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Evidence for Circadian Regulation of Starch and Sucrose Synthesis in Sugar Beet Leaves[1]

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ABSTRACT

Starch accumulation and sucrose synthesis and export were measured in leaves of sugar beet (*Beta vulgaris* L.) during a period of prolonged irradiance in which illumination was extended beyond the usual 14-hour day period. During much of the 14-hour day period, approximately 50% of the newly fixed carbon was distributed to sucrose, about 40% to starch, and less than 10% to hexose. Beginning about 2 hours before the end of the usual light period, the portion of newly fixed carbon allocated to sucrose gradually increased, and correspondingly less carbon went to starch. By the time the transition ended, about 4 hours into the extension of the light period, nearly 90% of newly fixed carbon was incorporated into sucrose and little or none into starch. Most of the additional sucrose was exported. Gradual cessation of starch accumulation was not the result of a futile cycle of simultaneous starch synthesis and degradation. Neither was it the result of a decrease in the extractable activity of adenosine diphosphoglucose pyrophosphorylase or phosphoglucose isomerase, enzymes important in starch synthesis. Nor was there a notable change in control metabolites considered to be important in regulating starch synthesis. Starch accumulation appeared to decrease markedly because of an endogenous circadian shift in carbon allocation, which occurred in preparation for the usual night period and which diverted carbon from the chloroplast to the cytosol and sucrose synthesis.

Carbon that is exported from leaves of sugar beet at night or during times of slow photosynthesis comes mainly from diurnal starch reserves rather than from accumulated sucrose (8, 9, 11). In photosynthesizing sugar beet (*Beta vulgaris* L.) leaves, sucrose is exported essentially as fast as it is synthesized and therefore does not accumulate to a significant extent, even when plants are growing at irradiance levels that are half of full sunlight (9). Increasing photosynthesis by increasing CO_2 or decreasing O_2 concentrations can increase sucrose synthesis and export rate (10, 26), whereas it is difficult to increase the proportion of currently assimilated carbon that is exported (6). Darkening or excising a number of sugar beet source leaves markedly lessens the supply of assimilate to sinks; yet, neither export nor sucrose synthesis increases in the remaining leaves, nor is the portion of newly fixed carbon allocated to starch altered (6). Clearly, the proportion of newly fixed carbon that is allocated to starch is regulated in a manner that assures a continuous supply of carbon for sugar beet sinks throughout the night (8, 13).

Regulation of transitory starch accumulation in leaves depends not only on photosynthesis rate but also on photoperiod. The relative amount of recently assimilated carbon that is allocated to transitory starch for subsequent export is inversely related to the duration of the daily photosynthetic period (3, 19). The daily time course of starch accumulation in leaves increases and decreases with changes in the rate of photosynthesis throughout a natural day period (9). However, a similar delay in starch accumulation at the beginning of the light period and a decrease before the end of the day occur even when irradiance is kept at a constant intensity throughout the entire day period (7, 8). The similarity of the latter time course of starch accumulation to that observed under a natural day period points to possible endogenous circadian regulation of the allocation of recently assimilated carbon.

In this study, we examined possible mechanisms for endogenous circadian regulation of carbon assimilation, allocation, and export by prolonging illumination throughout a period comprising the usual 14-h day period and the following night period. Persistence of the decrease in starch accumulation, which began just before the usual end of the day even under steady irradiance, enabled us to obtain reliable measurements of export and starch accumulation rates during the transition. The data indicate that an endogenous circadian shift in carbon allocation, which appears to be an ordinary occurrence in preparation for the night period, diverted newly assimilated carbon to sucrose synthesis and markedly increased export at the expense of starch accumulation. However, the specific mechanisms have not been identified.

MATERIALS AND METHODS

Plant Material

Sugar beet plants (*Beta vulgaris* L., Klein E type, multigerm) were grown as described previously (9). Plants were maintained under a 14-h photoperiod at 25°C, approximately 60% RH during the day, and 17°C, approximately 75% RH at night, and used when they were 5 to 6 weeks old.

Gas Exchange

Two leaves, the seventh and eighth, were enclosed in separate aluminum leaf chambers connected to an apparatus for controlling the CO_2 of the atmosphere circulating over

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the leaves. The rate of CO_2 depletion in the circulating atmosphere was measured by an IRGA (LIRA 3000; MSA, Pittsburgh, PA), and the level of CO_2 was maintained at a concentration of 350 ± 10 μL L^{-1} by a mass flow controller (15). Values of g_s[3], E, and C_i were measured with humidity sensors as described previously (16). Air temperature in the chambers was controlled at 25°C by a heat exchanger, and RH was regulated to 70% by controlling the dew point of the air entering the leaf chambers. To avoid the effects on photosynthesis and leaf metabolism of a sudden initiation of irradiance at the start of the day (15), illumination was gradually increased for the first 7 h, until it reached about 750 μmol m^{-2} s^{-1} (15). Illumination was then maintained at a steady level for the remainder of the 28-h light period.

Carbon Accumulation and Export

Accumulation of newly fixed carbon in a photosynthesizing source leaf was followed throughout the 28-h photoperiod by supplying $^{14}CO_2$ with a specific radioactivity of approximately 18 kBq mg^{-1} carbon and measuring ^{14}C accumulation in the leaf with a Geiger-Muller detector placed beneath the leaf (14). Absolute values for carbon accumulation were calculated from the specific radioactivity of the supplied $^{14}CO_2$ and the ^{14}C contained in sets of two 0.13-cm^2 leaf discs sampled from the leaf at hourly intervals. After the leaf discs were dried and oxidized, ^{14}C in the leaf discs was assayed by liquid scintillation counting (14). Carbon accumulated per unit area of leaf was determined by fitting the time-course data from leaf samples with the curve obtained by the Geiger-Muller detector. The time course for the cumulative amount of recently fixed carbon exported was determined by subtracting data of the time course for carbon accumulated in the leaf from the time course of cumulative net carbon fixed (14). In this experiment, sucrose was considered to be essentially the only organic compound transported from the leaf during the light period. The assumption appears to be reasonable but may overestimate sucrose export to some extent.

Labeling of Starch

Radioactivity was incorporated into starch by supplying air containing $^{14}CO_2$ to photosynthesizing leaves for a specific period and then switching to air containing $^{12}CO_2$ for the chase period. For labeling leaf starch grains throughout most of the usual day period, $^{14}CO_2$ with a specific radioactivity of approximately 18 kBq mg^{-1} carbon was supplied for the first 12 h of the light period and then followed with $^{12}CO_2$ for the subsequent 16 h. It is likely that this method will detect starch degradation if it occurs, because most of the starch formed during the previous day was depleted by the start of the day period, and practically all of the starch present by the end of the day was made during the 12-h labeling period. Two leaf discs were taken from the leaf at intervals throughout the entire light period, and both starch and radioactivity in starch were analyzed (8).

To label recently made starch heavily, $^{14}CO_2$ of high specific radioactivity was supplied during a 10-min pulse, 20 h after the start of the light period, and followed by air with unlabeled CO_2. Labeled CO_2 was introduced by injecting 1 mL of N_2 containing 260 kBq of $^{14}CO_2$ into the sealed leaf chamber to give a CO_2 specific radioactivity of 1.2 MBq mg^{-1} carbon. During the 10-min labeling period, depletion of CO_2 by carbon fixation was compensated for by adding $^{14}CO_2$ with the same specific radioactivity with a syringe pump. To begin the chase period, the chamber was flushed with air containing $^{12}CO_2$ at the end of the 10-min labeling period. The first leaf sample was taken 10 min later, which was 20 min after the start of labeling. Although the specific radioactivity for the short-term labeling was about 60 times that used for the long-term labeling, ^{14}C was incorporated into starch at only 1% of the rate during the long-term labeling because the starch synthesis rate was so low at 20 h of illumination.

Metabolite Analysis

Starch, sucrose, glucose, and fructose were measured by enzymic assays following extraction of the leaf samples as described by Fondy *et al.* (9). The metabolites PGA, UDPG, and esterified phosphate were extracted and measured as described by Servaites *et al.* (25).

Enzyme Assay

Leaf samples with a total area of 10 cm^2 were collected by clamping the tissue between two pieces of brass, which were precooled in liquid nitrogen until used. Enzymes were extracted by homogenizing leaf tissues in an ice-cold mortar with 1 mL of extraction buffer at pH 7.5 with final concentrations of 100 mM Hepes, 7 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 1.5 mM PMSF, and 1% (w/v) BSA. Following centrifugation at 12,000*g* for 5 min, the extract was gel filtered through a 0.5- × 10-cm column containing Sephadex G-50 equilibrated with extraction buffer. This partially purified extract was then used for the various enzyme assays.

ADPG pyrophosphorylase was assayed as described by Rao *et al.* (24). Final concentrations in the reaction mixture were 80 mM Hepes-NaOH (pH 7.4), 10 mM $MgCl_2$, 0.2 mg mL^{-1} BSA, 1 mM ADPG, 2 mM PPi, 0.6 mM $NADP^+$, 10 μM glucose-1,6-bisphosphate, 0.025 mM PGA, 20 units mL^{-1} phosphoglucomutase, and 1 unit mL^{-1} G6P dehydrogenase; the reaction was initiated by addition of leaf extract. Total activity of PGI in leaf tissue was assayed in 100 mM Tris-HCl buffer (pH 7.8), 7 mM $MgCl_2$, 2 mM fructose-6-phosphate, 0.4 mM $NADP^+$, 1 unit mL^{-1} of G6P dehydrogenase, and 0.5 mg mL^{-1} BSA. The reaction was initiated by addition of leaf extract and reduction of $NADP^+$ was measured as a change in A_{340}. Plastid PGI activity was calculated by subtracting the activity present in a sample that had been heated to 55°C for 10 min from the total activity (20). SPS activity was measured under limiting assay conditions as described previously by Servaites *et al.* (25).

[3] Abbreviations: g_s, stomatal conductance; E, transpiration rate; C_i, leaf internal CO_2 concentration; PGA, phosphoglyceric acid; UDPG, uridine diphosphoglucose; ADPG, adenosine diphosphoglucose; G6P, glucose-6-phosphate; PGI, phosphoglucose isomerase; NCE, net carbon exchange; SPS, sucrose phosphate synthase.

RESULTS

Photosynthesis during a Prolonged Light Period

The rate of NCE increased with irradiance, reaching a maximum value by 6 h, and then decreased slightly to a lower level during the remainder of the experiment (Fig. 1). The rate of NCE began to decrease gradually at the time the night period usually began and reached a rate of about 75% of the maximum level by 28 h of illumination.

Leaf Gas Exchange during a Prolonged Light Period

As light intensity increased, stomata opened gradually as judged by the increase in the values for g_s and E (Fig. 2). As photosynthesis increased, C_i decreased from the initial value of 340 to 250 μmol mol^{-1}. Fluctuations in g_s, E, and C_i were observed at 12 to 14 h of illumination, the time of the usual light-dark transition, and again at 24 h, the time when illumination usually begins.

Starch and Sugar Accumulation during a Prolonged Light Period

Accumulation of starch was detected approximately 2 h after the beginning of illumination, at which time the NCE rate was about 0.5 μg carbon cm^{-2} min^{-1}. Starch accumulation increased with irradiance and reached a maximal rate by 4 h (Fig. 1B). The rate of starch accumulation then remained relatively steady until approximately 2 h before the usual end of the day, when it began to decline noticeably. By 18 h of illumination, 4 h after the start of the usual night period, starch accumulation essentially stopped, decreasing to <0.1 μg carbon cm^{-2} min^{-1}. By the end of the 28-h photoperiod, the leaf starch level was only slightly higher than that observed at 14 h of illumination, the usual end of the day (Fig. 1A). Sucrose did not accumulate to a significant extent in sugar beet leaves throughout the usual 14-h day; nor did it do so when starch accumulation stopped during the extended light period. A small increase in leaf sucrose occurred as irradiance increased early in the day (Fig. 1A), but the maximum rate was only 0.07 μg carbon cm^{-2} min^{-1} (Fig. 1B). By 28 h of illumination, the sucrose level was approximately 40 μg cm^{-2}, only 12% of the level of accumulated starch (Fig. 1A). The time courses for accumulation of glucose and fructose were similar to that of sucrose.

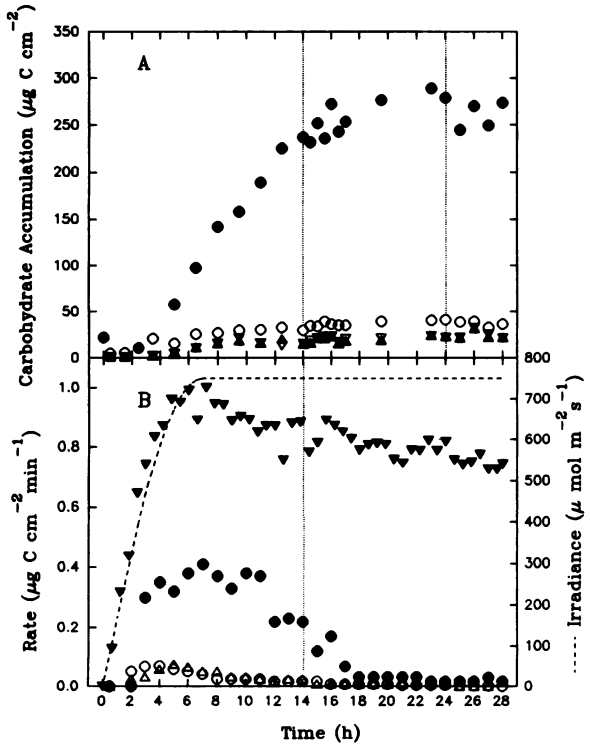

Figure 1. Photosynthesis and carbohydrate accumulation in leaves of sugar beet during a prolonged light period. A, Levels of starch (●), sucrose (○), glucose (▲), and fructose (▽). B, Irradiance (- - - - -), rate of NCE (▼) and rates of starch (●), sucrose (○), and hexose (△) accumulation are shown for the usual 14-h day and the extended light period.

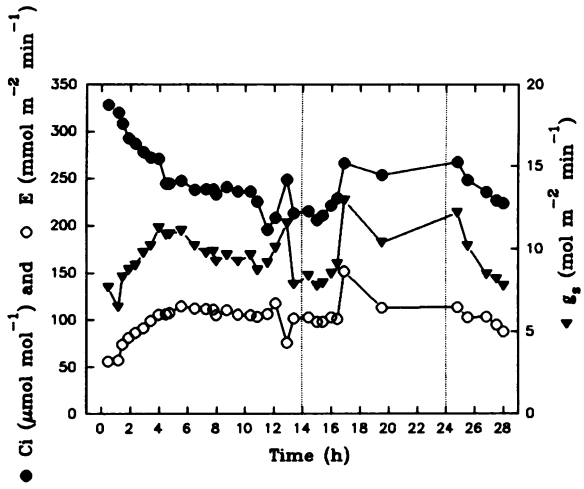

Figure 2. Gas exchange for leaves of sugar beet during a prolonged light period. Values for C_i (●), g_s (▼), and transpiration rate (○) are for the usual 14-h day and the extended light period.

Carbon Accumulation and Export by Leaves during a Prolonged Light Period

A substantial amount of the newly fixed carbon accumulated in exporting leaves throughout the first 12 h of the day period (Fig. 3A), with starch accounting for most of this carbon (Fig. 1A). Just before the usual end of the day, the export rate began to increase. When irradiance was continued into the usual night period, export reached a new rate of 0.7 μg carbon cm^{-2} min^{-1} after about 4 h and remained steady for the rest of the experiment. The marked increase in export was accompanied by a corresponding decrease in starch accumulation (Fig. 3B).

Sugar beet leaves photosynthesizing near the maximum irradiance level usually allocate approximately 40% of newly fixed carbon to starch, 55% to sucrose, and <5% to other products, including hexoses (Figs. 1 and 3B). However, near the end of the usual day period, this ratio began to change, and by 4 h under the extended light period, nearly 90% of

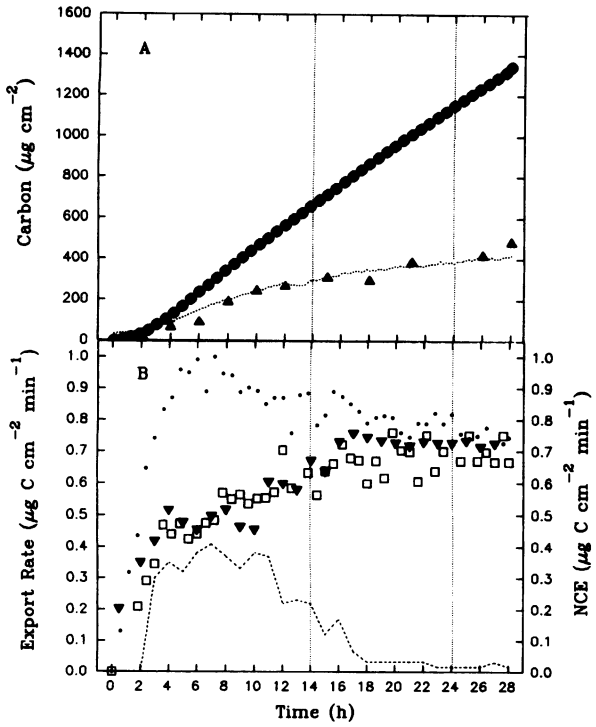

Figure 3. Carbon assimilation and carbon accumulation and export rate in a sugar beet source leaf during a prolonged light period. A, Cumulative net carbon assimilated (●); carbon accumulated in the leaf, measured in leaf samples (▲); or as tracer carbon accumulated, measured by a Geiger-Muller detector (· · · · ·). B, Photosynthesis rate (·) and carbon export rate, measured (□) or estimated from carbon potentially available for export (▼). The latter is the rate of net carbon assimilation minus the sum of the rates of starch, sucrose, and hexose accumulating in the leaves. Starch accumulation rate (- - - - -) is from Figure 1B.

newly fixed carbon was allocated to export, presumably as sucrose, and nearly none to starch. Carbon accumulation decreased to zero and failed to resume during the remainder of the light period.

Lack of Starch Degradation during a Prolonged Light Period

Accumulation of labeled carbon in starch followed the same pattern as that of starch accumulation during the 12-h labeling period (Fig. 4). No loss of radioactivity from starch was observed during the subsequent chase period when $^{14}CO_2$ was no longer supplied. Neither was radioactivity lost during the chase period following a short-term labeling of starch with $^{14}CO_2$ of high specific radioactivity (Fig. 4). In contrast, radioactivity was lost quickly from the water-soluble fraction soon after the end of the labeling period (data not shown), which is consistent with the rapid turnover as newly formed sucrose is exported from the leaves. The amount of radioactive starch per unit leaf area became more variable at the time of the usual day-night transition (Fig. 4), possibly as a result of endogenous diurnal changes in leaf water status and consequent changes in leaf area at these two transition times (Fig. 2).

Enzyme Activities and Metabolite Levels during a Prolonged Light Period

Activities of both ADPG pyrophosphorylase and plastid PGI in gel-filtered extracts of leaf samples showed no marked changes throughout the light period (Fig. 5A). With the start of illumination, the PGA level first decreased and then increased throughout the usual day period, reaching its highest level, 60 nmol mg^{-1} protein, by the end of a normal day (Fig. 5B). The level remained high during the extended light period. Total esterified phosphate also increased more than twofold during the course of the usual day and then remained relatively steady during the extended light period (Fig. 5B).

The activity of SPS in gel-filtered extracts of leaf samples increased rapidly during the first 2 h and remained relatively stable for the remainder of the light period (Fig. 6). G6P increased slowly throughout the usual day period and then remained high through the extended light period. Changes in UDPG, a substrate of SPS, showed no identifiable trend throughout a normal day and into the extended light period.

DISCUSSION

The increase in the proportion of newly fixed carbon allocated to sucrose and the corresponding decrease in starch accumulation, which began near the end of the usual day period and continued for the remainder of the experiment (Figs. 1 and 3), provide striking evidence that carbon allocation in sugar beet leaves is under endogenous circadian control. Ordinarily, starch accumulates in sugar beet leaves whenever photosynthetic carbon assimilation is above a particular threshold rate (11). However, about 2 h before the end of the day period, starch accumulation began to decrease (Fig. 1B), even though irradiance remained high and the photosynthesis rate was well above the value of 0.4 to 0.5 μg carbon cm^{-2} min^{-1} needed for starch synthesis (9). Moreover, when the light period was extended beyond its usual

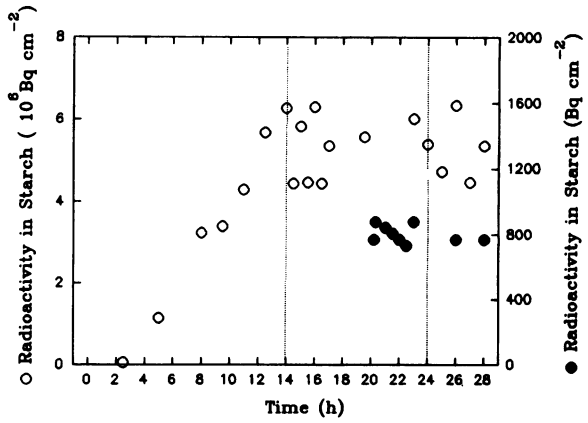

Figure 4. Label in starch during a prolonged light period. In one experiment, starch (○) was labeled with $^{14}CO_2$ from 0 to 12 h. During the subsequent chase period, from 16 to 28 h, air with $^{12}CO_2$ was supplied. In another experiment, starch (●) was labeled with $^{14}CO_2$ at high specific radioactivity for 10 min, starting at 20 h of illumination. The subsequent chase period continued until the end of photoperiod.

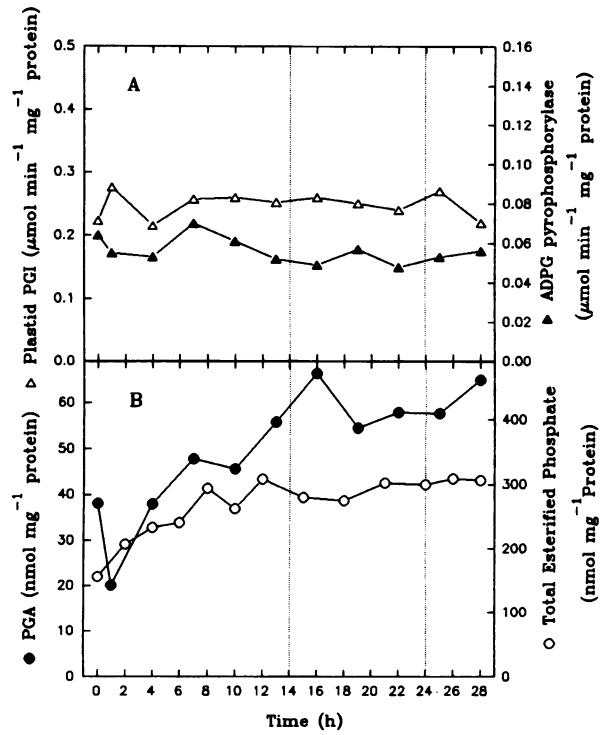

Figure 5. Enzyme activities and metabolite levels related to starch synthesis in a sugar beet during a prolonged light period. A, ADPG pyrophosphorylase (▲) and plastid PGI (△) activities. B, PGA (●) and total esterified phosphate (○) levels.

time, starch accumulation continued to decrease and essentially ceased by 4 h after the end of the usual day period (Fig. 1B). These results support the conclusion by Britz (1) that regulation of starch synthesis in a number of plants has a diurnal circadian component.

In previous studies (7, 8), a comparable decrease in starch accumulation in sugar beet leaves was observed near the end of the day period. A striking similarity exists between the decrease in foliar starch accumulation near the end of a day period when irradiance and NCE are undiminished (Fig. 1B; refs. 7 and 8) and the obvious shift from starch accumulation to degradation that occurs in leaves photosynthesizing under a natural day period (9). Starch accumulation ceased at the same time of the photoperiod in both cases, even though there was a marked difference in photosynthesis rates between the two situations. These data demonstrate that this circadian regulation is an ordinary occurrence in plants, which prepares metabolism for the accustomed day-night transition. Circadian regulation of starch accumulation is likely related to the strict regulation of transitory starch accumulation in relation to photosynthetic duration and the length of the night period (3, 6, 19).

Decreased starch accumulation could result from either an increased rate of starch degradation or a decreased rate of starch synthesis. It has been proposed that starch degradation is an unregulated process (23) and that starch accumulation represents the net balance between simultaneous synthesis and degradation (28). The occurrence of diurnal fluctuations in the activity of amylase (22) and of endogenous circadian periods of starch degradation rate during an extended light period (21) could be taken to indicate that starch degradation is ordinarily initiated by an endogenous circadian rhythm. However, the absence of a significant loss of ^{14}C from sugar beet leaves during chase periods following either long-term or short-term labeling (Fig. 4) demonstrates that the decrease in starch accumulation seen at the end of the day in this study is not caused by starch degradation. This conclusion is further supported by the fact that, in plants under the usual 14-h photoperiod, starch degradation does not start until about 30 min after the transition from the daytime level of irradiance to darkness (7). The occasional periods of starch degradation observed by Kruger *et al.* (21) in pea leaves during a prolonged light period are not consistent with our data, which demonstrate that the decrease in starch accumulation is not the result of an increase in starch degradation.

The cause for the observed slowing of starch synthesis is not clear. It has been suggested that starch synthesis may be inactivated when carbohydrates accumulate to a high level in leaves (18). However, feedback inhibition did not seem to occur under the present conditions, based on data from a previous study (8). When export of sucrose was drastically decreased by heat killing a length of the petiole, starch accumulation resumed to a rate about 60% of that observed during the middle of the day and continued for the remainder of the extended light period. Also, the reduction in starch accumulation cannot be accounted for by the slight decrease in photosynthesis rate (Fig. 1B), which, in any event, would not explain the increase in export. The slight decrease in the rate of photosynthesis that occurred when the light period was extended may be an endogenous circadian response (2, 12) that is too small to affect starch synthesis markedly (11).

The observed decrease in starch synthesis may have resulted from a reduction in the activity of one or more critical enzymes involved in regulation of the starch synthesis pathway (4, 23). The activities of ADPG pyrophosphorylase and plastid PGI in gel-filtered extracts did not decrease at the end

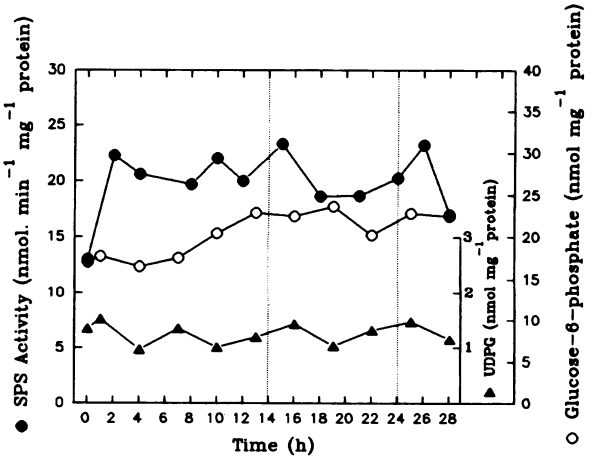

Figure 6. Enzyme activities and metabolite levels related to sucrose synthesis in a sugar beet during a prolonged light period. SPS activity (●), G6P level (○), and UDPG level (▲).

of the day or during the extended light period (Fig. 5A), indicating that a decrease in the amount or activity of these enzymes did not cause the decrease in starch accumulation. These observations do not rule out a decrease in some other enzyme of the starch synthesis pathway. It also is possible that a regulatory metabolite or inhibitor may reduce the activity of a key enzyme *in vivo* and may be removed by gel filtration.

Based on the assumption that total phosphate in the leaf cell is relatively constant (17), the observed increase in the level of esterified phosphate indicates that the Pi level decreased throughout the day up to the time of the usual day-night transition and then remained steady (Fig. 5B). The time course of the PGA/Pi ratio, inferred from the change in Pi and the measured increase in PGA level, would actually favor an increase in the *in vivo* activity of ADPG pyrophosphorylase (23). Resumption of starch synthesis when export was blocked (8) indicates that the decrease in starch accumulation probably did not result from inactivation of one or more enzymes of the starch synthesis pathway.

An alternative hypothesis is that entry of additional carbon into the sucrose synthesis pathway prevented carbon from entering the starch synthesis pathway. Such a mechanism appears to be consonant with the nature of the regulatory mechanisms involved in coordinate control of carbon allocation between sucrose and starch synthesis. Stitt and Quick (29) concluded that control of starch and sucrose synthesis from photoassimilate is such that, if some factor causes starch synthesis to slow, events within the chloroplast are not communicated to the sucrose synthesis pathway in the cytosol. Consequently, sucrose synthesis does not increase, but the altered conditions within the chloroplast cause the rate of NCE to decrease instead. However, when the rate of sucrose synthesis is altered by some factor, the change in carbon allocation will be communicated to the chloroplast stroma, and starch synthesis will change. The fact that the slowing of starch synthesis was accompanied by increased sucrose synthesis and not a decrease in photosynthesis indicates that promotion of sucrose synthesis triggered the change in carbon allocation.

Possible causes for additional carbon entering the sucrose synthesis pathway include an increase in the activity of one or more key enzymes of the sucrose synthesis pathway or activation of a path that conducts additional carbon from the chloroplast to sucrose synthesis in the cytosol (25). Allocation of carbon to sucrose synthesis is a major factor in the regulation of export from sugar beet leaves (7), and therefore, diversion of additional carbon to sucrose likely would cause export to increase. The activity of SPS in the leaves appears to be high enough to support the additional synthesis of sucrose. After light activation during the first 2 h of the photoperiod, extractable SPS activity was at a constant high level throughout the day period and into the extended light period (Fig. 6). Activity was about 2.5 times what is needed to support the highest sucrose synthesis rate observed during the prolonged light period. The increased ratio of G6P/Pi observed during the extended day (Figs. 5B and 6) is consistent with increased *in vivo* SPS activity (5). Likewise, the low level of UDPG during the entire light period is consistent with the absence of downward regulation.

Our current working hypothesis to explain decreased starch accumulation at the end of the day is that endogenous, diurnal activation of an additional path for exit of carbon from the chloroplast, separate from the phosphate translocator and fructose bisphosphatase, prepares leaf metabolism for the synthesis of sucrose from starch. In previous studies, it was proposed that a precursor of sucrose synthesis is transported from the chloroplast to the cytosol at night or under low photosynthesis by a mechanism other than the one that operates during the day under regulation by fructose-2,6-bisphosphate (9, 25). This additional translocator, which may transport hexose or a hexose phosphate from the chloroplast (27), appears to function simultaneously with the phosphate translocator at the beginning and end of the usual day period (25). When the light period is prolonged, both pathways continue to function and contribute to the higher rate of sucrose synthesis. Because export from sugar beet leaves is regulated by the availability of sucrose (6, 8) and because its leaves are capable of exporting sucrose at rates much faster than those found during the usual day period (26), the additional sucrose is exported.

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