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# Maintenance Carbon Cycle in Crassulacean Acid Metabolism Plant Leaves[1]

## SOURCE AND COMPARTMENTATION OF CARBON FOR NOCTURNAL MALATE SYNTHESIS

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### ABSTRACT

**The reciprocal relationship between diurnal changes in organic acid and storage carbohydrate was examined in the leaves of three Crassulacean acid metabolism plants. It was found that depletion of leaf hexoses at night was sufficient to account quantitatively for increase in malate in *Ananas comosus* but not in *Sedum telephium* or *Kalanchoë daigremontiana*. Fructose and to a lesser extent glucose underwent the largest changes. Glucose levels in *S. telephium* leaves oscillated diurnally but were not reciprocally related to malate fluctuations.**

**Analysis of isolated protoplasts and vacuoles from leaves of *A. comosus* and *S. telephium* revealed that vacuoles contain a large percentage (>50%) of the protoplast glucose, fructose and malate, citrate, isocitrate, ascorbate and succinate. Sucrose, a major constituent of intact leaves, was not detectable or was at extremely low levels in protoplasts and vacuoles from both plants.**

**In isolated vacuoles from both *A. comosus* and *S. telephium*, hexose levels decreased at night at the same time malate increased. Only in *A. comosus*, however, could hexose metabolism account for a significant amount of the nocturnal increase in malate. We conclude that, in *A. comosus*, soluble sugars are part of the daily maintenance carbon cycle and that the vacuole plays a dynamic role in the diurnal carbon assimilation cycle of this Crassulacean acid metabolism plant.**

A unique characteristic of CAM tissues is the maintenance of two large, metabolically active pools of reduced carbon viz. storage carbohydrate and organic acid. At night, carbon mobilized from the carbohydrate pool is oxidized via glycolysis to PEP,[4] which then is carboxylated and reduced subsequently to form malate. The malate accumulates and is stored as malic acid in the vacuole (3). During the day, malate or oxaloacetate is decarboxylated in the cytoplasm to form $CO_2$ for photosynthesis plus either pyruvate or PEP which is recycled via gluconeogenesis and accumulates as the storage carbohydrate pool. CAM plants invest a large percentage (10–20%) of their total organic constituents in the maintenance of this carbon cycle, which alternates between pools of malate at night and carbohydrate in the day (2, 15, 16).

In all CAM species studied, malate is the predominant organic acid that accumulates in CAM leaf tissue at night and is depleted during the day. Nocturnal $^{14}CO_2$ assimilation profiles indicate that various metabolites are labeled, especially aspartate, alanine, and intermediates of the citric acid cycle (17, 23). The percentage of the label that accumulates in these components relative to malate, however, is low. In some species, citrate fluctuates diurnally but the amplitude is three orders of magnitude less than that exhibited by malate (10, 15, 20, 25).

Some variation in the form of stored carbohydrate exists in CAM plants. In early studies, a decrease in starch seemed to accommodate entirely the observed increase in malate (16). Later, Pucher *et al.* (15), working with *Bryophyllum calycinum*, showed that starch alone could not account for all the malate accumulated at night; however, decreases in soluble glucan (ethanol-soluble polyglucose) could provide the balance. Sutton (21) found that in *Kalanchoë daigremontiana* and *K. tubiflora*, starch could donate only two-thirds of the carbon required for PEP synthesis at night. Soluble sugars underwent little diurnal change in these plants, but a fluctuation of soluble glucan indicated the latter was the source of the remaining one-third of the necessary carbon.

A third source of storage carbohydrate for malate is apparent from studies with *Ananas comosus* (pineapple) in which Sideris *et al.* (20) first observed that the nocturnal decrease in starch could account for only about 7% of the accumulated malate. The levels of soluble sugars in these leaves changed in phase with starch; however, the amplitude of the sugar fluctuations was much larger. Sideris *et al.* (20) concluded from this that soluble sugars were providing the major proportion of the carbon precursors for malate synthesis.

In a recent study on pineapple, starch turnover was estimated to accommodate approximately 14% of the nocturnally synthesized malate and, moreover, starch plus soluble glucan could account for only 33% of the total malate pool (2). We concluded that the remaining two-thirds of the required carbon could arise from the soluble sugar pool primarily composed of glucose, fructose, and sucrose. In pineapple, soluble sugars exhibited diurnal changes in fluctuations similar to the glucan and starch pools.

At least three important questions remain concerning the involvement of soluble sugars in the CAM maintenance carbon cycle. (a) To what extent do individual sugars contribute to malate synthesis? (b) What is the subcellular localization of sugar pools? (c) How widespread in CAM plants is the involvement of soluble sugars in the diurnal carbon maintenance cycle? In this investigation we have determined the daily patterns and amounts of various soluble sugars and acids as well as their subcellular

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[4] Abbreviations: PEP, phosphoenolpyruvate; BSTFA, *N-O*-bis(trimethylsilyl)trifluoroacetamide; DMF, dimethylformamide; TMS, trimethylsilyl.

localization in the leaves of three CAM plant species. We conclude first that glycolytic catabolism of glucose and fructose alone provides a large percentage of the 3-carbon precursor of malic acid synthesis in pineapple leaves but not in *K. daigremontiana* or *S. telephium* and second, that the major pools of these hexoses are located in the vacuole.

## MATERIALS AND METHODS

**Plant Material.** *Kalanchoë daigremontiana*, *Sedum telephium*, and *Ananas comosus* were isogenic and derived by vegetative propagation of a single parent plant. The plants were grown in a greenhouse in an artificial potting medium consisting of pine-bark, vermiculite, and granular fertilizer, including micro and macro nutrients. All plants were watered every 2 d and supplemented weekly with either a commerical N-P-K (15-15-15) fertilizer or one-half strength Hoagland solution.

One to two months prior to the start of the experiments, plants were transferred to growth chambers maintained at 30°C/15°C, 15 h/9 h day/night thermo/photo period and 70 to 80% RH. Light intensity (400–700 nm) was 200 to 350 $\mu$E $m^{-2}$ $s^{-1}$ within the canopy. The 1 to 2 month adaption period allows the plants to acclimate to these day/night cycles and ensured a large diurnal organic acid fluctuation characteristic of CAM.

Fully expanded, mature leaves were used in each experiment. The leaf ages were 1.5 to 3 months for *S. telephium* and *K. daigremontiana* and 8 to 12 months for *A. comosus*.

**Preparation of Whole Leaf Material for GC.** Entire leaves were removed at various times, rinsed briefly in distilled $H_2O$, blotted dry, and then plunged immediately into liquid $N_2$. The tissue was ground to a fine powder in a liquid $N_2$-chilled mortar and pestle and then was lyophilized immediately. After freezing, the tissue temperature was kept at −70°C until lyophilization was completed. Lyophilized tissue was stored desiccated at −20°C until used.

The one-step gas chromatographic method developed by Severson *et al.* (19) for the analysis of major tobacco leaf components was used to determine levels of the organic acids and simple sugars during the diurnal cycles. The components in dry leaf samples were extracted and converted to GC-volatile trimethylsilyl ethers/esters in one step. Briefly, the extraction-derivatization reagent, a 1:1 mixture of BSTFA and DMF (Pierce Chemical Co.) containing the internal standard l-octadecanol (0.64 mg/ml) was prepared daily. The lyophilized leaf samples were brought to room temperature under desiccation and 10 to 30 mg amounts were weighed into derivatization vials and sealed with Teflon-lined serum caps. The extraction-derivatization reagent then was added to the vials (approximately 25 mg tissue/ml of reagent). The mixture was sonicated in a bath sonicator (model SC100, Ultrasonic Industries) for 45 min, and then heated for 45 min at 76°C in a heat block. After cooling, the vials were spun for 5 min in a clinical centrifuge and 1 to 2 $\mu$l of the supernatant were injected in the GC.

**Packed-Column GC.** The samples were analyzed on a Hewlett-Packard 5710A GC on a 2 mm i.d. × 1.22 m Pyrex glass column packed with 5% Dexsil 300 on Chromosorb WAW using a temperature program of 90°C for 2 min, 90 to 290°C at 8°/min, and 4 min hold at 290°C and a helium flow rate of 30 ml/min (injection port temperature, 200°C; flame ionization detector temperature, 350°C). Drop-in Pyrex glass injection port liners were changed twice daily. Peak areas and retention time data were obtained using a Hewlett-Packard 3390A recorder-integrator. Component elutions were determined by authentic standard spiking techniques. Chromatographic response data relative to octadecanol were determined daily using known amounts of authentic standards. Sample component levels were determined using conventional internal standard calculations.

**Capillary GC.** High-resolution capillary GC was performed on a HP 5830 GC that was modified for capillary GC as described by Severson *et al* (18). The capillary column was made of 0.3 mm i.d. × 20 m fused silica glass which was statically coated using 3.0 mg/ml SE-54 in methylene chloride according to the method of Arrendale *et al.* (1). Samples were analyzed using a split-mode (100 ml/min split) with a $H_2$ carrier gas linear velocity of 35 cm/s and a temperature program of 80 to 280°C at 2°/min. Injection port temperature was 250°C and flame ionization detector temperature was 300°C. Component retention time data were confirmed by internal spiking with authentic standards.

**Preparation of Protoplasts and Vacuoles.** *Sedum* protoplasts and vacuoles were prepared by a previously published method (8). Pineapple protoplasts and vacuoles were prepared by a modification of this procedure as described elsewhere (5).

Isolated and purified protoplasts and vacuoles were washed by dilution into a 25-fold excess of 0.6 M mannitol, counted with a hemocytometer, and were concentrated by centrifugation 250 *g* × 10 min. Both protoplasts and vacuoles were lysed in water and 100 $\mu$l aliquots were removed, frozen in dry ice, and lyophilized in 0.1 ml Reacti-vials (Pierce Chemical Co.). Samples were derivatized as for whole leaves except that from 50 to 100 $\mu$l of BSTFA/DMF were added to the lyophilized tissue. Again, octadecanol was included in all samples as an internal standard for quantitation.

## RESULTS

**Diurnal Changes in Sugars and Organic Acids of Pineapple Leaves.** The extent of the diurnal changes in sugars and organic acids of pineapple leaves was determined by harvesting leaves every 3 h for 27 h and analyzing whole-leaf TMS derivatives by GC. Two representative chromatograms of extracts from pineapple leaves harvested just before the lights were turned on (6 PM) and just before they were turned off (9 AM) in the growth chamber are illustrated (Fig. 1). Large differences were found in the leaf contents of malate, citrate, glucose, fructose, and sucrose.

Under the chromatographic and derivatization conditions used, the monovalent anion of malic acid produced a peak at 6.3 min and the free acid eluted at 8.2 min. Chromatographic analysis of authentic standards of the free acid and the monovalent acid salt of malic acid gave peaks at 8.2 and 6.3 min, respectively. Addition of free malic acid to dry leaf powder before derivatization resulted in an increase in the peak at 6.3 min and very little change in the peak at 8.2 min, suggesting that during derivatization, malic acid was changed to the monovalent anion. The conjugate base of malate did not form a GC-volatile component. For simplicity, the peaks will be combined and referred to as malate. Multiple peaks were not observed with the various salts of either citric, isocitric, ascorbic, or succinic acids whether alone or combined with leaf samples.

During the day there was an overall increase in fructose (450 $\mu$mol/g dry weight and $\alpha$- and $\beta$-glucose (250 $\mu$mol/g dry weight) in pineapple leaves (Fig. 2). Concurrently, malate, citrate, and sucrose decreased 1000, 135, and 20 $\mu$mol/g dry weight, respectively, while ascorbate levels remained unchanged. The cyclical accumulation and depletion of malate in these pineapple leaves is characteristic of plants operating in full CAM. Reciprocal fluctuations of hexose and malic acid in pineapple leaves were substantiated by analyzing three separate leaf samples taken at 6 PM (high acid) and 9 AM (low acid). The results indicated that the change in glucose and fructose together could account for 145% of the nocturnal accumulation of malate, assuming that 1 mol of hexose provides 2 mol of triose via glycolysis (Table I).

**Diurnal Changes in *Kalanchoë diagremontiana* and *Sedum telephium* Leaf Sugars and Organic Acids.** In an effort to determine how widespread the concurrent fluctuations of malic acid and sugars were in CAM plants, two additional plants, *K. daigremontiana* and *S. telephium* were analyzed. Both sugars and

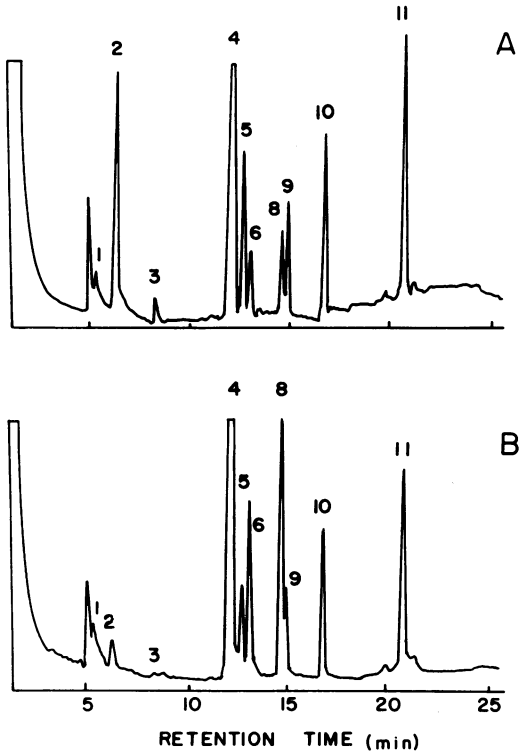


FIG. 1. Packed-column gas chromatographic profiles of TMS derivatives of whole pineapple leaves harvested at the end of the dark period (high acid, A) and at the end of the light period (low acid, B). Peak numbers are as follows: 1, succinate; 2 and 3, malate; 4, fructose; 5, citrate/isocitrate/quinate; 6, α-glucose; 8, β-glucose; 9, ascorbate; 10, octadecanol; 11, sucrose.

acids in *K. daigremontiana* decreased dramatically in the light while other components changed very little (Fig. 3). In *S. telephium*, sugars changed only slightly while organic acids decreased dramatically during the day (Fig. 4).

In *K. daigremontiana*, the increase in malate was nearly 50 times the change in any hexoses (Fig. 3). Sucrose decreased only slightly in the day and increased slightly at night (Fig. 3). In *S. telephium*, both malate and β-glucose showed an overall decrease in the light (Fig. 4). The level of β-glucose, however, oscillated during both day and night. Other sugars appeared to change only slightly (Fig. 4). The peak identified as isocitrate/citrate decreased significantly in the day and increased at night for both *S. telephium* and *K. daigremontiana* (Figs. 3 and 4).

Citrate has been shown to undergo relatively small daily changes compared to malate in the leaves of some species of CAM plants (10, 15, 20, 25). Conversely, isocitrate, which is a major component of the leaves of *Kalanchoë pinnatum* and *S. telephium*, has been reported to undergo little diurnal fluctuation (3, 15, 24).

**Capillary GC.** Recently, in a comparative study of eight species of *Sedum*, Knopf and Kluge (7) suggested that the amounts of both isocitrate and quinate changed dramatically and reciprocally in *S. telephium*. Their data indicated that during the day malate and isocitrate decreased, quinate increased, and citrate remained unchanged (7). It was important, therefore, to resolve this discrepancy and to establish the identity of the acid(s) in the packed-column 13 min peak of *S. telephium*.

TMS derivatives of authentic standards of citrate, isocitrate, isocitrate lactone, and quinate co-elute on our packed column (Fig. 1); however, they were well resolved on a high resolution capillary column. Fructose and citrate, however, which were clearly separated on the packed columns (Fig. 1), co-eluted on the capillary column but were resolved from isocitrate. The glucose peaks eluted well after these acids and therefore, did not interfere with their analysis. The TMS-derivatized acids from *S. telephium* leaves harvested at 6 PM and 9 AM were analyzed by capillary GC (Fig. 5, A and B). The results suggested that isocitrate was the component undergoing the largest diurnal change in *S. telephium* (Figs. 4 and 5, A and B). Citrate underwent very little change and isocitrate lactone and quinate were virtually undetectable at any time. When *K. daigremontiana* leaves were analyzed, isocitrate also showed a dramatic decrease in the light (Fig. 5, C and D). Analysis of the components of pineapple leaves indicated only trace amounts of isocitrate. The changes in intensity of peak 5 (Fig. 1) suggest that a large diurnal fluctuation of citrate occurs in pineapple.

**Compartmentation of Leaf Constituents.** The diurnal and reciprocal fluctuation of soluble sugars and organic acids in pineapple leaves suggested that at least part of the nocturnal supply of PEP as a precursor of malate may be fulfilled by glycolytic catabolism of hexoses. If hexoses in CAM plants are used as storage carbohydrate for nighttime production of PEP, then sequestration from cytoplasmic glycolytic enzymes is nec-

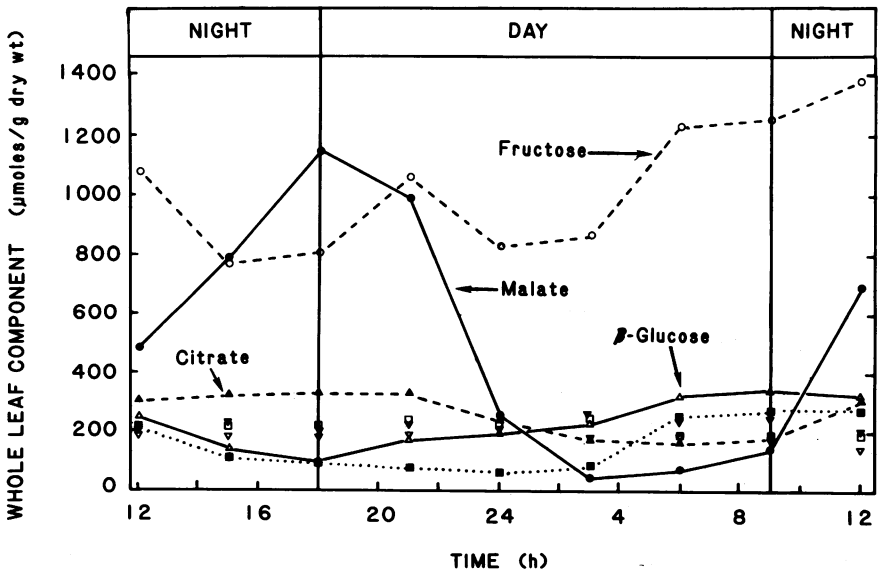


FIG. 2. Composite of diurnal changes of sugars and organic acids in whole pineapple leaves. Lines are as labeled except sucrose (□), ascorbate (▽), succinate (▼), and α-glucose (■).

Table I. *Quantitative Nocturnal Changes in Hexose and Malate in Pineapple Leaves*

Three separate leaf samples were taken just before the growth chamber lights were turned on (high acid) or off (low acid). The values are means of three determinations of each metabolite.

| Metabolite | Low Acid | | High Acid | | Nocturnal Change in Metabolite | |
|---|---|---|---|---|---|---|
| | *μg/mg dry wt* | *μmol/g dry wt* | *μg/mg dry wt* | *μmol/g dry wt* | *μg/mg dry wt* | *μmol/g dry wt* |
| Malate | 13.8 | 103.1 | 109.9 | 820.2 | +96.1 | +717 |
| Fructose | 169 | 940.4 | 122.2 | 679.1 | −46.8 | −260 |
| α + β-Glucose | 79.3 | 440.6 | 32.5 | 180.3 | −46.8 | −260 |

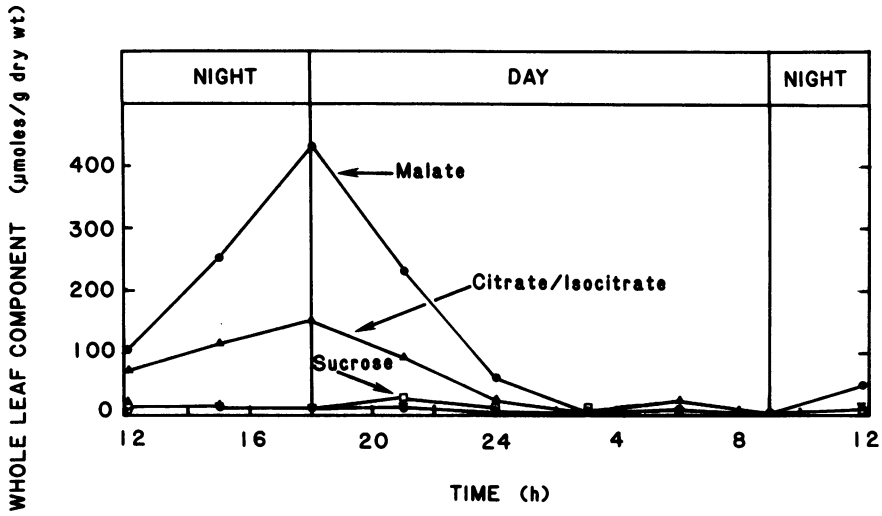


FIG. 3. Diurnal changes in sugars and organic acids in whole *K. daigremontiana* leaves. The unlabeled lines are as follows: β-glucose (△), fructose (○), and ascorbate/succinate (▽).

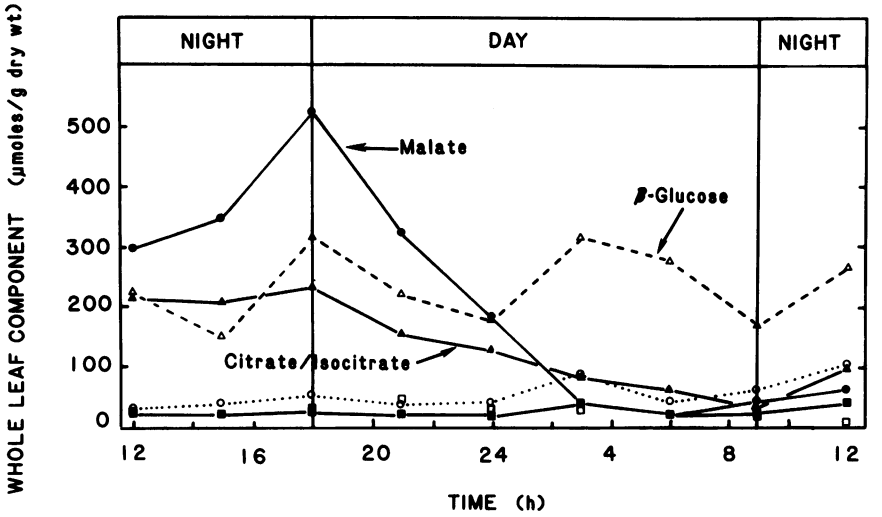


FIG. 4. Composite of diurnal changes in whole-leaf sugars and organic acids in *S. telephium*. The unlabeled lines are as follows: α-glucose (■), fructose (○), and sucrose (□).

essary. Soluble sugars, primarily hexoses and sucrose, have been localized in the vacuoles of a number of plants (8, 11, 12, 22, 26, 28). Thus, it is possible that sugars may be stored temporarily in the vacuole during the day.

To test this hypothesis, protoplasts and vacuoles were isolated and purified from pineapple leaves at two different times of the day. Because it took approximately 6 h to isolate vacuoles from pineapple, leaves were harvested for cell fractionation 3 h before the time of interest. After protoplasts were isolated (~3.5 h), they were cooled to 4°C to retard metabolism and to minimize subsequent changes in levels of cellular constituents.

Analysis of sugars and acids in pineapple protoplasts indicated many similarities but also some striking differences from the whole leaf (Figs. 1 and 6). The chromatographic peak at 5 min was much larger in the protoplast than the whole leaf. This peak was due in part to an unidentified artifact consistently generated by derivatization of the plant material (data not shown). The succinate peak was merged into the 5 min peak and could be resolved only by dilution and/or injection of a smaller sample. The peak at 13.5 min was due to residual mannitol used as an osmoticum during protoplast isolation. Mannitol was virtually absent from whole pineapple leaf extracts (Fig. 1). The absence of a peak at 6.5 min (elution time of the TMS-derivative of Tris buffer) indicated that the protoplasts, which are purified in Tris, were probably washed free of contaminating soluble components. It was striking that only very small levels of sucrose were ever found in protoplasts, whereas it was a dominant component of whole leaves (Figs. 1 and 6).

Analysis of vacuole components yielded a pattern similar to that of protoplasts suggesting that vacuoles are a major storage

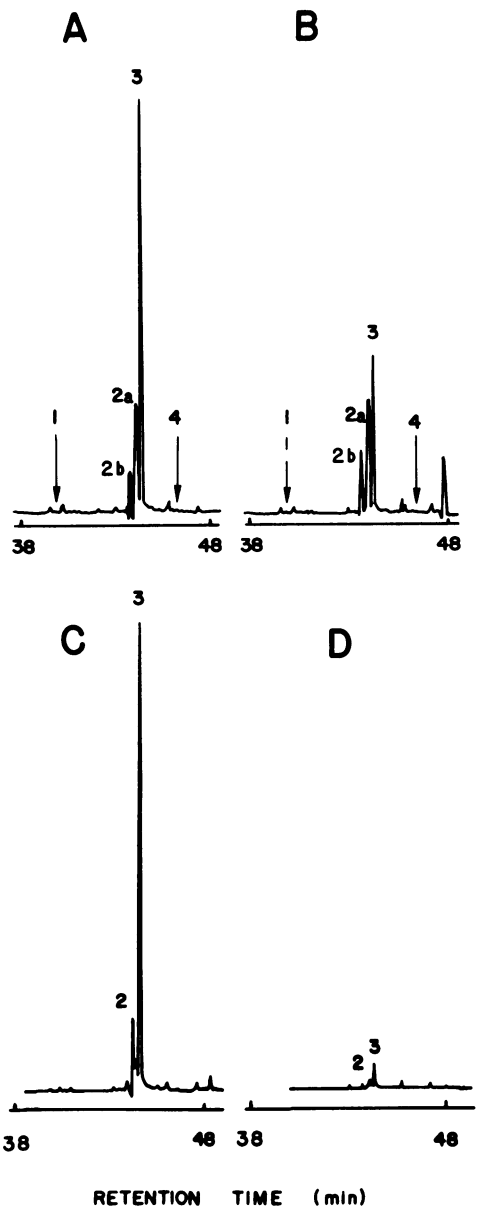


FIG. 5. Capillary gas chromatographic profiles of extracts from whole leaves of *S. telephium*, high acid (A) and low acid (B), and *K. daigremontiana*, high acid (C) and low acid (D). Only the portion of the chromatograms containing the acids is given. The peaks are: 1, isocitrate lactone; 2a, fructose; 2b, citrate + fructose; 3, isocitrate; and 4, quinate. Equal quantities of lyophilized leaf material were extracted and analyzed.

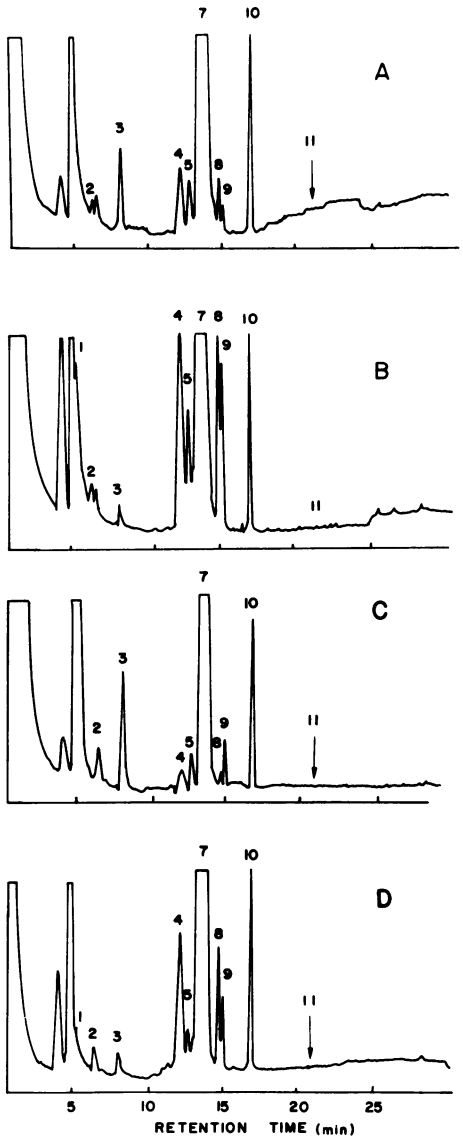


FIG. 6. Packed-column chromatograms of the TMS derivative of components of protoplasts at end of dark (A) and light (B) and vacuoles at end of dark (C) and light (D) isolated from pineapple leaves. Peaks are: 1, succinate; 2 and 3, malate; 4, fructose; 5, citrate; 7, mannitol; 8, β-glucose; 9, ascorbate; 10, octadecanol; 11, sucrose.

site for these compounds in CAM leaves (Fig. 6). This was confirmed by the high recovery of both sugars and acids in vacuoles (Table II). Sucrose was virtually absent from the vacuoles.

**Contribution of Vacuolar Hexose to Malic Acid Formation.** The potential contribution of vacuolar hexose to nocturnal malate formation was estimated by comparative analysis of organic acids and hexoses in protoplasts and vacuoles prepared from pineapple leaves harvested at the beginning and end of the night. At night, the amount of malate in protoplasts and vacuoles increased 3.7 and 3.4 $\mu$mol/$10^6$ protoplasts or vacuoles while fructose decreased by 2 and 1.6 $\mu$mol/$10^6$ protoplasts or vacuoles and glucose decreased by 0.7 and 0.6 $\mu$mol/$10^6$ protoplasts or vacuoles (Table II). The trend indicated that a depletion of vacuolar hexose occurred at the same time malate increased. Quantitatively, the decrease in vacuole hexose could potentially accommodate approximately 129% of the malate accumulated at night (Table II).

In *S. telephium*, like pineapple, there are a few differences between *Sedum* protoplasts and whole leaves (data not shown). Sucrose is present in protoplasts, but at a low level compared to leaves. The contents of protoplasts and vacuoles derived from them appeared qualitatively similar, again indicating that *S. telephium* leaf vacuoles are major storage repositories for acids and sugars (Table II). The small amount of sucrose in the protoplasts appears to be largely nonvacuolar (Table II). Consistent with the data illustrated in Figure 4, vacuole hexose can contribute only slightly towards the overall accumulation of malate at night (Table II) in *S. telephium*.

## DISCUSSION

The present study confirms and extends the findings of Sideris *et al.* (20) and Black *et al.* (2) to include both a quantitative

Table II. *Summary of Diurnal Changes in Organic Acids and Sugars of Protoplasts and Vacuoles Isolated from Pineapple and S. telephium Leaves*

| Metabolite | High Acid | | Low Acid | | Recovery | | Diurnal Change | |
|---|---|---|---|---|---|---|---|---|
| | Protoplasts | Vacuoles | Protoplasts | Vacuoles | High | Low | Protoplasts | Vacuoles |
| | *μmol/10⁶* | | | | *% in vacuoles* | | *μmol/10⁶* | |
| A. Pineapple[a] | | | | | | | | |
| Malate | 4.2 | 4.8 | 0.5 | 1.4 | 114 | 265 | −3.7 | −3.4 |
| Fructose | 2.2 | 0.8 | 4.2 | 2.4 | 35 | 57 | +2.0 | +1.6 |
| Glucose | 0.5 | 0.2 | 1.2 | 0.8 | 34 | 69 | +0.7 | +0.6 |
| Citrate | 1.2 | 0.5 | 0.9 | 0.7 | 43 | 72 | −0.3 | +0.2 |
| Ascorbate | 0.5 | 0.5 | 1.0 | 1.2 | 88 | 120 | +0.5 | +0.7 |
| Sucrose | ND[b]–0.07 | ND | 0.01 | ND–0.002 | ~0 | ~0 | | |
| B. *S. telephium*[a] | | | | | | | | |
| Malate | 19.3 | 17.7 | 1.4 | 1.3 | 92 | 90 | −17.9 | −16.4 |
| Fructose | 3.6 | 2.2 | 2.7 | 2.5 | 61 | 91 | −0.9 | +0.3 |
| Glucose | 5.6 | 4.3 | 4.9 | 6.2 | 76 | 125 | −0.7 | +1.9 |
| Isocitrate/Citrate | 20.7 | 16.2 | 6.3 | 10.1 | 78 | 162 | −14.4 | −6.1 |
| Succinate | 1.6 | 1.3 | 0.6 | 1.6 | 81 | 267 | −1.0 | +0.3 |
| Sucrose | 0.3 | 0–0.03 | 0.3 | 0.03 | 10 | 10 | 0 | 0 |

[a] Data are averages from three to four experiments each for high and low acid samples.
[b] Not detected.

estimate of the individual sugars in the reserve carbohydrate pool and its subcellular localization. The derivatization technique used in this study allowed a simultaneous assessment of the amount of both sugars and organic acids in the samples. Unlike *K. daigremontiana* and *S. telephium*, the nocturnal depletion of leaf hexose pools in pineapple was sufficient potentially to make a significant contribution to acid synthesis (Table I; Fig. 2, 3, and 4). This is in agreement with previous work with *K. daigremontiana* that indicated that carbon from starch degradation was supplemented by polyglucose glucan and not by soluble sugars (4, 21). It must be emphasized that while changes in sugars and organic acids in pineapple appear to be correlated, evidence for the unequivocal involvement of hexoses in acid synthesis must await radio-tracer studies.

The maintenance of high levels of total leaf sucrose levels throughout the day in CAM plants implies that sucrose synthesis and transport is balanced in CAM leaves. This finding corroborates other data from CAM plants showing minimum diurnal changes in sucrose in whole leaves (15). The relative contribution of starch and hexose to sucrose synthesis and to translocation at night is unknown. Coordinate sucrose synthesis and glycolysis for acid production suggests that a refined control of these metabolic pathways exists in the CAM plant leaf cells.

The protoplasts and vacuoles of pineapple were found to have many components identical to those recovered from whole leaves (Figs. 1 and 6). Undoubtedly some leakage from both protoplasts and vacuoles must occur during isolation; however, the qualitative similarity in the chromatograms indicates that the amount of leakage is acceptable. Estimates of various protoplast components in isolated vacuoles shows that in CAM leaf tissue vacuoles store approximately 50% of the hexoses and 50 to 100% of the organic acids (Table II). The excessive recovery of some of the protoplast components in isolated vacuoles illustrates a problem with quantitating vacuole and protoplast constituents on the basis of number. Both vacuoles and protoplasts are fragile and breakage may cause an underestimation of the quantity used for analysis.

Interestingly, sucrose is found in very low levels in isolated protoplasts and is virtually undetectable in pineapple vacuoles (Table II). The findings suggest that the small amount of sucrose found in protoplasts is stored in the cytoplasm, and the majority of the leaf sucrose is not stored in the mesophyll cells. A more plausible explanation is that sucrose might be exported or leak from the mesophyll cell during protoplast isolation. Why sucrose and not other compounds found in the leaves would leak selectively from the mesophyll cells during isolation is unclear.

When pineapple vacuoles are isolated at two extremes in the diurnal acid cycle, they are found to contain different amounts of both sugar and organic acids (Table II). The pattern of nocturnal accumulation of acid and depletion of sugars is similar in whole leaves, protoplasts, and vacuoles (Tables I and II). The quantitative change in the vacuolar sugar pool alone can account for more than the total change in vacuolar acid. These results suggest that, during the day, the vacuole acts as a temporary storage pool of hexose which then is removed at night and catabolized to become the three-carbon precursor of malate.

High percentages of both sugars and acids also are recovered in vacuoles from *S. telephium* leaves, indicating that this organelle also is a major intracellular storage site for these metabolites (Table II). Protoplasts and vacuoles show a marked diurnal change in both acids and sugars but, quantitatively, the change in sugar cannot account for more than a small percentage of the total acid changes in accord with what is observed in whole leaves.

High-resolution capillary GC permitted the components of the 13 min peak (Fig. 1, peak 5) on packed-column GC to be resolved and analyzed. Isocitrate levels appeared to fluctuate diurnally in both *S. telephium* and *K. daigremontiana* while citrate fluctuated in pineapple (Figs. 1–5). Analysis of leaves from *B. calycinum* with chromatography (24) and *S. telephium* with isocitrate dehydrogenase (3) indicated that no change in leaf isocitrate content occurred. In contrast, there is precedent for fluctuations in citrate levels in CAM plant leaves (25) analogous to pineapple. Saltman *et al.* found that dark $^{14}CO_2$ fixation resulted in only a small amount of label accumulating in citrate (~5%) and isocitrate (~8%) in *B. calycinum* after 2 h (17). It is unlikely, therefore, that intermediates of the Krebs cycle play more than a minor role in nocturnal carbon assimilation in CAM plants; however, no explanation for the fluctuations in citrate or isocitrate in this study is apparent.

In conclusion, the data demonstrate that, in pineapple, and perhaps to a minor extent in *S. telephium*, hexoses contribute

much of the carbon for the nocturnal synthesis of malate. Furthermore, these hexoses appear to be sequestered in the vacuole away from catabolic reactions during the day. Lack of significant changes (compared to malate) in hexoses with *K. daigremontiana* leaves indicate that the contribution of soluble hexoses to malate synthesis is not a ubiquitous feature of CAM plants. Thus the source of carbon for the maintenance of CAM in specific plant species can be either soluble sugars or starch or lower mol wt glucans.

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