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Transport of Phosphoenolpyruvate by Chloroplasts from *Mesembryanthemum crystallinum* L. Exhibiting Crassulacean Acid Metabolism[1]

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ABSTRACT

Chloroplasts from CAM-*Mesembryanthemum crystallinum* can transport phosphoenolpyruvate (PEP) across the envelope. The initial velocities of PEP uptake in the dark at 4°C exhibited saturation kinetics with increasing external PEP concentration. PEP uptake had a V_{max} of 6.46 (±0.05) micromoles per milligram chlorophyll per hour and an apparent K_{mPEP} of 0.148 (±0.004) millimolar. The uptake was competitively inhibited by Pi (apparent K_i = 0.19 millimolar), by glycerate 3-phosphate (apparent K_i = 0.13 millimolar), and by dihydroxyacetone phosphate, but malate and pyruvate were without effect. The chloroplasts were able to synthesize PEP when presented with pyruvate. PEP synthesis was light dependent. The prolonged synthesis and export of PEP from the chloroplasts required the presence of Pi or glycerate 3-phosphate in the external medium. It is suggested that the transport of pyruvate and PEP across the chloroplasts envelope is required during the gluconeogenic conversion of carbon from malate to storage carbohydrate in the light.

During the light, many plants with Crassulacean acid metabolism decarboxylate the bulk of their stored malate to CO_2 and pyruvate using NADP malic enzyme. Most of the pyruvate is transferred to storage carbohydrate by gluconeogenic processes (13), the first of which is the phosphorylation of pyruvate to PEP[3] by pyruvate, Pi dikinase. Of the enzymes involved in the decarboxylation of malate and the subsequent transfer of carbon from pyruvate to 3-PGA or triose-P, NADP malic enzyme is located in the cytoplasm, NAD malic enzyme is mitochondrial, pyruvate, Pi dikinase is exclusively chloroplastic, and enolase and PGA-mutase are cytosolic (26, 28). It has been proposed, on the basis of this intracellular location, that chloroplasts from CAM plants in which NADP malic enzyme is the predominant decarboxylase should have a substantial capacity for the transport of pyruvate and PEP across their envelopes (7, 22). Some indirect evidence exists for the transport of pyruvate and PEP across chloroplasts from CAM-*Mesembryanthemum* and *Sedum praealtum* (4, 23). In *Mesembryanthemum*, light-dependent $^{14}CO_2$ uptake is inhibited by pyruvate (5). It was suggested that pyruvate enters the chloroplasts and is phosphorylated to PEP, thus sequestering chloroplastic Pi and retarding the photosynthetic carbon reduction cycle. This pyruvate inhibition could be reversed by the addition of Pi.

In this report we demonstrate that chloroplasts from CAM-*Mesembryanthemum crystallinum* can transport PEP across their chloroplast envelopes at rates equivalent to the observed rates of malate loss. The characteristics of this PEP transport indicate the involvement of a Pi translocator with a high affinity for PEP. When presented with external pyruvate in the light, the chloroplasts were able to synthesize and export PEP.

METHODS

Plant Growth Conditions. *M. crystallinum* plants were grown from seed collected from a natural population in Israel by Prof. K. Winter (Würzburg, F.R.G.). The procedures for germination, hydroponic growth, and induction of CAM were those described by Winter *et al.* (28).

Isolation of Protoplasts. Protoplasts were isolated using a method modified from Demmig and Winter (5) and Monson *et al.* (19). Between 20 to 30 g of fully expanded leaves were diced into 2 mm · 2 mm pieces following the removal of the midribs and upper epidermis. Following immersion in a wash-medium of 800 mM sorbitol and 0.5 mM $CaCl_2$ the segments were suspended in 100 ml of digestion medium consisting of 800 mM sorbitol, 20 mM Mes-HCl (pH 5.2), 0.5 mM $CaCl_2$, 2% (w/v) Onozuka SS cellulase, and 0.5% (w/v) Macerozyme R-10. The tissue was subjected to three 5 s vacuum-infiltration treatments before incubation for 3 h at room temperature and laboratory light. The Petri dish containing the tissue was gently agitated twice per hour. After separation of the protoplasts through a tea-sieve, the remaining tissue was gently shaken in 40 ml 800 mM sucrose. The combined filtrates were decanted into two 50 ml Babcock flasks and were overlain successively with 1.5 ml 800 mM sorbitol in 50 mM Hepes-NaOH (pH 8.0), and 1.5 ml 700 mM sorbitol in 100 mM Hepes-NaOH (pH 7.6). Following 5 min centrifugation at 80*g* in a swinging-bucket rotor, the protoplasts which collected in the two upper layers were gently removed with a Pasteur pipette.

Isolation of Chloroplasts. After the protoplasts were broken by a double passage through a 25-gauge needle, the chloroplasts were sedimented by 30 s centrifugation at 200*g*. The pellet was suspended in 1 ml test medium containing 750 mM sorbitol, 50 mM Hepes-NaOH (pH 7.8), 10 mM EDTA, and 15 mM NaCl. The centrifugation procedure was repeated and the pellet was gently resuspended in the same medium and stored on ice until required.

[1] Dedication: This manuscript is dedicated to Prof. P. F. Brownell on the eve of his retirement.

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[3] Abbreviations: PEP, phosphoenolpyruvate; DHAP, dihydroxyacetone phosphate; DIDS, 4,4′-diisothiocyano-2,2′-disulfonic acid stilbene; F-1,6-P_2ase, fructose-1,6-bisphosphatase; *p*-CMS, *p*-chloromercuriphenyl sulfonate; 3-PGA, glycerate 3-phosphate.

Characterization of Chloroplasts. Light- and CO_2-dependent O_2 evolution was measured at 25°C and 120 W m^{-2} in a Bachofer O_2 electrode. Chloroplast intactness was measured after Lilley *et al.* (18), and Chl content after Arnon (2).

The stromal volume was determined by measuring the relative uptake by the chloroplasts of ^{14}C-sorbitol and ^3H_2O over 60 s (12). The uptake of ^{14}C-sorbitol reached saturation after 30 to 60 s whereas ^3H_2O uptake required less than 10 s. The addition of ^{14}C-sorbitol to the chloroplasts increased the total sorbitol concentration from 750 to 755 mM, a change unlikely to alter the stromal volume significantly. The chloroplasts were separated from the radioactive incubation solution by centrifugation through 100 μl AP 200 silicon oil (Wacker, Munich) into 20 μl of 7% (v/v) $HClO_4$ for 25 s in a Beckman Microfuge. Radioactivity in the fractions above and below the silicon oil was measured in a scintillation counter following corrections for background counts, counting efficiency and quenching.

PEP Measurements. PEP was measured by monitoring, with a Pye Unicam fluorescence spectrophotometer, the bioluminescence at 549 nm and 20°C produced by a coupled assay containing 250 μl ATP-bioluminescence CLS solution (Boehringer, Mannheim; containing Hepes, luciferase from *Photinus pyralis*, D-luceferin, $MgCl_2$, EDTA, DTT, and AMP), 6.6 mM Hepes-NaOH (pH 7.8), 3.3 mM $MgSO_4$, 0.04 mM ADP, sample, and 30 nkat pyruvate kinase in a volume of 1 ml. As ADP contained approximately 1 to 2% ATP, bioluminescence was observed in the absence of pyruvate kinase. This preluminescence was allowed to decay before the pyruvate kinase was added to the cuvette. The bioluminescence was porportional to the amount of PEP between 0 to 600 pmol PEP ml^{-1} and was not affected by the concentrations of Chl, NaF, or metabolites present in the samples from the different treatments.

Measurements of PEP Uptake. The method was modified from that of Huber and Edwards (16). In a 400 μl Eppendorf tube, 180 μl buffer, containing effectors when required, were layered over 100 μl AP 200 silicon oil below which were 50 μl 750 mM sucrose. The tubes and all solutions were kept at 4°C. After the addition of 20 μl chloroplasts (6–9 μg Chl), the tubes were spun for 25 s at approximately 10,000*g*, and were heated for 10 min at 100°C. The PEP content in the chloroplast-containing fraction was then determined.

Measurements of PEP Formation. Chloroplasts were warmed to 25°C for 5 min before addition to the Eppendorf tubes; if necessary, they were illuminated with 9000 lux provided by a projector lamp. The supernatant and chloroplasts were separated as described above.

Enzyme Activities. Enzymes were measured at 25°C. PEP carboxylase (EC 4.11.31), reversible NAD glyceraldehyde-3-P dehydrogenase (EC 1.2.1.13), and pyruvate, Pi dikinase (EC 2.7.9.1) were assayed after Holtum and Winter (14); enolase (EC 4.2.1.11) and glutamate dehydrogenase (EC 1.4.1.3) after Bergmeyer (3); and catalase (EC 1.11.1.6) after Aebi (1).

RESULTS

Chloroplast Integrity. Chloroplasts were capable of HCO_3^- and light-dependent O_2 evolution rates of between 80 and 115 μmol · mg^{-1} Chl · h^{-1}; the rates were linear with Chl concentration up to at least 30 μg Chl · ml^{-1} in the O_2 electrode. Intactness measurements using ferricyanide indicated an average intactness of about 89%. On the basis of ^{14}C-sorbitol and ^3H_2O uptake, chloroplast stromal volumes under our conditions were 28 ± 5 μl · mg^{-1} Chl.

Chloroplast preparations, before washing, contained 280 nkat · mg^{-1} Chl of the chloroplast-marker enzyme reversible glyceraldehyde-3-P dehydrogenase, 18.7 nkat · mg^{-1} Chl of the cytoplasmic marker PEP carboxylase, 1.2 nkat · mg^{-1} Chl of the mitochondrial marker glutamate dehydrogenase, and 830 nkat · mg^{-1} Chl of the peroxisomal marker catalase. These activities represented 96.5, 8.3, 21.5, and 12.5%, respectively, of the activities present in whole protoplasts. After a single wash, the chloroplasts contained about 14 nkat · mg^{-1} Chl PEP carboxylase activity, this represented about 6.2% of that present in the parent protoplasts. Additional washes resulted in both reductions in PEP carboxylase activity and unacceptable losses of O_2 evolution capacity. A single wash reduced the chloroplast-bound enolase activity of 0.08 nkat · mg^{-1} Chl to less than 1 pkat · mg^{-1} Chl. The presence of 2 mM NaF, which was routinely in our tests, reduced enolase activity even further without substantially affecting O_2 evolution which was inhibited by 7%. The low contamination in our chloroplast preparations of enzymes capable of converting PEP to 3-PGA is illustrated by the observation that when PEP was supplied to chloroplasts in the light, in the absence of CO_2, no O_2 evolution was observed. Furthermore, total levels of PEP supplied to chloroplast preparations in the light and in the dark remained constant.

Uptake of PEP. *Mesembryanthemum* chloroplasts accumulated PEP from the external medium in the dark. A plot of the initial rates of PEP uptake against increasing external PEP concentrations gives a saturation curve expected for facilitated transport (Fig. 1). A double-reciprocal plot of this data indicates a V_{max} (4°C) of 6.46 (±0.05) μmol · mg^{-1} Chl · h^{-1} and an apparent K_{mPEP} of 0.148 mM (Fig. 1, inset).

Effects of Pi and 3-PGA on PEP Uptake. Both Pi and 3-PGA inhibited the initial rates of PEP uptake in a competitive manner (Figs. 2 and 3). The apparent $K_{i\ Pi}$ was 0.19 mM (4°C) and the apparent $K_{i\ 3-PGA}$ was 0.13 mM (4°C). DHAP also inhibited PEP uptake.

Effects of Malate and Pyruvate on PEP Uptake. Neither malate nor pyruvate affected the initial rates of PEP uptake (Fig. 4).

PEP Synthesis in Mesembryanthemum Chloroplasts. Intact chloroplasts synthesized PEP when presented with pyruvate. Half-maximal rates of synthesis were observed at an external pyruvate concentration of 1.3 mM; maximal rates required about 12 mM. This ability was light-dependent (Fig. 5) and was stimulated by 3-PGA or Pi. Maximal rates of PEP synthesis required about 0.8

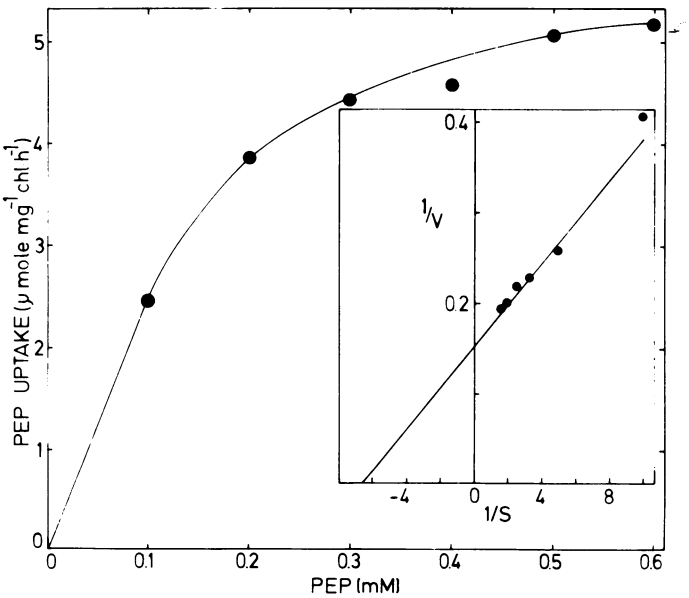

FIG. 1. Influence of external PEP concentrations on the initial rates of PEP uptake by chloroplasts from CAM-*Mesembryanthemum* in the dark. Incubation time was 5 s. The inset is a double reciprocal plot of the data; the *x*-axis intercept indicates an apparent $K_{m\ PEP}$ of 0.15 mM, the *y*-axis intercept indicates a V_{max} of 6.46 μmol · mg^{-1} Chl · h^{-1}.

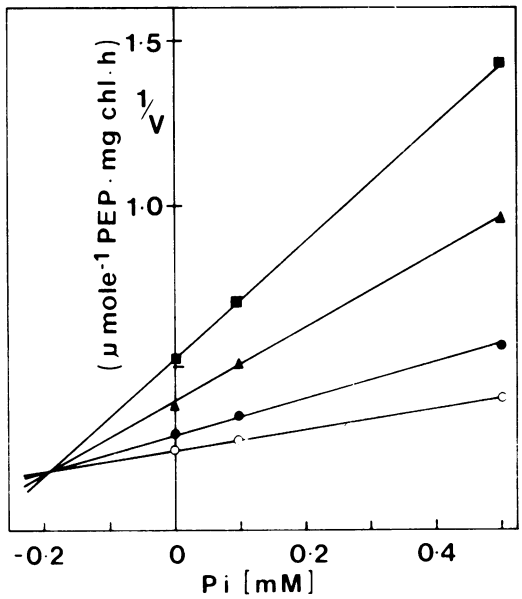

FIG. 2. The effect of Pi on the initial rates of PEP uptake in the dark; 0.7 mM PEP (○), 0.5 mM PEP (▲), 0.3 mM PEP (●), and 0.2 mM PEP (■). The common intersect indicates competitive inhibition, a line subtending the intersect to the *y*-axis gives the apparent $K_{i\ Pi}$ of 0.19 mM.

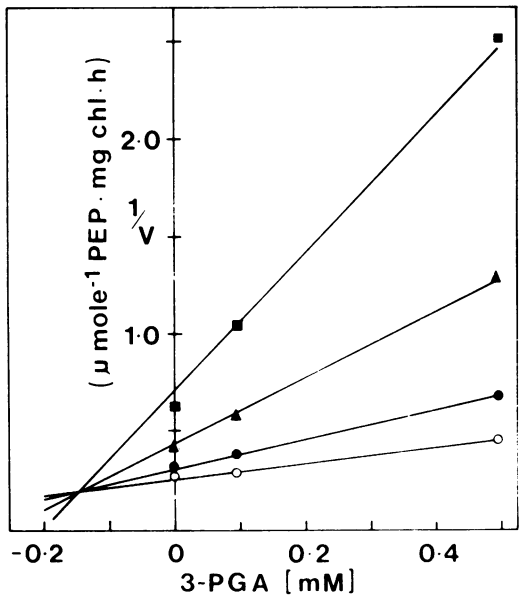

FIG. 3. The effect of 3-PGA on the initial rates of PEP uptake in the dark; 0.7 mM PEP (■), 0.5 mM PEP (▲), 0.3 mM PEP (●), and 0.2 mM PEP (○). The apparent $K_{i\ 3-PGA}$ is 0.14 mM.

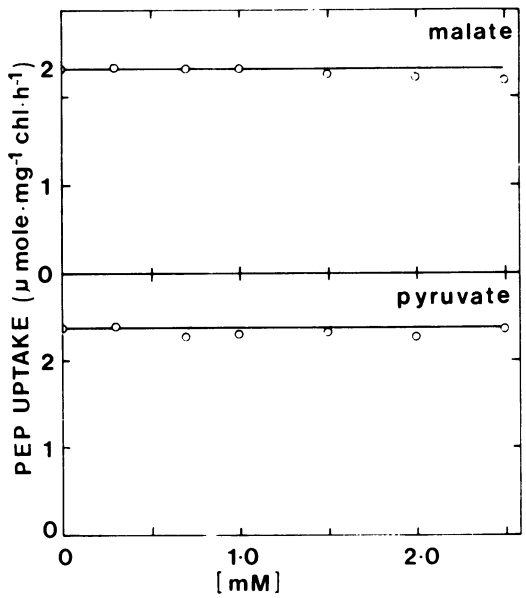

FIG. 4. The influence of pyruvate and malate on the initial rates of PEP uptake in the dark. External PEP concentration was 0.1 mM, incubation time was 5 s.

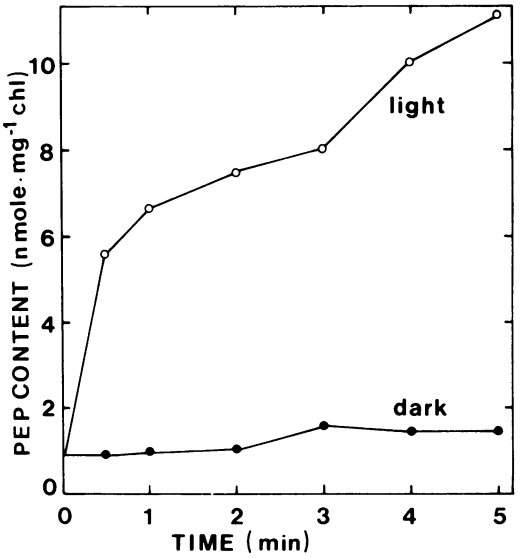

FIG. 5. The influence of illumination on the pyruvate-dependent synthesis of PEP by CAM-*Mesembryanthemum* chloroplasts. Incubation medium contained, in addition to buffer, 2 mM pyruvate, 5 mM HCO_3^-, 2 mM NaF, and chloroplasts (6–9 μg Chl).

mM Pi; half-maximal rates required around 0.05 mM Pi. In the presence of 2 mM pyruvate and 1 mM 3-PGA, the PEP concentration in the chloroplasts was approximately 0.12 mM after 10 to 300 s in the light (Fig. 6) during which period the concentration in the external medium increased from 0.13 to 1.3 μM. PEP synthesis was unaffected by the presence of 1 mM malate in the external medium but ceased completely in the presence of 5 μM of the uncoupler CCCP.

Inhibitor Studies. Attempts to assess the effects of the known Pi-translocator inhibitors *p*-CMS, DIDS, and pyridoxal 5′-phosphate on PEP uptake were inconclusive due to the interactions of these compounds with the bioluminescence test system.

DISCUSSION

The Transport of PEP. PEP enters chloroplasts from CAM-*M. crystallinum* at appreciable rates in the dark. The observation that the initial rates of uptake exhibit saturation kinetics with increasing external PEP concentrations suggests that the uptake of PEP is not a simple diffusive process. The affinity for PEP, apparent K_m = 0.15 mM at 4°C, is an order of magnitude greater than that observed for PEP transport across spinach chloroplasts ($K_{i\ PEP}$ = 4.7 mM at 4°C [10]), and similar to or greater than those observed for transport across C_4 mesophyll chloroplasts (for *Zea mays* $K_{i\ PEP}$ = 1.6 mM at 15°C [4]; for *Digitaria sanguinalis* $K_{i\ PEP}$ = 0.43 mM at 4°C [16]). O_2 evolution studies indicate lower affinities for PEP uptake, $K_{i\ PEP}$ = 21 mM for

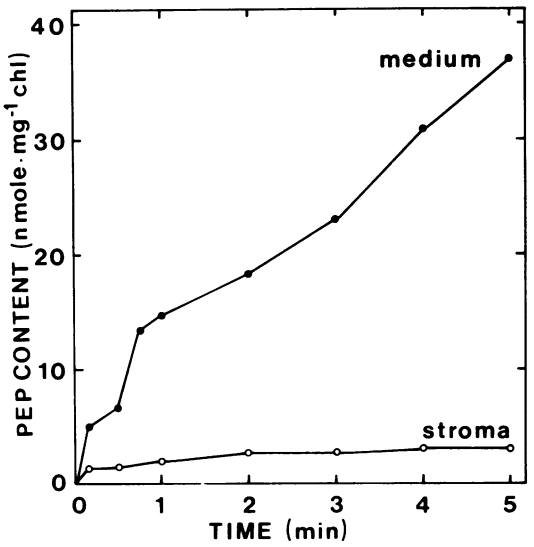

FIG. 6. PEP contents of the stroma and external medium in the light. The 200 μl medium contained, in addition to buffer, 2 mM pyruvate, 1 mM 3-PGA, 5 mM HCO_3^-, 2 mM NaF, and chloroplasts (0.19 μl stromal volume).

spinach at 25°C, and $K_{i\ PEP}$ = 3.0 mM for *Digitaria* mesophyll chloroplasts at 30°C (25). The V_{max} for PEP transport by *Mesembryanthemum* chloroplasts of 6.46 μmol · mg^{-1} Chl · h^{-1} at 4°C is consistent with a rate of around 30 μmol · mg^{-1} Chl · h^{-1} at 25°C, such a rate would be similar to the maximum rates of malate loss observed in *Mesembryanthemum*.

In common with PEP transport across C_4 chloroplasts, the transport of PEP into *Mesembryanthemum* chloroplasts exhibits characteristcs similar to those expected for transport across a Pi translocator. The uptake of PEP is inhibited competitively by both Pi and 3-PGA. The K_i values for Pi and 3-PGA at 4°C, of 0.19 and 0.13 mM, respectively, are similar to the K_m values of these compounds for the Pi translocators from spinach ($K_{m\ Pi}$ = 0.2 mM, $K_{i\ 3-PGA}$ = 0.15 mM also measured at 4°C [10]), *Z. mays* ($K_{i\ Pi}$ = 0.6 mM, $K_{i\ 3-PGA}$ = 0.35 mM measured at 15°C [5]), and *Digitaria* ($K_{m\ 3-PGA}$ = 0.13 mM, estimated at 30°C from O_2 evolution measurements [25]).

The inability of malate or pyruvate, both tested at concentrations of up to 2.5 mM, to either stimulate or retard the initial rate of PEP uptake by *Mesembryanthemum* chloroplasts in the dark is a further indication of the relative specificity of the carrier system for PEP, Pi, 3-PGA, and triose-P. In mesophyll chloroplasts from *Digitaria*, pyruvate has been reported to stimulate the uptake of ^{32}Pi and to reduce the steady state concentrations of ^{32}Pi in the stroma (16).

The Production of PEP from Pyruvate. In the presence of external pyruvate, CAM-*Mesembryanthemum* chloroplasts synthesized and exported PEP. This PEP formation was relatively insensitive to malate; at a subsaturating pyruvate concentration of 2 mM, PEP synthesis was inhibited only 3.3% by 1 mM malate. The synthesis of PEP was light dependent, as one might expect if pyruvate, Pi dikinase was involved in the catalysis, and was minimal in the absence of either 3-PGA or Pi. The presence of either, or both, of these compounds promoted the export of PEP into the surrounding medium. The formation of PEP in the chloroplasts presumably sequesters chloroplastic Pi and thus, in the absence of an external Pi source or a means of recycling this Pi within the chloroplast, further PEP formation cannot occur. In the presence of 5 mM $HClO_3^-$, 2 mM pyruvate and 1 mM 3-PGA at 25°C, the stromal PEP concentration was relatively stable at 0.12 mM which is in the region of the $K_{m\ PEP}$ of 0.15 mM (determined at 4°C).

Although the V_{max} of the PEP transport system is probably adequate to handle the rates of malate decarboxylation observed *in vivo*, the rates of PEP synthesis observed in the light when the chloroplasts were presented with pyruvate were well below the rates required. A number of factors could be responsible for this shortfall. First, the conditions, in particular an external medium pH of 7.8, may have not been optimal for pyruvate uptake by CAM-*Mesembryanthemum* chloroplasts in the light. For maize mesophyll chloroplasts, however, the optimum pH for pyruvate uptake is about 7.8 (20). Second, the activity of pyruvate, Pi dikinase measured in our chloroplast preparations, about 1 nkat · mg^{-1} Chl, was considerably lower than the 30 nkat · mg^{-1} Chl observed in whole leaf extracts. This low activity may be a result of cold inactivation of the enzyme during storage of the chloroplasts on ice. Nevertheless, as the maximum rates of PEP synthesis observed were only one-quarter of the activity of pyruvate, Pi dikinase in the same chloroplasts, it is possible that the activity of pyruvate, Pi dikinase was not the single rate-limiting step for the production of PEP from pyruvate in our experimental system.

The mechanism by which pyruvate enters *Mesembryanthemum* chloroplasts has not been studied but it is likely that, in common with the system in chloroplasts from C_4 mesophyll cells, uptake is electrogenic and light stimulated (11, 15, 20). In the light, the V_{max} for pyruvate uptake by chloroplasts from C_4 mesophyll cells is in the order of 20 to 40 μmol · mg^{-1} Chl · h^{-1} (11, 20); K_m values range from 0.2 to 0.4 mM for *Panicum miliaceum* (20) to 0.6 to 1.0 mM for *Digitaria* (15), and 0.8 to 0.9 mM for maize (11). In the dark, the V_{max} for pyruvate transport in the above species is always below 2 μmol · mg^{-1} Chl · h^{-1}. Chloroplasts from C_3 species and from C_4 bundle-sheath cells exhibit lower capacities and affinities; for pea K_m = 0.33 mM and V_{max} = 6.5 μmol · mg^{-1} Chl · h^{-1} (24), for wheat K_m = 0.5 mM and V_{max} = 1.8 μmol · mg^{-1} Chl · h^{-1}, and for the bundle sheath chloroplasts of *Panicum* K_m = 0.5 to 0.7 mM and V_{max} = 2.1 to 4.5 μmol · mg^{-1} Chl · h^{-1} (20). If pyruvate transport in CAM cells is analogous to that in C_4 mesophyll cells, then the puzzling requirement by CAM plants for micronutrient levels of Na^+ could be explained by the requirement of pyruvate transport for Na^+ (21; S Boag, personal communication).

It is not yet known whether chloroplasts from C_3-*Mesembryanthemum* also have a capacity for PEP or pyruvate transport. It is difficult to predict a role for such a capacity since the activities of pyruvate, Pi dikinase, PEP carboxylase, and NADP malic enzyme in leaves of these plants are extremely low (14, 28). The possibility that the capacity for the transport of PEP and pyruvate across the chloroplast envelope develops during the induction of CAM is currently under investigation, as are the optimal conditions for the production of PEP from pyruvate in the light.

The observation that chloroplasts from CAM-*Mesembryanthemum* can import pyruvate and can synthesize and export PEP adds credence to the postulate that, in CAM plants in general, the 3-carbon products of malate decarboxylation in the light are converted to storage carbohydrates by gluconeogenic processes (7, 14, 22). Since *Mesembryanthemum* transfers much of the carbon in malate to the chloroplasts where it is stored as starch, it follows that the carbon in the intermediate PEP, which is formed in the chloroplast and is exported into the cytoplasm during deacidification, must eventually reenter the chloroplast. It is most likely that PEP is exported from the chloroplasts in exchange for 3-PGA or triose-P (Fig. 7). No data are available as to the cytosolic concentrations of these intermediates during deacidification, nor for that matter during any other part of the day-night cycle in CAM plants. Some circumstantial evidence does exist to suggest that they may be appreciable. In comparison to the rates of malate decarboxylation, and thus the rates of

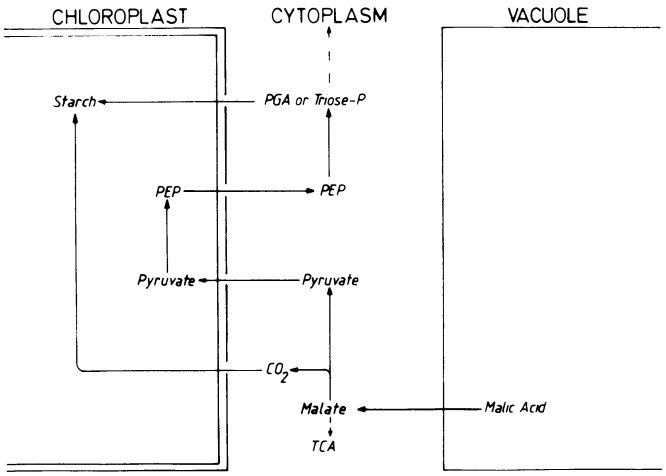

FIG. 7. Proposed principal pathway by which carbon from malate is transferred to storage carbohydrate during the light. The cytoplasmic energy requirements would be fulfilled if one malate in nine is metabolized in the mitochondria (7).

production of 3-PGA and possibly triose-P in the cytoplasm, the activities of cytosolic F-1,6-P_2ase and PPi-dependent fructose-6-phosphate-1-phosphotransferase are low in CAM-*Mesembryanthemum* (8). Furthermore, the cytosolic F-1,6-P_2ase appears to exhibit a low affinity for F-1,6-P_2 (17), rather like the C_4 enzyme (27). High cytoplasmic concentrations of 3-PGA and triose-P would also favor the low levels of F-2,6-P_2, of about 25 pmol · mg^{-1} Chl, which have been observed in this, and some other, CAM tissues during deacidification (8, 9, 17).

Although the data presented here and that available for C_4 plants indicate that PEP is transported by a system with all the appearances of a modified Pi-translocator, the data are insufficient to allow a conclusion as to whether the chloroplast envelope contains a single carrier population with a high affinity for PEP, or whether a population with a low affinity for PEP (the C_3 type) is also present. This possibility is being pursued.

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