory of Chemistry, Faculty of Medicine, Teikyo University, Ohtsuka, Hachioji, Tokyo, Japan 192-03.

[3] Present address: E. I. duPont de Nemours, Agricultural Products Department, Crop Research Laboratory; P.O. Box 30, Newark, DE 19714.

[4] Abbreviations: SPS, sucrose phosphate synthase; CER, carbon exchange rate; DHAP, dihydroxyacetone-phosphate; F16BP, fructose 1,6-bisphosphate; F16BPase, fructose 1,6-bisphosphatase; F26BP, fructose 2,6-bisphosphate; F26BPase, fructose 2,6-bisphosphatase; F6P, fructose 6-phosphate; F6P,2K, fructose 6-phosphate,2-kinase; G6P, glucose 6-phosphate; UDPG, UDP-glucose.

Measurement of Metabolites. Sampling of the leaf tissue for the determination of metabolites was carried out at the same time that other measurements were made (11). Half of the same leaves, which were used for the studies to determine enzyme activities, were used for these studies. Samples of leaf segments were frozen as quickly as possible in liquid N_2 in the light or in darkness (under a low light intensity green safe light). Frozen leaf material was extracted with 3% $HClO_4$ and neutralized samples were used for the enzymic determination of metabolites as described previously (21) with the following exceptions. Charcoal treatment was omitted for the measurement of UDPG because charcoal adsorbs this metabolite. UDPG was determined in a mixture (1.0 ml) of 350 mM glycine-KOH (pH 8.7), 2.5 mM NAD, 6.3 mM EDTA and about 0.1 unit of UDPG dehydrogenase (Sigma U-7251).[5] For all metabolites other than F26BP, the pellet of the $HClO_4$ extract was saved for determination of pheophytin (25).

For the determination of F26BP, a slightly alkaline aqueous extraction medium was used, essentially as described in (9). Frozen leaf material (100–120 mg fresh weight) was ground in a mortar with about 0.5 ml of a cold extraction medium containing 0.1 M Hepes-KOH (pH 8.0), 5 mM EDTA, and 5 mM KF. The mortar and pestle were rinsed with 2.0 ml of the cold extraction medium. The extract was then placed in a water bath at 80°C for 5 min, followed by centrifugation at 1,500*g* for 5 min. The pellet was washed with 0.5 ml of the extraction medium and the suspension was again centrifuged at 1,500*g* for 5 min. The volume of the combined supernatant was determined and Chl was measured in both the supernatant and pellet. An aliquot of the combined supernatant was used for the determination of F26BP after charcoal treatment and centrifugation at 36,000*g* for 10 min. F26BP was measured in the bioassay using potato tuber pyrophosphate:fructose-6-P phosphotransferase (24). The recovery of authentic F26BP and all other measured metabolites (added prior to tissue extraction) was consistently greater than 85% (see also Ref. 21) and no correction for recovery was made.

Chl Determination. Chl was measured by the method of Arnon (2). Pheophytin was extracted from $HClO_4$ residues into 80% acetone using a Teflon/glass homogenizer. Pheophytin was measured by the method of Vernon (25) and converted to Chl units.

RESULTS AND DISCUSSION

Diurnal changes in the levels of metabolites related to sucrose synthesis are shown in Figure 1. The diurnal changes in net CER (1) are shown for comparison (Fig. 1A). The amount of DHAP varied greatly between light and dark which is consistent with previous findings (13, 21). The amount of DHAP was very low in the dark and increased markedly after sunrise, reached a maximum level around noon, and then gradually decreased to a very low level in the dark (Fig. 1B). The diurnal changes in the level of DHAP clearly paralleled the changes in the CER. The maximum concentration of DHAP on the basis of Chl (about 350 nmol mg^{-1}Chl) (see also Fig. 3A) was significantly higher than that found in the C_3 plant spinach (50 nmol mg^{-1}Chl) (16). The concentration of DHAP in spinach leaves also varied greatly between light and dark; however, the level of DHAP during the day was almost constant in spinach leaves (16). In that study, diurnal changes in CER were not shown, thus the direct comparison of the level of DHAP in relation to photosynthetic capacity between C_3 and C_4 plants is not possible. The fluctuations in the levels of G6P and F6P were not so marked as that

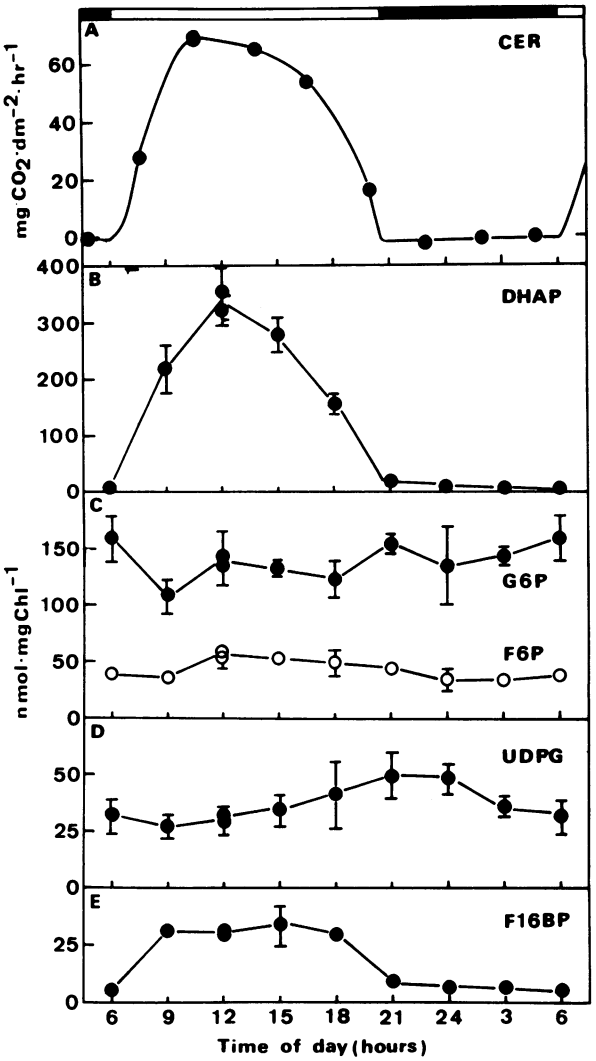

FIG. 1. Diurnal changes in CER and metabolite levels related to sucrose synthesis in maize leaves. CER data are taken from the companion paper (11). For further details, see Figure 1 in Ref 11. The results of metabolite levels are mean ± SD of four separate samples. Light conditions are indicated at the top of the figure: open bar, light; closed bar, dark.

of DHAP (Fig. 1C). The G6P level in the dark was slightly higher than that in the light, whereas the reverse was true for F6P. F6P increased during the late morning and stayed constant for the afternoon and then gradually decreased in the early night and stayed constant thereafter. The amount of UDPG slowly increased throughout the day and into the night, and then decreased before sunrise (Fig. 1D). The diurnal fluctuation in the level of F16BP was pronounced (Fig. 1E). The F16BP level increased significantly after sunrise and stayed at a constant level during most of the day, then decreased during the late afternoon. The almost constant level in F16BP from 0900 h to 1800 h was in contrast to the changes in DHAP content (Fig. 1B) that tended to parallel CER.

F6P and UDPG were present in significant amounts even in the dark; however, the levels of DHAP and F16BP were very low in the dark. These trends are essentially consistent with the results found in C_3 plants (16).

Diurnal changes in the level of the regulatory metabolite, F26BP, were also investigated (Fig. 2). The amount of F26BP varied greatly between light and darkness; high at night and low during the day. After sunrise, F26BP decreased significantly and

[5] Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the United States Department of Agriculture and does not imply its approval to the exclusion of other products that may also be suitable.

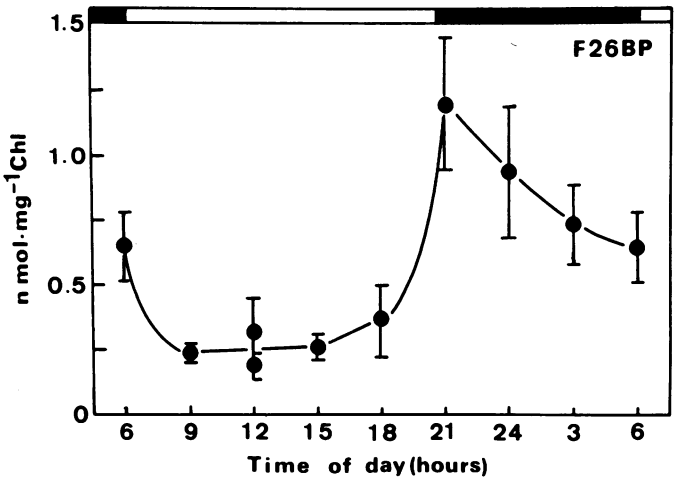

FIG. 2. Diurnal changes in leaf F26BP concentration in maize leaves. Values are the mean ± SD of four separate samples. Light conditions are indicated at the top of the figure: open bar, light; closed bar, dark.

then remained relatively constant at about 0.25 nmol mg^{-1}Chl. Toward the end of the day, F26BP concentration gradually increased, and after sunset increased dramatically up to 1.20 nmol mg^{-1}Chl. During the night, F26BP concentration decreased gradually to about 0.65 nmol mg^{-1}Chl at 0600 h. These results are fundamentally different from the diurnal changes in spinach leaves (16), where the level of F26BP decreased transiently upon illumination but then increased to a level above that found in the dark.

We focused our attention on the changes in the level of DHAP, which is an initial precursor of sucrose, and F26BP, an important regulatory metabolite in sucrose synthesis (6, 8, 20). Stitt and Heldt (17) estimated that most of the F26BP, and about 85% of the DHAP was in the mesophyll cells of illuminated maize leaves. This finding allowed us to consider the regulation of sucrose synthesis and also F26BP synthesis in maize leaves using the results obtained in the present study, where metabolite content of whole leaf tissue was measured without further subfractionation. The relationship between leaf CER and the concentration of F26BP and DHAP is shown in Figure 3. During the day, the level of DHAP was positively correlated with CER (Fig. 3A). These results indicate that photosynthetic activity reflected the supply of DHAP available for sucrose synthesis. On the other hand, the concentration of F26BP was inversely related to CER (Fig. 3B). Stitt *et al.* (18) found a similar relationship in spinach and proposed that the increase in F26BP, when photosynthesis is low, can be accounted for by low concentrations of metabolites (DHAP and 3-P-glycerate) which inhibit the synthesis of F26BP (15). The relationship between F26BP and DHAP levels in maize leaves during the day are plotted in Figure 4. As expected, DHAP and F26BP concentration varied reciprocally (Fig. 4). These results indicated that DHAP could be an important regulator of the synthesis of F26BP in C_4 plants as it is in C_3 plants. However, the following observations suggest that DHAP regulation of F26BP in maize leaves may not be simple. F6P,2K in maize leaves was reported to be inhibited by DHAP with half-maximal inhibition at 1 mM; however, maize F26BPase was also inhibited by DHAP, unlike the spinach enzyme (14). Furthermore, maize F6P,2K and F26BPase were also quite sensitive to inhibition by P-enolpyruvate (14). Further investigations are needed to clarify the regulation of the level of F26BP not only in C_4 plants but also in C_3 plants.

To consider how metabolite levels may influence sucrose synthesis, the rate of sucrose synthesis *in vivo* was estimated as the sum of the rate of sucrose accumulation in the mature leaf tissue (calculated from Fig. 2A in Ref. 11) and the rate of export assimilate at the same time (calculated from Fig. 1 in Ref. 11). We assume that all of the exported carbohydrate is in the form of sucrose. The relationships between the estimated rate of sucrose synthesis and DHAP and F26BP leaf concentrations are shown in Figure 5. The estimated rate of sucrose synthesis during

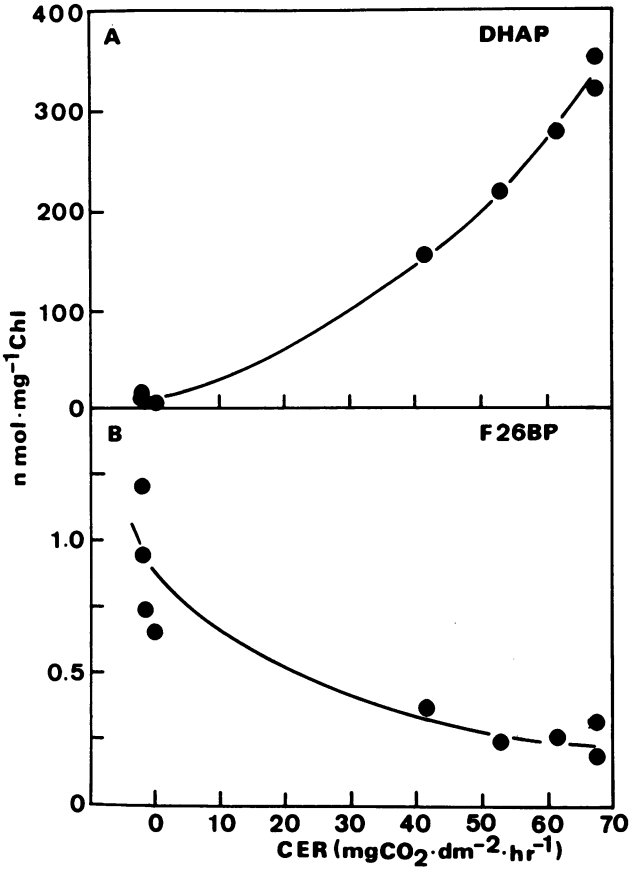

FIG. 3. Relation between CER and leaf concentration of (A) DHAP and (B) F26BP in maize during a day-night cycle. CER at the time of measurement of metabolite levels was estimated from the plot in Fig. 1A. Data for DHAP and F26BP concentrations are taken from Figures 1B and 2.

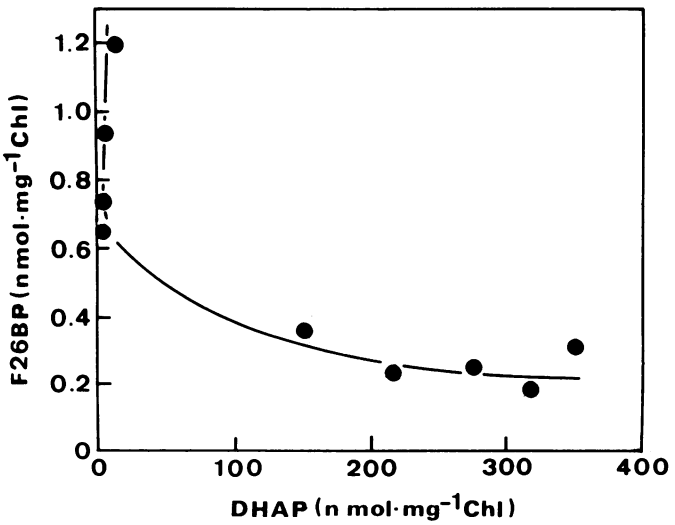

FIG. 4. Relation between the concentrations of DHAP and F26BP in maize leaves during a day-night cycle. Data are from Figures 1B and 2.

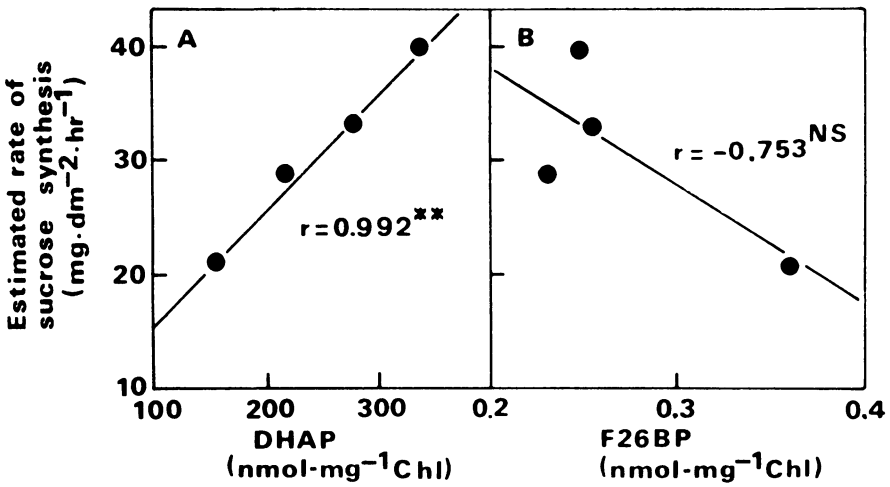

FIG. 5. Relation between the estimated rate of sucrose synthesis *in vivo* and leaf concentrations of DHAP and F26BP during the day in maize. Sucrose synthesis rate was estimated as the sum of the rate of sucrose accumulation and the rate of assimilate export. For further details, see text. Data for metabolite levels are from Figures 1 and 2. **, $P < 0.01$. Where $n = 4$, significance at the 1% level occurred at $r = 0.950$.

the day was positively correlated with the content of DHAP (Fig. 5A), which suggests that the rate of sucrose synthesis is regulated by the ability of photosynthesis to supply triose-P. The cytosolic F16BPase activity in C_3 plants varies strikingly depending on the availability of DHAP and there appears to be a 'threshold' level of DHAP, below which the cytosolic F16BPase is inactive, and above which activity is increased by very small increases in DHAP concentration (6). The cytosolic F16BPase in maize leaves has a high K_m for F16BP compared to spinach, but is controlled by F26BP in a manner analogous to the C_3 enzyme (17). Together these results suggest that availability of DHAP could be one of the major factors influencing the rate of sucrose synthesis in maize leaves through the same regulatory mechanisms as in C_3 plants.

The relationship between leaf F26BP concentration and the rate of sucrose synthesis was not statistically significant (Fig. 5B), but we suggest that a reciprocal relationship does exist. In the present study, we did not obtain enough samples which had an intermediate level of F26BP between the level in the dark (more than about 0.65 nmol mg^{-1}Chl) and the level in the light (about 0.18–0.36 nmol mg^{-1}Chl). Therefore, it is premature to exclude the possibility that the rate of sucrose synthesis is regulated by F26BP. However, F26BP in the dark was significantly higher than in the light, which suggests that cytoplasmic F16BPase *in situ* was severely inhibited in the dark. Consequently, it is unclear how starch can be converted to sucrose in the dark in maize leaves. One possible way is a route involving starch breakdown followed by cytoplasmic F16BPase action within the bundle sheath cell. Recently it was reported that there was substantial activity of F16BPase in isolated maize bundle sheath cells in the dark (23). The activity observed could be due to the cytoplasmic form of the enzyme. If this is so, the enzyme would be free from regulation of F26BP, which occurs mainly in the mesophyll (17). However, the regulation of starch breakdown and sucrose synthesis in maize leaves at night remains a topic for future investigation.

SPS is another point of regulation in the sucrose biosynthetic pathway. The substrates of this enzyme, F6P and UDPG, fluctuated only moderately over a 24 h light-dark cycle, and it is noteworthy that there is a significant relationship between these metabolites and the rate of sucrose synthesis (data not shown). Furthermore, G6P, an allosteric activator of spinach SPS (3) showed only minor fluctuations (Fig. 1C). Although the actual concentrations of these metabolites in the mesophyll cytoplasm, where SPS is localized (22), was not determined, these results nonetheless suggest that fluctuations in SPS substrate and G6P concentration are not major factors that control the rate of sucrose synthesis. However, it is possible that the concentration of Pi fluctuates diurnally. Because Pi is an inhibitor of SPS (3),

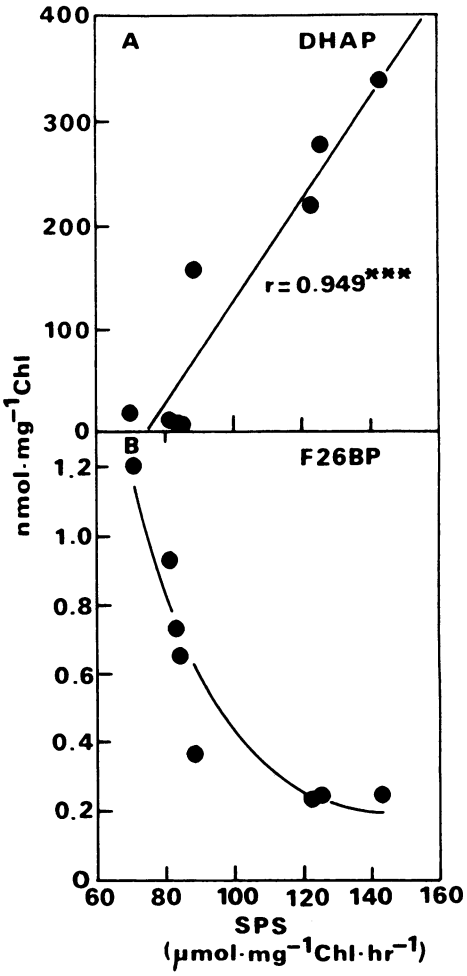

FIG. 6. Relation between SPS activity and leaf concentrations of (A) DHAP and (B) F26BP in maize during a day-night cycle. Data for SPS activities are from Figure 3, curve A in Ref. 11. Leaf concentration of DHAP and F26BP at noon was averaged from the results obtained in two separate experiments. ***, $P < 0.001$. Where $n = 8$, significance at the 0.1% level occurred at $r = 0.925$.

the changes in Pi concentration may influence enzyme activity *in situ*. A factor which may have influenced the rate of sucrose synthesis was the activity of SPS, which changed dramatically during day and night (11). These results indicated that SPS activity is modulated by light, but the exact mechanism of the

light modulation of SPS is not known at present. The relationship between the concentration of DHAP or F26BP and the activity of SPS was studied using the results obtained in this study and an accompanying study (11) (Fig. 6). The leaf concentration of DHAP was positively and significantly correlated with the activity of SPS. On the other hand, F26BP concentration was inversely related to the activity of SPS. Neither DHAP nor F26BP had any effect on the activity of maize SPS *in vitro* (data not shown). However it is possible that the level of either or both of these metabolites may be involved in the light modulation of SPS *in vivo*. Further studies will be required to understand the mechanism of light modulation of SPS, and whether metabolites are involved in the process.

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