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Leaf Phosphate Status, Photosynthesis and Carbon Partitioning in Sugar Beet

II. Diurnal Changes in Sugar Phosphates, Adenylates, and Nicotinamide Nucleotides

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ABSTRACT

Sugar Beets (*Beta vulgaris* L. cv F58-554H1) were cultured hydroponically in growth chambers. Leaf orthophosphate (Pi) levels were varied nutritionally. The effect of decreased leaf phosphate (low-P) status was determined on the diurnal changes in the pool sizes of leaf ribulose 1,5-bisphosphate (RuBP), 3-phosphoglycerate (PGA), triose phosphate, fructose 1,6-bisphosphate, fructose-6-phosphate, glucose-6-phosphate, adenylates, nicotinamide nucleotides, and Pi. Except for triose phosphate, low-P treatment caused a marked reduction in the levels of leaf sugar phosphates (on a leaf area basis) throughout the diurnal cycle. Low-P treatment decreased the average leaf RuBP levels by 60 to 69% of control values during the light period. Low-P increased NADPH levels and NADPH/NADP$^+$ ratio but decreased ATP; the ATP/ADP ratio was unaffected. Low P treatment caused a marked reduction in RuBP regeneration (RuBP levels were half the RuBP carboxylase binding site concentration) but did not depress PGA reduction to triose phosphate. These results indicate that photosynthesis in low-P leaves was limited by RuBP regeneration and that RuBP formation in low-P leaves was not limited by the supply of ATP and NADPH. We suggest that RuBP regeneration was limited by the supply of fixed carbon, an increased proportion of which was diverted to starch synthesis.

Photosynthesis is inhibited in plants grown with an insufficient supply of Pi (3–6, 9, 19, 22, 25, 29). Photosynthetic CO_2 fixation in low-P plants may be limited by either RuBPCase[1] activation or by RuBP regeneration (4, 5, 22). RuBP regeneration in turn may be limited by ATP supply, either because low-P treatment diminishes photosynthetic electron transport capacity, or because there is insufficient Pi available for the phosphorylation of ADP to ATP. With regard to the latter point, several researchers have proposed that the rate of Pi regeneration (from the conversion of phosphorylated intermediates to starch and sucrose) may limit the rate of photosynthesis under certain conditions (15, 24, 27, 33).

In part I of this series (22), we showed that the reduction in the rate of photosynthesis in low-P leaves did not appear to be due to effects on RuBPCase activity, suggesting that low-P effects on photosynthesis were mediated through RuBP regeneration. This same study showed that the activities of several other key enzymes of the Calvin cycle were significantly affected by low-P (22). Based on these and other results (20, 21), we proposed that the decrease in photosynthesis with low-P may be due to the diversion of fixed carbon from RuBP regeneration toward starch synthesis (21, 22). This hypothesis is further explored in the present study by measuring changes in leaf metabolites. We show that low-P decreased photosynthesis through an effect on RuBP regeneration and that this effect is not due to ATP and NADPH limitation. Because very few studies monitor the changes in the levels of leaf metabolites over time during the 24 h cycle, and because biochemical pathway patterns may change with time during the light and dark (*e.g.* starch and sucrose syntheses and degradations), we followed the changes in leaf metabolites diurnally.

MATERIALS AND METHODS

Plant Culture

Sugar beets (*Beta vulgaris* L. cv F58-554H1) were cultured hydroponically in growth chambers at 25°C, 500 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PFD and a 16 h photoperiod (28). Low-P and control plants were obtained by growing the plants at Pi concentrations of 0.05 and 1.0 mM, respectively (22). All measurements were carried out using recently expanded leaves.

Leaf Sampling and Extraction of Metabolites

Samples were prepared after 8 h of continuous darkness at 25°C in the growth chamber for the extraction of leaf metabolites in intact leaf tissue. The plants were then illuminated in the growth chamber at 500 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PFD and samples were prepared at 2, 4, 6, 10, and 16 h after illumination and 2 and 8 h after darkness. At each time point, four leaf discs (3.88 cm^2 each) were punched and frozen rapidly in liquid N_2 using a custom-built leaf punch machine. Adenylates (ATP, ADP, AMP), oxidized nicotinamide nucleotides (NADP$^+$, NAD$^+$), RuBP, PGA, triose-P, FBP, F6P, and G6P were

[1] Abbreviations: RuBPCase, ribulose 1,5-bisphosphate carboxylase/oxygenase; ADPG, adenosine 5′-diphosphoglucose; F6P, fructose-6-phosphate; FBP, fructose 1,6-bisphosphate; G3P, glyceraldehyde-3-phosphate; G6P, glucose-6-phosphate; IMP, inosine 5′-monophosphate; K_{cat}, catalytic constant; PGA, 3-phosphoglycerate; F_A, assimilatory force; Ru5P, ribulose 5-phosphate; RuBP, ribulose 1,5-bisphosphate; triose-P, dihydroxyacetone phosphate + glyceraldehyde-3-phosphate; UDPG, uridine 5′-diphosphoglucose.

extracted by grinding the leaf material in 12% $HClO_4$ (four leaf discs/4 mL) in a liquid N_2-cooled mortar and pestle. The extracts were left for 1 h on ice and centrifuged at 10,000*g* for 10 min at 4°C. The supernatant was then neutralized with 10 N KOH. The $KClO_4$ precipitate was removed from the extract by centrifugation in a microfuge (Eppendorf: model 5414). Reduced nicotinamide nucleotides (NADPH, NADH) were extracted in 1 N NaOH instead of $HClO_4$. The extracts were boiled for 5 min and rapidly cooled before centrifugation. The supernatants were neutralized with 6 N HCl. All the extracts except for leaf adenylates were decolorized with activated charcoal (20 mg/1.2 mL) before centrifugation.

Assay of Metabolites

RuBP was determined by ^{14}C incorporation into PGA as described in Badger *et al.* (2) using purified RuBP carboxylase (Sigma). Assays were run in 300 μL total volume (100 mM Bicine-NaOH [pH 8.0], 20 mM $MgCl_2$, 13 mM $NaH^{14}CO_3$ (50 Bq $nmol^{-1}$), 50 μg of purified RuBP carboxylase [EC 4.1.1.39] with 100 μL of sample per assay. Duplicate assays were run for 30 min at room temperature; the reactions were terminated by adding 0.2 mL of 6 N acetic acid. The samples were dried under an air stream and counted by liquid scintillation spectrometry. PGA was determined as described in Usuda (31). The reaction mixture contained: 40 mM Hepes-KOH (pH 7.8), 5 mM ATP, 0.2 mM NADH, 5 mM phosphocreatine, 10 units/mL of creatine phosphokinase (EC 2.7.3.2), 5 units/mL of NAD-G3P dehydrogenase (EC 1.2.1.12), and 5 units/mL of PGA-kinase (EC 2.7.2.3). The reaction was initiated by the addition of an aliquot of sample. Triose phosphates were determined by the addition of 5 units/mL triose phosphate isomerase (EC 5.3.1.1) and 0.4 units/mL glycerophosphate dehydrogenase (EC 1.1.1.8) to the assay medium containing: 50 mM Hepes-KOH (pH 8.0), 20 mM $MgCL_2$, 10 mM KCl, 1 mM EDTA, 0.3 mM NADH, and an aliquot of sample (14). FBP, F6P, and G6P levels were determined in an assay consisting of: 50 mM Hepes-KOH (pH 7.6), 5 mM $MgCl_2$, 1 mM EDTA, 0.2 mM $NADP^+$, an aliquot of sample, and the sequential addition of 0.2 units/mL glucose-6-P dehydrogenase (EC 1.1.1.49) for determining G6P, 0.4 units/mL phosphoglucoisomerase (EC 5.3.1.9) for determining F6P, and 0.05 units/mL of FBPase (EC 3.1.3.11) for determining FBP (14). Leaf adenylates were determined according to Fader and Koller (8). Leaf nicotinamide nucleotides were determined according to Maciejewska and Kacpereska (16). The leaf Pi levels were estimated as described before (9). By grinding leaf samples in the mortar with and without addition of standards, the percentage recovery of each metabolite was estimated. The recovery of the various metabolites in the above procedure was higher than 78%. The data presented are not corrected for calculated loss of metabolites.

Chemicals

All compounds used were purchased from Sigma Chemical Co. with the exception of $NaH^{14}CO_3$ (Amersham).

RESULTS

Diurnal Changes in Leaf Sugar-P

Leaf RuBP levels in control plants increased to high levels in less than 2 h and then remained fairly constant with time up to 16 h (Fig. 1). With the onset of darkness, RuBP levels decreased substantially within 2 h. RuBP levels in low-P leaves changed similarly with time. However, low-P treatment caused a marked reduction in RuBP during the light: it decreased the average leaf RuBP levels (over 2–16 h) by 60 to 69% of control values on a leaf area basis during the light period. Low-P treatment did not affect RuBP levels in darkness.

Leaf PGA levels in control leaves changed with time in a manner similar to the changes with time for RuBP but the changes were much less pronounced (Fig. 1B). In low-P leaves, PGA levels exhibited no significant increase with time. Unlike RuBP, PGA levels were markedly diminished by low-P treatment in darkness as well as light. These results show that low-P treatment decreased leaf PGA more than the leaf RuBP: during the light period, the average PGA level was decreased by 79% while in darkness it was decreased by 69%.

Low-P treatment had much less effect on leaf triose-P levels (Fig. 1C) than it did on PGA and RuBP levels. On average,

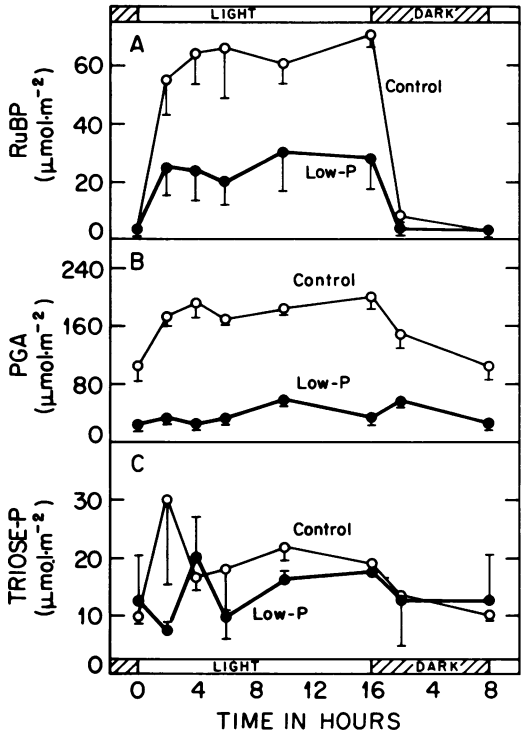

Figure 1. Effect of low-P treatment on the diurnal changes in RuBP (A), PGA (B), and triose-P (C) levels in sugar beet leaves. Values are mean ± SD for three replications. (○), Control; (●), low-P. The mean Chl content of leaves for control and low-P treatment were 428 mg·m^{-2} and 529 mg·m^{-2}, respectively. Note that 10 μmol·m^{-2} of a metabolite is equivalent to 23.4 and 18.9 nmol·mg^{-1} Chl for control and low-P leaves, respectively. This content is equal to an intracellular concentration of 0.94 mM (control) and 0.76 mM (low-P) if confined to the chloroplast or cytosol with a volume of 25 μL·mg^{-1} Chl.

the triose-P levels in light were slightly lower in low-P *versus* control leaves, and in darkness there was no difference between the two treatments: the average light values were 20 and 14 $\mu mol \cdot m^{-2}$ in control and low-P leaves, respectively.

Low-P treatment decreased PGA much more than it decreased RuBP as is illustrated by the changes in PGA/RuBP ratio (Fig. 2A). Despite the reduction in PGA, low-P treatment had little effect on the level of triose-P and the triose-P/PGA ratio was much higher in low-P leaves (Fig. 2B). The RuBP/triose-P ratio was lower in low-P leaves over most of the light period but there was no difference in darkness (Fig. 2C).

The data show that FBP increased during the first 2 h of illumination and then remained fairly constant with time during the next 14 h (the variations with time are not statistically significant) (Fig. 3A). With the onset of darkness, FBP in low-P leaves decreased to its minimum night level within 2 h while in control leaves it required longer than 2 h for this to occur. The changes with time for FBP mirrored those for RuBP. Low-P treatment decreased the level of leaf FBP both in light and darkness: the average FBP level in the light decreased by 41% while the average dark level decreased by 58%.

The changes with time and low-P treatment for F6P and G6P (Fig. 3, B and C) were similar to those for PGA (Fig. 1) in that there were relatively small increases in the light in control plants, and in that low-P treatment decreased dark values of these metabolites substantially. Low-P treatment had a much greater effect on F6P and G6P levels than it did on FBP levels (Fig. 3, B and C). If one averages the data over 2 to 16 h of the light period, low-P treatment decreased F6P by 64% and G6P by 77%. The F6P and G6P data also differed from the FBP data in that there was much less change between light and dark. By comparing the average light value (data from 2–16 h of light) with the average dark value (data at beginning of light period and at the end of the dark period), we determined that there were significant increases (on illumination) for F6P (but not G6P) in control as well as in low-P leaves. The average light FBP/F6P ratio in low-P leaves was increased by 63% while the G6P/F6P ratio was decreased by 32% when compared to control values. The data with time were too variable to determine precisely how F6P and G6P changed with time but they suggest that, in control leaves, both these hexose phosphates increased over the first 4 h of light and that they remained high for 2 h of darkness before dropping to their minimum night values.

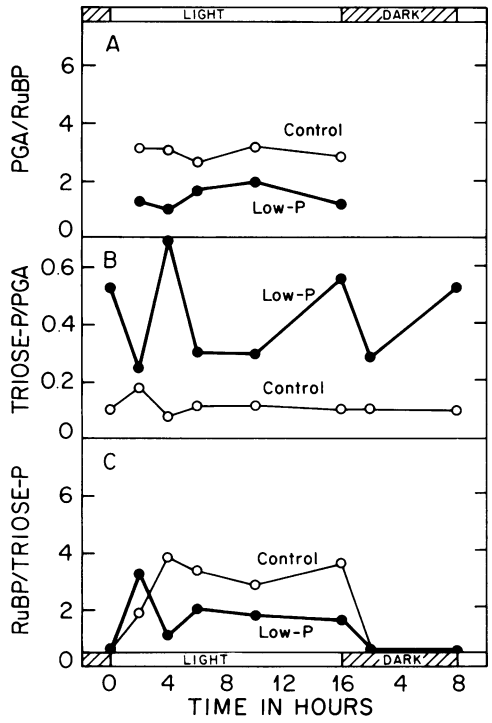

Figure 2. Effect of low-P treatment on the diurnal changes in the ratios of PGA/RuBP (A), triose-P/PGA (B), and RuBP/triose-P (C) in sugar beet leaves. (○), Control; (●) low-P.

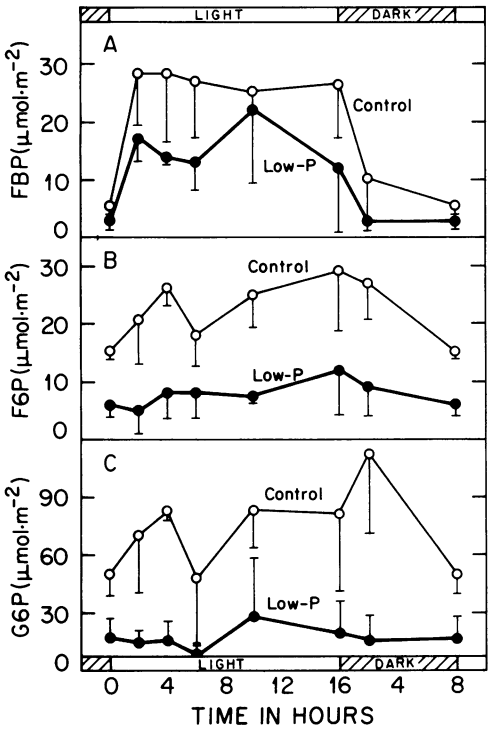

Figure 3. Effect of low-P treatment on the diurnal changes in FBP (A), F6P (B), and G6P (C) levels in sugar beet leaves. Values are mean ± SD for three replications. (○), Control; (●), low-P.

Diurnal Changes in Leaf Adenylates

Low-P treatment decreased leaf ATP (Fig. 4A) and total adenylates (Fig. 4C) appreciably but had little effect on the ATP/ADP ratio (Fig. 4B). In control leaves ATP levels increased over the first 2 h then remained fairly constant with time until darkness when there was a slow decline. In low-P leaves, ATP levels appeared to increase slowly with time over the first 10 h of the light period, then become constant before declining slowly in darkness. Somewhat similar patterns with time for each treatment occurred for total adenylates. The average ATP value over 2 to 16 h of light was 22.5 $\mu mol \cdot m^{-2}$; the average total adenylate value over the same period was 46 $\mu mol \cdot m^{-2}$. With low-P treatment, the average

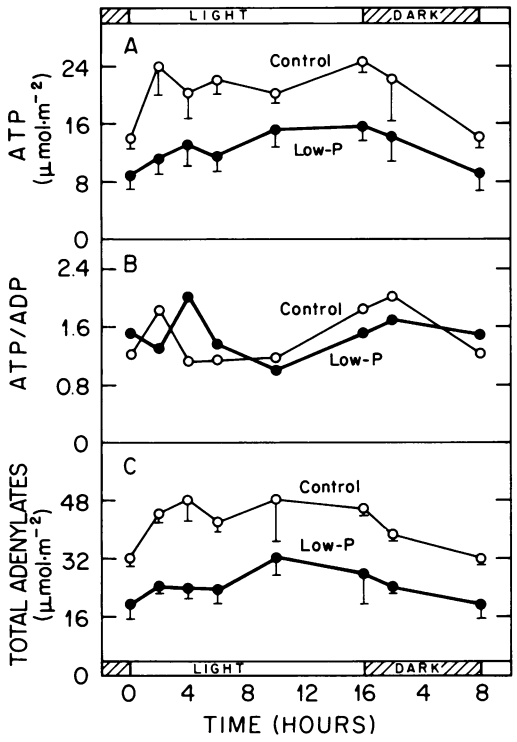

Figure 4. Effect of low-P treatment on the diurnal changes in ATP (A), ATP/ADP ratio (B), and total adenylates (C) in sugar beet leaves. Values are mean ± SD for at least three replications. (○), Control; (●), low-P.

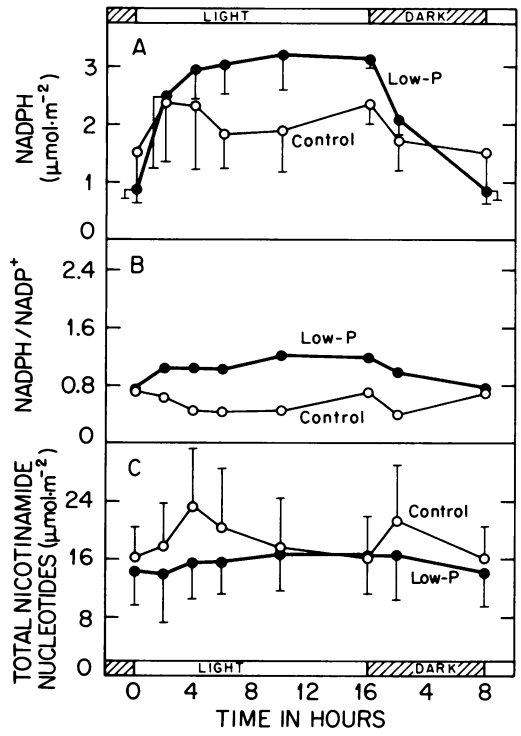

Figure 5. Effect of low-P treatment on the diurnal changes in NADPH (A), NADPH/NADP$^+$ ratio (B), and total nicotinamide nucleotides (C) in sugar beet leaves. Values are mean ± SD for at least three replications. (○), Control; (●), low-P.

values were decreased by 40% for ATP and by 43% for total adenylates.

Diurnal Changes in Leaf Nicotinamide Nucleotides

Leaf NADPH levels increased sharply during the first 2 h of illumination (Fig. 5A). Surprisingly, the increase was more pronounced in low-P than in control leaves. Low-P treatment caused a marked increase in leaf NADPH which continued up to 10 h of illumination (in control leaves the increase peaked at 2 h). The average NADPH value over 2 to 16 h was 37% higher in the low-P compared to the control leaves. In darkness, the average value was 22% lower in low-P compared to control leaves.

The increase in NADPH with low-P treatment was due to an increase in NADPH/NADP$^+$ ratio (Fig. 5B) and to an increase in NADP$^+$ + NADPH/NAD$^+$ + NADH ratio (data not shown); it was not due to an increase in total nicotinamides (Fig. 5C). The NADPH/NADP$^+$ ratio was greater over most of the 24-h cycle. The total nicotinamide nucleotides were slightly higher in the control *versus* the low-P leaves, the average value over the 24 h period being 18.3 μmol·m^{-2} in the control and 15.2 μmol·m^{-2} in low-P leaves.

DISCUSSION

The results suggest that low-P treatment affects photosynthesis more through RuBP regeneration than through the activation of RuBPCase. We estimate the RuBPCase binding site concentration in sugar beets to be 46 μmol·m^{-2} for controls and 48 μmol·m^{-2} for low-P leaves. These values for binding site concentration were estimated assuming a catalytic turnover number (K_{cat}) of 3.25 mol CO_2 fixed per mol enzyme sites per second (32) and a total activity of the enzyme of 151 and 157 μmol CO_2·m^{-2}·s^{-1} for control and low-P leaves, respectively (22). Assuming a stromal volume of 25 μL·mg^{-1} Chl (13) and a Chl concentration of 428 mg·m^{-2} for control and 529 mg·m^{-2} for low-P, this yields a binding site concentration of 4.3 mM for the control and 3.6 mM for low-P leaves. Our calculations show that in the light the RuBP levels in the control (about 65 μmol·m^{-2}, Fig. 1A) were approximately 1.4 times the binding site concentration (consistent with previous reports, 2, 18, 32, 33) while in low-P leaves, RuBP (about 25 μmol·m^{-2}, Fig. 1A) was about half the binding site concentration. Thus, these results clearly suggest that photosynthesis in low-P plants was limited by RuBP regeneration rather than by RuBPCase activation state (although in fairness it should be pointed out that our calculations assume that the values for K_{cat} and stromal volume were unchanged by low-P treatment).

Assuming that low-P treatment influences photosynthetic rate via RuBP regeneration, how is RuBP regeneration affected in turn by low-P? One possibility is that RuBP regeneration is limited via effects of low-P on the light reactions, and, in particular, on the supply of ATP and NADPH to the stromal enzymes of the Calvin cycle. Earlier research (1) showed that low-P treatment does not impair the capacity of the photochemical apparatus and the present work, especially

in regard to NADPH, supports that view: NADPH, and the NADPH/$NADP^+$ ratio, were increased by low-P treatment, suggesting that there was an excess production of reducing power relative to its consumption in the reactions of the Calvin cycle. Total nicotinamide nucleotides were unchanged by low-P treatment and the increase in NADPH with low-P treatment was due to the reduction of $NADP^+$ leading to high ratios of NADPH/$NADP^+$, and not to increases in $NADP^+$ + NADPH.

Low-P treatment could also have diminished RuBP regeneration via the level of ATP, either because there was insufficient electron transport capacity or because there was insufficient Pi for photophosphorylation (10, 12). The absolute amount of ATP was substantially decreased by low-P treatment. Working with isolated chloroplasts, Giersch and Robinson (12) proposed that with insufficient Pi there was insufficient ATP formation for the phosphorylation of PGA to triose phosphate and/or for the phosphorylation of Ru5P to RuBP. Here, with sugar beet leaves, low-P treatment did not decrease the amounts of triose-P and substantially increased the triose-P/PGA ratio, suggesting that the decreased amounts of ATP in low-P leaves did not impair the capacity of leaves to phosphorylate PGA to triose-P. Furthermore, if the supply of ATP was not limiting the reduction of PGA, it is unlikely that it would limit the phosphorylation of Ru5P since the K_m for ATP is markedly lower for the Ru5P kinase reaction than for PGA kinase [K_m(ATP) for PGA kinase and Ru5P kinase are 0.7 mM and 35–65 μM, respectively] (15, 23). However, it is possible that Ru5P kinase limited RuBP formation since its initial activity was decreased by low-P treatment (22).

An alternative approach to evaluating whether the supply of photochemical energy is limiting RuBP regeneration is embodied in the concept of F_A developed by Dietz and Heber (7). Using their equations and a pH value of 7.8 (7, 11), we calculated F_A from steady state concentrations of triose-P, PGA, and H^+. Low-P treatment increased the F_A throughout the light period (the average light values of F_A for control and low-P leaves were 94 and 254 M^{-1}, respectively). Thus, the increased triose-P/PGA and F_A values in low-P leaves clearly indicate that the synthesis of triose-P was not limited by the supply of ATP and NADPH.

There was no evidence from these results that low-P led to less ATP due to insufficient Pi for the phosphorylation of ADP to ATP. Rather, the data show that on illumination of low-P leaves, net adenylate accumulation was reduced. Similar effects of low-P were observed with *Lemna gibba* (30). The low adenylate levels observed in low-P leaves may be due to the combined effect of lower rates of adenylate synthesis and higher rates of adenylate degradation (17). Adenylates are synthesized via the IMP pathway which utilizes ribose-5-P for its precursor. Since low-P treatment seems to decrease sugar P to a marked degree, especially those involved in RuBP regeneration from triose-P, it seems likely that the reduction in adenylates may have been attributable to a reduction in ribose-5-P.

Since low-P treatment did not appear to affect RuBP regeneration via the supply of ATP and/or NADPH, how might low-P impair RuBP regeneration? In addition to lower levels of RuBP in low-P leaves, there was also lower levels of FBP, F6P, and G6P. Triose-P, on the other hand, were not affected by low-P. This suggests that RuBP formation may have been reduced by diversion of photosynthates away from RuBP formation. Since low-P plants have increased activities of FBPase, FBP aldolase (22), and ADPG pyrophosphorylase (21), it is possible that there was an increased flow of carbon to starch with a decreased flow of carbon toward RuBP synthesis. Other research from this laboratory (9, 21) suggests that low-P treatment results in an increased partitioning of fixed carbon in starch (compared to sucrose synthesis). According to this view, low-P treatment would reduce RuBP regeneration because a greater proportion of incoming carbon is going to starch synthesis than in the controls.

Another question posed in this paper is whether or not the pattern of biochemical responses to low-P changes during the day. Previous studies on P nutrition tested the effects only at one time point during light period (4, 6, 25). The levels of most metabolites measured increased during the light period and decreased in darkness. For the most part, the levels of metabolites remained fairly constant after the first 2 h of light and did not change with time. Thus, there was no evidence that the factors controlling photosynthesis (*i.e.* RuBP *versus* RuBPCase activation state) changed during the 16 h light period. Both RuBP and FBP changed substantially from light to dark suggesting that RuBPCase and FBPase were subject to tight on-off regulation with light-dark transitions (15, 33).

The diurnal changes in leaf metabolites observed in this research with sugar beet exposed to a 16 h daylength were very similar to those observed by Gerhardt *et al.* (11) for spinach plants exposed to a 9 h photoperiod. They observed that RuBP, FBP, and DHAP were barely detectable in the dark and increased 20- to 100-fold in the light. For these three metabolites, only small changes occurred during the day, whereas light/dark changes were very large. A second group of metabolites, which included PGA, F6P, G6P, and UDPG, was present in the dark in considerable amounts and rose

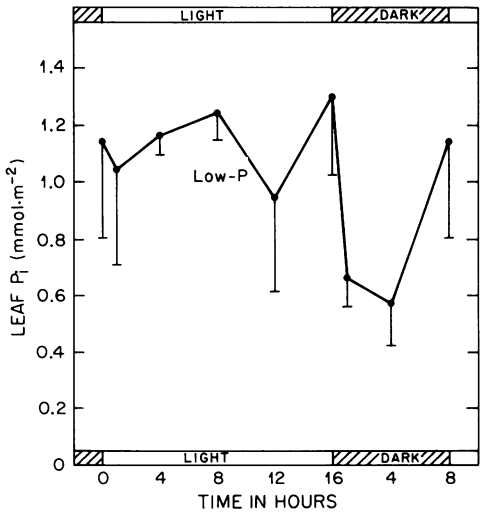

Figure 6. Diurnal changes in Pi levels of low-P sugar beet leaves. Values are mean ± SD for three replications. (The leaf Pi levels in control leaves did not change during the diurnal cycle; see also Sicher and Kremer [25].)

only slightly after illumination. Triose-P in our study was present in a greater amount in the dark, possibly because our dark period (8 h) was much shorter than theirs (15 h) so that there was less time to diminish to very low levels.

Working with P-deficient barley seedlings, Sicher and Kremer (25) observed a light-induced decrease in leaf Pi. They assumed that under low-P conditions, most of the Pi in the leaf was nonvacuolar. They therefore attributed the decrease in leaf Pi in the light to the photosynthetic conversion of Pi to organic-P. With low-P sugar beet, however, we did not observe a decrease in leaf Pi on illumination (Fig. 6). This may be because the leaf Pi values here included a substantial vacuolar component. The data of Figure 6 show a decrease in leaf Pi from light to dark. This may be due to transport of Pi out of the leaf to other sinks in darkness or to the consumption of Pi in such processes as the phosphorolysis of starch.

Based on our measurements of ATP, ADP, NADPH, $NADP^+$, triose-P, and PGA in leaves, we estimated the nonvacuolar Pi concentration in control and low-P leaves using the same equation that was used to calculate F_A (7). We assumed a pH value of 7.8 and a nonvacuolar volume of 40 $\mu L \cdot mg^{-1}$ Chl (15, 26). The average light Pi concentrations in nonvacuolar compartments were 0.39 $\mu mol \cdot mg^{-1}$ Chl (10.2 mM) and 0.30 $\mu mol \cdot mg^{-1}$ Chl (7.8 mM) for control and low-P leaves, respectively. However, these calculations are based on the assumption that the extent of binding for adenylate and nicotinamide nucleotide pools was similar for control and low-P treatments. These estimated values suggest that plants growing under low-P conditions may conserve nonvacuolar Pi to maintain metabolism: in fact, Pi concentrations were high enough to support fairly high rates of photosynthesis (65% at light saturation) (19, 22).

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