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Changes in Metabolite Levels in *Kalanchoë daigremontiana* and the Regulation of Malic Acid Accumulation in Crassulacean Acid Metabolism[1]

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ABSTRACT

Changes in glucose-6-P, fructose-6-P, fructose-1,6-diP, 6-phosphogluconate, phosphoenolpyruvate, 3-phosphoglycerate, and pyruvate levels in the leaves of the Crassulacean plant *Kalanchoë daigremontiana* Hammet et Perrier were measured enzymically during transitions from CO_2-free air to air, air to CO_2-free air, and throughout the course of acid accumulation in darkness. The data are discussed in terms of the involvement of phosphoenolpyruvate carboxylase in malic acid synthesis and in terms of the regulation of the commencement of malic acid synthesis and accumulation through the effects of CO_2 on storage carbohydrate mobilization and its termination through the effects of malic acid on phosphoenolpyruvate carboxylase activity.

It has become increasingly clear that the synthesis of malic acid in darkness by the CAM[2] plant *Kalanchoë daigremontiana* involves a single carboxylation step (4, 5, 8, 15). The presence of large amounts of PEP carboxylase in CAM plants (16) and the relationship between CO_2 concentration and malic acid accumulation (18) compared with the relationship between CO_2 concentration and malic acid synthesis by isolated enzymes, have been taken as indications that PEP carboxylase is responsible for the dark carboxylation step in CAM. Malic enzyme, also present in large quantities in CAM plants, has characteristics which suggest that it may not play a substantial role in carboxylation reactions leading to malate synthesis (13).

More recent studies on the properties of enzymes isolated from CAM plants (9) indicated that PEP carboxylase is inhibited by malic acid and this property has been implicated in models accounting for the feedback control of the termination of malic acid accumulation. G6P activates CAM-type PEP carboxylases *in vitro* and its effects may also be involved in the control of malate synthesis (16). Mukerji and Ting (12) have shown PEP carboxylase to be inhibited by high CO_2 or bicarbonate concentration, and this also could be involved in the regulation of CAM. Sutton (14) has presented evidence that a phosphorylase which may be involved in the mobilization of carbohydrate reserves in CAM plants is inhibited by PEP. Sutton also suggests that inhibition of PEP carboxylase by malic acid toward the end of acid accumulation would cause an increase in PEP level. This rise in PEP level would, by its effect on the phosphorylase, inhibit carbohydrate utilization. At a sufficiently high PEP level, higher than that which affected the phosphorylase, phosphofructokinase would also be inhibited (6, 7).

These various hypotheses describing the path of carbon in CAM and its regulation depend upon changes within the cells in the levels of metabolic intermediates, and some measure of their validity can be obtained by determining whether or not appropriate changes in the levels of key intermediates occur as predicted.

Careful measurements of changes in levels of several keto acids, PEP and malic, citric and isocitric acids have been made by Milburn *et al.* (11) in a comparative study of a variety of CAM plants growing under natural tropical conditions. However, their data on changes in intermediate levels must include changes related to all of the many environmental changes associated with the natural transition between day and night. Furthermore, acid accumulation was incomplete during the natural dark periods involved in the experiments in which detailed investigations of intermediate changes were made. Although valuable from a comparative viewpoint, their data on intermediate levels are difficult to interpret in terms of the metabolic regulation of CAM.

The synthesis of the carboxylation acceptor of CAM from stored carbohydrate has received some attention in experiments involving attempts to trace the path of carbon after feeding tissues with radioactive carbohydrates (2, 4, 13). The data from these experiments were complex and inconclusive although in the main consistent with the supposition that the reactions encompass part of the glycolytic sequence.

The present work, involving enzymic measurements of suspected CAM intermediates during experimental perturbation of the course of malic acid accumulation by alteration of CO_2 concentration, and during the entire dark phase of CAM, was carried out to test existing hypotheses and to extend information on the nature and metabolic regulation of the path from reserve carbohydrate to malate.

MATERIALS AND METHODS

Plant Material. *Kalanchoë daigremontiana* Hammet et Perrier plants were grown from leaf propagules in a constant environment of day length 20 hr (illumination was by warm white fluorescent tubes giving an irradiance of 8×10^3 ergs/cm^2-sec at the plant tops), temperature 25 C, and relative humidity 60%.

Sample Preparation. All experiments were performed using leaf slices prepared by removing the midrib and leaf edges of whole *K. daigremontiana* leaves and slicing the remaining leaf material into 1-cm slices with a razor blade. In all of the experiments involving assay of intermediates, 20-g samples of leaf slices were used.

[1] The authors are grateful for financial support from the Science Research Council.

[2] Abbreviations used: CAM: Crassulacean acid metabolism; G6P: glucose-6-P; F6P; fructose-6-P; 6PG; 6-phosphogluconate; FDP; fructose-1,6-diP; 3PGA; 3-Phosphoglycerate; PEP: phosphoenolpyruvate; Pyr: pyruvate; TraEDTA: triethanolamine EDTA/HCl.

Killing and Extraction. For the determination of acid content, the leaf slices were killed and extracted by boiling water.

Where enzymic assays were to be performed, the leaf fragments were immersed in liquid N_2, ground to powder while still frozen, then extracted with cold 5% (v/v) perchloric acid. The resulting slurry was centrifuged for 2 min at 26,000*g*, the supernatant fluid adjusted to pH 7.6 at 2 C with cold saturated K_2CO_3, and the resulting perchlorate precipitate removed by a 2-min centrifugation at 26,000*g*. Norit N. K. decolorizing charcoal (200 mg) was added to the supernatant fluid, and after 20 min at 0 C, the charcoal was removed by centrifugation at 26,000*g* for 20 min. Part of the extract was used immediately for the measurement of amounts of PEP and Pyr and the remainder stored at −10 C.

Enzymic Assays. G6P, F6P, 6PG, Pyr, and PEP were assayed by the methods of Bergmeyer (1). Triose phosphates, FDP, and 3PGA were assayed as follows.

One-tenth ml of 0.1 M TraEDTA (pH 7.6), 0.05 ml of 5 mM NADH, 0.1 ml of 50 mM ATP, 0.02 ml of 0.1 M $MgCl_2$, and 1 ml of extract were mixed in a 1.5-ml quartz cuvette. Intermediates were then assayed by the successive addition of a mixture of α-glycerophosphate dehydrogenase (EC 1.1.1.8) and triose phosphate isomerase (EC 5.3.1.1) followed by aldolase (EC 4.1.2.13), glyceraldehyde-3-P dehydrogenase (EC 1.2.1.12) and 3-P-glycerate phosphokinase (EC 2.7.2.3). The concentration of each intermediate was calculated from the changes in absorption at 340 nm.

The absorbance reproducibility of the Unicam SP800 spectrophotometer used was 0.005 *A* units and no data are included in this paper from intermediate assays in which the maximum change was less than 0.015 units. Corrections based on internal standardization were applied to all assays.

The reproducibility and precision of the extraction and assay methods were determined by assaying replicate samples. The numbers of replicate extractions and assays performed, the mean concentration of each intermediate, and the standard errors of the means are shown in Table I.

Each point in Figure 1 represents the mean of results from two separate experiments.

In long term time course experiments, it did not prove possible to control the experimental conditions with a precision sufficient to prevent minor changes in the time scale of intermediate level changes. Thus, although repeat experiments yielded qualitatively identical data, mean values derived from a number of such experiments increase the apparent time over which intermediate level changes occur and decrease the precision of the results. For this reason, the data presented in Figure 2 refer to a single experiment.

Titratable Acid Determination. A boiling water extract of a 5- to 10-g sample of leaf slices was titrated while still hot with 0.01 N NaOH to the phenolphthalein end point.

RESULTS

Transition from Air to CO_2-free Air. Figure 1, a-e shows changes in intermediate levels in *K. daigremontiana* leaf fragments following a rapid transition in darkness at 14 C from laboratory air (≃0.03% [v/v] CO_2) to CO_2-free air. Control samples were maintained in air. Illumination of the leaf material prior to the experiment ensured that the fragments were potentially able to accumulate acid. PEP and G6P increased and remained above the control level. Pyruvate, F6P, and FDP showed transient increases followed by a decline to, and in the case of pyruvate and FDP, below the control level.

Table I. *Reproducibility of Metabolite Extraction and Assay*

Means and standard errors of the means of metabolite concentrations in six replicate leaf samples maintained at 14 C in darkness in CO_2-free air for 3 hours prior to extraction.

Metabolite	Mean Concentration μmol/100 g fresh wt	Standard error of mean
G6P	7.37	0.53
F6P	0.98	0.064
FDP	5.4	0.22
Triose phosphates	0.85	0.04
PGA	1.65	0.16
PEP	2.65	0.18
Pyruvate	2.85	0.21

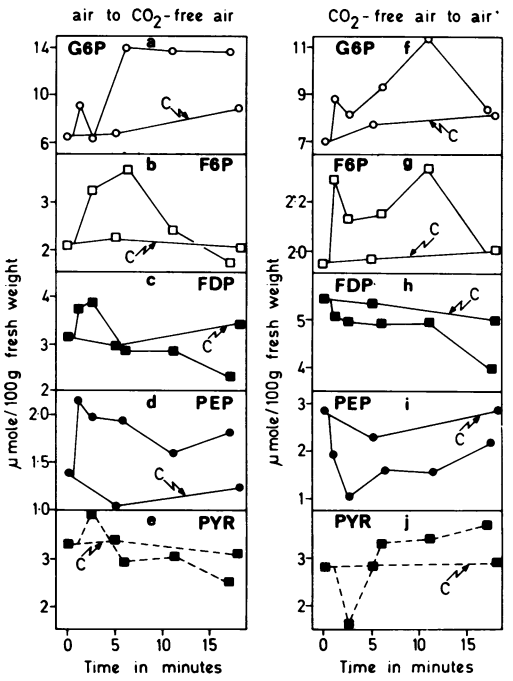

FIG. 1. Changes in intermediate levels in leaf material following transition from air to CO_2-free air (a-e and from CO_2-free air to air (f-j). Control samples (C) were maintained in air (a-e) or CO_2-free air (f-j).

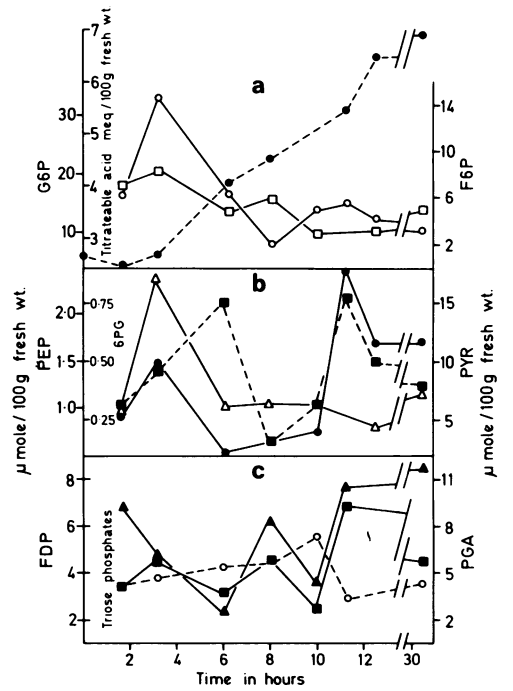

FIG. 2. Changes in levels of G6P(○——○), F6P (□——□), PEP (●——●), 6PG (△——△), PYR (■- - -■), FDP (■——■), PGA (▲——▲), triose phosphates (○- - -○) and titratable acid (●- - -●) during the course of acid accumulation in darkness.

Transition from CO_2-free Air to Air. Experimental procedures were as described for the air to CO_2-free transition except that the atmosphere surrounding the leaf tissue was rapidly changed from CO_2-free air to air. PEP and FDP decreased and remained below the control level and pyruvate showed a transient decrease. G6P and F6P increased initially then declined to the control level (Fig 1, f-j). Satisfactory measurements of changes in levels of PGA and triose phosphates were beyond the scope of the methods used.

Time Course of Acid Accumulation. Intact leaves were illuminated overnight to prepare them for an acid accumulation phase. Leaf slice samples were cut, then placed in darkened tubes and incubated in a stream of moist air at either 11 or 20 C. Samples were taken at the times indicated in Figure 2 which also shows changes in levels of malate and intermediates throughout the course of acid accumulation at 11 C.

The G6P and 6PG levels increased during the first 3 hr, then began to fall to a low level which was maintained until the end of the experiment.

An initial increase was also observed in F6P and FDP. 3PGA decreased during the first 6 hr of the experiment, then increased during the remaining 20 hr. The levels of triose phosphates remained more or less constant throughout the experiment. PEP exhibited an initial increase similar to that in G6P and F6P. This initial increase was followed by a fall to a low level which was maintained throughout the period of maximum acid accumulation both at 20 C and 11 C. As acid accumulation slowed and ceased, a rapid and large increase in the PEP level was observed. Pyruvate increased in amount during the first 6 hr throughout the onset of acid accumulation. This rise was followed by a transient fall to a low level which coincided with the transient changes in 3PGA and FDP. Recovery from this decrease in level commenced about 2 hr before the increase in PEP mentioned above.

Apart from variations in timing and magnitudes of changes, essentially similar results were obtained regardless of whether the incubations were carried out at 11 C or 20 C.

DISCUSSION

When the CO_2 concentration in the atmosphere surrounding *K. daigremontiana* leaf tissue in darkness increased, there was an immediate fall in the level of PEP. Conversely, when the CO_2 level was reduced, there was an increase in PEP level (Fig. 1). These results are consistent with the involvement of PEP as a substrate in carboxylation reactions catalyzed by PEP carboxylase. Pyr increased transiently in response to the change from air to CO_2-free and and decreased temporarily on transition from CO_2-free air to air. Such transient changes can not reflect a simple precursor-product relationship between PEP and Pyr since the changes in PEP levels were maintained throughout the experiment.

Application of the same arguments as used above in relation to PEP carboxylase suggest the implication of malic enzyme in the changes in Pyr level. However, as Figure 2 shows, the changes in PEP correlate more closely with the course of CO_2 fixation than do the changes in Pyr. The level of PEP is low throughout the period of most rapid accumulation of acid. Pyr, on the other hand, decreases in amount some time (3 hr at 11 C) after maximum acid accumulation commences, and begins to increase about 5 hr before the acid accumulation rate declines. These data are therefore consistent with involvement of Pyr and therefore the role of malic enzyme in acid accumulation, being at most a minor one. The amount of malic acid to which malic enzyme has access may well vary during the course of acid accumulation and this could at least partly account for the changes in Pyr level.

The decrease in level of FDP which followed the transition from CO_2-free air to air can be interpreted as resulting from an increased flux of carbon through this intermediate leading ultimately to malate. Coincident with the decrease in FDP level, there was an increase in the levels of G6P and F6P. Thus, an increase in CO_2 promotes the synthesis of G6P and F6P or, less likely, reduces their consumption. It appears that CO_2 concentration is involved in the regulation of the mobilization of stored carbohydrate in CAM. The site of action can be located between carbohydrate and phosphofructokinase, but the data do not allow a distinction to be made between direct effects of CO_2 and secondary effects resulting, for example, from a CO_2-induced fall in PEP level.

Transition from air to CO_2-free air also resulted in increases in levels of G6P, F6P, and FDP. These are interpreted as resulting from a "backing-up" of intermediates caused by the interruption of PEP consumption which follows the reduction of substrate CO_2 level.

All of the compounds assayed are involved in metabolic processes other than CAM, including respiration and photosynthetic carbon reduction, and it is probable that there exist, in the various cellular compartments, more or less separate pools of intermediates associated with particular pathways (3, 10). Information on the individual behavior of these separate pools is difficult to obtain, and therefore few firm conclusions concerning CAM can be reached from perturbation experiments of the kind so far discussed which are likely to affect more than a single metabolic pathway.

In experiments on malic acid accumulation in CAM, it is possible to avoid this problem by following changes in intermediate levels under constant conditions during the course of acid accumulation in the dark (Fig. 2). Without any change in external conditions, the darkened tissue commences a CO_2 fixation and acid accumulation cycle. Any changes in intermediate levels which occur during this internally regulated time course are likely to be associated exclusively with CAM. The reactions of CAM can, in a sense, be biochemically dissected from a constant background of other metabolic activities.

During the first 5 hr of darkness, transient increases were observed in G6P, 6PG, F6P, PEP, and FDP which may relate to an increase in the flow of carbon through to PEP. The quesion of what triggers the increase in carbon flux cannot yet be answered, but it is likely that an effect of the light/dark transition is involved. As well as the direct effects on intermediate levels caused by cessation of photosynthetic carbon reduction, transition from light to dark will result in an increase in internal CO_2 concentration which the CO_2 transition data described above indicate is involved in the mobilization of reserve carbohydrate.

Whatever its cause, the increase in G6P could activate PEP carboxylase *in vivo* as it has been shown to do *in vitro* (16). The resulting increased utilization of PEP would account for the falls in PEP, G6P, and 6PG which accompany the commencement of the phase of rapid acid accumulation (Fig. 2). The decline in PEP may exert a secondary effect by relieving an inhibition of phosphofructokinase (6, 7, 14) which would further contribute to an increased carbon flow to PEP.

As acid accumulation proceeds, the level of G6P decreases steadily until it is well below the level at which we have suggested it might stimulate acid synthesis by activating PEP carboxylase. The low internal CO_2 concentration which is maintained during rapid acid accumulation may (since high CO_2 concentration inhibits PEP carboxylase) be of significance in maintaining PEP carboxylase activity in the absence of high levels of G6P. The maintenance of the high carbon flux induced initially by high CO_2 concentration may similarly depend upon the replacement of the effects of CO_2 by the effects of some change characteristic of the rapid acid accumulation phase such as low PEP level.

As the rate of acid accumulation falls, the most obvious change in intermediate level is a relatively rapid increase in PEP. This suggests a change in PEP carboxylase activity. Kluge and Osmond (9) and Ting and Osmond (16) showed that malic acid

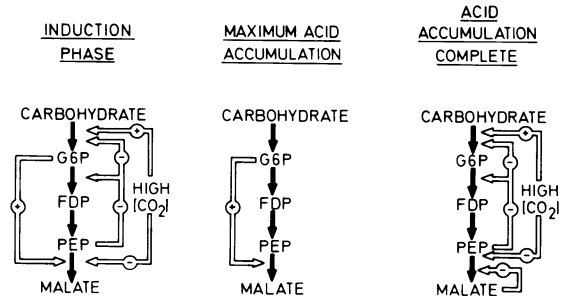

FIG. 3. Intermediate and Enzyme interactions which may be involved in the regulation of malic acid accumulation in CAM. Induction phase: following transfer of tissue from light to dark, respiration causes an increase in internal CO_2 concentration. This, in turn, causes an increase in carbohydrate mobilization which leads to an increase in G6P level which activates PEP carboxylase. Activation of PEP carboxylase leads to a reduction in CO_2 and PEP concentrations which leads to the maximum acid accumulation phase. Maximum acid accumulation phase: reduced CO_2 concentration further activates PEP carboxylase and reduced PEP results in increased phosphofructokinase activity. Acid accumulation proceeds until it reaches a level which leads to the commencement of the final accumulation phase. Acid accumulation complete: malate inactivation of PEP carboxylase results in a reduction in the rate of malate synthesis and an increase in CO_2 which further reduces malate synthesis by inhibiting PEP carboxylase. Concomitant increases in PEP levels may also inhibit phosphofructokinase and the carbohydrate-mobilizing enzymes.

inhibits Crassulacean PEP carboxylase *in vitro* and suggested that the switch-off mechanism of acid accumulation acts through malic acid inhibition of carboxylation. Cessation of acid accumulation is also accompanied by a fall in FDP level consistent with the inhibition of phosphofructokinase by the increased PEP level. The decrease in F6P may also reflect a decrease in carbohydrate mobilization associated with the rising PEP level which accompanies the decline in CO_2 fixation.

Figure 3 collates the interactions described above between PEP, malic acid, G6P, and CO_2, which may regulate the synthesis and accumulation of malic acid by reactions which involve PEP carboxylase and which encompass part of the glycolytic sequence.

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