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Leaf Carbon Metabolism and Metabolite Levels during a Period of Sinusoidal Light[1]

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ABSTRACT

Photosynthesis rate, internal CO_2 concentration, starch, sucrose, and metabolite levels were measured in leaves of sugar beet (*Beta vulgaris* L.) during a 14-h period of sinusoidal light, which simulated a natural light period. Photosynthesis rate closely followed increasing and decreasing light level. Chloroplast metabolite levels changed in a manner indicating differential activation of enzymes at different light levels. Starch levels declined during the first and last 2 hours of the photoperiod, but increased when photosynthesis rate was greater than 50% of maximal. Sucrose and sucrose phosphate synthase levels were constant during the photoperiod, which is consistent with a relatively steady rate of sucrose synthesis during the day as observed previously (BR Fondy *et al.* [1989] Plant Physiol 89: 396–402). When starch was being degraded, glucose 1-phosphate level was high and there was a large amount of glucose 6-phosphate above that in equilibrium with fructose 6-phosphate, while fructose 6-phosphate and triose-phosphate levels were very low. Likewise, the regulatory metabolite, fructose, 2,6-bisphosphate was high, indicating that little carbon could move to sucrose from starch by the triose-phosphate pathway. These data cast doubt upon the feasibility of significant carbon flow through the triose-phosphate pathway during starch degradation and support the need for an additional pathway for mobilizing starch carbon to sucrose.

During a period of sinusoidal light, which simulates a natural diurnal light period, measured changes occur in rates of leaf gas exchange and allocation of newly fixed carbon between starch and sucrose (9). However, when the photoperiod is started and stopped abruptly with high light in a stepped light regime, responses of photosynthesis rate, starch level, export rate and sucrose level are rapid during the initial and final 1 to 2 h of the light period, but practically no change in metabolism occurs during the major part of the photoperiod (7, 8). The level of chloroplastic and cytosolic metabolites also increase rapidly at the onset of high light (25). A sudden change in irradiance can under certain conditions bring about a series of large oscillations in photosynthesis rate and related metabolite levels which dampen out gradually as control is restored (25, 31). With the exception of a recent study conducted on maize (29), little is known of how intermediary metabolite levels change in response to a sinusoidal light regime.

The purpose of this study was to identify and characterize the mechanisms regulating NCE[2] rate and carbon allocation between starch and sucrose during a 14-h period of gradually increasing and decreasing light. Our approach was to compare changes in the rates of transpiration, photosynthesis, and sucrose synthesis and export as measured previously (9), to changes in the levels of leaf metabolites present at various times during a single period of sinusoidal light. The data indicate that during the period of gradually changing light an ordered, sequential activation of enzymes occurs, resulting in an efficient induction of carbon flow through the Calvin cycle to starch and sucrose. Because the change in light level during a natural regime is slow and steady, it appears that orderly activation of enzymes is needed to maintain metabolite levels and allow carbon metabolism to pace changes in light level.

MATERIALS AND METHODS

Plant Material

Sugar beet (*Beta vulgaris* L. Klein E type multigerm) plants were grown as described previously (9) under a combination of metal halide and sodium vapor lamps (Sunbrella, EGC, Chagrin Falls, OH) at a maximal PPFD of 0.6 mmol photons m^{-1} s^{-1} and 14-h photoperiod (25°C day/15°C night).

Gas Exchange Measurement

The day before measurements were made a mature leaf was sealed into an aluminum leaf chamber for measuring NCE. The leaf chamber was connected to a closed system containing an infrared gas analyzer (Mine Safety Co., Pittsburgh, PA) for measuring rate of CO_2 depletion in the system, a reservoir, and a controller to maintain CO_2 concentration between 325 and 375 μL/L. Relative humidity was maintained at 70% by means of a condensor and leaf temperature was maintained at 24 ± 2°C. Air was circulated at a rate of 1.8 L min^{-1}. The experimental period comprised a 14-h period of sinusoidal light generated using the apparatus described previously (10).

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[2] Abbreviations: NCE, net carbon exchange; C_i, leaf internal CO_2 concentration; rubisco, ribulose 1,5-bisphosphate carboxylase/oxygenase; F26BP, fructose 2,6-bisphosphate; FBP, fructose 1,6-bisphosphate; PGA, 3-phosphoglyceric acid; RuBP, ribulose-1,5-bisphosphate; F6P, fructose 6-phosphate; SPS, sucrose 6-phosphate synthase; G6P, glucose 6-phosphate; G1P, glucose 1-phosphate.

Transpiration rate was determined from the difference in the water vapor content of the air entering the leaf chamber and that surrounding the leaf, measured with a humidity probe (Vaisala Humicap, Helsinki, Finland). Leaf temperature was measured with a 150-μm diameter iron-constantan thermocouple. Intercellular CO_2 concentration was calculated from measurements of NCE, transpiration, rate of air flow over the leaf, air and leaf temperature as described by Fields *et al.* (6).

Metabolite Extraction and Measurement

Two sugar beet leaves, adjacent to the leaf for measuring NCE, were sampled during the light period by freeze-clamping 5-cm^2 sections of leaf tissue between liquid N_2-chilled pieces of brass (21). The time required to freeze the leaf section was about 0.1 s. Perchloric acid extracts were prepared as described previously (21). RuBP was measured by the incorporation of $^{14}CO_2$ into acid-stable products in the presence of purified rubisco (15). Other metabolites were measured spectrophotometrically in coupled enzyme assays according to Lowry and Passonneau (17). Wheat rubisco (EC 4.1.1.39) was a gift from M. Parry (Rothamsted Experiment Station, Harpenden, Herts, UK). Other enzymes were purchased from Boehringer-Mannheim (Indianapolis, IN).

For F26BP measurements, freeze-clamped leaf tissue was extracted with 1.2 mL of a mixture of 50 mM Tris base, 5 mM EDTA, and 10 mM 2-mercaptoethanol:methanol:chloroform (20:65:25). After centrifugation (12,000*g*, 5 min), the pellet was mixed vigorously for 1 min with 1 mL of 1 M NH_4HCO_3 (pH 10) (12). The supernates were combined and 1 mL of chloroform added. After mixing and centrifugation to separate the layers, the organic lower layer was removed, taken to 10 mL with ethanol, and Chl concentration (μg/mL) determined spectrophotometrically at A_{654} (21). The aqueous layer was removed and evaporated to dryness under reduced pressure at 40°C. The residue was dissolved with 1 mL of water. F26BP was assayed according to van Schaftigan *et al.* (30) using 6-phosphofructokinase (pyrophosphate-dependent) (EC 2.7.1.90) purified from potato tubers (30) and internal standards of 1.25, 2.5, and 5 pmol of F26BP. Recovery of F26BP (5 pmol) added to the tissue during extraction was about 75 $\pm$ 10%. Elimination of the NH_4HCO_3-extraction step resulted in recoveries of only about 45%.

SPS Measurement

Leaf samples (10 cm^2) were collected by freeze-clamping and stored in liquid N_2 until assay. Extraction, gel filtration, and assay of the leaf tissue was the same as that described by Sicher and Kremer (23). Protein was determined using a dye-binding assay as described previously (21).

RESULTS AND DISCUSSION

Response of NCE to Sinusoidal Light

Net carbon exchange rate increased gradually with increasing light level (Fig. 1A) and sometimes saturated at about 0.6 mmol photons m^{-1} s^{-1} before the maximum light level was reached. Intercellular CO_2 concentration decreased with increasing light level initially during the first hour (Fig. 1B), remained constant for the next 8 h, and then increased as the light level decreased.

In the experiment shown in Figure 1, the NCE rate was higher at a given light level with increasing light than with decreasing light. In other plants a symmetric response to increasing and decreasing light is seen (9). The asymmetric response of NCE to increasing and decreasing light is unexpected and its cause is unknown. An NCE rate which was higher earlier in the day, than later in the day was also observed in alfalfa (1) and *Eucalyptus* (14) measured under a period of natural irradiance. The rate of NCE was higher with increasing light even though C_i was about the same or even a little lower in the morning hours (Fig. 1) (14), indicating that the cause is biochemical in nature. In a separate experiment, a light response curve was constructed at various times during a sinusoidal day (data not shown). The response of NCE to a given light level decreased during the day, implying that a change in the quantum efficiency of the leaf occurred as the day progressed. Brown *et al.* (1) attributed the lower NCE in the afternoon to a higher concentration of leaf starch present at that time.

Response of Leaf Carbohydrate Levels to Sinusoidal Light

Starch level declined during the first hour of the light period, but then increased when NCE had reached a rate of 0.5 μg C cm^{-2} min^{-1} (Fig. 1C). Changes in the rate of starch accumulation followed closely changes in the rate of NCE. The onset of starch breakdown in the afternoon occurred at the same NCE rate at which starch began to accumulate. Sucrose levels were similar to those observed in the previous study (9), about 10 to 15 μg C/cm^2 or 200 to 300 nmol/mg protein, and remained remarkably constant throughout the entire period of measurement (Fig. 1D). In the previous study, Fondy *et al.* (9) observed that the rate of sucrose synthesis in sugar beet leaves during a sinusoidal light period was relatively steady, being higher during the middle of the day at higher light and NCE rates. The data presented here and previously (9, 13) indicate that at the beginning and end of the photoperiod, when NCE rate is insufficient to support sucrose synthesis rate, starch degradation contributes carbon to sucrose synthesis.

Calvin Cycle Function during Sinusoidal Light

Some of the metabolite data in Figure 1 represent the sum of both chloroplastic and cytosolic pools. RuBP and most of the FBP and PGA are localized in the chloroplast, while the bulk of the triose-P, F6P, G6P, and G1P are in the cytosol (11, 32). Upon increasing light level the first noticeable changes in metabolite levels were a drop in PGA from its high night time level (Fig. 1E) and a rise in the level of triose-P (Fig. 1G). FBP (Fig. 1F) was low in the dark, underwent a transient increase at the beginning of the light period, followed by a decrease in level. These initial events imply an easy flow of carbon from PGA to triose-P and FBP following a light-induced rise in the levels of ATP and NADPH. The conversion of PGA to triose-P is usually not far from equilibrium

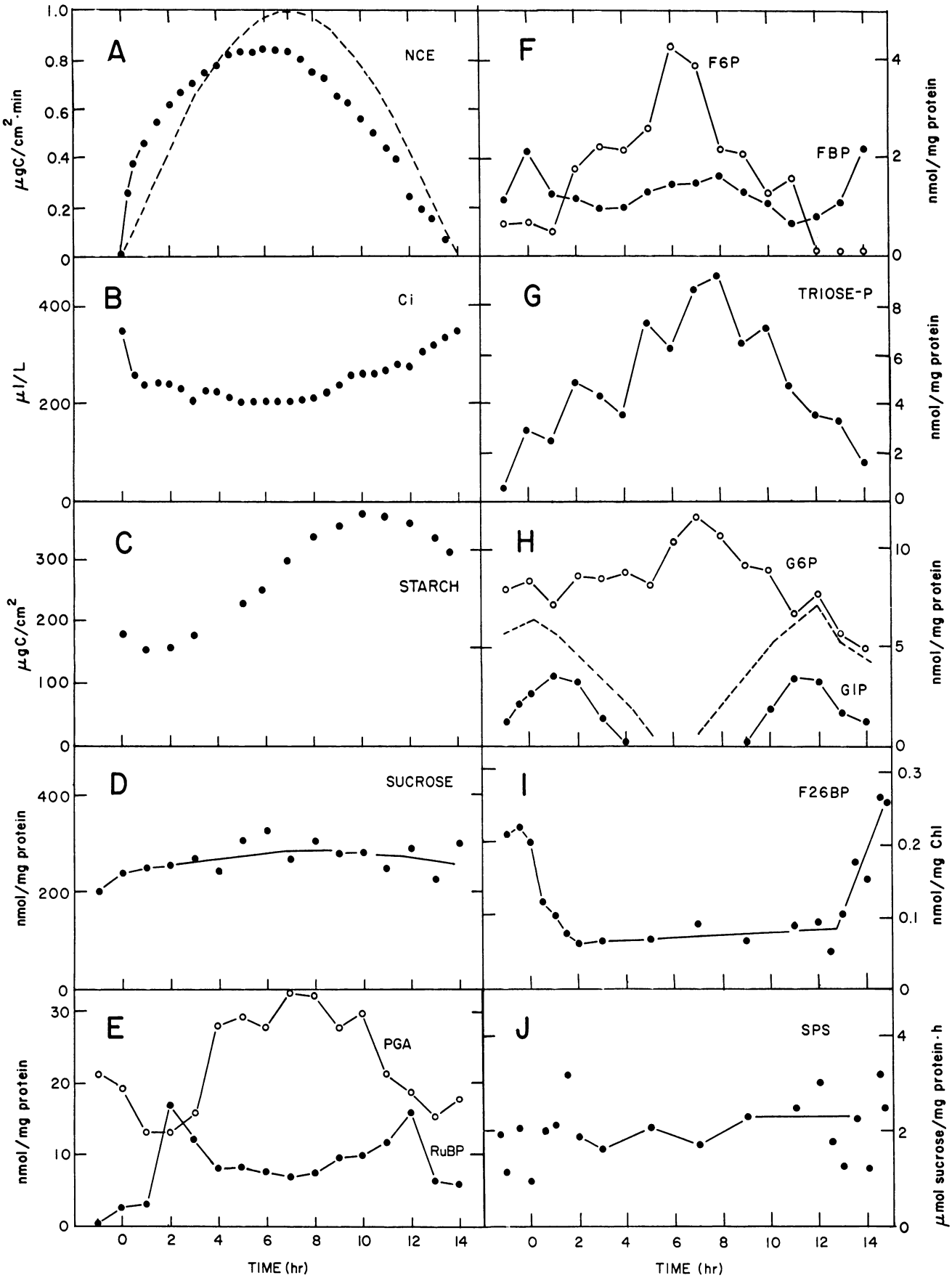

Figure 1. Time course of rate of net carbon exchange, intercellular CO_2 level, starch, sucrose, and metabolite levels, and SPS activity during a 14-h period of sinusoidal light. The dashed line in (A) represents relative light level with maximal PPFD being 0.6 mmol m^{-2} s^{-1}. The dashed line in (H) represents the nonequilibrium amount of G6P, and is the difference between the G6P level (H) and three times the F6P level (F). Light began at 0 time.

(3) and, therefore, the ratio of PGA to triose-P changes in direct proportion to the phosphorylation potential, ATP/ADP+Pi, and redox ratio of NADPH/$NADP^+$ (4).

As light level continued to increase rapidly, the FBP level continued to drop. This was followed by a sharp increase in RuBP (Fig. 1E) and F6P (Fig. 1F). The level of a metabolite is controlled both by the ease with which it is synthesized and the ease with which it is metabolized. Often, metabolite pool sizes are small in comparison to their rates of turnover and, consequently, a small imbalance between rates of synthesis and degradation can have a great effect on pool size. An important factor affecting the ease with which carbon flows through the Calvin cycle and, consequently, the levels of various metabolites is the light-activation of enzymes involved in the synthesis and breakdown of these metabolites (4). The decline in FBP level and the accompanying rise in the F6P level imply an increased activation of fructose bisphosphatase. In spinach (3), the chloroplastic FBP/F6P ratio also increases and then decreases very rapidly with increasing light. Dietz and Heber (3) concluded that chloroplastic fructose bisphosphatase is only a limitation to carbon flow at very low light intensities. The early and rapid changes observed in the levels of FBP and F6P (Fig. 1F) support this conclusion. An increasing level of assimilatory power, as indicated by the decreasing PGA level, coincided with the sharp increase in RuBP (Fig. 1E). The rise in RuBP could also imply an activation of phosphoribulokinase. Later in the day, PGA level rose as assimilatory power declined at high NCE rate.

Between 3 and 4 h after the beginning of the sinusoidal light period, RuBP level decreased and PGA level rose indicating a continuing activation of rubisco. During the middle of the day, the RuBP level was maintained at about 10 nmol/mg protein, which was near the level at which it could limit NCE rate in sugar beet (21). Under gradually increasing light, the rate of RuBP synthesis was nearly matched to its use rate and both these rates were maintained at the point of being nearly colimited at high light. Because the RuBP pool size is small in relation to the rate of flux through the pool, a small imbalance in either the rate of synthesis or use would result in a transient change in pool size. However, if the two activities remained constant or increased or decreased in a balanced manner, then no change would be observed in the RuBP level. The observed imbalance probably resulted from differences in the degree of light activation of phosphoibulokinase and rubisco.

Abrupt changes in illumination can cause wide shifts in RuBP level (18). We observed previously (21) that when the photoperiod was started with stepped light, the RuBP level quickly rose to 20 nmol/mg protein and was maintained at this level throughout the light period. Because adenylate energy charge and rubisco activation remained constant, the high RuBP level was maintained throughout the photoperiod. Under a period of sinusoidal light, RuBP level (Fig. 1E) also increased to 20 nmol/mg protein, but later declined to a near-limiting level. We presume that this occurred because of decreased synthesis, perhaps from decreasing energy charge, and increased rate of use, resulting from increased activity of rubisco.

Sucrose Synthesis from Newly Fixed Carbon

The level of F26BP, a potent inhibitor of fructose bisphosphatase (2), gradually decreased during the morning transition period, the first 2 h of the photoperiod, and then increased correspondingly during the evening transition period (Fig. 1I). A decreasing level of F26BP results in an increasing activity of cytosolic fructose bisphosphatase and an increasing flux of newly fixed carbon from the chloroplast into sucrose synthesis. If the movement of triose-P out of the chloroplast is too rapid, then the chloroplast would be quickly drained of triose-P as a result of the high dark-level of cytosolic Pi (26). Insufficient triose-P would remain to build and maintain the level of Calvin cycle intermediates. During the transition periods, when the contribution of carbon from starch to sucrose is decreasing, there is a compensating increase in the contribution of newly fixed carbon to sucrose synthesis with increasing NCE (9). The fall in F26BP level with increasing NCE very closely matched the rise in rate of sucrose synthesis from newly fixed carbon (Fig. 2A). Hence, it would appear that F26BP has an important role in phasing in of synthesis of sucrose from newly fixed carbon with increasing NCE rate during a sinusoidal light period. This phasing in occurs even as sucrose synthesis from starch degradation is decreasing. Regulation of F26BP level by changing NCE rate is also evident when NCE rate is changed by altering CO_2 concentration (22).

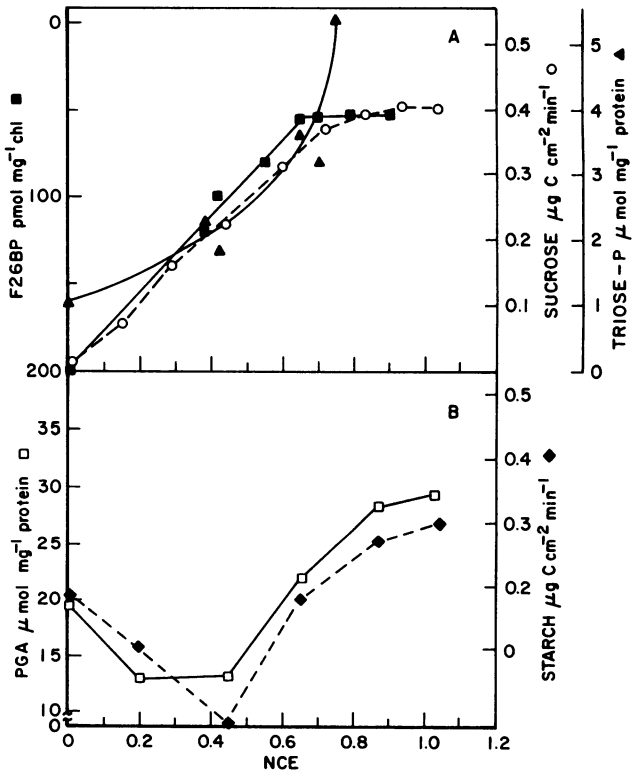

Figure 2. Changes in (A) F26BP and triose-P levels and rate of sucrose synthesis from newly fixed carbon, and (B) P-glyceric acid level and starch degradation and synthesis rate as a function of change in NCE. Data for sucrose synthesis rate is from Fondy *et al.* (Fig. 3 of Ref. 9). Starch degradation is shown by a dashed line.

Starch Synthesis From Newly Fixed Carbon

Starch degradation ceased and synthesis began when a certain NCE rate was exceeded, likewise synthesis stopped and degradation began when NCE rate was below this rate (Fig. 1, A and C). The declining rate of starch degradation coincided with a decreasing PGA level (Fig. 1D). The close relationship between PGA level and starch synthesis rate can be seen when the two are compared as a function of NCE rate (Fig. 2B). If most of the esterified phosphate in the chloroplast is present as RuBP and PGA and the total Pi level in the chloroplast is constant over a short term basis, then Pi level should decrease as esterified phosphate increases (11). The metabolite changes during a sinusoidal light period would result in a low PGA/Pi ratio during periods of low NCE and starch degradation and a high PGA/Pi level during the middle of the day when starch was being rapidly synthesized. During the transition between starch breakdown and synthesis, RuBP level was highest and PGA level was lowest. At this time the PGA/Pi ratio was momentarily very high. Although there is much evidence to support regulation of starch synthesis in isolated chloroplasts via a changing PGA/Pi ratio (for review, see Preiss [19]); the data presented here would support the occurrence of this mechanism in intact leaves. Further progress in understanding the role of the PGA/Pi ratio in controlling the rate of starch synthesis must await better estimates of the actual stromal Pi level in the chloroplast at a given rate of starch synthesis. The data shown here, although consistent with the PGA/Pi theory of starch regulation, do not rule out the possibility that other mechanisms are involved in the regulation of starch synthesis and breakdown.

Sucrose Synthesis From Previously Fixed Carbon

During the transition period, when photosynthesis rate is insufficient to provide the entire carbon requirement for sucrose synthesis, previously fixed carbon, which was temporarily stored as starch, is broken down to provide additional carbon for sucrose synthesis (9). Extractable SPS activity (Fig. 1J) was present at about four times the rate of *in vivo* sucrose synthesis and remained relatively constant, indicating that leaves of sugar beet have a high capacity to synthesize sucrose. *In vivo* SPS activity likely was high because the level of G6P, a known positive effector of SPS (5), also remained high during the entire period (Fig. 1H).

Although starch is known to be degraded by both amylolytic and phosphoryllytic pathways in the chloroplast (19, 24), little is known of the pathway and regulation of carbon flow from starch to sucrose in leaves. In isolated chloroplasts, starch was broken down predominantly via phosphorylase and the major products were triose-P and PGA (16, 27). Metabolism of a major portion of the starch carbon from hexoses to triose-P in the chloroplasts of exporting leaves, followed by resynthesis to hexoses in the cytosol appears unlikely. Our data and those of others (28, 29) indicate that the cytosolic FBPase is probably inhibited when starch is being degraded because of the generally high levels of F26BP present at that time. Furthermore, the levels of triose-P and F6P were low when starch was being degraded rapidly, but increased with increasing NCE (Fig. 1, F and G) (28), indicating that their origin was exclusively from newly fixed carbon. This conceptual difficulty could be resolved if starch carbon were converted to sucrose by a pathway that did not involve triose-P or cytosolic fructose bisphosphate. In addition, a second pathway would provide a means for coordinating sucrose synthesis from two different carbon sources. Such a pathway might involve conversion of starch carbon to the hexose level in the chloroplast, followed by transit across the chloroplast envelope, and subsequent metabolism to sucrose in the cytosol. Although the transport of free sugars across the chloroplast envelope is not rapid (20) and details of such a pathway are lacking, the high concentrations of glucose phosphates observed during starch breakdown provides evidence that hexoses might be involved. G1P, the product of starch phosphorylase, was present in measurable quantities only during the periods of starch breakdown (Fig. 1H). In addition, the G6P:F6P ratio was high. The amount of G6P present in excess of G6P-F6P equilibrium value (17) was considerable (as shown by the dashed lines in Fig. 1H) and coincided with measurable amounts of G1P, a likely source of the excess G6P. The ratio of G1P:G6P:F6P when sucrose was being made from starch was considerably different from that when newly fixed carbon was the source, indicating differences in flux at various points of entry in the pathway to sucrose synthesis.

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