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# Soluble Sugars as the Carbohydrate Reserve for CAM in Pineapple Leaves[1]

## Implications for the Role of Pyrophosphate:6-Phosphofructokinase in Glycolysis

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### ABSTRACT

**Neutral ethanol-soluble sugar pools serve as carbohydrate reserves for Crassulacean acid metabolism (CAM) in pineapple (*Ananas comosus* (L.) Merr.) leaves. Levels of neutral soluble sugars and glucans fluctuated reciprocally with concentrations of malic acid. Hexose loss from neutral soluble-sugar pools was sufficient to account for malic acid accumulation with about 95% of the required hexose accounted for by turnover of fructose and glucose pools. Hexose loss from starch or starch plus lower molecular weight glucan pools was insufficient to account for nocturnal accumulation of malic acid. The apparent maximum catalytic capacity of pyrophosphate:6-phosphofructokinase (PPi-PFK) at 15°C was about 16 times higher than the mean maximum rate of glycolysis that occurred to support malic acid accumulation in pineapple leaves at night and 12 times higher than the mean maximum rate of hexose turnover from all carbohydrate pools. The apparent maximum catalytic capacity of ATP-PFK at 15°C was about 70% of the activity required to account for the mean maximal rate of hexose turnover from all carbohydrate pools if turnover were completely via glycolysis, and marginally sufficient to account for mean maximal rates of acidification. Therefore, at low night temperatures conducive to CAM and under subsaturating substrate concentrations, PPi-PFK activity, but not ATP-PFK activity, would be sufficient to support the rate of glycolytic carbohydrate processing required for acid accumulation. These data for pineapple establish that there are at least two types of CAM plants with respect to the nature of the carbohydrate reserve utilized to support nighttime $CO_2$ accumulation. The data further indicate that the glycolytic carbohydrate processing that supports acidification proceeds in different subcellular compartments in plants utilizing different carbohydrate reserves.**

The nocturnal acidification phase of CAM is supported by the glycolytic conversion of large quantities of reserve carbohydrate to PEP.[2] PEP is subsequently carboxylated and the product, oxaloacetate, is reduced to form the store of malic acid that accumulates in the vacuoles of chloroplast-containing cells of CAM plants. During the day, malic acid is remobilized from the vacuole and decarboxylated. The $CO_2$ so generated is fixed via the reductive pentose phosphate pathway. The 3-carbon product of the decarboxylation reaction, either PEP or pyruvate, is converted gluconeogenically to carbohydrate, thereby replenishing the reserve carbohydrate pools. The coupling of reserve carbohydrate turnover, in large part, to the production of large intracellular stores of organic acids rather than to the synthesis of sucrose for export from the cell is a unique feature of CAM. CAM plants commit as much as 5% to 17% of their total dry weight (*e.g.* pineapple and *Bryophyllum calycinum* [22], respectively) to the CAM cycle. Based upon $O_2$ studies of CAM plants (*e.g.* Refs. 1, 20), we estimate that the relative quantity of carbohydrate committed to support nocturnal acidification in these species is 20 to 170 times the carbohydrate catabolized at night to meet respiratory energy needs.

The current concept of CAM, especially the nature of the carbohydrate pools that support malate biosynthesis during the dark period, is based almost entirely on data for species of Crassulaceae. In most of these species, starch is the source of hexose for malate synthesis. For at least two species of Crassulaceae and one of Cactaceae, however, lower mol wt glucan pools supplement hexose loss from starch and contribute significant quantities of hexose for conversion to PEP, and hence, to malate (25, 30). The behavior of low mol wt sugar pools in these species precludes their involvement as carbohydrate sources for malate production (22, 25, 29, 32).

In contrast, the present report demonstrates that neutral soluble sugar pools serve as carbohydrate reserves for CAM in pineapple (*Ananas comosus* (L.) Merr., Bromeliaceae). Pronounced diurnal changes in the concentration of ethanol-soluble sugars in pineapple leaves were reported by Sideris *et al.* (23), however, since the carbon fixation pathway utilized by pineapple had not been identified, the significance of the observation was not apparent. In the present report we confirm the early observation of Sideris *et al.*, identify the low mol wt sugars that fluctuate diurnally, characterize the dynamics of pool size changes of ethanol-soluble sugars, starch, and low mol wt glucans, and interpret the results with respect to the operation of CAM in pineapple. We show that although

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[2] Abbreviations: ATP-PFK, nucleoside triphosphate dependent 6-phosphofructokinase; PEP, phospho*enol*pyruvate; PPi-PFK, pyrophosphate dependent 6-phosphofructokinase (pyrophosphate:6-phosphofructose 1-phosphotransferase); gfw, gram fresh weight.

glucan fractions in pineapple leaves fluctuate diurnally, fructose and glucose pools provide the majority, if not all, the hexose required to support nocturnal $CO_2$ accumulation. A preliminary report (2) of these results has been presented.

The turnover of neutral soluble sugar reserves to support $CO_2$ assimilation and malic acid accumulation at night has important implications with respect to the nature and the compartmentation of the glycolytic reactions that mediate the transfer of carbon between these two pools. Plant cells contain cytoplasmic and plastid isozymes of nucleoside-triphosphate-dependent phosphofructokinase (10, 16) and a cytoplasmically localized PPi-dependent phosphofructokinase (*e.g.* Refs. 2, 4, 7, and 19). These enzymes catalyze the first unique step of glycolysis, *i.e.* the phosphorylation of Fru-6-P to form Fru-1,6-$P_2$, using ATP or PPi, respectively, as the energy source and phosphate donor.

Extractable activities of PPi-PFK from pineapple leaves are 15 to 20 times higher than activities of ATP-PFK. PPi-PFK activities in CAM plants of four other families likewise are higher (4–70-fold) than respective activities of ATP-PFK (5). The properties of ATP-PFK in plants suggest that it is an important regulatory enzyme and that it contributes substantially to control of carbon flow through glycolysis (27). The high activities of PPi-PFK with respect to ATP-PFK in pineapple and other CAM plants, however, suggest that PPi-PFK participates in glycolytic carbon catabolism in these plants especially at night when demand for carbon processing via glycolysis to support $CO_2$ accumulation, respiratory energy needs, the production of required metabolites and the biosynthesis of exportable carbohydrates is high. To test this hypothesis, we determined the range of mean maximum rates of glycolysis in pineapple leaves during the acidification phase of CAM by measuring nocturnal changes in carbohydrate and malic acid pools, respectively. These estimates of hexose processing via glycolysis were then compared to the catalytic capacities of PPi-PFK and ATP-PFK in pineapple leaves.

## MATERIALS AND METHODS

### Plant Material

Isogenic *Ananas comosus* (L.) Merr. plants were grown in the Botany Department greenhouse, University of Georgia, Athens, GA. Plants were transferred to a Conviron controlled environment chamber three to four weeks before use and acclimated to a 15 h/30°C/60 to 70% RH day and 9 h/15°C/90% RH night. Quantum flux density at canopy height was 250 $\mu E/m^2 \cdot s$. Plants were fertilized once a week with a commercial 15:15:15 ($N:P_2O_5:K_2O$) fertilizer at a rate of 1.8 g/L.

### Reagents

Perchloric acid, starch, and sucrose were purchased from J. T. Baker. Starch was dialyzed against distilled water for 24 h with three changes of water and dried in a desiccator. D-Glucose and resorcinol were obtained from Fisher Scientific Co. Anthrone, glucose analysis kit No. 510, β-D-fructose, invertase, and L-malate were obtained from Sigma Chemical Co.

### Tissue Sampling and Extraction

Three successively aged leaves were sampled for each of six plants every 3 h for a 24-h period. Samples were obtained by taking successive transverse cuts beginning 15 cm from the leaf tip and ending approximately 35 cm from the tip. The composite sample of three leaf sections per plant per time point weighed about 1 g. Control experiments showed that starch, titratable acid, and soluble sugar levels in samples taken at the extremes of similar sample areas early and late in the light period were comparable. Likewise, acid and carbohydrate levels in the three leaves were similar.

The leaf pieces were washed, blotted dry, halved longitudinally, then separated into two equivalent subsamples. One subsample was stored at −20°C and used later for titratable acid determination and malate assay. The remaining subsample was immediately sliced into small pieces and homogenized in about 5 mL 80% ethanol/g tissue. The homogenate was quantitatively transferred to a centrifuge tube (final ethanol:tissue volume was at least 10:1) and quickly brought to 75°C in a water bath. Particulate material was ground against the tube wall throughout the 5 min extraction period. The extract was centrifuged for 3 min at 1400*g*. The supernatant was removed and the pellet reextracted three additional times with 80% ethanol at 75°C for 5 min with grinding as before. The supernatants from the hot 80%-ethanol extractions plus two 80%-ethanol washes of the pellet were combined and centrifuged at 22,000*g* at 4°C for 20 min. The composite supernatant volume was adjusted to 25 mL with 80% ethanol. Control experiments indicated more than 99% of the 80% ethanol-soluble sugars were extracted with this procedure. Ethanol-insoluble materials from all centrifugations were combined and resuspended in 5 mL of water. Ethanol-soluble and insoluble fractions were stored at −20°C.

### Glucan Extraction and Starch Precipitation

Ethanol-insoluble fractions were thawed, then boiled with refluxing for 15 min. After cooling, 52% $HClO_4$ was added to give a final concentration of 29.4% (13). Samples were ground against the tube wall for 5 min, then intermittently for the next 15 min. The extract was centrifuged at 12,000*g* for 30 min. The pellet was reextracted in 6.75 mL of 29.4% $HClO_4$ with grinding as before. Supernatants from the two perchlorate extractions and two distilled water washes of the pellet were combined and the volume adjusted to 25 mL with distilled water. The $HClO_4$-solubilized carbohydrate was termed total glucan.

Iodine-precipitable glucans (herein defined as starch) were precipitated from a 10-mL aliquot of each $HClO_4$ extract as described by Hassid and Neufeld (13). Ethanolic NaCl washes were repeated twice. The starch precipitate was resuspended in hot water and the volume adjusted to 3 mL after cooling. In control experiments, mean recovery (±SE) of added authentic starch aliquots with this procedure was 98% ± 2% ($n = 6$).

Total glucan and starch were determined as glucose equivalents in the $HClO_4$ extract and the starch preparation, respectively, using 0.1% anthrone in 72% $H_2SO_4$ (11, 13). Samples were diluted, if necessary, with 20.3% $HClO_4$ and

distilled water, respectively. Samples and anthrone reagent were precooled on ice. After mixing, assay tubes were kept on ice 10 min, then immersed in a 100°C bath for 7.5 min, then rapidly cooled in an ice bath. $A_{620}$ was determined after samples warmed to room temperature. Glucose standard solutions, prepared in 20.3% $HClO_4$ or water, as appropriate, were assayed with three dilutions of each sample in the standard curve range. Data for all glucan fractions were expressed as μmol hexose eq/gfw.

### Sugars Soluble in 80% Ethanol

A 20-mL aliquot of the ethanol-soluble fraction was evaporated under vacuum at 50°C to a small volume. The final volume of the concentrated sample plus several water washes of the flask was about 5 mL. A neutral soluble sugar fraction was prepared from 2 mL of the latter extract according to the method of Sutton (25). Glucose, sucrose, and fructose were assayed. Appropriate standards and blanks were assayed with three dilutions of each sample in the standard curve range for each set of samples.

Glucose was determined with a Glucostat kit (Sigma) with the following modifications: 2.5 mL of reagent A″ (Sigma) were added to a sample appropriately diluted in a final volume of 0.5 mL. The reaction was terminated with 50 μL of 4 N HCl after 20 min at 30°C and the $A_{425}$ determined.

Sucrose was hydrolyzed for 30 min at 30°C in a 0.5 mL reaction mixture containing the sample, 25 μmol of potassium acetate/acetic acid buffer pH 4.5, and 20 units of invertase. Control experiments indicated sucrose hydrolysis was virtually complete in 15 min. Total glucose was determined as described above. Glucose derived from sucrose was determined by subtraction. Sucrose concentrations were expressed as μmol hexose eq/gfw.

Fructose and fructosans were assayed according to the method of Huber (15). The sensitivity of this assay initially necessitated fructose determination in concentrated extracts that had not been purged of ions. Control experiments indicated phosphorylated fructose compounds did not contribute significantly to fructose estimates in these samples. After discovering that reducing the resorcinol concentration to 0.1% increased the assay sensitivity 3x, we were able to determine fructose concentrations in neutral extracts. The extract fraction utilized for fructose analysis for various plants is noted in pertinent figures.

### Chromatography

The predominant sugars in neutral ethanol-soluble sugar extracts of pineapple leaves were identified by one-dimensional paper chromatography. Fifty μg of standard authentic sugars in 80% ethanol were spotted separately and in a mixed preparation on Whatman No. 1 paper along with aliquots of the neutral sugar fractions from two early and two late light period pineapple leaf samples. Assays indicated between 15 μg and 40 μg of glucose and sucrose were applied in the various aliquots. The chromatogram was developed by descending solvent flow at 22°C in 4:1:5 butanol:acetic acid:water. Sugars were detected with aniline oxalate, 1% $KMnO_4$ in 3% $H_2SO_4$, and with modified Dische's reagent (3).

### Determination of Titratable Acidity

Leaf samples were sliced into small pieces, then ground in a small volume of $CO_2$-free water in a glass mortar. The homogenate was quantitatively transferred to a centrifuge tube, brought to a volume of nearly 10 mL, and boiled for 10 min. After cooling, the extract was centrifuged at 12,000*g* for 10 min. The pellet was washed twice with $CO_2$-free water. The three supernatants were combined and the volume adjusted to 25 mL. An aliquot was titrated to pH 8.3 with 4 mM NaOH. Data were expressed as μmol malic acid/gfw by applying a 2 μeq/μmol malic acid conversion.

### Malate Determination

Malate was determined according to the method of Gutmann and Wahlefeld (12). Duplicate assays were performed on each extract.

### Phosphofructokinase Extraction and Assay

ATP-PFK and PPi-PFK were extracted and assayed as described previously (5), but with 5 mM cysteine replacing β-mercaptoethanol. For determinations of $Q_{10}$, PPi-PFK was assayed in the presence of 0.5 μM Fru-2,6-$P_2$ and the ATP-PFK assay contained 5 mM DTT. The temperature of each assay mixture was determined immediately following the assay.

## RESULTS

### Diurnal Changes in Titratable Acidity and Malic Acid

Concentrations of titratable acids, malic acid and various carbohydrates in pineapple leaves were determined every 3 h for a 24-h period (Figs. 1 and 2). Malic acid concentrations estimated from titratable acid determinations were slightly lower, but highly correlated ($r^2 = 0.96$ ($n = 18$)) with malic acid levels determined by assay (Fig. 1, A and B). Since the underestimate of malic acid from titratable acid determinations was consistent, accurate determination of malic acid changes over any time interval could be determined from these data. Consequently, only titratable acids were monitored in subsequent work. Mean titratable acid levels decreased from approximately 105 μmol malic acid eq/gfw to 12 μmol/gfw during the first 8 h of the light period, rose slightly but remained low during the second half of the light period, and increased to about 100 μmol/gfw during the dark period (Fig. 1A). The rate of malic acid accumulation increased progressively throughout the dark period. Malic acid accumulation late in the dark period was approximately 1.6 times higher than during the earlier hours of the night (Figs. 1A and 3).

### Diurnal Changes in Glucans

The pattern of diurnal changes in total glucans was the inverse of changes in malic acid (Fig. 1, A and C). Starch comprised 70% to 85% of the total glucan fraction throughout

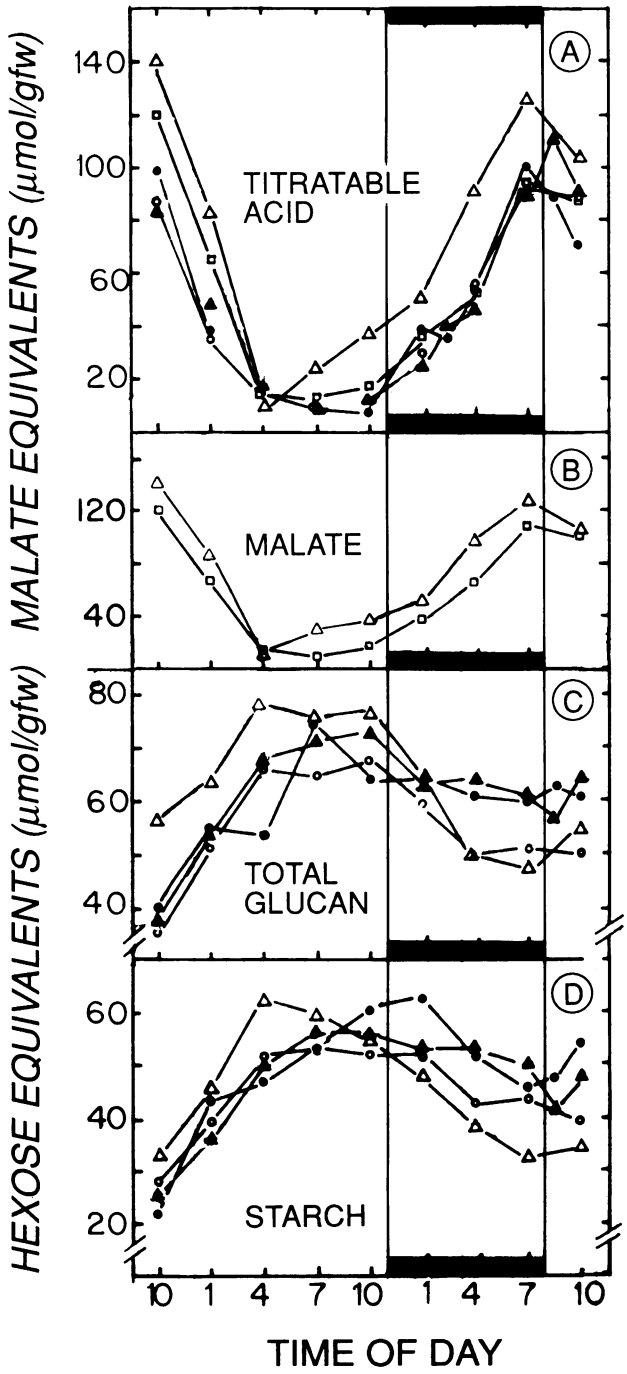


**Figure 1.** Pool sizes of (A) titratable acid, (B) malic acid, (C) total glucan, and (D) starch in pineapple leaves during a 24-h period. Data are given for four to six plants. Titratable acids are given as μmol malate eq/gfw (conversion based on 2 μeq acid per μmol malate). Starch and total glucans (starch plus low mol wt glucans) are given as μmol hexose eq/gfw. Dark bars indicate the 9-h dark period.

the 24 h period and changes in total glucan levels were primarily attributable to changes in starch (Fig. 1, C and D). Starch levels accounted for 83% of the variance in total glucan levels while changes in lower mol wt glucan pools explained only 6% of the variance ($n = 42$). The rate of hexose turnover from total glucans was relatively constant (~2.9 μmol hexose eq/gfw·h) during the first 5 h of the dark period, but decreased dramatically to ~0.4 μmol hexose eq/gfw·h later in the dark period (Figs. 1C and 3). Early in the dark period hexose turnover from low mol wt glucans accounted for approximately two-thirds of the decline in total glucan (Fig. 3). After the first 2 h of darkness, however, the turnover of hexose from starch accounted for all hexose turnover from total glucans. During the last 3 h of the dark period decreases in the starch pool were countered by increases in lower mol wt glucan pools (Fig. 3) indicating starch conversion to smaller polymers.

### Fructosans

No fructose polymers were detected.

### Diurnal Changes in Neutral Ethanol-Soluble Sugars

The three sugars detected in neutral ethanol-soluble sugar fractions of pineapple leaves co-chromatographed with fructose, glucose, and sucrose (4). The dynamics of diurnal changes in pool sizes of these sugars were subsequently examined. Differences in absolute total soluble sugar levels (herein defined as the sum of fructose, glucose, and sucrose levels) and in pool sizes of component soluble sugars among plants were evident (Fig. 2); these between-plant differences were maintained throughout the 24 h period. Hence, turnovers of total soluble sugars, fructose, and glucose during the dark period were very consistent, as evidenced by the low SE for the mean turnover of these components (Table I). Some between-plant variability was attributed to different prehistories of the two sets of plants examined. The three plants exhibiting higher hexose levels, but lower sucrose levels (Fig. 2, B–D) were moved from the greenhouse to the environment chamber in the fall; the three plants with lower hexose but higher sucrose concentrations were placed in the chamber and acclimated to standard conditions in the spring of the following year.

Glucose and fructose concentrations changed reciprocally with changes in malic acid levels (Figs. 1A and 2, B and C). Sucrose concentration, however, remained relatively constant during the light period when malic acid levels were declining rapidly (Figs. 1A and 2D). A small but consistent decline in sucrose was noted early in the dark period. Thereafter, sucrose levels increased to predark levels at the same time the tissue was accumulating malic acid (Figs. 1A and 2D).

Fructose content of leaf tissues ranged from 2.5 to 3.5 times larger than glucose content. Nearly equivalent amounts of hexose, however, were depleted from each of these pools during the night (Fig. 2, B and C; Table I). The mean rate of decline of total soluble sugars was lower early and late in the dark period (~4.7 and 3.8 μmol hexose eq/gfw·h, respectively), and highest from 2 to 5 h into the night (5.5 μmol hexose eq/gfw·h) (Figs. 2A, 3).

### Stoichiometry of Carbohydrate and Malic Acid Changes

A 1:1 stoichiometry of hexose depletion from soluble-sugar pools and hexose required to support malic acid accumulation

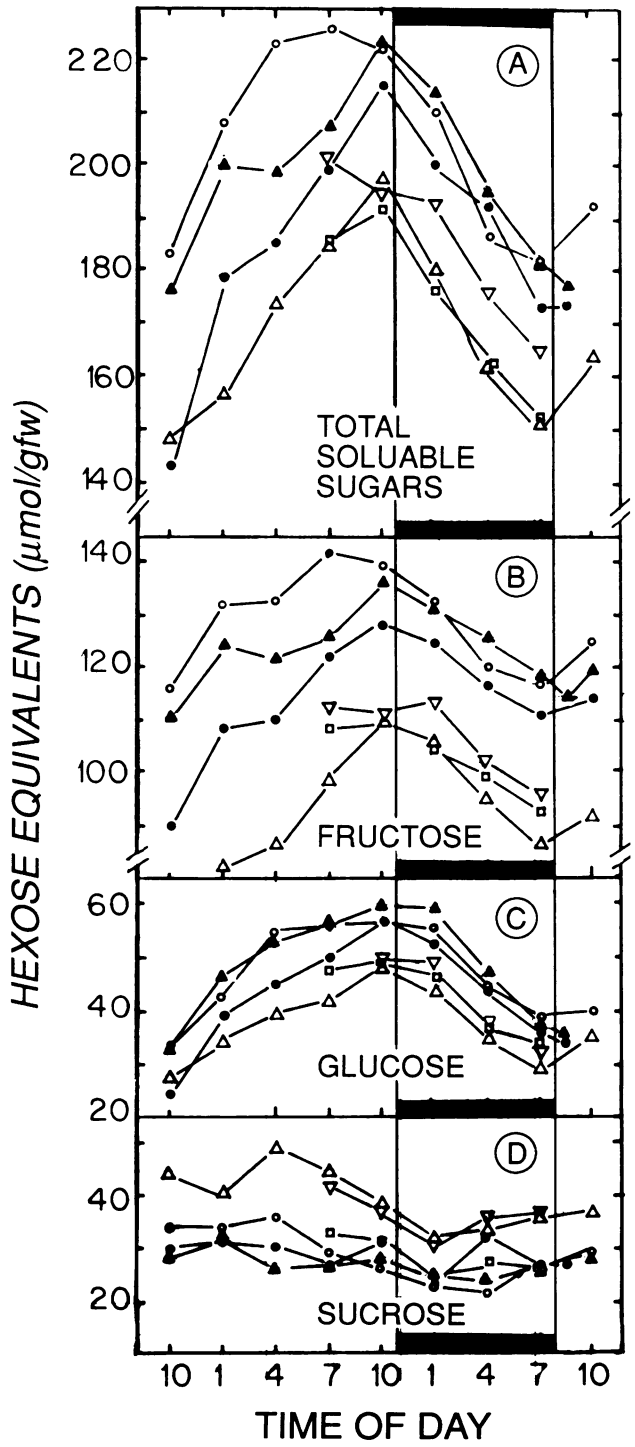


**Figure 2.** Pool sizes of (A) total soluble sugars (sum of fructose, glucose, and sucrose); (B) fructose; (C) glucose; and (D) sucrose in pineapple leaves during a 24-h period. Data are given for six plants. For three plants (△, □, ▽) fructose was determined for neutral (Dowex treated) extracts (see "Materials and Methods"). Data for all soluble sugar fractions are given as μmol hexose eq/gfw. Dark bars indicate the 9-h dark period.

**Table I.** *Increase in Malic Acid and Decrease in Various Carbohydrate Fractions in Pineapple Leaves during a 9-h Dark Period*

| Fraction | Change, Mean ± SE (*n*) |
|---|---|
| | *μmol/gfw* |
| Malate | 84.0 ± 3.3 (5) |
| Total glucan | 18.6 ± 6.4 (4) |
| Starch | 15.6 ± 2.5 (4) |
| Total soluble sugars | 41.8 ± 1.2 (5) |
| Glucose | 19.1 ± 1.2 (5) |
| Fructose | 20.2 ± 1.3 (5) |
| Sucrose | 2.5 ± 1.0 (5) |

at night (1 hexose:2 malic acid) was observed (Table I). In four of five plants examined hexose turnover from soluble sugar pools was completely sufficient to account for malic acid accumulation. In the fifth plant, soluble sugar turnover could supply 91% of the triose-P required for conversion to PEP. In contrast to the very consistent turnover in total soluble sugars at night, considerable variability in turnover of glucan pools was noted among plants (Table I, Fig. 3). On the average, hexose derived from glucans could account for approximately 44% of the malic acid that accumulated. Overall, hexose depletion from all carbohydrate pools examined exceeded hexose required to support malic acid synthesis by about 40%.

### Rates of Glycolysis and Activities of PPi-PFK and ATP-PFK

The maximum rate of malic acid accumulation occurred during the last 3-h sampling period in the dark (Figs. 1A and 3). The mean maximum rate of malic acid accumulation of about 14 μmol/gfw·h (Table II) required glycolytic catabolism of hexose to proceed minimally at a rate of approximately 7 μmol hexose/gfw·h. The measured mean maximum rate of hexose depletion from all carbohydrate pools of 9.4 μmol hexose/gfw·h occurred between 2 and 5 h into the dark period (Table II). These two estimates of the mean maximal rates of glycolysis over a 3-h period during the acidification phase of CAM were considered to represent the range of mean maximal rates of glycolysis occurring at night.

The mean substrate-saturated activities (±SE) of PPi-PFK and ATP-PFK from pineapple leaves at 30°C were 290 ± 13.9 μmol Fru-1,6-$P_2$ produced/gfw·h ($n$ = 5) and 16 ± 1.3 μmol Fru-1,6-$P_2$/gfw·h ($n$ = 5), respectively. $Q_{10}$ values (±SE) for PPi-PFK and ATP-PFK determined from sequential assays at 30°C and about 15°C were 1.86 ± 0.06 ($n$ = 5) and 1.79 ± 0.04 ($n$ = 4), respectively.

## DISCUSSION

### Malic Acid

In pineapple, as in other CAM plants, malic acid is the organic acid that shows dramatic changes in concentration during CAM (Fig. 1, A and B) (20). Citrate levels in pineapple leaves may change diurnally; however, the direction of change varies with respect to changes in malic acid (21, 23). Isocitrate

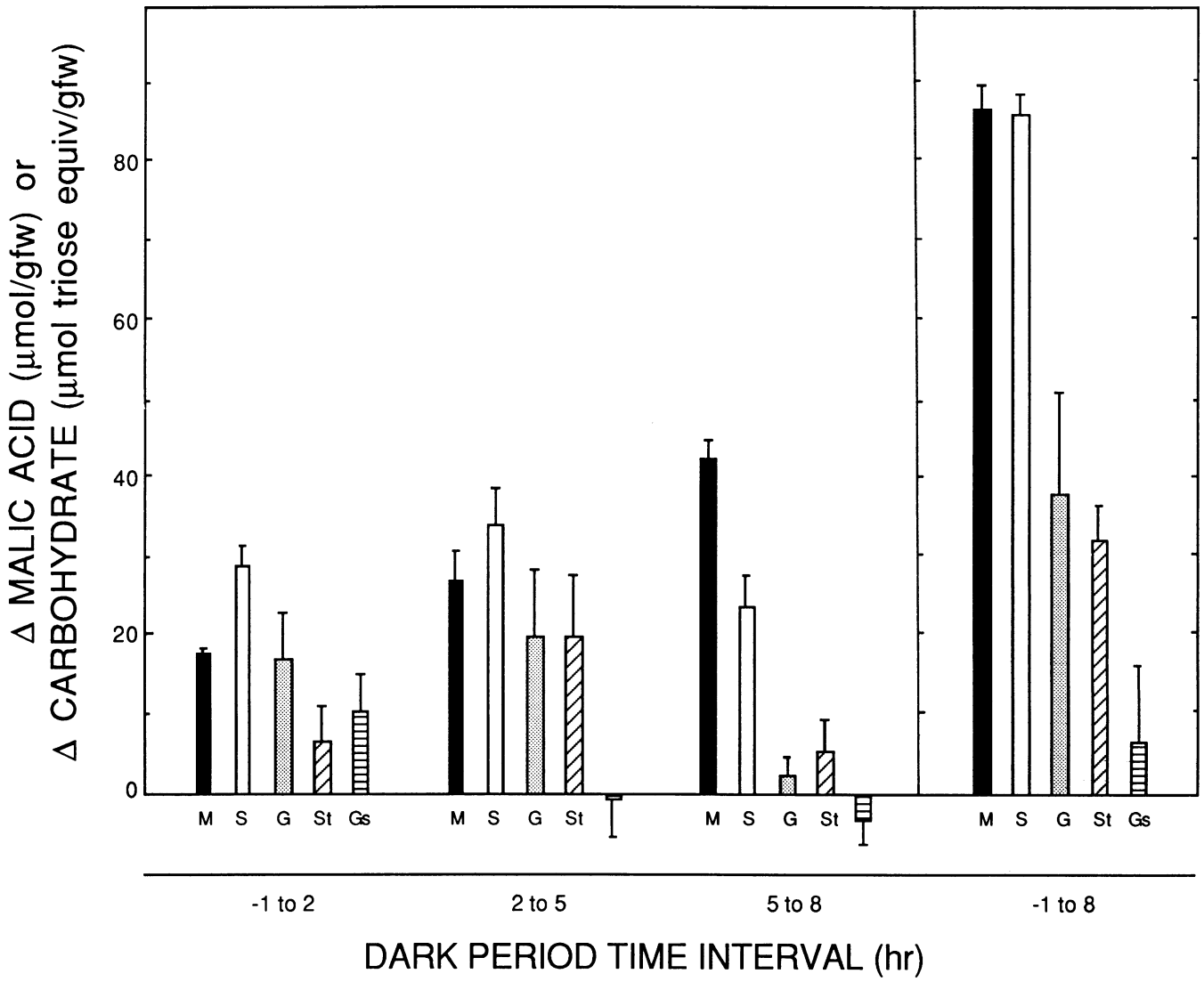


**Figure 3.** Increase in pool size of malic acid (μmol/gfw) and decreases in pool sizes of various carbohydrates (given as μmol triose eq/gfw) in pineapple leaves during the dark phase of CAM. Data (mean ± SE) are pool size changes occurring during three 3-h time intervals beginning 1 h before the onset of the dark period (−1 h) and ending 8 h into the dark period. Cumulative pool size changes from −1 h to 8 h are also presented. For low mol wt glucans a value less than "zero" denotes an increase in the pool size during the time interval indicated. ■M, malic acid ($n$ = 5); □S, total soluble sugars (sum of fructose, glucose, and sucrose) ($n$ = 5); □G, total glucan (starch plus low mol wt glucans) ($n$ = 4). ▨St, starch ($n$ = 4); ▤Gs, low mol wt glucans ($n$ = 4).

**Table II.** *Mean Maximum Rates*[a] *of Malic Acid Accumulation and Carbohydrate Depletion in Pineapple Leaves during the Dark Phase of CAM*

| Fraction | Mean Maximum Rate of Change, Mean ± SE ($n$) |
|---|---|
| | *μmol malic acid or hexose/gfw/h* |
| Malic acid | 14.0 ± 0.8 (5) |
| Total soluble sugars | 6.1 ± 0.6 (5) |
| Total glucan | 3.8 ± 1.2 (4) |
| Total carbohydrate | 9.4 ± 2.0 (4) |

[a] Mean maximum rate is mean hourly rate of change during the 3 h period in the dark during which maximum turnover of the selected fraction occurred.

is detectable only at low concentrations and changes in keto acids contribute little to changes in titratable acidity (21).

### Glucans as Carbohydrate Sources for Malate Synthesis

It is generally accepted in the literature on CAM that glucan pools constitute the sources of hexose that support $CO_2$ accumulation at night. This is clearly not the case for pineapple. Although glucan pools in pineapple leaves fluctuate reciprocally with malic acid levels (Fig. 1, C and D), the turnover of hexose from these pools could, on the average, supply only about 44% of the hexose required to support malic acid synthesis (Table I). *Aloë arborescens*, a CAM plant that, like pineapple, uses PEP carboxykinase as the major enzyme for malate decarboxylation, likewise shows insufficient turnover

of hexose from glucan pools to account for malic acid accumulation (28). In contrast, hexose depletion from either starch alone or starch plus low mol wt glucans is sufficient to account for all the malic acid that accumulates in species of Crassulaceae (22, 25, 32) and in *Opuntia aurantiaca* (22).

Hexose depletion from starch reserves in pineapple leaves accounts for over 80% of the decrease in total glucans at night (Table I). Net removal of hexose from low mol wt glucan pools occurs only in the early hours of the dark period (Fig. 3). These pools increase later in the dark period at the expense of larger mol wt polymers, *i.e.* starch. Starch pools previously reported for pineapple leaves showed little diurnal variation (23). Our calcuations using data of Sideris *et al.* (23) indicate that only about 2% of the increase in titratable acidity reported could be accounted for by removal and processing of hexose from starch reserves. The differences in these results may be due to differences in techniques used to extract and solubilize starch. Starch pools in plants used in the present study were considerably larger than those determined by *Sideris et al.* (23). Alternatively, differences in growth conditions in the two studies may have influenced the relative allocations of carbon to starch and sucrose. Indeed, higher sucrose levels and a larger turnover of sucrose in pineapple leaves at night were obserbed by Sideris *et al.* (23) than in our study. Since very little sucrose is located in protoplasts of pineapple mesophyll cells (18), the larger sucrose turnover observed by Sideris *et al.* (23) indicates that larger amounts of carbohydrate were being exported.

### Neutral Soluble Sugar Pools as Carbohydrate Sources for Malate Synthesis

Pineapple leaves contain large soluble sugar pools (150–250 μmol hexose eq/gfw) that fluctuate diurnally in parallel with glucan fluctuations and reciprocally with changes in malic acid levels (Figs. 1 and 2A). The 1:2 stoichiometry of hexose turnover from soluble sugar pools and malic acid accumulation (or 1:1 stoichiometry of triose-P derived from soluble hexose reserves and malic acid) at night suggests that hexose turnover from these reserves is strongly coupled to malate biosynthesis (Table I, Fig. 3). The data of Sideris *et al.* (23), when extrapolated to the end of the dark period, likewise indicate that the depletion of soluble sugar reserves is sufficient to account for malic acid accumulation. In contrast to these data for pineapple, soluble sugar pools in *Bryophyllum tubiflorum* and *Kalanchoë daigremontiana* are small (~5 μmol/gfw) and remain constant throughout the day/night cycle (25). Furthermore, although hexose derived from $^{14}C$-labeled starch supports malate biosynthesis, it is essentially excluded from flow through soluble sugar pools (25). Soluble sugar pools in *B. calycinum*, while larger than in the latter two species (30 μmol/gfw), behave inconsistently with respect to changes in malic acid (22, 29), again indicating that these pools are not coupled to malic acid accumulation.

Given the magnitude of soluble sugar concentrations in pineapple leaves it is unlikely that these sugars are sequestered in the cytosol or smaller organelles. Vacuoles isolated from pineapple leaves contain substantial amounts of glucose and fructose and diurnal changes in vacuolar hexose levels are consistent with the interpretation that these pools are coupled to CAM (18). The relative contributions of various soluble sugar and glucan pools to malic acid formation at night remains to be determined, but it is clear that soluble sugar pools provide the majority, if not all, triose-P required.

### Phosphofructokinase Activities and Estimated Rates of Glycolysis

The turnover of fructose and glucose pools in pineapple leaves to provide hexose for conversion to PEP, and hence malate, is significant with respect to the compartmentation of glycolytic carbon flow during acidification and the involvement of PPi-PFK and ATP-PFK in hexose catabolism during CAM. Starch degradation to triose-P occurs within the chloroplast (assuming permeability features of chloroplast envelopes are similar in CAM and $C_3$ species). Fru-6-P generated in the chloroplast is therefore inaccessible to PPi-PFK and its phosphorylation to Fru-1,6-$P_2$ must occur via the chloroplast isozyme of ATP-PFK. The mobilization of monosaccharides from vacuoles at night, however, provides a source of hexose to the cytoplasm, thereby increasing the likelihood that PPi-PFK, a cytoplasmically localized enzyme (2, 4, 7, 19), is involved in hexose catabolism coupled to malic acid accumulation. It is perhaps noteworthy that species of Crassulaceae that couple starch degradation to malic acid accumulation exhibit PPi-PFK activities that are either similar to or lower than ATP-PFK activities, while PPi-PFK activities in pineapple are about 15 to 20 times higher than activities of ATP-PFK (5).

Substrate-saturated activities of pineapple leaf PPi-PFK at 30°C are 30 to 40 times the estimated mean maximum rates of glycolysis at night in pineapple (Table II). Activities of total leaf ATP-PFK at 30°C range from just sufficient to about twice the activity required to account for rates of malic acid accumulation. Several factors, however, make it unlikely that sufficient ATP-PFK activity is present in pineapple leaves to catalyze the phosphorylation of Fru-6-P at rates required during the acidification phase of CAM. First, acidification is promoted by low night temperatures. Plants in this study experienced a night temperature of 15°C. The $Q_{10}$ values determined for PPi-PFK and ATP-PFK indicate that activities of these enzymes at 15°C are 39% and 42% (*i.e.* 114 and 6.7 μmol Fru-1,6-$P_2$/min·gfw) of the respective catalytic capacities observed at 30°C. Therefore, at 15°C ATP-PFK activity is nearly sufficient to account for rates of hexose turnover required for malic acid accumulation, but alone, is insufficient to account for rates of total hexose turnover. PPi-PFK activity at 15°C, however, is an order of magnitude higher than that required to account for observed rates of hexose turnover.

Second, neither PPi-PFK nor the isozymes of ATP-PFK are expected to be substrate saturated *in vivo*. Metabolite levels in a few CAM plants have been reported (*e.g.* Ref. 6), however, levels of metabolites in various subcellular compartments of CAM plants are not known. Fru-2,6-$P_2$ concentrations in pineapple leaves increase at night (9) to levels that are consistent with full activation of pineapple PPi-PFK (4). Reported Fru-6-P concentrations in the cytoplasm and chloroplasts of $C_3$ plants (*e.g.* Ref. 31) are near the apparent $K_m$ (Fru-6-P) for Fru-2,6-$P_2$-activated pineapple PPi-PFK (0.35 mM) (4) and the apparent $K_m$ (Fru-6-P) for ATP-PFK from

**Table III.** *Energy Requirements for Malic Acid Synthesis and Accumulation during the Dark Phase of CAM: Starch as the Hexose Donor, ATP-PFK as the Catalyst for the First Committed Reaction of Glycolysis*[a]

| | | |
|---|---|---|
| Starch ($Glc_n$) + $PO_4^{-2}$ | ⟷ | Glc-1-$P^{-2}$ + $Glc_{n-1}$ |
| Glc-1-$P^{-2}$ | ⟷ | Glc-6-$P^{-2}$ |
| Glc-6-$P^{-2}$ | ⟷ | Fru-6-$P^{-2}$ |
| Fru-6-$P^{-2}$ + $ATP^{-4}$ | ⟷ | Fru-1,6-$P_2^{-4}$ + $ADP^{-3}$ + $H^+$ |
| Fru-1,6-$P_2^{-4}$ | ⟷ | G3$P^{-2}$ + DHA$P^{-2}$ |
| DHA$P^{-2}$ | ⟷ | G3$P^{-2}$ |
| 2 G3$P^{-2}$ + 2 $Pi^{-2}$ + 2 $NAD^+$ | ⟷ | 2 1,3-bisPG$A^{-4}$ + 2 NADH + 2 $H^+$ |
| 2 1,3-bisPG$A^{-4}$ + 2 $ADP^{-3}$ | ⟷ | 2 3-PG$A^{-3}$ + 2 $ATP^{-4}$ |
| 2 3-PG$A^{-3}$ | ⟷ | 2 2-PG$A^{-3}$ |
| 2 2-PG$A^{-3}$ | ⟷ | 2 $PEP^{-3}$ + 2 $H_2O$ |
| 2 $CO_2$ + 2$H_2O$ | ⟷ | 2 $HCO_3^-$ + 2 $H^+$ |
| 2 $PEP^{-3}$ + 2 $HCO_3^-$ | ⟷ | 2 $OAA^{-2}$ + 2 $PO_4^{-2}$ |
| 2 $OAA^{-2}$ + 2 NADH + 2 $H^+$ | ⟷ | 2 $Malate^{-2}$ + 2 $NAD^+$ |
| 2 $ATP^{-4}$ | ↷ | 2 $ADP^{-3}$ + 2 $PO_4^{-2}$ + 2 $H^+$ |
| 2 $Malate^{-2}_{cyt}$ + 4 $H^+_{cyt}$ | ⟷ | 2 Malic $acid_{vac}$ |
| Net: 1 Glc + 2 $CO_2$ + $ATP^{-4}$ | ⟶ | 2 Malic $acid_{vac}$ + $ADP^{-3}$ + $Pi^{-2}$ + $H^+$ |
| Net energy per mol malic acid accumulated: 0.5 ATP | ⟶ | 0.5 ADP + 0.5 Pi + 0.5 $H^+$ |

[a] DHAP, dihydroxyacetone phosphate; PGA, phosphoglyceric acid; OAA, oxaloacetic acid; cyt, cytoplasm; vac, vacuole.

**Table IV.** *Energy Requirements for Malic Acid Synthesis and Accumulation during the Dark Phase of CAM: Free Hexose as the Hexose Donor, PPi-PFK as the Catalyst for the First Committed Reaction of Glycolysis*[a]

| | | |
|---|---|---|
| Hexose + $ATP^{-4}$ | ⟷ | Hexose-$P^{-2}$ + $ADP^{-3}$ + $H^+$ |
| Fru-6-$P^{-2}$ + $PPi^{-4}$ | ⟷ | Fru-1,6-$P_2^{-4}$ + $Pi^{-2}$ |
| Fru-1,6-$P_2^{-4}$ | ⟷ | G3$P^{-2}$ + DHA$P^{-2}$ |
| DHA$P^{-2}$ | ⟷ | G3$P^{-2}$ |
| 2 G3$P^{-2}$ + 2 $Pi^{-2}$ + 2 $NAD^+$ | ⟷ | 2 1,3-bisPG$A^{-4}$ + 2 NADH + 2 $H^+$ |
| 2 1,3-bisPG$A^{-4}$ + 2 $ADP^{-3}$ | ⟷ | 2 3-PG$A^{-3}$ + 2 $ATP^{-4}$ |
| 2 3-PG$A^{-3}$ | ⟷ | 2 2-PG$A^{-3}$ |
| 2 2-PG$A^{-3}$ | ⟷ | 2 $PEP^{-3}$ + 2 $H_2O$ |
| 2 $CO_2$ + 2 $H_2O$ | ⟷ | 2 $HCO_3^-$ + 2 $H^+$ |
| 2 $PEP^{-3}$ + 2 $HCO_3^-$ | ⟷ | 2 $OAA^{-2}$ + 2 $PO_4^{-2}$ |
| 2 $OAA^{-2}$ + 2 NADH + 2 $H^+$ | ⟷ | 2 $Malate^{-2}$ + 2 $NAD^+$ |
| 2 $ATP^{-4}$ | ↷ | 2 $ADP^{-3}$ + 2 $PO_4^{-2}$ + 2 $H^+$ |
| 2 $Malate^{-2}_{cyt}$ + 4 $H^+_{cyt}$ | ⟷ | 2 Malic $acid_{vac}$ |
| Net: hexose + 2 $CO_2$ + $ATP^{-4}$ + $PPi^{-4}$ | ⟶ | 2 Malic $acid_{vac}$ + $ADP^{-3}$ + 3 $Pi^{-2}$ + $H^+$ |
| Net energy per mol malic acid accumulated: 0.5 ATP + 0.5 PPi | ⟶ | 0.5 ADP + 1.5 Pi + 0.5 $H^+$ |

[a] Abbreviations as in Table III.

CAM and $C_3$ plants (16, 17, 26). PPi concentrations reported for pineapple leaves (14 nmol/gfw) (24) are near the apparent $K_m$ (PPi) of 16 $\mu$M for Fru-2,6-$P_2$-activated pineapple PPi-PFK (4). ATP concentrations in chloroplasts and in the cytoplasm (14, 31) are 5 to 10 times higher than apparent $K_m$ (ATP) values reported for ATP-PFK for $C_3$ or CAM plants (17, 26). Based on these estimates of substrate concentrations, ATP-PFK and PPi-PFK would at best catalyze the phosphorylation of Fru-6-P at 25% to 50% of the maximum velocity predicted. Therefore, at 15°C and with reasonable estimates of substrate concentrations, ATP-PFK activity is clearly insufficient to account for the rate of malic acid accumulation. PPi-PFK activity, however, is sufficient to support glycolysis at rates required.

A third consideration regarding the ability of ATP-PFK to sustain glycolysis at rates observed in pineapple at night is the relative proportion of hexose derived from soluble sugar *versus* glucan pools. If extrachloroplastic free hexose is the sole source of hexose for malic acid accumulation, then ATP-PFK is even less capable of catalysis at required rates because only about 45% of the ATP-PFK activity is localized in the cytoplasm of pineapple mesophyll cells (4).

### Models for Glycolysis Supported by Starch Reserves *versus* Free Hexose Reserves during CAM

Lüttge *et al.* (20) have argued against hexose pools serving as hexose sources for PEP formation during the acidification phase of CAM in two species of *Kalanchoë*. These researchers conclude that predicted rates of ATP production in these species can not meet energy requirements for malate biosynthesis and transport into the vacuole unless polysaccharides are the source of hexose for acidification and hexose moieties are released by phosphorolysis. Table III outlines the energetics of malic acid production and accumulation in the vacuole when starch is degraded by phosphorolysis and ATP-PFK catalyzes the phosphorylation of Fru-6-P. The net energy requirement to convert 0.5 mol hexose-P to 1 mol malate and accumulate 1 mol malate and 2 mol $H^+$ in the vacuole is 0.5 mol ATP. One-half mol ADP, 0.5 mol Pi and 0.5 mol $H^+$ are the extravacuolar products (Table III). Input of hexose into glycolysis via hexokinase instead of starch phosphorylase would increase the ATP requirement to 1 mol ATP per 1 mol malic acid accumulated. Since the average rate of malic acid accumulation in *K. tubiflora* is nearly twice the predicted rate of ATP production (20), the model depicted in Table III is the only model involving ATP-PFK consistent with respiratory rates and rates of malic acid accumulation.

The model presented in Table IV outlines the energetics of malic acid accumulation when free hexose pools donate hexose for triose-P production and PPi-PFK catalyzes the phosphorylation of Fru-6-P. One-half mol ATP and 0.5 mol PPi are consumed per mol malic acid accumulated in the vacuole. One and one-half mol Pi, 0.5 mol ADP, and 0.5 mol $H^+$ are generated in the cytoplasm. If respiratory rates in pineapple are similar to those in *K. tubiflora*, night-time acidification rates in pineapple can be supported by turnover of hexose from glucose and fructose pools as long as PPi-PFK, but not ATP-PFK, catalyzes the phosphorylation of Fru-6-P and either a sufficiently large PPi pool exists or PPi production is not at the expense of ATP generated oxidatively at night. If neither of the latter conditions is met, the ratio of rates of dark respiration to malic acid accumulation must be higher in pineapple than in *Kalanchoë*.

The model in Table IV predicts that when energy requirements for malic acid accumulation balance ATP production 1 mol Pi is generated per mol malic acid accumulated in the vacuole. The model in Table III predicts no net Pi change under the same circumstance. Interestingly, Daley and Vines (8) report that Pi levels in pineapple leaves increase at night, although not with the stoichiometry predicted if all hexose utilized for malic acid production were derived from soluble hexose reserves. Overall, the malic acid to Pi stoichiometry would be modulated by the degree to which starch is utilized for malate production and/or ATP-PFK is involved in glycolytic processing of hexose for malate synthesis.

Although we cannot estimate the percentage of carbohydrate turnover occurring via PPi-PFK, the data strongly support the involvement of this enzyme in carbohydrate processing during acidification. Further work is therefore required to address the question of the source of PPi for this enzyme. Carbohydrate turnover at night in pineapple leaves exceeds that required for acidification by about 40% (Table I). A small proportion of this excess will sustain respiration at rates reported for CAM plants. The remainder of the carbohydrate turnover is channeled to biosynthesis. PPi is perhaps generated in sufficient quantity as a product of metabolite activation reactions associated with the synthesis of sucrose and various polymers.

## CONCLUSIONS

Neutral soluble sugar pools serve as carbohydrate reserves providing hexose to support malic acid accumulation during CAM in pineapple. In pineapple leaves, levels of glucose and fructose fluctuate in parallel with starch and reciprocally with respect to malic acid (Figs. 1 and 2). Soluble sugar turnover at night is very consistent among plants and is sufficient to account for all hexose required to support malic acid accumulation (Table I). Total glucan turnover at night is less consistent among plants and could account, on the average, for about 44% of the malic acid that accumulates.

A strong case can be made for the involvement of PPi-PFK in hexose turnover during the acidification phase of CAM in pineapple. First, a source of hexose-P is available to this cytoplasmic enzyme. Second, the catalytic capacity of PPi-PFK at low temperatures that promote acidification and under non-substrate saturating conditions is many fold in excess of rates of glycolysis required to support malic acid accumulation, energy and/or other biosynthetic needs. The catalytic capacity of ATP-PFK in pineapple is not sufficient to meet these needs. Third, the energetics of malic acid accumulation at the expense of free hexoses are consistent with dark respiration rates reported for CAM plants (1, 20) if PPi-PFK but not ATP-PFK catalyzes the phosphorylation of Fru-6-P.

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