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Characterization of Diurnal Changes in Activities of Enzymes Involved in Sucrose Biosynthesis[1]

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THOMAS W. RUFTY, JR., PHILLIP S. KERR, 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 27650

ABSTRACT

Experiments were conducted with vegetative soybean plants (*Glycine max* [L.] Merr., 'Ransom') to determine whether the activities in leaf extracts of key enzymes in sucrose metabolism changed during the daily light/dark cycle. The activity of sucrose-phosphate synthase (SPS) exhibited a distinct diurnal rhythm, whereas the activities of UDP-glucose pyrophosphorylase, cytoplasmic fructose-1,6-bisphosphatase, and sucrose synthase did not. The changes in extractable SPS activity were not related directly to photosynthetic rates or light/dark changes. Hence, it was postulated that the oscillations were under the control of an endogenous clock. During the light period, the activity of SPS was similar to the estimated rate of sucrose formation. In the dark, however, SPS activity declined sharply and then increased even though degradation of starch was linear. The activity of SPS always exceeded the estimated maximum rate of sucrose formation in the dark. Transfer of plants into light during the normal dark period (when SPS activity was low) resulted in increased partitioning of photosynthate into starch compared to partitioning observed during the normal light period. These results were consistent with the hypothesis that SPS activity *in situ* was a factor regulating the rate of sucrose synthesis and partitioning of fixed carbon between starch and sucrose in the light.

Sucrose is the primary transport carbohydrate in most higher plants (11). In leaves of C_3 plants, synthesis of sucrose occurs in the cytoplasm of mesophyll cells (3, 25, 30). The principal precursors for the sucrose biosynthetic pathway are triose phosphates which are generated within the chloroplast and exported to the cytoplasm by way of the phosphate translocator (17, 30). In the light, triose phosphates originate from concurrent photosynthesis and in the dark from degradation of starch. Decreased export of assimilates and lower sucrose levels in leaf tissue (8, 10, 12, 16) suggest that triose-P availability and the rate of sucrose formation are decreased considerably in the dark.

It is unclear whether the diurnal changes in sucrose formation are associated simply with altered availability of triose-P precursor or whether coordinated regulatory adjustments occur in the activities of sucrose biosynthetic enzymes. Cytoplasmic FBPase[2] and SPS appear to be two important enzymes in the control of sucrose formation (9, 14, 22). Coordination between precursor availability and activities of FBPase and SPS *in situ* could be achieved either through a system of metabolic 'fine' regulation involving activators and inhibitors (1, 14, 24), or a system of 'coarse' regulation involving alterations in net synthesis or modification of pre-existing enzyme which would affect the total enzyme activity in leaf extracts. In previous unpublished experiments, decreased activities of SPS were observed in extracts of leaves harvested in the dark. The purpose of the present study was to determine whether the activities in leaf extracts of SPS, cytoplasmic FBPase, and UDPG pyrophosphorylase, key enzymes in sucrose biosynthesis, changed during the daily light-dark cycle, and if so, whether such diurnal changes reflected light modulation (*i.e.* rapid light/dark response) or a type of endogenous control.

MATERIALS AND METHODS

A series of four experiments was conducted to investigate diurnal variations in activities of enzymes involved in sucrose biosynthesis in leaf extracts of vegetative soybean plants (*Glycine max* [L.] Merrill, 'Ransom'). All four experiments were conducted similarly in an attempt to allow replication over time. The plants were grown in a standard soil mix in a greenhouse in August and September, 1982. Plants were watered in the morning with tap water, and in the afternoon of alternate days with approximately 600 ml of a standard nutrient solution which contained 7.5 mM NO_3^-, 0.5 mM $H_2PO_4^-$, 3.0 mM K^+, 2.5 mM Ca^{2+}, 1.0 mM Mg^{2+}, 1.0 mM SO_4^{2-}, and trace elements at the concentration of Hoagland solution.

The experiments were conducted on clear days when the third trifoliolate was fully expanded. In each experiment, four plants were sampled every 2 or 3 h over the 24-h light-dark cycle. At each harvest, leaf discs (4.4 cm^2) were removed from the third trifoliolate for starch analysis. The discs were immediately submerged in 80% ethanol and placed in a freezer at −10°C. The third trifoliolate was then excised, weighed, sliced into segments, and immediately frozen at −80°C for later enzyme analysis. Stomatal resistances and CERs of the third trifoliolate of randomly selected plants were monitored throughout the 24-h period.

To determine whether the leaf enzyme activities responded quickly to light/dark transitions, eight plants were moved from the greenhouse at 1300 h into a dark growth chamber maintained at the same temperature as the greenhouse, and at 0300 h, a set of eight plants was moved from the dark greenhouse into a growth chamber at a similar temperature and with an irradiance of 550 $\mu E\ m^{-2}\ s^{-1}$ at the leaf canopy. Following placement of plants into the growth chambers, measurements and sampling of the third trifoliolate were done as before in four control and transferred plants at 2.5 and 5.0 h after the transfer. Data are

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[2] Abbreviations: FBPase, fructose-1,6-bisphosphatase; SPS, sucrose-phosphate synthase; FBP, fructose-1,6-bisphosphate; F6P, fructose 6-phosphate; G1P, glucose 1-phosphate; G6P, glucose 6-phosphate; UDPG, uridine 5′-diphosphoglucose; CER, net CO_2 exchange rate.

presented as means of the two harvests. Leaf discs were removed at the time of transfer and at each harvest which allowed measurement of starch accumulation or degradation throughout the 5-h period.

Photosynthesis and Resistance Measurements. Photosynthetic rates were measured using a Beckman model 865 differential IR CO_2 analyzer[3] equipped with a clamp-on Plexiglas cuvette enclosing the upper and lower surfaces of a 10-cm^2 area of the appropriate leaf. Air at the same temperature and CO_2 concentration of the ambient air was passed through the cuvette for 30 to 45 s at a flow rate of 1.5 l/min. Differences between CO_2 concentration in incoming and exhaust air streams were monitored and used to calculate CERs. Stomatal resistances were measured with a Li-Cor transient porometer.

Starch Analysis. Leaf discs were extracted with hot 80% ethanol until the tissue was free of pigment. Particulates including starch were pelleted by centrifugation and then suspended in 1.0 ml of 0.2 N KOH and placed in boiling water for 30 min. After cooling, the pH of the mixture was adjusted to about pH 5.5 with 200 μl of 1.0 N acetic acid. To each sample, 1.0 ml of dialyzed amyloglucosidase solution (from *Aspergillus oryzae* [Sigma], 35 units/ml in 50.0 mM Na-acetate buffer, pH 4.5) was added, and the tubes were incubated at 55°C for 15 min. After digestion, the tubes were placed in boiling water for 1 min, centrifuged, and the glucose in the supernatant was analyzed enzymically using hexokinase and G6P dehydrogenase (21).

Enzyme Extraction and Assays. The frozen leaf tissue was ground with a Polytron high speed homogenizer in a medium (8.0 ml of medium/g fresh weight) containing 50 mM Hepes-NaOH (pH 7.2), 5 mM $MgCl_2$, 1 mM EDTA, 2.5 mM DTT, 2% PEG-20 (w/v), and 1% BSA (w/v). The brei was then filtered through eight layers of cheesecloth, and cells were disrupted by passage through a French pressure cell (330 kg/cm^2). Debris was pelleted by centrifugation at 38,000*g* for 10 min and enzyme assays were conducted on the supernatant fluid.

Sucrose-P synthase was assayed by measurement of fructose-6-P-dependent formation of sucrose (+ sucrose-P) from UDP-glucose (3, 22). The assay mixture (70 μl) contained 7.5 mM UDP-glucose, 7.5 mM fructose-6-P, 15 mM $MgCl_2$, 50 mM Hepes-NaOH (pH 7.5), and an aliquot of leaf extract. The assay mixture for sucrose synthase was the same, except that fructose replaced fructose-6-P. Mixtures were incubated at 25°C, and reactions were terminated after 10 min by addition of 70 μl 1.0 N NaOH. Unreacted F6P (or fructose) was destroyed by placing the tubes in boiling water for 10 min. After cooling, 0.25 ml of 0.1% (v/v) resorcinol in 95% ethanol and 0.75 ml of 30% HCl were added, and the tubes incubated at 80°C for 8 min. The tubes were allowed to cool, and the A_{520} was measured.

Cytoplasmic FBPase was assayed by measuring F6P formation by a continuous spectrophotometric assay under conditions where the chloroplast enzyme is virtually inactive. The 1.0-ml reaction mixture contained 50 mM Hepes-NaOH (pH 7.0), 5 mM $MgCl_2$, 100 μM FBP, 0.2 mM $NADP^+$, 2 units each of phosphoglucoisomerase and G6P dehydrogenase, 1 unit of 6-phosphogluconate dehydrogenase, and an aliquot of leaf extract. Production of NADPH was monitored at A_{340}. UDPG pyrophosphorylase activity was determined by measuring PP_i-dependent GIP formation from UDPG using a coupled spectrophotometric assay. Contents of the 1.0 ml assay were 50 mM Hepes-NaOH (pH 7.5), 5 mM $MgCl_2$, 5 mM UDPG, 2 mM PP_i, 0.3 mM $NADP^+$, 1.5 units of phosphoglucomutase, 1 unit of 6-phosphogluconate dehydrogenase, 5 units of G6P, and 50 μl of leaf extract. The reaction was initiated with the leaf extract and the production of NADPH monitored by changes in A_{340}.

The tissue extract also was used for hexose and sucrose determinations. Following centrifugation, an aliquot of the extract was removed, diluted with redistilled H_2O, boiled for 1 min, and analyzed enzymically (21).

Statistical Analysis. The results of all four experiments were similar with one notable exception which is referred to in "Results." The data points presented in Figures 1, 2, and 3 represent means from two of the experiments with a total of eight replications. Variances in the two experiments were homogeneous, and therefore a pooled estimate of variance was used to calculate LSD values at the 0.05 level of probability. The LSD included on each graph can be used in comparisons among all points. The results of one experiment are shown in Figure 4 and Table I, and SE are included with each data point.

RESULTS

Diurnal Effects. On the days the diurnal experiments were conducted in the greenhouse, the photosynthetic period extended from about 0700 to 1800 h (Fig. 1). Photosynthesis was greatest during the morning hours and then tended to decline gradually. During most of the photoperiod, starch accumulated almost linearly and the leaf sucrose content was maximized (Fig. 2). By 2000 h, net degradation of starch had begun and leaf sucrose had decreased noticeably. During most of the dark period, leaf starch was degraded steadily, whereas the leaf sucrose content declined rapidly during the initial 6 h of darkness and thereafter remained at a nearly constant level (Fig. 2). Because tissue sucrose concentrations generally have been found to reflect the rate of sucrose formation and export out of source leaves (11), the reduction in the leaf sucrose content in the dark presumably reflected equilibration of tissue sucrose pools with the reduced rate of sucrose biosynthesis.

The activities of UDPG pyrophosphorylase (Fig. 3A), cytoplasmic FBPase (Fig. 3B), and sucrose synthase (not shown) in leaf extracts remained relatively constant throughout the 24-h light-dark cycle. The activity of SPS, however, changed markedly (Fig. 3C). The minimum activity was observed at 0300 h at about the middle of the dark period. Thereafter, activity started to increase and tended to plateau through most of the light period. Maximum activities were observed 2 to 3 h after the end of the light period, and then dropped sharply to the minimum activity. The diurnal rhythm in SPS activity has been clearly discernible in each greenhouse experiment, with the only major variation being the extent of the increase in activity in the early part of the light period. The activity at 0900 has been as high as 90% of the maximal activity at 2000 h which gave the dirunal rhythm of activity the appearance of a bimodel system with two peaks separated by 12 h.

Light-Dark Transitions. During the normal photoperiod, the changes in SPS activity (Fig. 3C) were not closely aligned with irradiance or photosynthetic rate (Fig. 1). To investigate further the possibility of an association between SPS activity and carbon fixation, plants were moved into the dark during the light period and others were exposed to light during the dark period. The light-dark transitions had no immediate effect on the activity of SPS, as compared to control plants remaining in the normal greenhouse conditions (Fig. 4). The activities of cytoplasmic FBPase and UDPG pyrophosphorylase also were monitored (data not shown), but no changes were observed.

In plants transferred from the light into the dark, leaf starch was degraded at a rate similar to that in leaves of plants in the normal dark period (Table I, columns B and C). Similarly, transfer of plants into the light during the normal dark period resulted in photosynthetic carbon fixation (Table ID), but at lower rates compared to those of control plants during the normal

[3] 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.

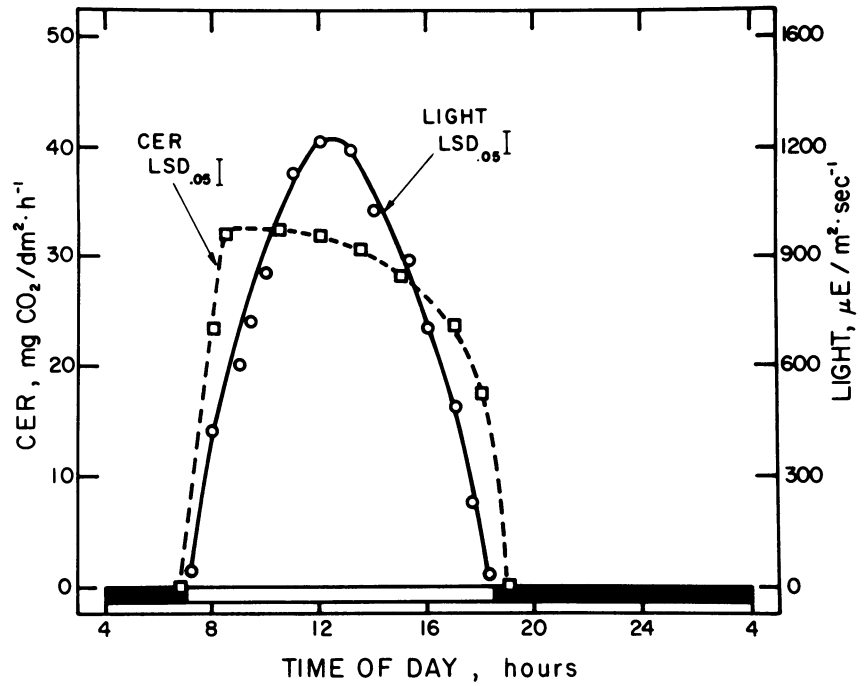

FIG. 1. Irradiance and CERs of the third trifoliolate of vegetative soybean plants during the daily light-dark cycle.

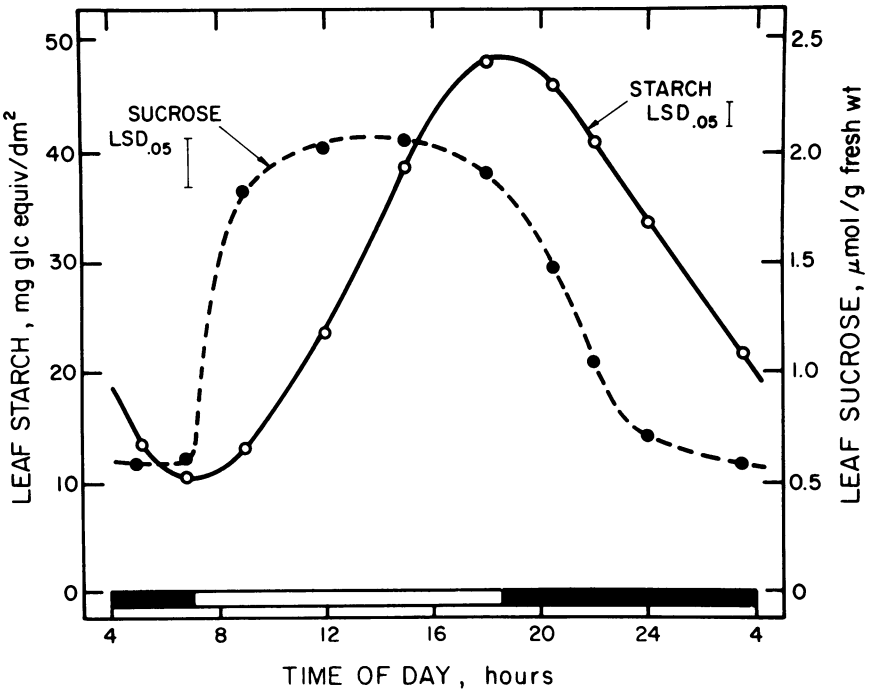

FIG. 2. Leaf starch and sucrose content of the third trifoliolate of vegetative soybean plants during the daily light-dark cycle.

light period (Table IA). The lower photosynthetic rates were associated with increased stomatal resistance. Even though photosynthetic rates were lower in plants moved into the light, the mean rate of starch accumulation during the 5-h period was increased slightly (compare Table I, D and A). Therefore, in plants transferred into the light during the normal dark period, a larger proportion of the photosynthate was partitioned into starch (34 *versus* 20%) at a time coincident with the lowest activities of SPS (Fig. 4).

DISCUSSION

The activity of SPS in leaf extracts clearly changed during the daily light-dark cycle, which contrasted with the diurnal stability of the activities of UDPG pyrophosphorylase, cytoplasmic FBPase (Fig. 3), and sucrose synthase. The changes in SPS activity were not coupled directly to photosynthetic rates or light-dark alterations. Consequently, enzyme activity was not controlled by light modulation which has been observed with certain chloroplast enzymes (4) and cytoplasmic G6P dehydrogenase (2).

The observation that the diurnal oscillations in extractable activity of SPS were not directly coupled to light effects suggests that the oscillations were under the control of an endogenous clock (5, 15). The activity of SPS increased prior to sunrise in each greenhouse experiment (*cf.* Figs. 3 and 4), which is consistent with an endogenous diurnal rhythm. The absence of a direct

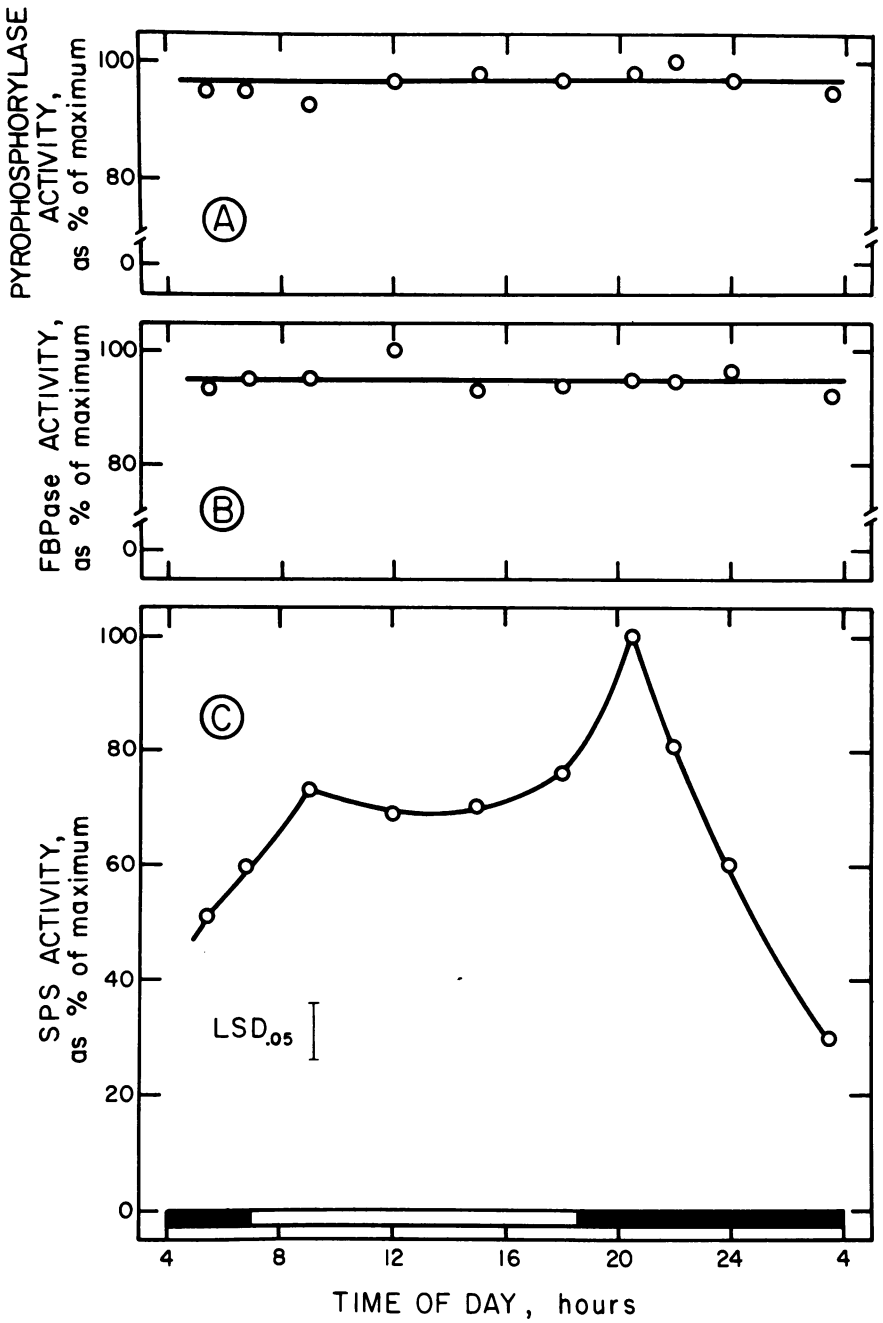

FIG. 3. The activities of (A) UDPG pyrophosphorylase, (B) FBPase, and (C) SPS in extracts of the third trifoliolate of vegetative soybean plants during the daily light-dark cycle; the maximal activities of the three enzymes were 410, 38, and 33 μmol of product/g fresh weight h^{-1}, respectively.

relationship between SPS activity and light effects does not preclude the possibility that light effects acted as timing agent(s) for the rhythm (5). Endogenously controlled diurnal rhythms have been reported in the activities of other enzymes which have metabolic importance (*e.g.* 7, 13, 28).

Metabolic Implications. It has been suggested that the extractable activity of SPS reflects the capacity of the sucrose biosynthetic system in the light (9, 14, 22). The results from the present study are generally consistent with that contention. In the experiment shown in Table I, for example, plants in the normal photoperiod had CERs of about 26 mg of CO_2 or 591 μmol of C/dm^2 h^{-1} (Table IA). Starch was accumulated at a rate of 3.7 mg of glucose or 123 μmol of C/dm^2 h^{-1}. If it is assumed that 70% of the remaining photosynthate was partitioned into sucrose (19, 29), the rate of sucrose formation can be estimated at about 328 μmol of C/dm^2 h^{-1}. The activity of SPS during the same time interval was about 25 μmol of sucrose/g fresh weight h^{-1} (Fig. 4) which was equivalent to 300 μmol of C/dm^2 h^{-1}. Therefore, the measured activities of SPS were very close to the calculated rates of photosynthetic sucrose formation.

The rate of sucrose formation in the light apparently can regulate partitioning of photosynthate between starch and sucrose by influencing how effectively triose phosphates are diverted from biosynthesis of starch in chloroplasts (18, 27). The metabolic consequence of the diurnal rhythm in SPS activity on the rate of sucrose formation and partitioning of photosynthate apparently could be seen following transfer of plants during the normal dark period into the light, when extractable activities of SPS were low (Fig. 4). Although activity of SPS was changing, the mean activity during the time interval (204 μmol of C/dm^2

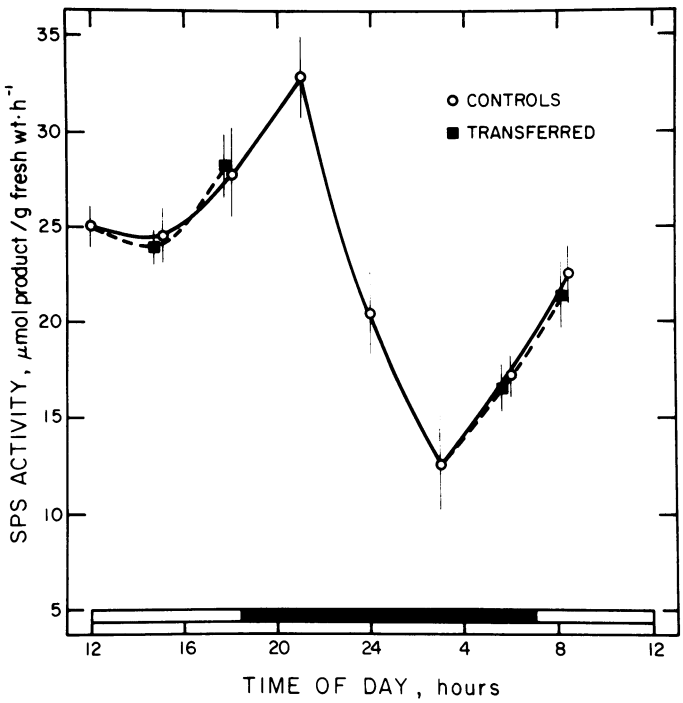

FIG. 4. The effect of light-dark transition on activity of SPS in extracts of the third trifoliolate of vegetative soybean plants. Plants were transferred either into the dark in the normal light period or into light in the normal dark period. Each data point represents the mean of four replications.

Table I. *The Effect of Light-Dark Transitions on CER, Stomatal Resistance, and Change in Leaf Starch and Sucrose Content of the Third Trifoliolate of Vegetative Soybean Plants*

Plants were transferred into the dark for 5 h in the normal light period or into the light for 5 h in the normal dark period. Data represent means from harvests 2.5 and 5.0 h after the transfer.

	Light Period[a]		Dark Period[b]	
	Control (A)	Transfer to Dark (B)	Control (C)	Transfer to Light (D)
CER (mg CO_2/ dm^2 h^{-1})	26.0 ± 0.7			18 ± 0.3
Stomatal resistance (s/cm)	1.7 ± 0.1	16.3 ± 0.8	10.2 ± 0.4	4.4 ± 0.2
Change in tissue starch (mg Glc/dm^2 h^{-1})	+3.7 ± 0.3	−1.9 ± 0.1	−2.1 ± 0.1	+4.2 ± 0.4
Leaf sucrose (μmol/g fresh wt)	2.8 ± 0.2	1.3 ± 0.1	1.1 ± 0.1	2.3 ± 0.3

[a] Measurements made between 1300 and 1800 h.
[b] Measurements made between 0300 and 0800 h.

h^{-1}) was similar to the calculated rate of sucrose formation (188 μmol of C/dm^2 h^{-1}). Associated with these reduced rates, a noticeably larger proportion of photosynthate was partitioned into starch (34%) compared with plants in the normal light period (20%). This observation is somewhat analogous to that of Silvius *et al.* (27). When soybean plants were transferred into higher irradiance, the activity of SPS in leaf extracts and translocation of carbon were unchanged, but photosynthetic rates and accumulation of starch were increased appreciably.

Additional evidence lends further support for the notion that total SPS activity is a factor which limits the capacity of the sucrose biosynthetic system in the light and the related partitioning of photosynthate between starch and sucrose. Source-sink manipulations which altered 'demand' for sucrose from source leaves resulted in corresponding changes in extractable activity of SPS within 1 h and reciprocal changes in partitioning of photosynthate into starch (26). Also, in species and genotype comparisons, activities of SPS in leaf extracts were reciprocally related to differences in partitioning of photosynthate into starch (20, 21). The reciprocal relationship between extractable activity of SPS and partitioning into starch has not been observed with other enzymes involved in sucrose synthesis, except in a few instances with cytoplasmic FBPase (26).

Even though the activity of SPS and the capacity of the sucrose biosynthetic system apparently can influence sucrose formation in the light, they appear to be of less regulatory importance in the dark. During the normal light-dark cycle in greenhouse experiments, the activity of SPS declined in the dark period until about 0300 h, and then increased (Figs. 3C and 4). In contrast, degradation of starch and, presumably, formation and export of sucrose were maintained at constant rates (Fig. 2). At the lowest point in the rhythm of SPS activity, sufficient activity still was present (119 μmol of C/dm^2 h^{-1}) even if all of the carbon mobilized in starch degradation (105 μmol of C/dm^2 h^{-1}) was utilized in synthesis of sucrose. The rate of sucrose formation in the dark likely was limited primarily by the rate of starch degradation and the associated generation of triose-P precursor.

The concepts developed in this paper do not exclude or discount other forms of regulation that may affect the rate of sucrose synthesis and partitioning of photosynthate between starch and sucrose in the light. Metabolic regulation of ADP-glucose pyrophosphorylase in the chloroplast (24), and of FBPase and SPS in the cytoplasm (1, 9, 14) could be involved. Over a 24-h period, net synthesis of starch and the rate of starch breakdown in the dark could be affected by changes in activities of starch-degrading enzymes which also may have endogenous diurnal rhythms (23). Nevertheless, an increasing amount of evidence suggests that in some leaf tissues regulation of total activity of SPS may be an important component of the control system. It is conceivable that alteration of the diurnal rhythm of SPS activity could be an important factor in the acclimation of carbohydrate metabolism which occurs when environmental conditions, *e.g.* photoperiod (6) or irradiance (27), are altered.

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