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Photoperiodism and Enzyme Activity

TOWARDS A MODEL FOR THE CONTROL OF CIRCADIAN METABOLIC RHYTHMS IN THE CRASSULACEAN ACID METABOLISM

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ABSTRACT

Metabolic readjustments after a change from long days to short days appear, in *Kalanchoe blossfeldiana*, to be achieved through the operation of two main mechanisms: variation in enzyme capacity, and circadian rhythmicity. After a lag time, capacity in phosphoenolpyruvate carboxylase and capacity in aspartate aminotransferase increase exponentially and appear to be allometrically linked during 50 to 60 short days; then a sudden fall takes place in the activity of the former. Malic enzyme and alanine aminotransferase behave differently. Thus, the operation of the two sections of the pathway (before and after the malate step) give rise to a continuously changing functional compartmentation in the pathway. Circadian rhythmicity, on the other hand, produces time compartmentation through phase shifts and variation in amplitude, independently for each enzyme. These characteristics suggest that the operation of a so-called biological clock would be involved. We propose the hypothesis that feedback regulation would be more accurate and efficient when applied to an already oscillating, clock-controlled enzyme system.

Most of the external climatic factors controlling physiological functions act as periodic signals, mainly linked directly or indirectly to the light-dark cycles. Such signals mark strongly the time structure of the organism and they can be expected to have a highly efficient and versatile action on physiological coordination.

In recent years a number of papers have dealt with phytochrome control of enzyme activity (23), but the connection between these results and photoperiodic normal life conditions is rarely discussed. A recent paper (2) has shown that the first steps of the control by photoperiodism of a morphogenetic process (flowering) and of a metabolic pathway (CAM)[1] could be achieved through similar mechanisms.

CAM appears as a very convenient system for studying the laws of metabolic readjustments to alterations in environmental conditions. The classic definition of this pathway describes it as a circadian periodical process of CO_2 exchange, according to the reactions:

$$\left.\begin{array}{l}\text{PEP} + CO_2 \xrightarrow{\text{PEPC}} \text{oxaloacetate} \quad (1)\\ \text{oxaloacetate} + \text{NADH} \xrightarrow{\text{MDH}} \text{malate} + \text{NAD} \quad (2)\end{array}\right\} \text{(in dark)}$$

$$\text{malate} + \text{NADP} \xrightarrow{\text{ME}} \text{pyruvate} + CO_2 + \text{NADPH (in light)} \quad (3)$$

As oxaloacetate does not accumulate, reactions (1) and (2) should be considered as one single functional unit in which flow is determined by reaction (1) (15, 25, 27). The oscillatory behavior of CO_2 fixation and of malate content in Crassulacean leaves is well known. Recently, Queiroz (15, 16) and Mukerji (10) have reported circadian oscillation in P-enolpyruvate carboxylase and malic enzyme activities in different succulents. The control of CAM by photoperiodism had been first demonstrated by Gregory *et al.* (4) by CO_2 exchange measurements. Malate content (13, 14) and enzyme activity (15, 16) were studied later under combined different photoperiodic and thermoperiodic conditions. More recent results (8) established that the oscillatory behavior is extended to alanine and aspartate aminotransferases connected with the pathway. This supports earlier work showing that malate and aspartate are the fastest labeled products of $^{14}CO_2$ fixation by Crassulacean plants in darkness (3, 5, 22) and that alanine is also a major product of the pathway (3, 9, 26).

From the available data on CAM, it can be emphasized that: (*a*) the physiological significance of the pathway could be the obtention, mainly during the day, of massive amounts of chemical species from inputs available mainly during the night: the physiological roles of the over-all reactions NADH (night) → NADPH (day) and fixed CO_2 (night) → produced CO_2 (day) would deserve further research in a broad context including climatic changes; (*b*) photoperiodism controls the operation of CAM; (*c*) thermoperiodism and photoperiodism modulate quantitative time characteristics of CAM through iterative action.

This paper shows that photoperiodism would act on the whole CAM pathway through both a circadian control and a cumulative (seasonal) control. The connections between rhythmic behavior and quantitative readjustments as a response to changes in external photoperiodic conditions are discussed.

MATERIALS AND METHODS

Plant Material and Photoperiodic Treatment. Kalanchoe *blossfeldiana* cv. Tom Thumb was grown from cuttings from

[1] Abbreviations: CAM: Crassulacean acid metabolism; PEPC: P-enolpyruvate carboxylase EC 4.1.1.31; MDH: malate dehydrogenase EC 1.1.1.37; ME: malic enzyme EC 1.1.1.40; AAT: aspartate aminotransferase EC 2.5.1.1; AlaAT: alanine aminotransferase EC 2.6.1.2; PEP: P-enolpyruvate.

a strain cultivated in the phytotron of Gif-sur-Yvette under noninductive long days of 16 hr. This strain proved to be stable for the past 10 years regarding flowering and CAM behavior. Plants were illuminated by combined incandescent and fluorescent light (Lumière du Jour de Luxe, 125 w), 100,000 ergs cm^{-2} sec^{-1}. After 2 months, the plants were transferred into inductive short day conditions (9 hr day) or into noninductive control conditions (9 hr day followed by interruption of the dark period with a single exposure to 250 ergs cm^{-2} sec^{-1} red light from Philips 40 w TL15 tubes). The effectiveness of the red light treatment on phytochrome has been proved by reversion with far red radiation. In all cases, temperature was 27 C during the day and 17 C during the night. This thermoperiod has proved to be the most convenient for development of these plants (13, 14).

Preparation of Enzyme Extracts and Measurements of Enzyme Capacity. For the purpose of this study (comparison between the activities of different steps of a pathway and their variation in time), changes in maximum enzyme activity present in total extracts (referred to as enzyme capacity) will be considered as affording an evaluation of the changes in enzyme potentiality of the tissues (21, 24). The utilization of total extracts for the measurement of enzyme activity presents the problem of possible artifacts due to variation in cellular components coming into contact with the enzymes during the extraction. The presence of phenolic compounds and of high amounts of acid in Crassulacean leaves presents well known difficulties for obtaining extracts suitable for enzymic studies. To prevent enzyme inactivation by phenolics, polyethylene glycol (mol wt 20,000) has been utilized according to the results of preceding studies dealing more specifically with this aspect of the problem (2). Malate content in the leaves of these plants is known to undergo large daily variations and, as the present results show, much larger seasonal variations according to the prevailing photoperiod. Composition of the extraction medium has been adjusted in order to cover buffering requirements determined from preliminary measurements of maximum acidity attained during the experiment. It is known that malate inhibits P-enolpyruvate carboxylase from these plants (15, 16). The possibility that the variation in malate content could introduce artifacts in the variations of the enzymic activity measured in the extracts has been considered and dismissed by the experimental results (see "Results" and Figs. 2, 4, and 5).

Extraction Media. In this material, young leaves are more sensitive to changes in photoperiodic conditions. Samples of young leaves (second pair from the apex) were taken according to strictly standardized criteria, and rapidly and thoroughly ground at 4 C in 0.2 M tris-HCl buffer, pH 7.4, with 3.5 mM $MgCl_2$ and polyethylene glycol (mol wt 20,000) 1% w/w of leaf fresh weight to prevent enzyme inactivation by phenolic compounds. The homogenate was subjected to successive centrifugations until all enzyme activity was extracted from the pellets.

Measurements of Enzyme Activity. The composition of the reaction media is checked throughout the successive stages of the experiment for maximum enzyme activity, measured spectrophotometrically by linear changes in absorbance at 30 C in 0.2 M tris-HCl buffer. Measurements were made at 340 nm, except for aspartate aminotransferase (280 nm). (*a*) P-enolpyruvate carboxylase: enzyme activity was coupled with reduction of the produced oxaloacetate by the endogenous malate dehydrogenase. The assay contained, in 3 ml, 10 μmoles $MgCl_2$, 5 μmoles NaF, 7 to 20 μmoles P-enolpyruvate, 0.36 μmole NADH, and enzyme extract corresponding to 0.10 to 0.25 mg dry weight of leaves. (*b*) Malic dehydrogenase: this was assayed in a final volume of 3 ml containing 5 μmoles $MgCl_2$, 3 to 6 μmoles oxaloacetate, 0.36 μmole NADH, and enzyme extract corresponding to 0.005 to 0.03 mg dry weight of leaves. (*c*) Malic enzyme: the assay contained, in 3 ml, 10 μmoles $MgCl_2$, 20 to 80 μmoles of malate, 0.36 μmole of NADP, and extract corresponding to 0.20 to 0.50 mg dry weight of leaves. (*d*) Alanine aminotransferase: this was measured by coupling with glutamate dehydrogenase. In 3 ml there were 45 μmoles ammonium acetate, 0.3 μmole of NADH, 50 to 100 μmoles of pyruvate, 15 to 50 μmoles of glutamate, 2.4 units of glutamate dehydrogenase, and extract corresponding to 0.10 to 0.50 mg dry weight of material. (*e*) Aspartate aminotransferase: in 3 ml there were 3 to 9 μmoles of oxaloacetate, 20 to 70 μmoles of glutamate, and extract corresponding to 0.10 to 0.50 mg dry weight of leaves. The amount of oxaloacetate must be carefully controlled according to the amount of enzyme present in extracts, to avoid inhibition by excess substrate (8). The concentration of pyridoxal phosphate in the extracts is sufficient for the reaction. (*f*) Chemicals: these were obtained from Calbiochem or Sigma, except polyethylene glycol (mol wt 20,000) which was purchased from Touzart et Matignon, Paris.

Enzyme activity has been expressed on a dry weight basis as it was shown that under short day treatment the dry weight of leaves, sampled according to strict morphological criteria, changes slowly and linearly with time during the experiment.

Measurements of Malate and ^{14}C-Malate. Total content of malate in the leaves was measured titrimetrically after silica gel column chromatography, as previously described (13). Incorporation of $^{14}CO_2$ was carried on by leaves *in situ:* chambers of convenient volume, relative to the volume of the leaves, were sealed around the stem supporting the leaves. These double wall chambers in Plexiglas can be illuminated by light comparable to that received under growth conditions in the phytotron and were maintained under $\pm$0.5 C controlled temperature by circulating water inside the double wall. Air with constant composition (300 μl/l CO_2) was passed through the chamber at a convenient constant flow; the air leaving the chamber passed through an infrared CO_2 analyzer ONERA 80 thus providing eventual control of the leaves' behavior (4, 13, 17). During the labeling experiment (5 min) the chamber was momentarily isolated from the circuit and $^{14}CO_2$ was released from ^{14}C-$NaHCO_3$ (58–60 mc/mmole, Commissariat à L'Energie Atomique, Saclay, France) injected into lactic acid. The CO_2 content in the chamber did not change significantly during the 5-min experiment. The leaves were then removed and thoroughly extracted (13); organic acids were separated by column chromatography (13), and radioactivity was measured by liquid scintillation counting. Labeling in malate was expressed as cpm fixed per mmole in 5 min.

RESULTS

Control of CAM by Photoperiodism. Data on CO_2 exchanges, obtained by infrared gas analyzer (4, 13), or by labeling malate from $^{14}CO_2$ (22), as well as from data on total malate variations in the leaves (13, 14), show that plants grown in long days have a very low CAM activity. This activity is localized in the older (more than 3 months) slightly succulent leaves. In contrast, young leaves are thin in long days and display no apparent CAM activity.

During short days, strong CAM activity is present in all leaves, which are then very succulent even while they are young. Hence young leaves appear to be a very suitable material for the study of the induction and development of CAM according to changes in photoperiodic conditions.

It is an important feature that plants kept under short days but with interruption of the nights by a flash of red light, display strict nonCAM behavior. This "long day effect" of red light can be reversed by far red radiation: CAM appears as a typical phytochrome-dependent process (4, 13, 18).

Results of two aspects of the effect of photoperiodism on CAM will be considered: the day-night oscillation, which is a more complex mechanism than is usually assumed, and the cumulative effect of short days on plants previously grown under long days.

Variation in Malate Content. The typical day-night metabolic pattern for reactions 1, 2 and 3 in short days is illustrated by data in Figure 1. Variation in the amount of malate conforms with the recordings obtained by infrared CO_2 analyzer (4, 13, 17). There is a progressive increase in malate, corresponding to increasing CO_2 uptake by the leaves during the first few hours of the night; after about 6 hr of darkness, CO_2 uptake decreases and the rate of malate accumulation slows down. Part of the accumulated malate is decarboxylated during the following day. In older leaves and under favorable temperature conditions, a net CO_2 production by the leaves can be observed during a period of time ranging from about 1 to a few hours in the first part of the day, which corresponds to maximum speed of malate disappearance (see Fig. 1 in [13] and Fig. 4 in [17]).

Figure 2 shows the typical kinetics of the development of CAM in the young leaves of plants transferred from long day growing conditions into short day conditions. After a lag time of about 7 short days (2, 8), there is an exponential increase in malate content both at the end and at the beginning of the dark period (9 AM and 6 PM). The difference between these two curves corresponds to the day-night oscillation in malate content. In this typical experiment, the amplitude increases during the first 40 short days following the lag time, which means that both dark synthesis and day utilization of the malate increase with the number of short days. The former

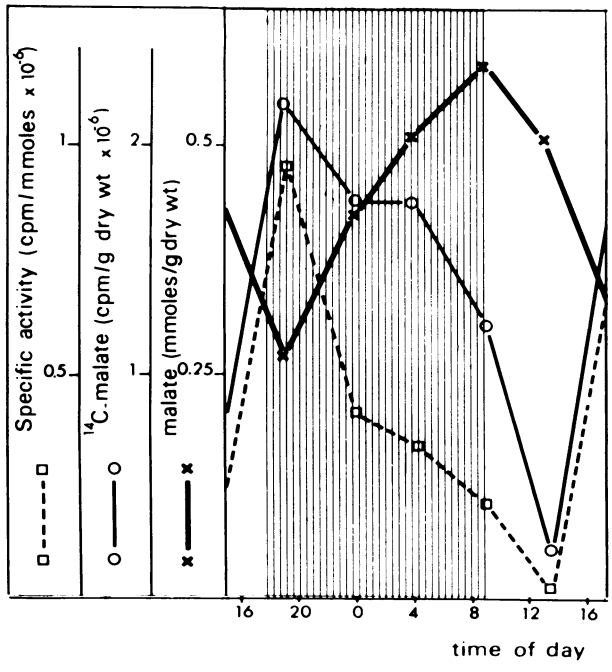

FIG. 1. Typical circadian oscillation in malate content compared to ^{14}C-malate produced in $^{14}CO_2$-feeding experiments (5 min) and to specific activity in malate. Measurements in young leaves after 30 short days, 27 C during the day, 17 C during the night, 75% humidity.

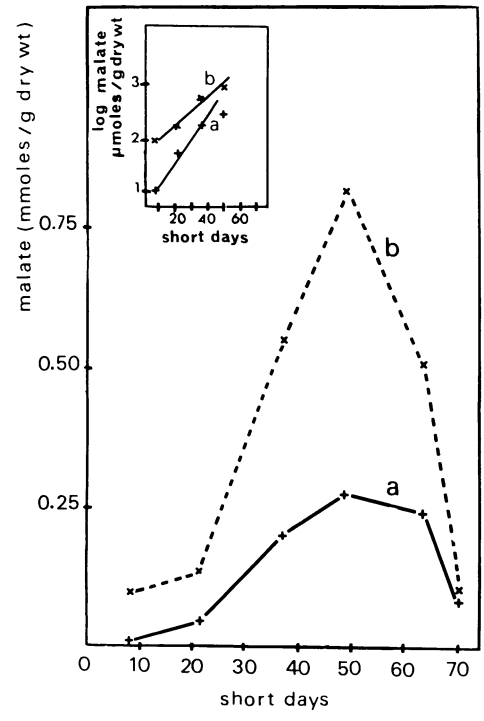

FIG. 2. Effect of short days on the variation in malate content expressed in linear and log plots (inset). Measurements at the beginning of the night (curve a) and at the end of the night (curve b).

increases faster, resulting in an over-all accumulation of malate. After about 50 short days, the stimulatory effect of the photoperiod on the pathway seems to stop operating, and the amount of malate decreases as a result of decrease in the activity of synthesis (Fig. 2). It must be emphasized that no sharp change appears in the steady, relatively slow linear increase of dry weight per leaf during the whole experiment.

VARIATION IN ENZYME CAPACITY

Validity of the Measurements. Figure 3 shows day-night oscillations in both P-enolpyruvate carboxylase and malic enzyme capacities. The shape of these variations is consistent with the curves for CO_2 exchanges (13, 17) and for oscillation in malate content (Fig. 1), and with the results of $^{14}CO_2$ fixation into malate *in vivo* (Fig. 1 and ref. 6).

The question that arises at first concerns the validity of these variations and the possibility that the measured enzymic daily oscillation might bear no relationship to the physiological situation but instead be an artifact resulting from the presence of variable amounts of malate in the extracts, because it is known that malate inhibits P-enolpyruvate carboxylase from these plants (15). In order to check this possibility, extracts obtained at 6 AM (highest malate content, relatively low enzyme capacity apparent) were mixed in different proportions with extracts obtained at 8 PM (lowest daily malate content, highest enzyme capacity); Figure 4 shows that no inhibition of the latter is observed even with high proportions of the 6 AM extract. The use of boiled 6 AM extracts leads to a similar result. It must be emphasized that the leaves used are young leaves (less than 400 mg fresh weight) containing relatively much lower amounts of malate than the leaves usually used by other authors in Crassulacean studies.

Moreover, we observed that when the plants were kept under short days the amount of malate increased exponentially in the leaves (Fig. 2); but Figure 5 shows that during the same

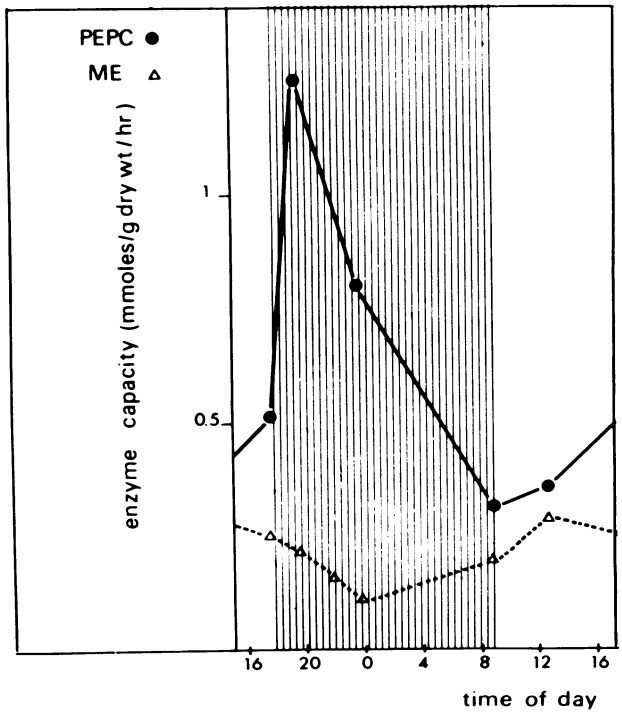

FIG. 3. Typical circadian oscillation in enzyme capacity of P-enolpyruvate carboxylase (PEPC) and malic enzyme (ME). The plants have received 30 short days. Compare with Figure 1.

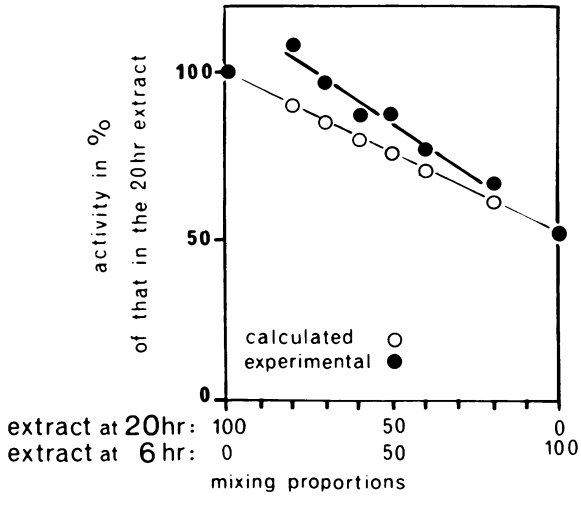

FIG. 4. Mixing experiments combining in different proportions extracts having high malate content and low PEPC activity (at 6 hr) with extracts having low malate content and high PEPC activity (at 20 hr).

time the activity of P-enolpyruvate carboxylase in the extracts also increased exponentially. If the decrease of P-enolpyruvate carboxylase activity, measured during the second part of the night (Fig. 3), was an artifact due to the malate present in the extracts, then the exponential increase in malate with the number of short days would be expected to depress the measured enzyme activity. The opposite is observed. Comparison of Figures 2 and 5 shows that both the measured activity of the enzyme and the amount of malate increase with the number of short days and hence, the observed daily variations of enzyme capacity in these leaves cannot be attributed to an artifact produced by the daily variations in malate. Moreover, circadian variations in the capacity of other enzymes connected to the pathway and not sensitive to malate (8) are observed, as presented in this paper.

It can be concluded that the observed enzyme oscillation is functionally coherent with the *in vivo* production of malate, concerning both the 24-hr cycle and the cumulative effect of short days.

Variations in P-Enolpyruvate Carboxylase Activity. The curves in Figure 5 show that the effect of short days was an exponential increase of capacity followed, after about fifty short days, by a sharp decrease. Comparison between extracts obtained at 6 PM (curve a) and at 9 AM (curve b) shows that there was a progressive change in amplitude of the day-night oscillation. Also, it can be expected from the crossing over of the two curves that the phase of the oscillation is not stable and may shift strongly after the 50th short day.

These changes in phase and amplitude have been confirmed by a systematic study of the variation in enzyme capacity during 24-hr cycles as a function of increasing number of short days (Fig. 6). During the first 60 cycles, daily maximums and minimums increased progressively with the number of short days, along with increases in amplitude and progressive phase shifting. After about 60 short days, there was a jump in the phase of the oscillation with sudden displacement of maximums and minimums, together with a sharp fall in the value of the maximum and in the amplitude of the oscillation. Experiments of incorporation of $^{14}CO_2$ into malate appear to confirm that this phase shifting in enzyme capacity reflects *in vivo* changes. Intact attached leaves were fed with $^{14}CO_2$ at a fixed time of the 24-hr cycle (7 PM), at different stages of the short day treatment. The rationale was that the shift in the peak of the enzymic oscillation implies changes of the values measured at a fixed time of the day-night cycle; if a correlation can be found between the *in vivo* CO_2 incorporation and these values, such correlation would indirectly confirm that the shifting in phase exists *in vivo*. Figure 7 shows that a good correlation actually

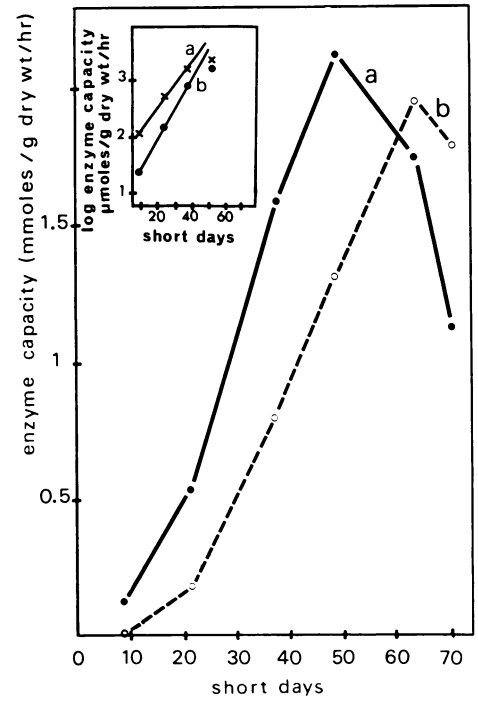

FIG. 5. Effect of short days on the variation in P-enolpyruvate carboxylase capacity, expressed in linear and log plots (inset). Measurements at the beginning of the night (curve a) and at the end of the night (curve b).

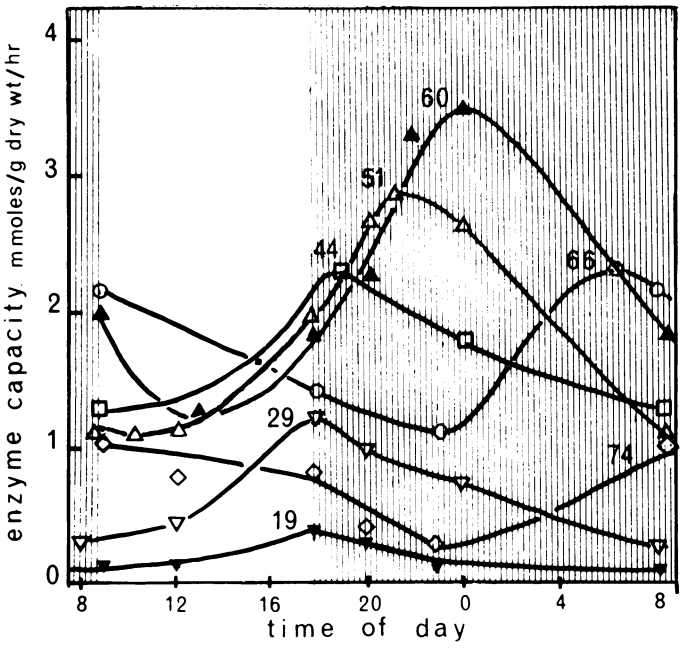

FIG. 6. Progressive change in circadian variation in P-enolpyruvate carboxylase with increasing number of short days. Oscillations during the 19th, 29th, 44th, 51st, 60th, 66th, and 74th short days.

exists between the production of labeled malate *in vivo* and the successive values of enzyme capacity measured *in vitro* at the same time.

Other Enzymes of the Pathway. As malate dehydrogenase capacity in these leaves is at any time up to several hundred times higher than the capacity of the other CAM enzymes (16), it appears that it will not be a control step of the metabolic flow.

Variations in capacity of malic enzyme, aspartate aminotransferase, and alanine aminotransferase afford data on the operation of the other steps of the pathway under persisting short day conditions. Data for the 29th, 51st, 60th and 66th cycles are presented in Figure 8, compared with the corresponding P-enolpyruvate carboxylase curves.

The general behavior of P-enolpyruvate carboxylase and aspartate aminotransferase appears to be very similar: big changes in capacity with the number of short days, circadian oscillation, parallel phase shift (except for the jump after the 60th short day which appears to be a specific feature of P-enolpyruvate carboxylase). Moreover, the increase in capacity of both enzymes appears to be related by an allometric law (Fig. 9), which is not the case between P-enolpyruvate carboxylase and the other two enzymes. Malic enzyme and alanine aminotransferase display a behavior different from the preceding pair of enzymes. This is emphasized by the over-all variation in mean enzyme capacity as it can be easily observed in Figure 8, and also by the curves plotting amplitude of oscillation against mean enzyme capacity (Fig. 10).

An important observation is that short day treatment produces no changes in K*m* values for any of the CAM enzymes measured after partial purification through Sephadex G25. Attempts, either through starch gel electrophoresis or diethylaminoethyl column separation, to find different isoenzymes or selective changes in the isoenzyme pattern with short day treatment has failed for the time being.

DISCUSSION

Coordination in CAM Operation. A survey of the literature shows that most results on characteristics and laws ruling CAM have been obtained through samples taken without any consideration of physiological timing or through short time experiments, and generalized as if it was assumed that the pathway displayed steady operation. As a matter of fact, Figures 6 and 8 show that CAM is metabolically a very flexible system, in which a steady state can hardly be defined. Hence the knowledge of the relations between the components of the system, and between the system and its environment requires long time experiments, performed under necessarily highly standardized growing conditions and experimental conditions, because metabolic balances can be expected to depend closely on the preceding history of the plant.

The present paper shows that variations in malate content *in vivo* and results of $^{14}CO_2$ feeding experiments are in good functional coherence with variations in enzyme capacity. Therefore, variation in enzyme capacity appears to afford a realistic timing evaluation of the changes in the enzymic potentialities of the pathway.

The results suggest that the block of CAM enzymic machinery must actually be split into two different parts, separated by the malate step. There is a functional coherence of variation and phase in the response of P-enolpyruvate carboxylase and aspartate aminotransferase that is lacking in the couple malic enzyme-alanine aminotransferase (Figs. 8 and 9).

The oxaloacetate step is a case of a branch point at which the enzymes starting the two branches have a higher capacity than the enzyme producing their common substrate. Hence P-enolpyruvate carboxylase appears to control the over-all

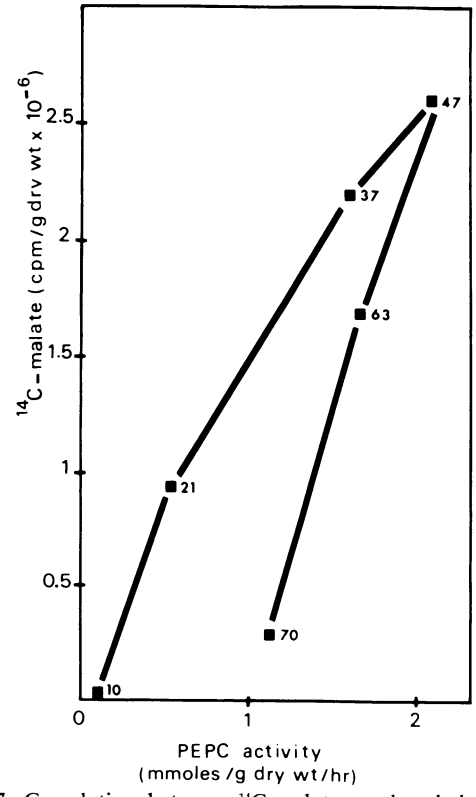

FIG. 7. Correlation between ^{14}C-malate produced during $^{14}CO_2$-feeding experiments and P-enolpyruvate carboxylase capacity. Data obtained at the beginning of the night period for the 10th, 21st, 37th, 47th, 63rd, and 70th short days.

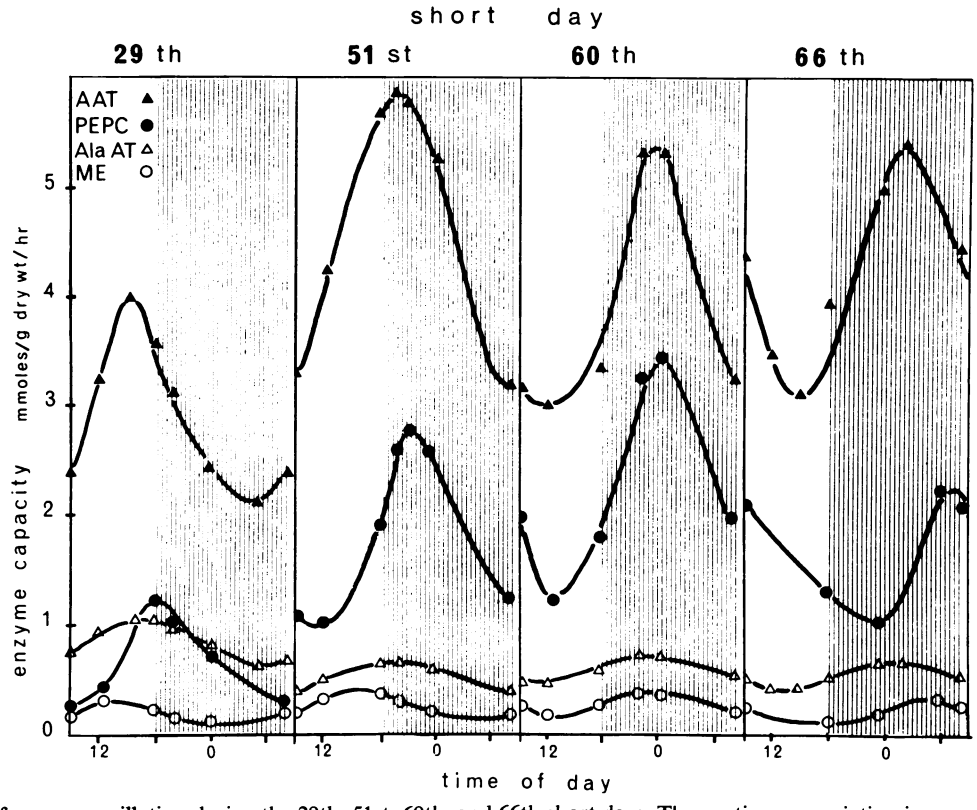

FIG. 8. Patterns of enzyme oscillation during the 29th, 51st, 60th, and 66th short days. The continuous variation in compared capacity, phase, and amplitude of oscillation produce, for the different enzymes, continuously changing ratios between activity during the night and activity during the day. For P-enolpyruvate carboxylase compare with Figure 6. Note that during the first 50 short days the shift in phase is similar for all the enzymes (about 0.3 hr/cycle), then the phase of malic enzyme starts shifting faster (0.7 hr/cycle). Later, a phase jump takes place in the oscillation of P-enolpyruvate carboxylase (about 1 hr/cycle between the 60th and the 66th short days).

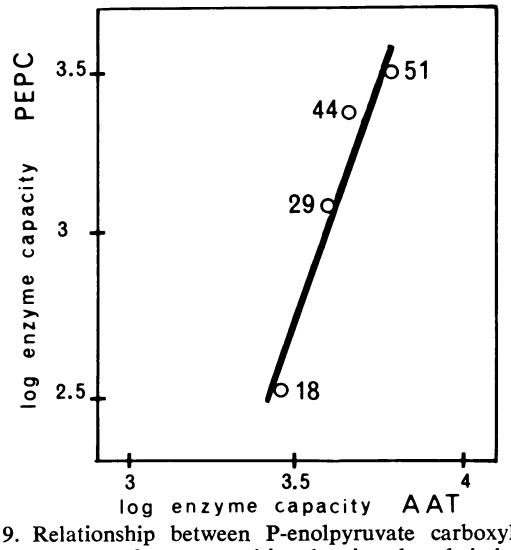

FIG. 9. Relationship between P-enolpyruvate carboxylase and aspartate aminotransferase capacities showing that their increase is linked by an allometric law; data for the 18th, 29th, 44th, and 51st short days.

flow, and this is confirmed by the absence of oxaloacetate accumulation at any stage of the process. The amount of produced malate and aspartate is at any time a function of the actual activity of P-enolpyruvate carboxylase and also of the competition between malate dehydrogenase and aspartate aminotransferase for oxaloacetate, assuming that no physical compartmentation or permeability barrier exists between the site of both enzymes as suggested by all available data for these leaves.

It is remarkable that the increase in the capacities of P-enolpyruvate carboxylase and aspartate aminotransferase ap-

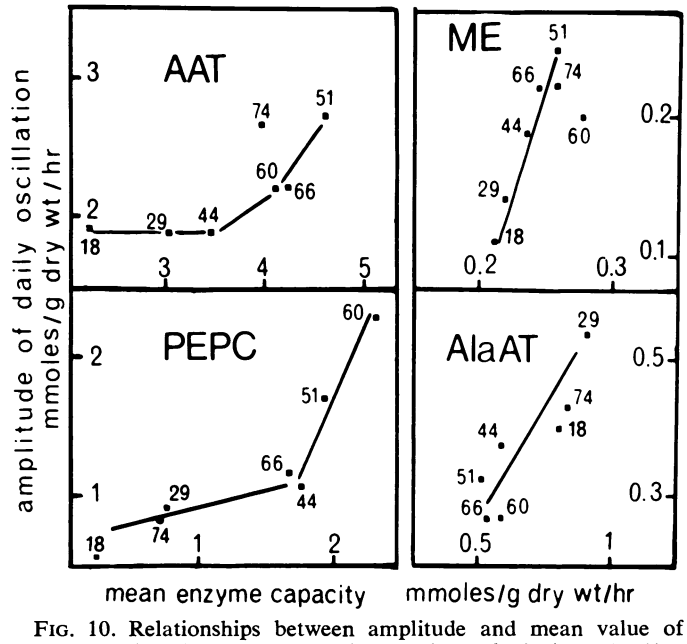

FIG. 10. Relationships between amplitude and mean value of daily oscillations in enzyme capacity for the 18th, 29th, 44th, 51st, 60th, 66th, and 74th short days. Compare with Figures 6 and 8.

pear to be related by an allometric law (Fig. 9). This law is considered to be an expression of optimality in correlated changes displayed by linked components of a developing system (19, 20). The present results show that alanine aminotransferase and malic enzyme do not follow this law relative to P-enolpyruvate carboxylase, while recent data (12) show that P-glyceraldehyde dehydrogenase (NAD-linked) does follow it. A discussion on the physiological meaning of these differences in behavior must wait for further investigation on changes in other enzymes directly or indirectly connected to CAM.

Mechanism of the Oscillations in CAM. The hypothesis that the oscillation in CAM could result from the action of a feedback control has been proposed (15, 16) on the basis of the inhibition of P-enolpyruvate carboxylase by malate. It was proposed that the oscillation in the activity of P-enolpyruvate carboxylase *in vivo* could result from feedback inhibition by the malate produced during the very active first part of the dark period; during the following day malate depletion by malic enzyme would decrease the amount of accumulated malate and thus would restore the activity of P-enolpyruvate carboxylase. Recent results were reported (7) on *Bryophyllum tubiflorum*, giving some support to this hypothesis: it was estimated that even a small fraction of the accumulated malate, if located in the cytoplasm, would bring the local concentration of inhibitor into the range of K*i* for malate.

But some of the present results do not fit with the rather simple feedback hypothesis: (*a*) the feedback mechanism cannot by itself explain the fact that the period of oscillation is a 24-hr period, which is a fundamental characteristic of CAM; the action of light could achieve this periodicity in different ways (18) but such action had not been demonstrated as yet; (*b*) the observed phase shifts of the oscillation (Fig. 6) cannot be explained by a simple feedback effect; (*c*) similarly, this effect cannot account for the oscillations observed for the other enzymes of the pathway (Fig. 8).

We conclude that if the feedback action of the malate is effective in the leaves, it cannot account for all the characteristics of the oscillation in CAM, and that other mechanisms must be present. Most available data on CAM characteristics clearly suggest the involvement of a so-called biological clock. The operation of CAM is sensitive to photoperiod, which is typical of biological clock-controlled events (1); moreover, the rhythm of dark CO_2 fixation as well as the rhythm of CO_2 output have the typical characteristics of the endogenous circadian rhythms, in response to light and temperature stimuli (11, 17, 30). The shape of P-enolpyruvate carboxylase variation (17) could be approached by Wever's equation for circadian rhythms in variables controlled by cumulative action of an external factor (28, 29). It is remarkable that the oscillations in capacity of the four enzymes follow similar phase shifting during about 50 short days (Fig. 8) as if they reflected the action of a single basic oscillator.

CONCLUSION

There is, for the moment, no clear complete explanation for the oscillatory characteristics of CAM. The induction and development of CAM in the young leaves of *Kalanchoe blossfeldiana* suggest that any integrative model attempting to explain the control of a pathway by photoperiodism probably includes: (*a*) an induction mechanism, as described (2), under the control of the so-called biological clock; (*b*) the potentiality for timing readjustments of metabolic flow through complex circadian rhythmical mechanisms at enzyme level, involving changes in V_{max}. The possibility that a feedback effect (such as malate inhibition of P-enolpyruvate carboxylase) would be more efficient if applied to an already oscillating (endogenous rhythm) enzyme activity is under current investigation.

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