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Diurnal Changes in Maize Leaf Photosynthesis[1]

III. LEAF ELONGATION RATE IN RELATION TO CARBOHYDRATES AND ACTIVITIES OF SUCROSE METABOLIZING ENZYMES IN ELONGATING LEAF TISSUE

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ABSTRACT

Maize (*Zea mays* L. cv. Pioneer 3184) leaf elongation rate was measured diurnally and was related to diurnal changes in the activities of sucrose metabolizing enzymes and carbohydrate content in the elongating portion of the leaf. The rate of leaf elongation was greatest at midday (1300 hours) and was coincident with the maximum assimilate export rate from the distal portion of the leaf. Leaf elongation during the light period accounted for 70% of the total observed increase in leaf length per 24 hour period. Pronounced diurnal fluctuations were observed in the activities of acid and neutral invertase and sucrose phosphate synthase. Maximum activities of sucrose phosphate synthase and acid invertase were observed at 0900 hours, after which activity declined rapidly. The activity of sucrose phosphate synthase was substantially lower than that observed in maize leaf source tissue. Neutral invertase activity was greatest at midday (1200 hours) and was correlated positively with diurnal changes in leaf elongation rate. There was no significant change in the activity of sucrose synthase over the light/dark cycle. Sucrose accumulation rate increased during a period when leaf elongation rate was maximal and beginning to decline. Maximum sucrose concentration was observed at 1500 hours, when the activities of sucrose metabolizing enzymes were low. At no time was there a significant accumulation of hexose sugars. The rate of starch accumulation increased after the maximum sucrose concentration was observed, continuing until the end of the light period. There was no delay in the onset of starch mobilization at the beginning of the dark period, and essentially all of the starch was depleted by the end of the night. Mobilization of starch in the elongating tissue at night could account for a significant proportion of the calculated increase in the tissue dry weight due to growth. Collectively, the results suggested that leaf growth may be controlled by the activities of certain sucrose metabolizing enzymes and may be coordinated with assimilate export from the distal, source portion of the leaf. Results are discussed with reference to diurnal photoassimilation and export in the distal, source portion of the leaf.

Regulation of leaf growth and development is a critical factor in crop productivitiy because these processes ultimately determine the total photosynthetic area available for light interception and carbon assimilation. The growth and development of maize leaves occurs in a manner unique to monocots; leaf growth occurs through the coordinated activities of three regions of the developing leaf. At the base of the leaf is the intercalary meristem—a region of rapid cell division. Adjacent and distal to the intercalary meristem is a region of cell expansion and chloroplast development. The remaining, distal portion of the lamina is photosynthetically competent and active in exporting photoassimilates to, and through, the growing proximal portion of the leaf (13, 14). Thus, the elongating maize leaf represents a gradient in tissue age and photosynthetic capacity; the oldest 'source' tissue occurs at the distal end of the leaf, while the proximal region of the leaf constitutes younger, sink tissue. This source/sink relationship of the elongating maize leaf has been demonstrated using $^{14}CO_2$ labeling. A significant proportion of ^{14}C label, originating in the distal portion of the leaf, can, in course of time, be recovered in the elongating sink tissue of the same leaf (9). Recently, we examined photosynthetic parameters in the distal, source portion of elongating maize leaves (12), and reported on pronounced diurnal changes in the rate of carbon fixation and assimilate export. We asked whether similar diurnal fluctuations exist in the catabolism and utilization of assimilates for leaf growth in the elongating portion of the leaf. Therefore the specific objectives of this study were to monitor diurnal changes in: (a) the rate of leaf elongation, (b) the activities of several sucrose metabolizing enzymes, and (c) the levels of nonstructural carbohydrates in the elongating portion of the maize leaf over a natural light/dark cycle.

MATERIALS AND METHODS

Plant Material. Maize plants (*Zea mays* L. cv Pioneer 3184) were grown in 15 L pots (two plants per pot), in the greenhouse during June and July in a medium containing Terra Lite,[2] sterile top soil, and perlite, (1:1:1). A commercial slow release fertilizer (Sta Green ProStart, 13-6-6, Brawley Seed Co., Mooresville, NC) was incorporated at the recommended rate into the soil at planting. Plants received aged tap water (to reduce chlorine content of city water) daily and at 10 d intervals, soil was supplemented with alternating treatments of 10 ml of 3.2 M KNO_3 and 1.6 M NH_4NO_3. Plants were grown under supplemental lights (Sylvania 1000 W metalarc lamps) which provided a fluence rate of 400 umol/m^2·s at plant level, in the absence of sunlight. Lights were on from 0.5 h after sunrise until 0.5 h before sunset. The plants used in this study were the same experimental population used in the studies reported by Kalt-Torres *et al.* (12) and Usuda *et al.* (16). The plants were 22 d old

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and at this stage of development, leaf position eight (acropetally numbered) was beginning to emerge from the whorl. The experiment was conducted on consecutive, sunny days.

Experimental Protocol. The study was conducted over a 27 h period (0900 h on d 1 to 1200 h on d 2) in the greenhouse. The time period was divided into 3 h intervals; for each interval, six different plants were used. A 10 cm segment of the elongating portion of leaf 6, starting from the ligule of the subtending leaf (5) was taken. At this stage of development, the tissue sampled completely encircled the stalk. A uniform portion of the tissue was frozen separately at −20°C for nonstructural carbohydrate analysis, and the remainder of the sample was immediately frozen at −80°C for enzyme activity measurements. The collection of tissue for enzyme and carbohydrate analysis actually spanned 2 consecutive days. However, results were combined such that data were plotted starting from 0600 h, which was around sunrise, until 0300 h, during the dark. Data were plotted in this way for ease of discussion, and this was possible because the overlapping points from d 1 and 2 were very close in all measurements. Thus most data points are means of 6 replicate samples, but those taken between 0900 h and noon are the mean of 12 replicates.

Elongation Measurements. Elongation measurements were taken over a 24 h period, and were also plotted starting from the beginning of the light period (0600 h). The elongation rate of leaf 6 was determined at 3 h intervals by measuring a segment from a mark that was made on the midrib of leaf 6 to the ligule of leaf 5. The mark was made with a felt tip marker, 5 cm up from the ligule of leaf 5 at the beginning of the experimental period. In one experiment, plants were transferred to a darkened growth chamber after the end of the normal dark period, and elongation measurements were taken over the following 12 h. To determine specific leaf weight changes during the day and night, elongating tissue samples were harvested at the end of the light and dark periods. Leaf area was determined, and leaf samples were lyophilized to measure dry weight changes of the samples.

Enzyme Extraction and Assay. Frozen leaf samples, without midribs (0.3–0.4 g), were ground in a chilled mortar with about 0.2 g sea sand and 2.5 ml extraction buffer (50 mM Hepes-NaOH [pH 7.5], 10 mM $MgCl_2$, 1 mM EDTA, 5 mM DTT, 2% [w/v] PEG-20, 0.5% [w/v] BSA, and 0.02% [w/v] Triton X-100). The extract was filtered through Miracloth (Behring Diagnostics, LaJolla CA) and centrifuged at 1100*g* for 2 min. The supernatant solution was desalted by centrifugal gel filtration (8), into a buffer containing 50 mM Hepes-NaOH (pH 7.5), 10 mM $MgCl_2$, 1 mM EDTA, 2.5 mM DTT, and 0.5% (w/v) BSA. All enzymes were measured in fixed point assays at 33°C. Product formation was measured at four different time points for each separate extract.

Sucrose synthase: Sucrose synthase was measured in the synthetic direction by quantitation of sucrose formation using the resorcinol method (2). Assays (350 μl) contained 7.0 μmol fructose, 3.5 μmol UDP-glucose dissolved in 50 mM Hepes-NaOH (pH 7.5), 15 mM $MgCl_2$ plus 135 μl desalted extract. The reaction was terminated after 0, 5, 10, and 20 min by removing a 70 μl aliquot of the reaction mixture and placing it in a tube containing 70 μl 1 N NaOH. Tubes were boiled for 10 min to destroy any remaining fructose, then 250 μl 0.1% (w/v) resorcinol in 95% ethanol plus 750 μl 30% (v/v) HCl were added. Tubes were incubuated at 80°C for 8 min and after cooling, the A_{520} was measured. Sucrose formation was quantitated by comparison to a sucrose standard curve after subtraction of A_{520} at 0 min (background).

Sucrose phosphate synthase: Sucrose phosphate synthase was assayed by essentially the same procedure as sucrose synthase, except that assays (350 μl) contained 3.5 μmol fructose 6-P and 8.75 μmol UDP-glucose dissolved in 50 mM Hepes-NaOH (pH 7.5), 15 mM $MgCl_2$ plus 135 μl desalted extract.

Acid invertase: Acid invertase was measured in a reaction mixture (300 μl) containing 30 μmol sucrose dissolved in 100 mM citrate-phosphate buffer (pH 5.0) and 80 μl desalted extract. Aliquots (50 μl) of the reaction mixture were removed after 0, 5, 10 and 20 min and the reaction was terminated by boiling for 30 s. The hexose sugar formed due to invertase activity was detected by measuring the NADPH formed in a 1 ml mixture containing 50 μl of the heat-killed sample plus 1 mM NADP, 1 mM ATP, 0.5 unit hexokinase, 2 units phosphoglucoisomerase and 3 units glucose-6-P dehydrogenase, 5 mM $MgCl_2$, 5 mM DTT, and 100 mM Hepes-NaOH (pH 8.0).

Neutral invertase: Neutral invertase was measured by the same procedure as the acid invertase except that 160 μl desalted extract was incubated wth 30 μmol sucrose in 50 mM Hepes-NaOH (pH 7.5).

Nonstructural Carbohydrate Analysis. Starch and soluble sugars (sucrose and hexose sugars) were measured in prefrozen tissue samples as described by Huber *et al.* (10).

Statistical Analysis. Diurnal changes in the measured parameters were analyzed statistically by one way analysis of variance. Values represent the means of six determinations ±SE.

RESULTS

Diurnal Changes in Maize Leaf Elongation. Figure 1, curve A, shows the diurnal changes in elongation rate of the sixth leaf of 22 d old maize plants measured over the natural light/dark cycle. Diurnal changes in the rate of leaf elongation were statistically significant and elongation rate was greater during the day than at night. During the day the elongation rate profile was fairly symmetrical, so that elongation rate changes were similar in the morning and afternoon and the maximum rate was observed at midday. Of the total leaf elongation (4.6 cm) that occurred during the 24 h period, approximately 70% occurred during the light period. Leaf six elongation was related to a total increase in dry weight, such that elongation during the light period (3.2 cm) was equivalent to an increase in dry weight of 54 mg in the elongating zone that was sampled. Approximately 20% of the increase in dry weight could be accounted for by an accumulation of starch (10.2 mg). During the dark period, elongation (1.4 cm) was equivalent to an increase in dry weight of 18.5 mg. A significant,

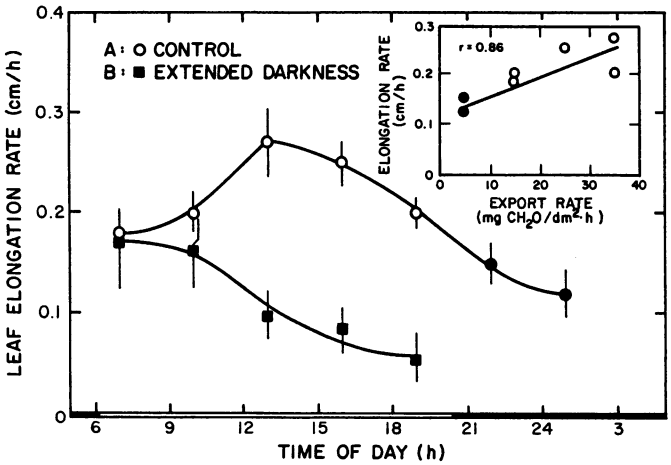

FIG. 1. Diurnal maize leaf elongation rate. Curve A (continuous line) indicates elongation rates of plants in the greenhouse, exposed to the normal light/dark cycle: (O), measurements made in the light; (●), measurements made in the dark. Curve B (■) indicates elongation rate of plants held in a darkened growth chamber after the end of the normal dark period. Inset: relation between leaf elongation rate (curve A) and assimilate export rate (12) from the distal portion of the leaf.

positive correlation was also apparent between the rate of leaf elongation and the rate of assimilate export from the distal portion of the leaf over the 24 h light/dark cycle (Fig. 1 inset). The elongation of leaf seven was also monitored (data not shown) and a similar diurnal pattern was observed, although the elongation rate of leaf seven was always greater than leaf six. At this stage of development leaf six was 62% fully elongated and leaf seven was 41% elongated.

In a separate experiment, plants were transferred to a darkened growth chamber at the end of the normal dark period, and leaf elongation was monitored during the following 12 h period (Fig. 1, curve B). After about 4 h, a decrease in the leaf elongation rate was apparent in these plants compared to plants in the light. In extended darkness, the rate of leaf elongation continued to decline for the entire period, and no midday peak in elongation rate was observed.

Diurnal Changes in Tissue Carbohydrate Concentration. Diurnal changes in the concentrations of sucrose, hexose sugars and starch in elongating maize leaf tissue are shown in Figure 2. There were significant diurnal changes in the concentration of sucrose and starch, and accumulation of these carbohydrates occurred almost exclusively during the afternoon. There was, however, no significant accumulation of hexose sugars. The level of sucrose remained essentially constant from the beginning of the light period until about 0900 h, then increased and reached a maximum at 1500 h (Fig. 2A). After 1500 h, the sucrose concentration decreased at a relatively constant rate and reached a minimum concentration during the middle of the night. The highest and lowest sucrose concentrations differed by about 3.5-fold. Hexose and sucrose levels were similar from about the middle of the dark period until 0900 h, when the sucrose concentration began to rise. The concentration of starch measured in the elongating tissue was higher than the level of sucrose or hexoses at all times during the experimental period (Fig. 2, A and B). The concentration of starch was relatively constant from the begining of the light period until 1200 h after which starch accumulated in the tissue (Fig. 2B). Starch content was greatest at the end of the light period, and this maximum concentration of starch represented a 6.7-fold increase over the minimum starch content observed at the beginning of the light period. Mobilization of starch at night occurred at a fairly constant rate from the beginning of the dark period until about half way through the night, after which the mobilization rate decreased. There appeared to be no delay in the initiation of starch mobilization at the beginning of the dark period and essentially all of the starch that was accumulated during the day was remobilized at night.

Diurnal Pattern of Sucrose Metabolizing Enzymes. Acid and neutral invertase, sucrose synthase and sucrose phosphate synthase were measured in extracts of tissue taken from the elongating portion of leaf 6 over the day/night cycle. Significant diurnal changes were observed in the activities of acid and neutral invertase, and sucrose phosphate synthase, but not sucrose synthase (Figs. 3 and 4). Figure 3 shows the diurnal activity change of acid invertase (panel A) and neutral invertase (panel B). Both the acid and the neutral invertase activities were highest during the light period; maximum acid invertase was observed at 0900 h, and neutral invertase activity was highest at noon. The acid invertase activity was higher than the neutral activity at all times during the diurnal cycle. At their respective maxima, acid invertase was about 2.4-fold higher than the neutral activity, and at their minima, acid exceeded neutral invertase activity by about 4-fold. After reaching a maximum at 0900 h there was a rapid decline in the acid invertase activity, so that the minimum activity was observed at 1500 h, 6 h after the point of maximum activity. Acid invertase activity then remained fairly constant from 1500 h until the beginning of the following light period. The neutral invertase activity decreased more gradually than the acid invertase activity and reached a minimum at the beginning of the dark period—about 9 h after maximum activity was

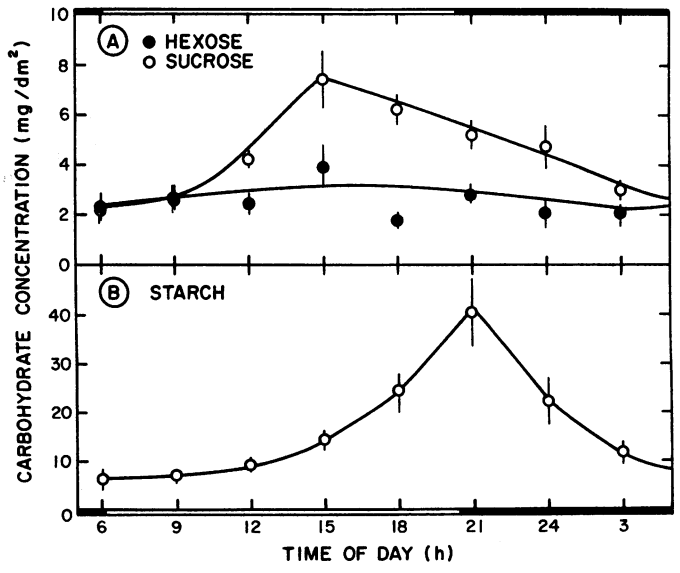

FIG. 2. Diurnal changes in maize leaf carbohydrate concentrations. A, Sucrose and hexose sugars (glucose + fructose, glucose 6-*P*, fructose 6-*P*); B, starch. Note the differences in ordinate scales between panels A and B. Bars at the top and bottom of the figure indicate the light conditions at the time of harvest: open bar, light, closed bar, dark.

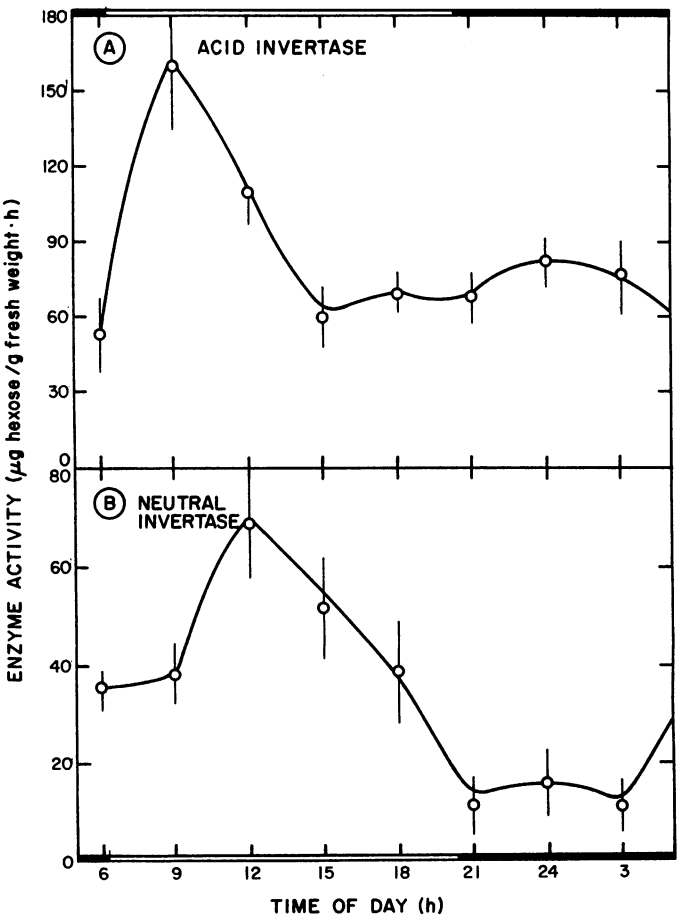

FIG. 3. Diurnal changes in the activities of (A) acid invertase and (B) neutral invertase. Note differences in the ordinate scale between the two panels.

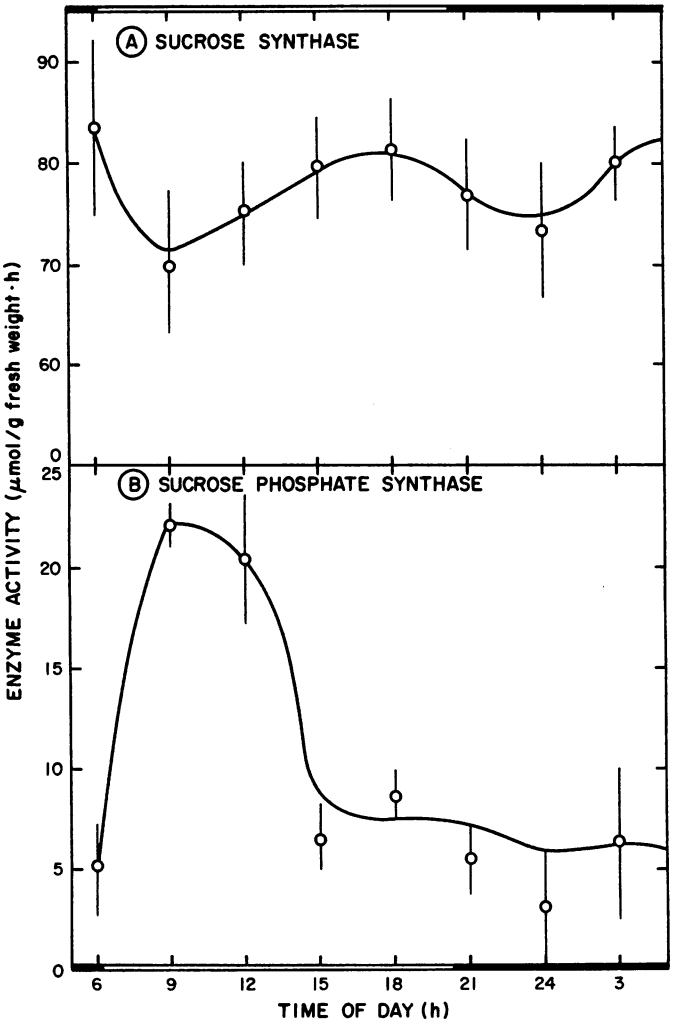

FIG. 4. Diurnal changes in the activities of (A) sucrose synthase and (B) sucrose phosphate synthase. Changes observed in sucrose synthase activity were not statistically significant. Note differences in the ordinate scale between panels A and B.

observed at noon. Neutral invertase activity remained low for the remainder of the dark period.

Diurnal changes in sucrose synthase and sucrose phosphate synthase are shown in Figure 4. Sucrose synthase activity was constant at approximately 80 μmol/min·g fresh weight (panel A). In contrast, pronounced diurnal changes in sucrose phosphate synthase activity were observed. Highest activity occurred at 0900 h, then sucrose phosphate synthase activity dropped fairly rapidly and remained essentially constant (about 6 μmol/min·g fresh weight) after 1500 h. Maximum sucrose phosphate synthase activity observed (about 22 μmol/min·g fresh weight) was lower than the activity of sucrose synthase (about 80 μmol/min·g fresh weight).

DISCUSSION

In maize, catabolism of carbohydrate in the elongating, proximal portion of the leaf provides the carbon necessary for leaf growth. In the growing region of the leaf, carbohydrate is derived primarily from import of photosynthate, from breakdown of stored reserves (sucrose and starch) and possibly from local photosynthesis. Hexose sugars derived from these carbohydrate sources are utilized in leaf growth by hexose conversion to cellular constituents or hexose catabolism for energy production. This study characterized some parameters of growth and carbohydrate utilization over a natural light/dark cycle. To our knowledge, diurnal characterization of leaf elongation, carbohydrate content and activities of sucrose metabolizing enzymes in young vegetative maize plants has not been previously reported.

Kalt-Torres *et al.* (12) have characterized diurnal carbon exchange rate and assimilate export rate in the distal, source portion of leaf 6. The maximum rate of CO_2 fixation and assimilate export occurred at midday, and was therefore coincident with the maximum rate of leaf elongation observed in this study. When leaf elongation rate was plotted against assimilate export rate, a significant positive correlation was apparent ($r = 0.86$) (Fig. 1, inset). Other lines of evidence support a possible relationship between assimilate export rate and the rate of leaf elongation. Data obtained by Hofstra and Nelson (9), indicate that the distal portion of the leaf supplies a significant proportion of photosynthate to the proximal portion of the elongating leaf. Twenty-four h after $^{14}CO_2$ labeling of the distal portion of leaf 6 of 3 week old maize plants, 64% of the labeled carbon remained in the proximal portion of the leaf, below the fed area. In our study, when plants were subjected to continued darkness after the end of the normal dark period, the leaf elongation rate was substantially decreased and no midday peak in elongation was observed (Fig. 1, curve B). Carbohydrate reserves were essentially depleted at the end of the normal dark period (Fig. 2, A and B), and continued darkness precluded carbon assimilation and export. Collectively, these results suggest that leaf elongation rate may be coordinated with the rates of CO_2 fixation and assimilate export from the distal, source portion of the leaf.

The profile of change in leaf elongation rate was fairly symmetrical between the morning and afternon hours, and the maximum rate was observed at midday. Assuming that the requirement for carbohydrate to sustain a given rate of leaf elongation was the same at different times of the day, it is interesting to note that essentially all of the carbohydrate (starch and sucrose) present in the tissue at the end of the day had accumulated in the afternoon. This result suggests that the rate of assimilate import into the tissue was greater in the afternoon than in the morning. It is possible that an accumulation of carbohydrate in other sink tissues also fed by the source portion of leaf 6 diminished their respective rates of assimilate import, and in this way may explain the observed carbohydrate accumulation in the elongation zone of leaf 6 during the afternoon hours.

At no point was there a significant accumulation of hexoses (Figs. 1 and 2) which suggested that available hexose sugars were immediately catabolized. Diurnal profiles of starch and sucrose concentration in the elongating tissue (Fig. 2, A and B) were markedly different than the diurnal changes in starch and sucrose concentrations in the distal, source portion of the leaf (12). In contrast to the profile of sucrose concentration shown in Figure 2A, a rapid accumulation of sucrose was observed at the beginning of the light period in the source portion of the leaf. The sucrose concentration continued to increase for 12 h during the day, and then decreased rapidly at the end of the light period (12). In the elongating tissue, however, sucrose accumulated for only 6 h, beginning at midmorning until 1500 h, then gradually decreased over the remainder of the light and dark period. The maximum concentration of sucrose observed in the source tissue was significantly higher (25 mg/dm^2) than in the elongating tissue (7.5 mg/dm^2). The maximum concentration of starch observed in the source tissue (12) was essentially the same as the level observed in the elongating tissue (Fig. 2B); (42 mg/dm^2 and 40 mg/dm^2, respectively). However, in the source tissue, the starch accumulation rate began to increase at 0900 h and slowed before the end of the light period, while in the elongating tissue, the rate of starch accumulation increased at noon, and accumulation was rapid until the end of the light period.

Starch accumulation accounted for 20% of the increase in specific leaf weight observed during the light period. This increase is equivalent to previously reported values for changes in specific maize leaf weight due to starch accumulation (3). By the end of the day, starch represented the major pool of carbohydrate in the elongating tissue, and there was no significant delay in the initiation of starch mobilization at the beginning of the dark period. During the night, leaf elongation continued at 45 to 60% of the maximum rate observed at midday (Fig. 1). Based on calculations of the increase in dry weight due to elongation at night and the degradation of accumulated starch at night, hexoses derived from starch may have accounted for as much as 73% of the calculated increase in dry matter due to leaf growth. This calculation does not account for respiratory consumption of carbon and is based on the assumption that no carbohydrate was exported from or imported into, the elongating tissue during the dark period. In this regard, the rate of assimilate export from the source portion of the leaf during the night was approximately 10% of the maximum rate observed during the day (12). Both the leaf elongation rate and starch content decreased at a relatively constant rate for most of the night (Figs. 1 and 2B).

The activities of sucrose phosphate synthase, acid invertase and neutral invertase displayed pronounced diurnal fluctuations in the elongating maize leaf tissue (Figs. 3, A and B, and 4B). Because the respective activity maxima of the neutral and the acid invertase activity were displaced by 3 h (Fig. 3, A and B), they were cleary not the same enzyme activity, functioning at pH 5.0 and 17.5. Only the activity of neutral invertase was significantly correlated with the rate of leaf elongation ($r = 0.93$). This result suggests that the diurnal changes in neutral invertase activity may affect the supply of hexoses available for leaf growth, and this observation thus warrants further study.

Compartmentation of specific enzymes and substrates is an important factor in the regulation of sucrose import, storage, and utilization and must therefore be considered when interpreting these results, because enzyme and carbohydrate measurements were done on whole tissue extracts. There is a paucity of information on the pathways of carbohydrate uptake and catabolism in maize leaf sink tissue, and extensive diversity is known to exist in the mechanism of sucrose uptake in different tissues. For example, Giaquinta *et al.* (6) provide evidence that in the roots of maize seedlings, uptake of exogenous sucrose from the apoplast requires sucrose hydrolysis by a cell wall-bound acid invertase, while translocated sucrose symplastically enters the cell and is hydrolyzed at the vacuole prior to metabolism. In sugar cane leaf sheaths, the vacuolar acid invertase has been shown to fluctuate diurnally (7), and may also be subject to metabolic regulation (*e.g.* product inhibition [15]). Although acid invertase results presented here provide no information regarding cellular compartmentation, this activity did fluctuate diurnally and appeared to be reciprocally related to sucrose content (Figs. 2A and 3A).

The uptake, metabolism, and storage of sucrose have been most thoroughly studied in sugar cane. It is proposed that in this tissue sucrose undergoes extracellular hydrolysis, is taken up by the cell as hexose sugars, and is then phosphorylated and reconverted to sucrose-phosphate by sucrose phosphate synthase (for review see Ref. 7). Sucrose thus formed can be stored in the vacuole or can be reconverted to hexose by the action of sucrose synthase or neutral invertase. Therefore, neutral invertase and sucrose synthase activities may regulate carbon partitioning between a storage (vacuolar) sucrose pool and a cytosolic hexose pool which would be available for metabolic maintenance and growth. Sucrose catabolism via sucrose synthase is important in leaf growth since one of the reaction products, a sugar nucleotide, is a precursor of structural and storage polysaccharides (1). It is not known whether similar pathways for sucrose import and catabolism exist in maize leaf sink tissue. Diurnal profiles of acid invertase and sucrose phosphate synthase activities were very similar (Figs. 3A and 4B), but their respective maxima were not coincident with maxima in leaf elongation rate (Fig. 1, curve A), or assimilate export rate in source portion of the leaf (12).

Results obtained in the present study provide evidence for a coordination among the processes of carbon assimilation, export and assimilate utilization for maize leaf growth. Leaf elongation appears to be regulated, in part, by the availability of carbohydrate (due to import or local photosynthesis) and the activity of neutral invertase. It is important to note however that other factors are also important determinants of the rate of maize leaf elongation. For example, leaf water potential (11), and temperature of the apical meristem (17), have been shown to affect the elongation rate of maize leaves.

The diurnal pattern of leaf elongation presented here (Fig. 1) is not common to all monocots. Unlike maize, leaf elongation rate in onion (*Allium cepa*) (4) and orchard grass (*Dactylis glomerata*) (5) was constant over the day/night cycle. Carbohydrate content was examined in the study using onion; an accumulation of nonstructural carbohydrate was observed before the beginning of the light period, and utilization of accumulated carbon commenced before the beginning of the dark period. Therefore, although the pattern of leaf elongation may be the same in all monocots (*i.e.*, a source/sink gradient in the growing leaf) other metabolic factors clearly influence the elongation rate over the day/night cycle.

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