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Properties of phosphoenolpyruvate carboxylase in rapidly prepared, desalted leaf extracts of the Crassulacean acid metabolism plant *Mesembryanthemum crystallinum* L.

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Abstract. Properties of phosphoenolpyruvate (PEP) carboxylase, obtained from leaves of *Mesembryanthemum crystallinum* L. performing Crassulacean acid metabolism (CAM), were determined at frequent time points during a 12-h light/12-h dark cycle. Leaf extracts were rapidly desalted and PEP carboxylase activity as a function of PEP concentration, malate concentration, and pH was measured within 2 min after homogenization of the tissue. Maximum velocity of PEP carboxylase was similar in the light and dark at pH 7.5 and pH 8.0. However, PEP carboxylase had as much as a 12-fold lower K_m for PEP and as much as a 20-fold higher K_i for malate during the dark than during the light periods, the magnitude of these differences being dependent on the assay pH. Assuming that enzyme properties immediately after isolation reflect the approximate state of the enzyme in vivo, these differences in enzyme properties reduce the potential for CO_2 fixation via PEP carboxylase in the light. A small decrease in cytoplasmic pH in the light would greatly magnify the above differences in day/night properties of PEP carboxylase, because the sensitivity of PEP carboxylase to inhibition by malate increased with decreasing pH. Properties of PEP carboxylase were also studied in plants exposed to short-term perturbations of the normal 12-h light/12-h dark cycle (e.g., prolonged light period, prolonged dark period). Under all light/dark regimes, there was a close correlation between change in properties of PEP carboxylase and changes of the tissue from acidification to deacidification, and vice versa. Changes in properties of PEP carboxylase were not merely light/dark phenomena because they were also observed in plants exposed to continuous light or dark. the data indicate that, during CAM, PEP carboxylase exists in two states which differ in their capacity for net malate synthesis. The "physiologically-active" state is distinguished by a low K_m for PEP and a high K_i for malate and favors malate synthesis. The "physiologically-inactive" state has a high K_m for PEP and a low K_i for malate and exists during periods of deacidification and other periods lacking synthesis of malic acid.

Key words: Crassulacean acid metabolism – *Mesembryanthemum* – Phosphoenolpyruvate carboxylase.

Introduction

Fixation of CO_2 at night via phosphoenolpyruvate (PEP) carboxylase (EC 4.1.1.31) in plants exhibiting Crassulacean acid metabolism (CAM) leads to accumulation of malic acid in the chloroplast-containing cells. During the day, this malic acid is decarboxylated, and the internally generated CO_2 is refixed by RuBP carboxylase and enters the photosynthetic carbon reduction cycle (Osmond 1978; Osmond and Holtum 1981). Efficient use of CO_2 from the malic acid pool requires that fixation of CO_2 via PEP carboxylase be supressed. It has been proposed that feedback inhibition of PEP carboxylase by malate could regulate PEP carboxylase activity in the light (Queiroz 1967; Kluge and Osmond 1972).

Recent studies with the inducible CAM plant *Mesembryanthemum crystallinum* have shown changes in properties of PEP carboxylase during a day/night cycle, indicating that PEP carboxylase may exist in more than one state during CAM (Greenway et al. 1978; Winter 1980a, 1981). PEP carboxylase was shown to be more sensitive to inhibition by malate

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Abbreviations: CAM = Crassulacean acid metabolism; PEP = phosphoenolpyruvate; PEPC = PEP carboxylase; RuBP = ribulose 1,5-bisphosphate; RH = relative humidity

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during the light than during the dark periods when assays were performed within a short period after homogenization of the leaves. However, PEP carboxylase extracted during the light periods rapidly lost its high sensitivity after isolation unless the enzyme extracts were kept at acid pH and/or in the presence of malate (Winter 1981). It was concluded that malate exerts a dual control on the activity of PEP carboxylase during the light: (1) by strongly inhibiting enzyme catalysis, its potential inhibitory effect being markedly increased by the sensitization of PEP carboxylase; and (2) by maintaining the highly malate-sensitive state of PEP carboxylase, particularly in combination with a lowered cytoplasmic pH.

These previous results were obtained for only a limited set of assay conditions, i.e., for mainly one pH value and for one substrate (PEP) and inhibitor (malate) concentration; they also refer, to a great extent, to experiments with non-desalted, crude leaf extracts. The investigations presented here examine in detail the effects of pH and various inhibitor and substrate concentrations on the activity of PEP carboxylase in rapidly prepared, desalted leaf extracts of the inducible CAM plant *M. crystallinum* exposed to a large variety of light/dark regimes. The results demonstrate both a decrease in K_i for malate and an increase in K_m for PEP during deacidification and other periods lacking net synthesis of malic acid. They also show that very small changes in pH may lead to large alterations in PEP carboxylase properties, which may allow for an efficient control of PEP carboxylase activity in vivo. Under all experimental conditions, changes in properties of PEP carboxylase were closely related to changes of the leaf tissue from acidification to deacidification and vice versa. Preliminary reports on the results presented in this publication were given at the 20th Annual Meeting of the Australian Society of Plant Physiology in Canberra in May 1979 and at the 5th International Congress on Photosynthesis in Kallithea in September 1980.

Material and methods

Plant material. Mesembryanthemum crystallinum L. was grown in a nutrient solution containing 400 mM NaCl, as described earlier (Winter 1980). Under these conditions plants performed pronounced CAM. Plants were kept in a growth cabinet with a 12-h light [25° C, 40–50% relative humidity (RH)]/12-h dark period (15° C, 60–70% RH). Illumination occurred between 07.00 and 19.00 h. Photosynthetically active radiation was 400 μmol photons m^{-2} s^{-1}.

Enzyme extraction. Extracts were prepared from the 3rd foliar leaves (expanded) of approx. 8-week-old plants. Leaves were cut into 2-mm-thick slices with a razor blade after the midrib had been removed. Slices of 2.5 g fresh weight were ground for 15 s with mortar and pestle in the presence of 0.4 g washed sand, 10 mg Polyclar AT, and 10 ml extraction buffer. In experiments in which properties of PEP carboxylase were compared in extracts obtained during the late light period (17.00–19.00 h, tissue deacidified), or during the first half of the dark period (22.00–24.30 h, tissue acidifying), the extraction buffer consisted of 200 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Hepes)/KOH, 0.2 mM ethylenediaminetetraacetate (EDTA), 0.5 mM dithiothreitol (DTT) and had a pH of either 7.0 for extractions in the light or 7.15 for extractions in the dark. The slightly increased pH of the extraction buffer for preparation of leaf extracts in the dark compensated for the increase in the malic acid content of the tissue. In other experiments in which properties of PEP carboxylase were followed over full 12-h light/12-h dark cycles, or over certain periods of a perturbated cycle, the extraction buffer consisted of 200 mM 2-(N-morpholino)ethanesulfonic acid (Mes)/KOH, 0.2 mM EDTA, 0.5 mM DTT, pH 7.0. The homogenate was quickly filtered through one layer of Miracloth (Calbiochem, La Jolla, Cal., USA). Two ml crude extract were rapidly desalted under pressure using a Sephadex G-25 (medium) column (0.9 · 20 cm). This procedure required 30 s. The first 2 ml of the 2.5 ml eluate containing PEP carboxylase were usually used for enzyme assays. This fraction was 99% free of low molecular-weight compounds. In some experiments in which complete removal of malic acid present in crude extracts appeared essential (e.g., when PEP carboxylase was assayed at pH 7.0), only the first 1.5 ml of the 2.5 ml eluate were used to assay PEP carboxylase activity. Desalting buffers were at pH 7.0 and contained either 50 mM Hepes/KOH, 0.2 mM EDTA, and 0.5 mM DTT or 50 mM Mes/KOH, 0.2 mM EDTA, and 0.5 mM DTT, depending on whether the extraction buffer contained Hepes or Mes. Aliquots of leaf extracts, either before or after passage through Sephadex G-25, were used for determination of chlorophyll after Arnon (1949). All materials were cooled at 4° C before use.

Assay for PEP carboxylase. The enzyme was assayed at 25° C. Unless otherwise stated, activity of PEP carboxylase (50 μl desalted extract per test) was measured 2 min after homogenization of the leaves. The spectrophotometric assay was based on the oxidation of NADH due to oxaloacetate reduction coupled to malate dehydrogenase (EC 1.1.1.37). Reaction mixtures (total volume 3 ml) contained 2 mM $KHCO_3$, 5 mM $MgCl_2$, 3 IU malate dehydrogenase (desalted), and varying amounts of PEP; reaction mixtures were buffered with 25 mM Mes, 25 mM N,N'-bis(2-hydroxyethyl)glycine (Bicine)/KOH pH 8.0, 7.5, or 7.0. $KHCO_3$ was added immediately prior to enzyme assays which were started by addition of enzyme extracts. The pH of the test solutions was determined when the assays were finished, and these values are referred to in the text. Five assays were performed in parallel using a Varian 634 S spectrophotometer with a rotating cell holder.

Experiments in which properties of PEP carboxylase were followed throughout a complete 24-h light/dark cycle or throughout perturbations of this cycle were repeated at least twice. Results were fully reproducible, and representative data are shown.

Some experiments (parts of Fig. 1) were repeated using a radiochemical assay for activity of PEP carboxylase in which the incorporation of $^{14}CO_2$ into oxaloacetate was determined. Assays were performed in scintillation vials. The total assay volume was 0.6 ml, and reaction mixtures contained 25 mM Bicine, 25 mM Mes (pH 8.0), 5 mM $MgCl_2$, 10 mM $NaH^{14}CO_3$, and 2 mM PEP. Reactions were started by addition of 10 μl desalted leaf extract and were terminated after 1 min by injection of 100 μl 6 M HCl saturated with dinitrophenylhydrazine. Scintillation vials were dried in a stream of air and the incorporation of radioactivity into the acid-stable fraction was determined by liquid scintillation counting.

Determination of pyruvate, PEP and 2-phosphoglycerate. To study the specificity of the spectrophotometric test for PEP carboxylase, reactions were stopped by addition of 200 μl 5 M HCl. After neu-

tralization with 5 M NaOH, reaction mixtures were centrifuged at 10,000 *g* in a Beckman microfuge for 1 min, and 0.1 ml supernatant was used for enzymic determination of pyruvate, PEP, and 2-phosphoglycerate, according to Czok and Lamprecht (1970). The total assay volume was 3 ml.

Determination of malate in leaf tissue. Tissue with a fresh weight of 1–2 g was extracted for 15 min with 10 ml 20% (v/v) boiling ethanol. The extract was filtered through cotton wool and an aliquot of the filtrate was used for determination of L-(−)-malate after Hohorst (1970).

Results

Properties of PEP carboxylase during a standard 12-h light/12-h dark cycle

Sensitivity to inhibition by malate. Leaves of *M. crystallinum* which were used for extraction of PEP carboxylase showed nocturnal accumulation and diurnal degradation of malate typical of tissues with CAM (Fig. 1 A). The activity of PEP carboxylase, measured at pH 8.0, 2 mM PEP and in the absence of added malate, remained unchanged during the light/dark cycle (Fig. 1 B). In the presence of 2 mM malate, PEP carboxylase was inhibited by 90% when extracted during the light period, but by only 30% when extracted during the dark period (Fig. 1 C).

K_m *for PEP.* Figure 2 shows typical Lineweaver-Burk plots of enzyme velocity versus PEP concentration in the absence and presence of the inhibitor malate for extracts prepared during the light and dark periods. At pH 8.0, the relationship between PEP carboxylase activity and PEP concentration followed the Michaelis-Menten formalism over PEP concentrations from 2 to 0.1 mM in approximately 80% of about 250 experiments which were performed in the course of the studies presented in this publication. In the remaining 20% of all experiments, analysis of data was complicated due to a scatter of data points. These results, which were disregarded, were probably due to pipetting errors during rapid addition of enzyme extracts, since the assays were started within 2 min after homogenization of the leaves. The double reciprocal plots in Fig. 2 indicate basically a competitive-type inhibition of PEP carboxylase by malate as this inhibitor increased the apparent K_m for PEP with little or no effect on the maximum velocity. The inhibitor concentrations depicted in Fig. 2 reduce the K_m PEP by approximately 50% and roughly represent the K_i for malate at pH 8.0.

The K_m for PEP was about 0.2 mM during the dark and ranged from 0.8 to 1.0 mM during the light period, while there was no significant change in V_{max} during the 12-h light/12-h dark cycle (Fig. 3).

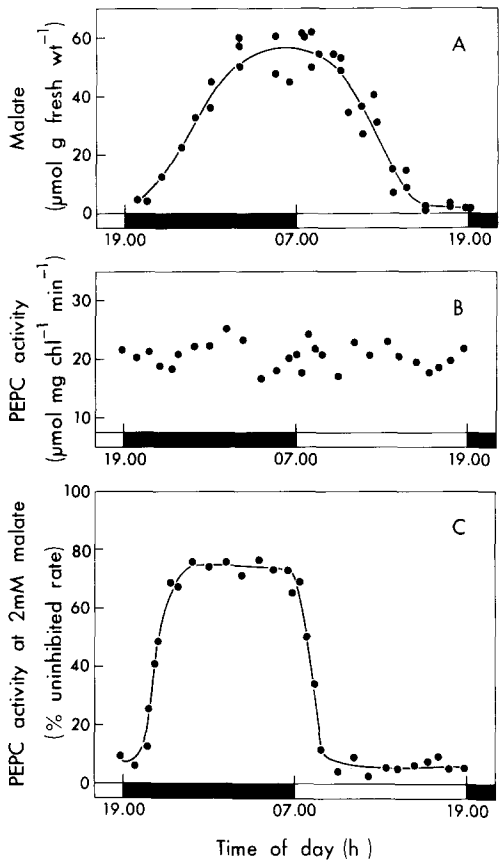

Fig. 1A–C. Change in sensitivity of PEP carboxylase to inhibition by malate during a 12-h light/12-h dark cycle. **A** Malate content of leaf tissue, **B** PEP carboxylase activity at pH 8.0 and 2 mM PEP, and **C** PEP carboxylase activity at pH 8.0, 2 mM PEP and 2 mM malate. Assays were performed 2 min after homogenization of leaves using desalted extracts. ■ dark period, □ light period

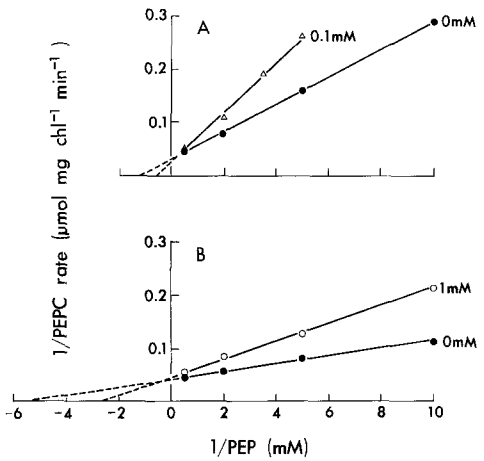

Fig. 2A, B. Lineweaver-Burk plots 1/enzyme velocity versus 1/PEP concentration of PEP carboxylase in the presence and absence of the inhibitor malate. Extracts were prepared in the late 12-h light period (**A**) and in the first half of the 12-h dark period (**B**). Assays were at pH 8.0 and were performed 2 min after homogenization of leaves using desalted extracts. The depicted malate concentrations roughly double the K_m for PEP

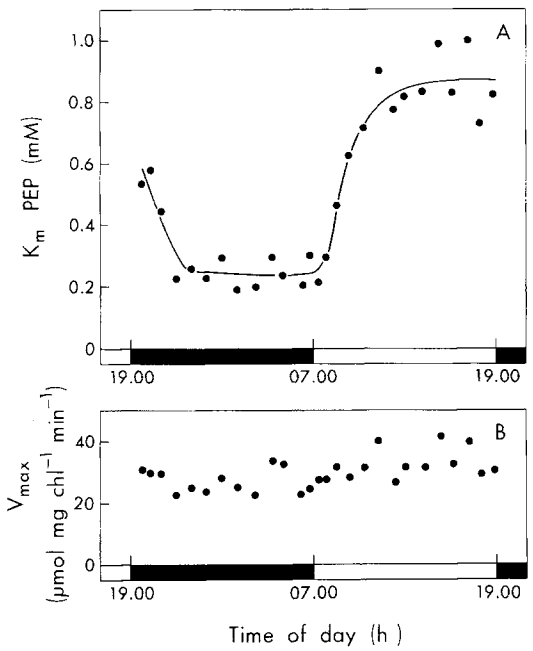

Fig. 3A, B. K_m for PEP (**A**) and maximum velocity (V_{max}) (**B**) of PEP carboxylase during a 12-h light/12-h dark cycle. Assays were at pH 8.0 and were performed 2 min after homogenization of leaves using desalted extracts. V_{max} was determined by extrapolation of Lineweaver-Burk plots. ■ dark period, □ light period

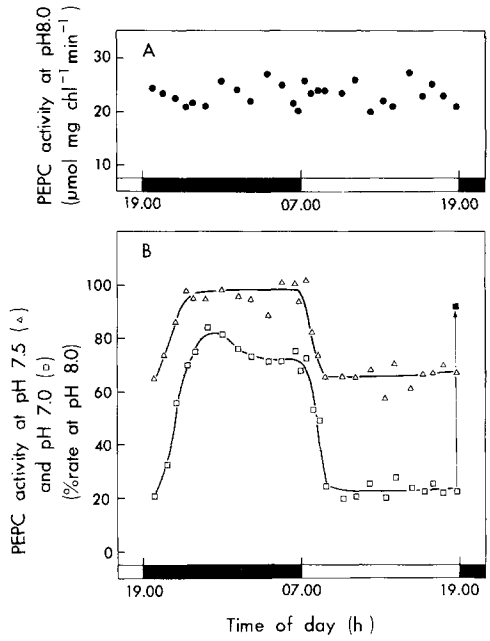

Fig. 5A, B. Change in activity of PEP carboxylase at 2 mM PEP as a function of the assay-pH during a 12-h light/12-h dark cycle. (**A**) pH 8.0, (**B**) pH 7.5 and pH 7.0. Assays were performed 2 min after homogenization of leaves using desalted extracts. In one case, activity was also determined 10 min after homogenization of leaves (pH 7.0, 18.30 h, black square). ■ dark period, □ light period

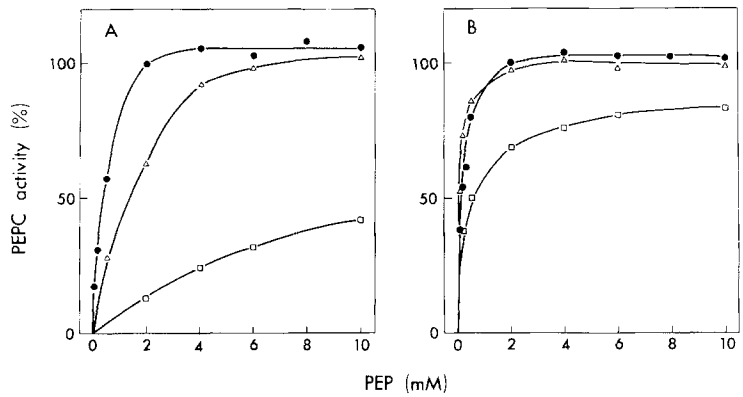

Fig. 4. Rate-curves of PEP carboxylase at pH 8.0 (●), pH 7.5 (△) and pH 7.0 (□) as a function of PEP concentration. Extracts were prepared in the late 12-h light period (**A**) and in the first half of the 12-h dark period (**B**). Assays were performed 2 min after homogenization of leaves using desalted extracts. Due to technical reasons only 4 PEP concentrations could be assayed per leaf extract. Therefore, each rate-curve refers to experiments with 2–3 leaf extracts (except pH 7.0, light). For determination of K_m-values, 4 PEP levels between 0 mM and the saturating concentration were used

pH effects. In the above experiments, PEP carboxylase was assayed at pH 8.0. In vivo, PEP carboxylase is probably exposed to somewhat lower pH values. Figure 4 shows PEP-response curves of enzyme activity, measured in the late-light and in the first half of the dark period, at pH 7.0 and pH 7.5 in comparison to pH 8.0. The enzyme activity at pH 8.0 and 2 mM PEP was taken as 100% because 2 mM PEP was saturating at this pH, both in the light and dark, and activities, expressed on a chlorophyll basis, were similar. At pH 7.0, considerably higher substrate concentrations were required to saturate PEP carboxylase. In the dark, V_{max} was reached around 6 mM PEP whereas the corresponding PEP level in the light was higher than 10 mM. At pH 7.5, the enzyme activities as a function of PEP concentration were intermediate between those at pH 7.0 and 8.0.

The data in Fig. 4, which were obtained for defined periods of the 12-h light/12-h dark cycle, were also valid for other parts of the light and dark periods. At 2 mM PEP, large fluctuations in PEP carboxylase activity at pH 7.0 occurred during a 24-h cycle with lower activity during the light than during the dark period (Fig. 5). Light/dark fluctuations in PEP carboxylase activity were less pronounced at pH 7.5 and absent at pH 8.0 which is consistent with the results

shown in Fig. 4. The shoulder in the pH 7.0-curve around 22.00 h (Fig. 5) was also observed in a second experiment, but was absent in a third experiment.

Tables 1 and 2 summarize some important kinetic properties of PEP carboxylase at pH 8.0, 7.5, and 7.0 immediately after homogenization of leaves in the late-light period and in the first half of the dark period. There was no significant change in V_{max} between light and dark at pH 8.0 and pH 7.5 and between both pH values. V_{max} was less at pH 7.0 and lower values were obtained in the light than in the dark (Table 1).

Table 1. Effect of assay pH on the maximum velocity (V_{max}) of PEP carboxylase. Leaf extracts were prepared from deacidified leaves in the late 12-h light period and from acidifying leaves in the first half of the 12-h dark period. Assays were performed 2 min after homogenization of leaves using desalted extracts. Differences in enzyme activity between light and dark at pH 8.0 and pH 7.5 and between both pH values are not significant (Chl= chlorophyll)

pH	V_{max} (μmol mg^{-1} Chl min^{-1})	
	Light	Dark
8.0	28.6	27.8
7.5	23.8	25.6
7.0	11.8	19.6

Table 2. Effect of assay pH on K_m PEP and K_i malate of PEP carboxylase. Extracts were prepared from deacidified leaves in the late 12-h light period and from acidifying leaves in the first half of the 12-h dark period. Assays were performed 2 min after homogenization of leaves using desalted extracts. Data represent the range of values obtained from at least five experiments. Data for pH 7.0 in the light are not given because assays were complicated by the extremely high sensitivity of PEP carboxylase to malate and low apparent affinity for PEP (see also text)

pH	K_m PEP (μM)		K_i Malate (μM)	
	Light	Dark	Light	Dark
8.0	700–1,000	200–300	73–75	400–900
7.5	900–1,250	90–220	4–5	60–86
7.0		310–420		4–9

At all three pH values, the K_m for PEP was lower for PEP carboxylase extracted in the dark than in the light (Table 2). Sensitivity to inhibition by malate increased with decreasing pH as indicated by a decreasing K_i. K_i values, determined at pH 8.0 and 7.5, were 5- to 12-times and 13- to 19-times higher in the dark than in the light, respectively. K_i values were determined assuming a competitive pattern of inhibition, although reciprocal plots 1/enzyme velocity versus 1/PEP-concentration, obtained for at least 2 malate concentrations, did not always intersect exactly in one point on the y-axis, which was probably due to the technical problem of initiating enzyme assays at exactly 2 min after homogenization of leaves. Nevertheless, at "infinite" (e.g., 20 mM) malate concentrations reciprocal plots crossed the intersection of x- and y-axes. At pH 7.5 and pH 7.0 in the light, reciprocal plots were not always linear since the rates at 0.1 mM PEP were frequently higher than predicted from linear kinetics. In such cases, the calculations were based on PEP concentrations above 0.2 mM. Reliable values for K_m PEP and K_i malate at pH 7.0 in the light were difficult to obtain because analysis of data had to be based on extremely small absorbance changes in the assay, due to the low apparent affinity of PEP carboxylase for PEP and the high sensitivity of the enzyme to inhibition by malate. Only very tentative values can be given for these conditions: K_m values varied from 1 to about 5 mM and K_i values were around 3–30 μM.

Change in PEP carboxylase properties after isolation. Since PEP carboxylase extracted during the light period may rapidly lose its initially high sensitivity to inhibition by malate after storage of extracts at 0° C (Winter 1981), it was of interest to evaluate whether such changes also occur in the K_m PEP. Little change in K_m PEP was observed at pH 8.0 after isolation

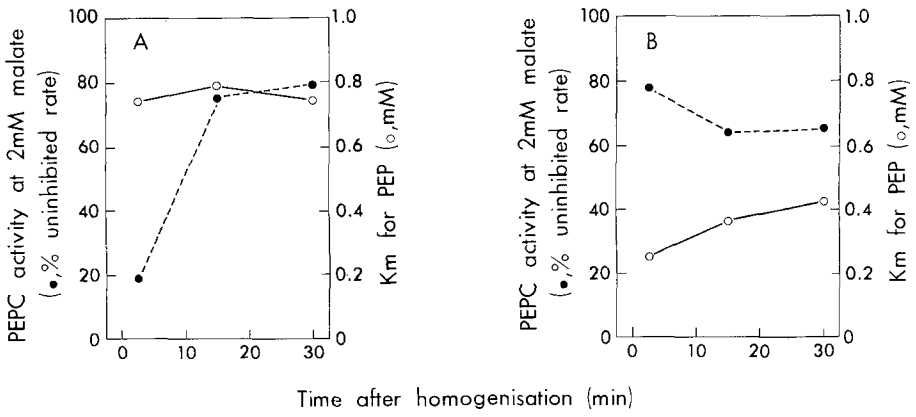

Fig. 6A, B. Sensitivity of PEP carboxylase to malate and K_m for PEP at pH 8.0 at various time points up to 30 min after homogenization of the leaf tissue. Extracts were obtained from deacidified leaves in the late 12-h light period (**A**) or from acidifying leaves in the first half of the 12-h dark period (**B**). Extracts were desalted immediately after homogenization of leaves and kept on ice during the 30-min period

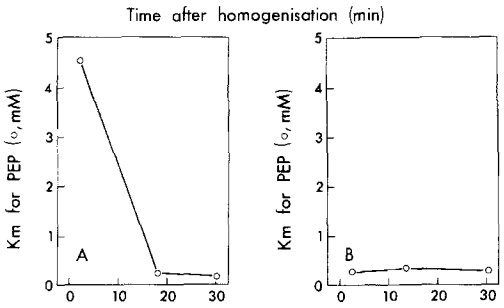

Fig. 7A, B. K_m for PEP of PEP carboxylase at pH 7.0 at various time points up to 30 min after homogenization of the leaf tissue. Extracts were obtained from deacidified leaves in the late 12-h light period (**A**) and from acidifying leaves in the first half of the 12-h dark period (**B**). Extracts were desalted immediately after homogenization of leaves and kept on ice during the 30-min period

(Fig. 6), but marked alterations occurred at pH 7.0 (Fig. 7).

At pH 8.0 (Fig. 6), loss of malate-sensitivity of PEP carboxylase extracted in the light and the results obtained in the dark were consistent with earlier experiments (Winter 1981). In contrast to these sensitivity changes of PEP carboxylase extracted during the light, the high and low K_m for PEP at pH 8.0 of PEP carboxylase extracted in the light and dark period, respectively, changed little or not at all during the 30-min storage period (Fig. 6). At pH 7.0, however, the high K_m observed immediately after preparation of extracts in the light rapidly decreased to the low level which prevailed in the dark-extracted enzyme (Fig. 7). This change in K_m PEP at pH 7.0 would explain the increase in activity of PEP carboxylase

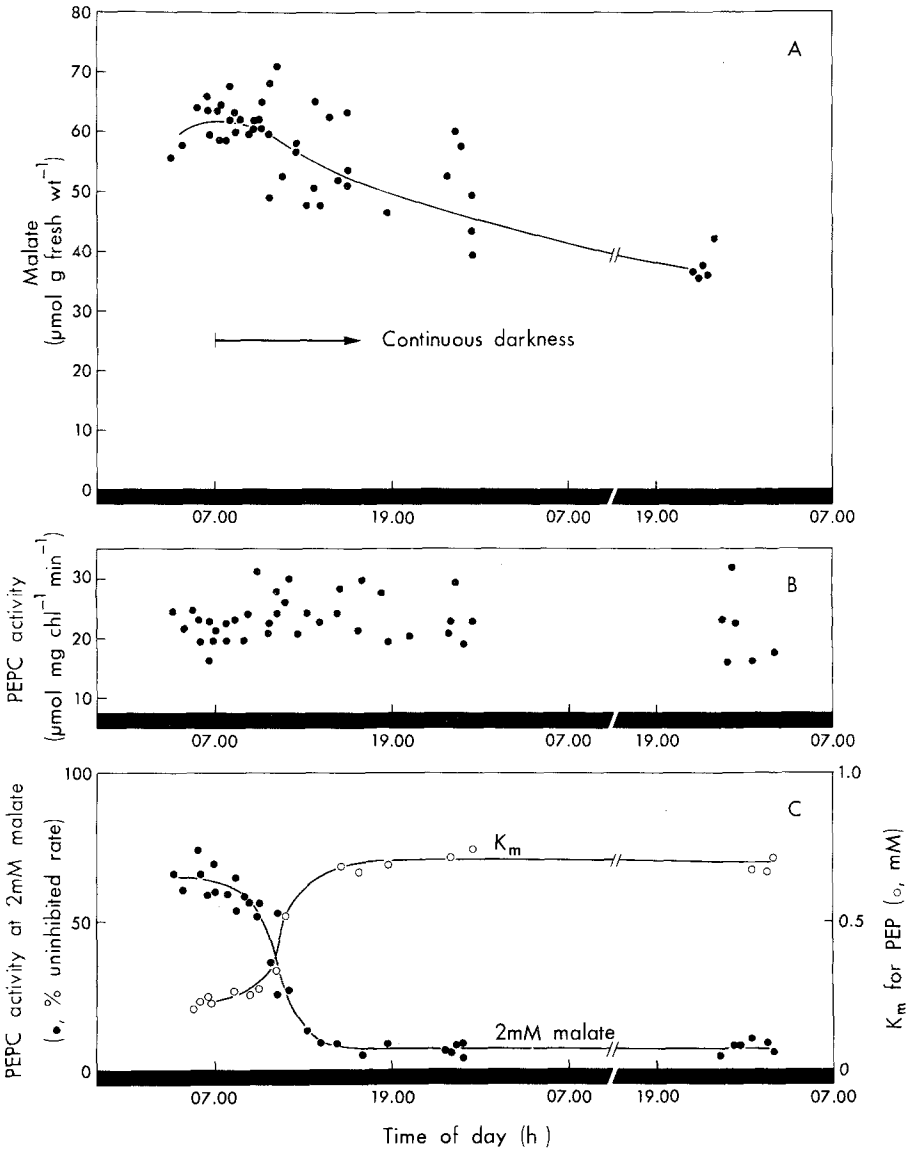

Fig. 8A–C. Change in properties of PEP carboxylase during a prolonged dark period.
A Malate content of leaf tissue,
B PEP carboxylase activity at pH 8.0 and 2 mM PEP,
C K_m PEP (○) and PEP carboxylase activity at pH 8.0, 2 mM PEP and 2 mM malate (●).
Assays were performed 2 min after homogenization of leaves using desalted extracts

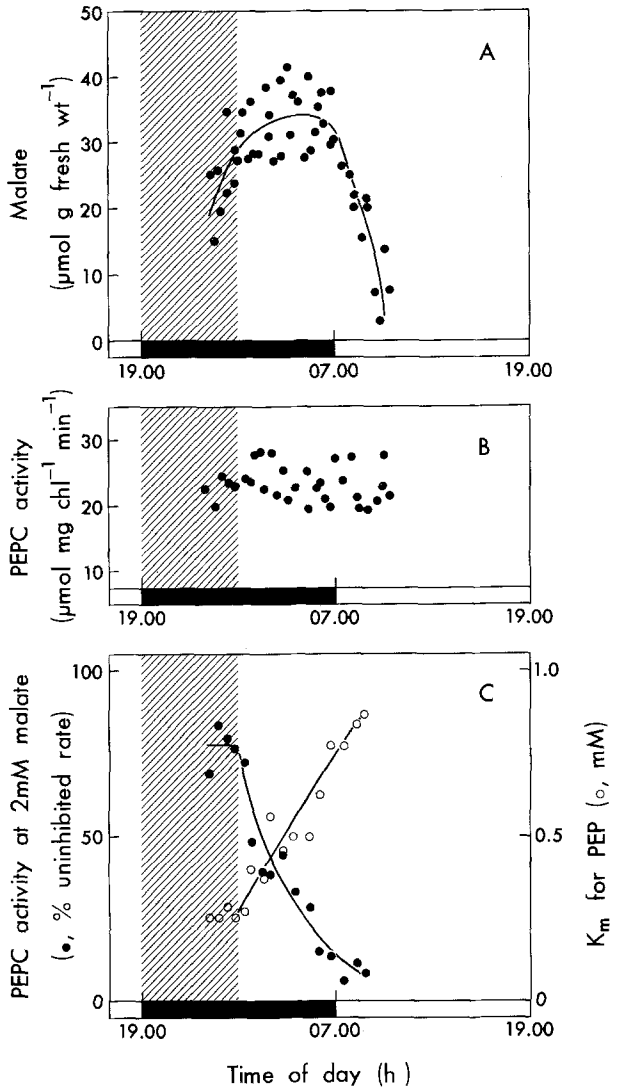

Fig. 9A–C. Change in properties of PEP carboxylase during a shortened dark period. **A** Malate content of leaf tissue, **B** PEP carboxylase activity at pH 8.0 and 2 mM PEP, **C** K_m PEP (○) and PEP carboxylase activity at pH 8.0, 2 mM PEP and 2 mM malate (●). Assays were performed 2 min after homogenization of leaves using desalted extracts. The black bar indicates the period of the normal 12-h dark period and the hatched area indicates the period of the actual dark period during the experiment

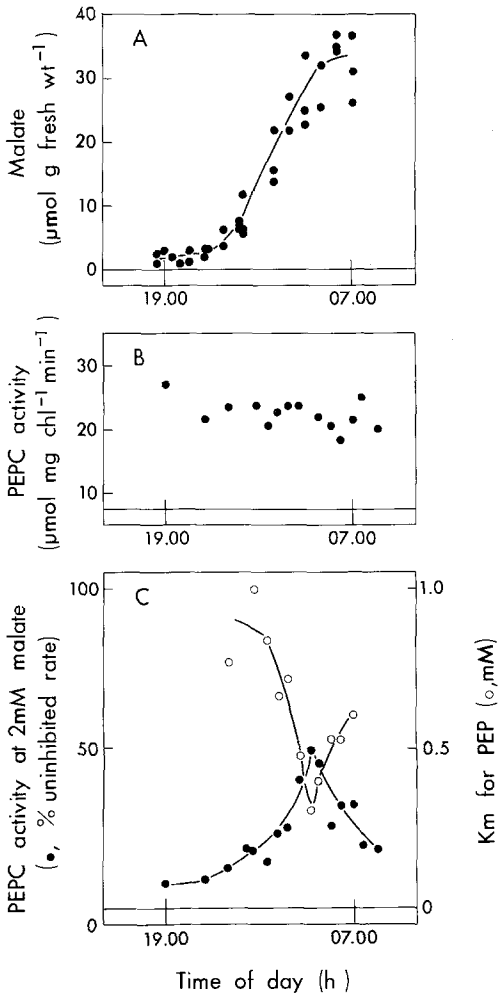

Fig. 10A–C. Change in properties of PEP carboxylase during a prolonged light period (19.00–07.00 h). Light was given at time of the normal dark period. **A** Malate content of leaf tissue, **B** PEP carboxylase activity at pH 8.0 and 2 mM PEP, **C** K_m PEP (○) and PEP carboxylase activity at pH 8.0, 2 mM PEP and 2 mM malate (●). Assays were performed 2 min after homogenization of leaves using desalted extracts

obtained during the light, at pH 7.0, and 2 mM PEP during a 10 min storage period of the enzyme extract at 0° C as shown in the experiment from Fig. 5B (18.30 h).

Properties of PEP carboxylase during perturbations of the standard 12-h light/12-h dark cycle

In the experiments described so far, PEP carbocylase was extracted from plants kept under a standard 12-h light/12-h dark cycle. In the following experiments, plants were exposed to short-term perturbations of this cycle, and changes in the malate content of the leaves and in the properties of PEP carboxylase, measured immediately after isolation, were examined. Studies of enzyme properties were restricted to determinations of the K_m PEP and of the sensitivity of PEP carboxylase to 2 mM malate at 2 mM PEP, both being measured at pH 8.0. In all experiments, the total activity of PEP carboxylase at pH 8.0 and saturating PEP was used as the control. This value did not significantly change under all conditions applied (Figs. 8B, 9B, and 10B).

Prolonged and shortened dark period. In continuous darkness, the leaves began to deacidify slowly only about 3–4 h after conclusion of the normal 12-h dark period (Fig. 8A). The sensitivity of PEP carboxylase

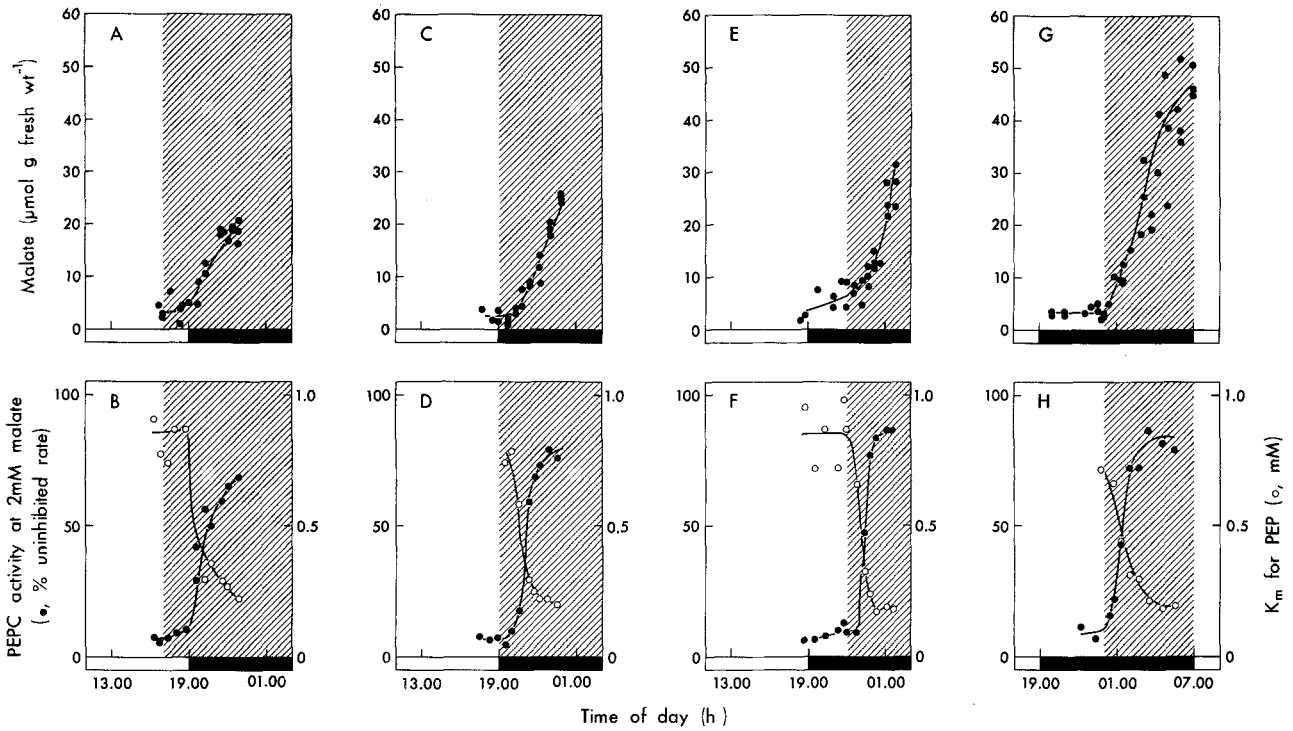

Fig. 11A–H. Effect of increasing length of the light period on change in properties of PEP carboxylase during the light/dark transition. Illumination was turned off 2 h before the end of the normal 12-h light period (**A, B**), at the end of the normal 12-h light period (**C, D**), and 3 (**E, F**) and 5 (**G, H**) h after conclusion of the normal 12-h light period. (**A, C, E, G**) Malate content of leaf tissue, (**B, D, F, H**) K_m PEP (○) and PEP carboxylase activity at pH 8.0, 2 mM PEP and 2 mM malate (●). Assays were performed 2 min after homogenization of leaves using desalted extracts. The black bar indicates the period of the normal 12-h dark period and the hatched area indicates the period of the actual dark period during the experiment

to inhibition by malate and the K_m PEP remained unchanged until malate accumulation had ceased. With the onset of malate degradation in prolonged darkness, properties of PEP carboxylase changed to those normally observed in the standard 12-h light period (Fig. 8C).

In the experiment shown in Fig. 9, lights were turned on in the middle of the 12-h dark period. There was an immediate response, as indicated by changes in the properties of PEP carboxylase, although the leaf tissue continued to show net malate synthesis at a very low rate. However, the change in properties of PEP carboxylase from those typical of the standard 12-h dark period to those typical of the standard 12-h light period required about 6 h from onset of illumination, in contrast to the 1- to 2-h duration after a normal dark/light transition (e.g., see Figs. 1 and 3). The leaf tissue started to deacidify rapidly exactly at that point in time (07.00 h) when the standard light period would normally have commenced.

Prolonged and shortened light period. Leaves showed malate accumulation and corresponding changes in PEP carboxylase properties not only in the dark, but also in the light, provided light was given at the time of the normal dark period. The amount of malate which accumulated over 12 h of prolonged light was only about 50% of the level usually observed at the end of a standard 12-h dark period (Fig. 10A). Synthesis of malate in the light was paralleled by a transient decrease in the sensitivity of PEP carboxylase to malate and in the K_m for PEP (Fig. 10C), even though this change in properties was less pronounced than after the onset of the dark period during a standard 12-h light/12-h dark cycle (Figs. 1 and 3).

The results shown in Fig. 11 demonstrate that the rate of net malate synthesis after the onset of the dark period and the rate in change of PEP carboxylase properties depends upon the length of the previous light period. When the dark period began 2 h earlier than normal (Figs. 11A and B), the onset of net synthesis of malate was delayed and the change in PEP carboxylase properties was much slower than after a normal light/dark transition (Figs. 11C and D). On the other hand, the rate of malate synthesis and of change in PEP carboxylase properties increased when the lights were turned off 3 and 5 h after the end of the standard 12-h light period, i.e., when the light period was extended into the dark period (Figs. 11E, F, G, and H). Although leaves were deacidified and

Table 3. Capacity for net malate synthesis in the dark as a function of the length of the light period. Plants were adapted to a 12-h light/12-h dark cycle. Data were taken from Fig. 11 (FW = fresh weight)

Onset of dark period	Net malate synthesis during first 4 h of darkness (μmol g^{-1} FW)	Rate of net malate synthesis between 3rd and 4th hour after onset of darkness (μmol g^{-1} FW h^{-1})
2 h before normal	14	3.5
normal	19	6.5
3 h after normal	33	12.5
5 h after normal	35	10.0

had similarly low levels of malate before every light/dark transition (Fig. 11), the capacity for malate synthesis clearly increased with increasing length of the light period (Table 3).

Discussion

PEP carboxylase assayed in rapidly prepared leaf extracts of the inducible CAM plant *M. crystallinum* shows marked increases in its sensitivity to inhibition by malate and in its K_m for PEP when extracts are obtained during the light period of a standard 12-h light/12-h dark cycle. Assuming that PEP carboxylase properties immediately after isolation approximate the properties of the enzyme in vivo, the above phenomena allow for an efficient control of PEP carboxylase activity. The increase in K_m PEP and the sensitization of PEP carboxylase to inhibition by malate clearly reduce the potential capacity for CO_2 fixation via PEP carboxylase in the light and favor CO_2 fixation via RuBP carboxylase.

PEP carboxylase from *M. crystallinum* and other CAM plants is a cytoplasmic enzyme (Spalding et al. 1979; Dittrich 1979; Ku et al. 1980; Winter et al. 1982), and the change in properties of PEP carboxylase would be amplified by a slight drop in cytoplasmic pH during the light period following the release of malic acid from the vacuoles. The consequence of a decrease in pH from 7.5 to 7.0 per se is illustrated in the following example: PEP carboxylase, extracted from *M. crystallinum* in the dark and assayed at pH 7.5 in the presence of 2 mM PEP and 0.1 mM malate, exhibits rates of about 20 μmol mg chlorophyll^{-1} min^{-1} which greatly exceed the maximum rates of malate synthesis observed in vivo. In contrast, no activity is detectable when PEP carboxylase is extracted in the light and assayed at pH 7.0 and at PEP and malate concentrations identical to the above values. Taking all three factors into consideration; i.e., (1) the sensitization of PEP carboxylase to malate, (2) the increase in K_m PEP, and (3) a possible small decrease in cytoplasmic pH during the light; it is conceivable that PEP carboxylase is not active in the light. Future research must be directed toward the evaluation of the actual cytoplasmic pH and malate concentration and the availability of PEP during the various phases of CAM.

Although properties of PEP carboxylase become more favorable for net malate synthesis at night, K_i values for malate of PEP carboxylase extracted in the dark (Table 2) are still much lower than previously reported values for PEP carboxylase from other CAM plants. Therefore, the results from Table 2 indicate a very efficient transport of the nocturnally produced malic acid into the vacuoles.

The change in PEP carboxylase properties in prolonged darkness (Fig. 8) and prolonged light (Fig. 10) shows that the interconversion between states of PEP carboxylase is not a light/dark-dependent effect. Still, direct effects of light cannot be ruled out completely in situations where plants were illuminated in the middle of the 12-h dark period (Fig. 9). Immediately following illumination, properties of PEP carboxylase started to change from those typical of the 12-h dark to those typical of the 12-h light period, although the malate level of the leaf tissue did not immediately decrease. PEP carboxylase properties changed very slowly, however, (e.g., compare data of Fig. 9 with those of Fig. 1) and probably still allowed net malate synthesis to occur at the observed low rate. A rapid decrease in leaf malate content at that point in time during the 24-h cycle when the lights are normally turned on was observed in two separate experiments (Fig. 9). Whether this result reflects rhythmic properties of CAM or whether it was fortuitous needs further clarification. The time-point at which deacidification began in prolonged darkness (Fig. 8) was somewhat variable and ranged from 2–5 h after conclusion of the normal dark period in 4 separate experiments. By and large, however, a certain rhythmic behavior of the physiological parameters studied here is undeniable. The results summarized in Figs. 9 and 11 and in Table 3 indicate that the capacity for net malate synthesis following onset of the dark period increases with increasing length of the light period, which is consistent with the above view and with recently published measurements of CO_2 and water vapour exchange in the CAM plant *Kalanchoe pinnata* (Winter 1980b).

The standard 12-h light/12-h dark treatments and perturbations of this light/dark cycle demonstrate a close relationship between change in PEP carboxylase properties and the transition from acidification to

deacidification and vice versa, i.e., transport of malate into and out of the vacuoles. Experiments designed to sensitize PEP carboxylase to inhibition by malate by extracting it in its relatively malate-insensitive state, i.e., during the dark period, in the presence of high malate concentration at acid pH have failed so far (Winter 1981). On the other hand, incubation of purified PEP carboxylase from *Kalanchoe daigremontiana* with malate in the assay medium and initiation of the spectrophotometric test with PEP led to substantially greater inhibition of PEP carboxylase activity than if reactions were initiated by addition of the enzyme extract (Nott and Osmond 1982).

Changes in K_m for PEP and in the sensitivity of PEP carboxylase to inhibition by malate occurred in parallel in most experiments (Figs. 1, 2, 8–11); i.e., an increase in the K_m for PEP was accompanied by an increase in the sensitivity of PEP carboxylase and a decrease in the K_m for PEP was accompanied by a decrease in the sensitivity of the enzyme. This finding still does not necessarily indicate that the change in both parameters is linked on a molecular basis. For example, PEP carboxylase extracted in the light and assayed at pH 8.0 rapidly lost its initially high sensitivity after isolation, and this loss in sensitivity was not paralleled by a change in K_m for PEP (Fig. 6A). Furthermore, K_i values decreased largely with decreasing pH, whereas K_m values were either not or much less affected (Table 2).

It may be argued that the obtained results are artifacts, which, e.g., are produced in the spectrophotometric PEP carboxylase assay based on NADH oxidation due to oxaloacetate reduction coupled to malate dehydrogenase. However, there are several arguments against this view:

(1) In the spectrophotometric tests of Fig. 1, there was no detectable conversion of PEP by enolase or by a PEP phosphatase. The decrease in the amount of PEP during the assay was equivalent to the amount of NADH oxidized. There was no NADH oxidation in the absence of PEP.

(2) Sensitization of PEP carboxylase to malate in the light was also observed in experiments in which PEP carboxylase activity was assayed using a radiochemical procedure in which the incorporation of ^{14}C radioactivity into oxaloacetate was measured (data not shown).

(3) Jones et al. (1978) made a comparison of the spectrophotometric and radioactive assay methods using purified PEP carboxylase from the constitutive CAM plant *Bryophyllum fedtschenkoi*. They found that the spectrophotometric system was valid under all conditions tested, e.g., up to malate concentrations of 20 mM.

(4) All experiments presented in this paper were performed with desalted leaf extracts. Therefore, interference of low molecular-weight compounds, including malate, with the assay can be ruled out.

Short-term exposure of PEP carboxylase to malate during extraction also does not explain the change in enzyme properties. For example, the change in properties during dark/light transitions or in prolonged darkness occurred at a point in time when the malate level of the tissue and, hence, in the crude homogenate remained fairly constant (Figs. 1, 3, 8).

The significance of the change in enzyme properties is also suggested by their close correlation to acidification and deacidification in vivo. This is particularly true in the experiment of Fig. 8, in which plants were kept in prolonged darkness and all environmental parameters remained constant, in contrast to most other experiments in which changes from light to dark, and vice versa, were accompanied by changes in leaf temperature. Environmental conditions were also constant when PEP carboxylase properties changed during prolonged light treatment (Fig. 10).

The results presented in this paper may help to explain some inconsistencies in results of previously published studies on PEP carboxylase in CAM plants. Light inactivation of PEP carboxylase was reported for some species of Mesembryanthemaceae (von Willert and von Willert 1979), and the degree of inactivation was correlated to the endogenous malate level of the leaves. These results may be largely due to the transfer of malate from non-desalted crude leaf extracts into the assay system, because a given malate concentration, which is not inhibitory in the dark, can become inhibitory in the light, due to the sensitization of PEP carboxylase (see also Winter 1980a). Furthermore, PEP carboxylase assays by von Willert and von Willert (1979) were performed at pH 7.0 and pH 7.5 at 1.5 mM PEP. At least for PEP carboxylase extracted from *M. crystallinum* in the light, this substrate concentration is far from saturating at both pH values (Fig. 4). Experiments with PEP carboxylase from *M. crystallinum* did not reveal light/dark differences in V_{max} at pH 8.0 and 7.5 under all conditions tested (Table 1). At pH 7.0, a decrease of V_{max} in the light became apparent, but the absolute rates in the light were still high compared to the rates of nocturnal acidification observed in vivo. Stimulatory effects of malate on the activity of PEP carboxylase from CAM plants have been reported by von Willert et al. (1979), but no such effects were revealed in the present investigations.

Greenway et al. (1978) were the first to suggest the existence of two states of PEP carboxylase during CAM. However, the authors observed similar malate inhibition of PEP carboxylase immediately following

extraction in the light and dark periods, which contrasts with the results presented in this paper and elsewhere (Winter 1980a, 1981). The change in the properties of PEP carboxylase following the onset of the dark period during a 12-h light/12-h dark cycle is complete only after about 2–3 h (Fig. 1). The "dark values" for malate inhibition of PEP carboxylase from the study done by Greenway et al. (1978) were probably obtained relatively soon after the onset of the dark period.

Conclusions

The data presented here indicate that, during CAM, PEP carboxylase exists in two states which differ in their capacity for net malate synthesis. The "physiologically active" state is distinguished by a low K_m PEP and a high K_i malate and favors net malate synthesis. The "physiologically inactive" state has a high K_m PEP and a low K_i malate and exists during periods of deacidification and other periods during which malic acid is not synthesized. To ultimately prove that two states of PEP carboxylase exist during CAM, it must be shown that purified PEP carboxylase can be manipulated to interconvert between states in vitro.

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