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Intracellular Localization of Some Key Enzymes of Crassulacean Acid Metabolism in *Sedum praealtum* [1]

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ABSTRACT

The intracellular locations of six key enzymes of Crassulacean acid metabolism were determined using enzymically isolated mesophyll protoplasts of *Sedum praealtum* D.C. Data from isopycnic sucrose density gradient centrifugation established the chloroplastic location of pyruvate Pi dikinase, the mitochondrial location of NAD-linked malic enzyme, and exclusively nonparticulate (not associated with chloroplasts, peroxisomes, or mitochondria) locations of phosphoenolpyruvate carboxylase, NADP-linked malic enzyme, enolase, and phosphoglycerate mutase. The consequences of this enzyme distribution with respect to compartmentalization of the pathway and the transport of metabolites in Crassulacean acid metabolism are discussed.

CAM is one of the three major variations in the over-all pathway of photosynthetic CO_2 assimilation, along with the so-called C_3 and C_4 pathways. CAM is probably the least well understood of the three. The path of carbon is fairly well established over most of the pathway, but the compartmentation of the pathway has not been established (20). Most proposed schemes for flow-through compartments make use of information from a few inconclusive reports of intracellular locations of some enzymes (7, 18) combined with assumptions based on enzyme distribution within cells of C_4 plants. Well documented information on the intracellular distribution of enzymes in CAM is needed in order to investigate regulation at the enzyme level. For this reason, this investigation was undertaken.

Two major problems inherent in such efforts with CAM plants are the large amount of phenolic substances normally found in succulent leaves and the high concentrations of organic acids associated with CAM. We have utilized enzymically isolated protoplasts to be able to protect organelles and enzymes against these two problems more readily than would be possible when homogenizing pieces of leaf tissue containing many cells each. It has also been reported (22) that a larger proportion of organelles are maintained intact by the relatively gentle homogenization procedure of protoplast rupture as compared to mechanical tissue homogenization. Through the use of *Sedum praealtum* protoplasts, the intracellular distribution of some key enzymes in the pathway of CAM is demonstrated.

MATERIALS AND METHODS

Chemicals. Cellulysin was obtained from Calbiochem. The dextran used was 15,000 to 20,000 mol wt fraction from U. S. Biochemical Corp., Cleveland, Ohio. Methylcellulose was obtained from Fisher Scientific, Pittsburgh. PEP[2] carboxylase was obtained from Worthington Biochemical Corp., Freehold, N. J. All other biochemicals were obtained from Sigma.

Plant Material. *S. praealtum* D.C. plants were grown under a 12-h photoperiod and a 30/15 C day/night temperature regime. The quantum flux density at soil level was about 600 μE m^{-2} s^{-1}. The plants were watered approximately every 3rd day and fertilized every 2 weeks. Young but fully expanded leaves were chosen for protoplast isolation (6th–9th leaf from apex). Isolations were begun in early morning just prior to the time lights would normally come on in the growth chamber. The temperature was kept at 30 C on the night preceding isolation to decrease both starch and acid content of the leaves.

Protoplast Isolation. The leaves were cut perpendicular to the leaf axis in slices about 1 mm thick with a sharp razor blade. The leaf slices were then washed once, vacuum-infiltrated, and again washed, all with 0.3 M sorbitol. The leaf slices were then incubated in an isolation medium containing 1.5% (w/v) Cellulysin and 0.3 M sorbitol at a pH of 5.0. After digestion at room temperature for 3 h, the mixture was swirled manually and passed through a nylon net with 210-μm apertures followed by another net with 155-μm apertures (Tetko, Inc., Elmsford, N. Y.) to remove undigested tissue, epidermal strips, and vascular strands. The protoplasts were allowed to settle out of the filtrate (about 15 min) over ice, and the loose pellet formed was used for protoplast purification.

Protoplast purification was accomplished by flotation on 10% (w/v) dextran. The loose protoplast pellet collected after settling was resuspended in a solution containing 20% (w/v) dextran, 0.3 M sucrose, 1% (w/v) PVP-40, 1 mM $CaCl_2$, and 50 mM Tris-HCl (pH 8.4). Layered on top of this was about 1 ml of a solution of the same composition except that the dextran concentration was 10% (w/v). A third layer of about 1 ml of the sorbitol medium (same as the breaking medium described below) was then added. This gradient was centrifuged in a refrigerated clinical centrifuge at 240*g* for 5 min. The intact protoplasts, which floated up to the interface between the 10% dextran solution and the sorbitol medium, were collected with a Pasteur pipette and resuspended in the breaking medium.

Differential Centrifugations. The protoplasts in a breaking medium containing 0.15 M sorbitol, 1% (w/v) PVP-40, 0.2% (w/v) methylcellulose, 5 mM EDTA, 5 mM DTT, and 250 mM Tris-HCl (pH 8.4) were ruptured by three passes through a 44-μm nylon net which was attached to a 2.5-ml syringe. Ruptured protoplast preparations were centrifuged for 2 min at 240*g* in a refrigerated clinical centrifuge. The supernatant was separated from the pellet and the pellet resuspended in the sorbitol medium.

Density Gradient Centrifugation. Sucrose density gradient centrifugations were run on protoplast extracts prepared by the same

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[2] Abbreviations: G3P: glyceraldehyde 3-phosphate; PEP: phosphoenolpyruvate; PGA: phosphoglycerate; RuBP: ribulose 1,5-bisphosphate.

procedure as for the differential centrifugations. The ruptured protoplast preparation (2.5 ml) was layered on top of a sucrose density gradient composed of a 4-ml cushion of 70% (w/w) sucrose, a 25-ml linear gradient from 60 to 40% (w/w) sucrose, and a 7-ml linear gradient from 40 to 10% (w/w) sucrose, all dissolved in 50 mM Tris-HCl, 1% (w/v) PVP-40, 5 mM DTT, and 5 mM EDTA (pH 8.0). The gradient was centrifuged at 25,000 rpm for 3.5 h at 4 C in a Beckman SW 27 rotor. At the end of the centrifugation, 1.2-ml fractions were collected using an ISCO model 640 density gradient fractionator while simultaneously monitoring the A_{280}.

Enzyme Assays. Pyruvate Pi dikinase (EC 2.7.9.1) was assayed spectrophotometrically as ATP-dependent activity essentially by the method of Hatch and Slack (10). Other enzymes assayed and reference to the method used were succinate dehydrogenase (EC 1.3.99.1) (4), RuBP carboxylase (EC 4.1.1.39) (16), NADP-triose-P dehydrogenase (EC 1.2.1.13) (15), PEP carboxylase (EC 4.1.1.31), and NADP-malic enzyme (EC 1.1.1.40) (24), NAD-malic enzyme (EC 1.1.1.38) (9), NAD-malate dehydrogenase (EC 1.1.1.37) (6), catalase (EC 1.11.1.6) (17), NAD-isocitrate dehydrogenase (EC 1.1.1.41) (3), hydroxypyruvate reductase (EC 1.1.1.29) (26), enolase (EC 4.2.1.11), and PGA mutase (EC 2.7.5.3) (14). Aliquots of fractions used for enzyme assays were routinely incubated for about 3 min in an equal volume of 0.1% (v/v) Triton X-100 to eliminate membrane integrity of organelles.

Other Analytical Methods. Chl was determined in 96% (v/v) ethanol by the method of Wintermans and De Mots (25). Sucrose concentration in each fraction of the gradient was determined by refractometry.

RESULTS

A typical preparation of purified mesophyll protoplasts from *S. praealtum* is illustrated in Figure 1. A similar preparation after three passes through a 44-μm nylon net is shown in Figure 2. If PVP was omitted from the breaking medium, chloroplasts at this stage appeared badly clumped and most of the activity of both mitochondrial and peroxisomal marker enzymes sedimented with the chloroplasts. Divalent cations (Mg^{2+}, Ca^{2+}), Pi, or a reduction in buffer concentration each also resulted in similar but less extreme co-sedimentation of marker enzymes for all three organelles. Distribution of some enzymes after a 240*g* centrifugation of extracts prepared in the optimal breaking medium is illustrated in Table I. In this experiment, Chl and NADP-triose-P dehydrogenase were used as chloroplast markers, hydroxypyruvate reductase and catalase were used as peroxisomal markers, and NADH-malate dehydrogenase was used as a marker for cytoplasm, mitochondria, and peroxisomes. NADP-triose-P dehydrogenase activity was found in the chloroplast pellet in approximately equal proportion to that of the Chl indicating sedimented chloroplasts are intact. Little activity of both PEP carboxylase and NADP-malic enzyme appeared in the chloroplast pellet which suggests that these enzymes are nonchloroplastic. The distribution of these two enzymes was consistent through all of several separations of chloroplasts by differential centrifugation.

Data from one of three consistent sucrose density gradient centrifugations are presented in Figure 3. The activity of peroxisomal marker enzymes (hydroxypyruvate reductase and catalase) which entered the gradient was primarily located in fractions 18 to 20 at a density of 1.24 to 1.26 g cm^{-3} (Fig. 3b). The mitochondrial marker enzymes, succinate dehydrogenase, and NAD-isocitrate dehydrogenase had a sharp peak of activity in fraction 11 at a density of about 1.20 g cm^{-3} (Fig. 3d). The chloroplast markers, RuBP carboxylase, NADP-triose-P dehydrogenase, and Chl had a peak of activity in fraction 28, which was the top of the cushion of 70% sucrose giving an equilibrium density between 1.31 and 1.36 g cm^{-3} (Fig. 3, a and c). Chl was found in an additional peak in fraction 9 at a density of 1.17 g cm^{-3} (Fig. 3a), but none of the assayed enzymes was associated to any extent with this peak. In addition, NAD-malate dehydrogenase activity was found to peak in the same fractions as the mitochondrial marker enzymes and, to a minor extent, with the peroxisomal and chloroplast markers. Among the enzymes of primary interest for their intracellular localization, PEP carboxylase, NADP-malic enzyme, enolase, and PGA mutase were found only at the top of the gradient (Fig. 3, e and f) and not associated with peroxisomal, mitochondrial, or chloroplast marker enzymes in the gradient. Pyruvate Pi dikinase activity in the gradient was found primarily in fraction 28 with

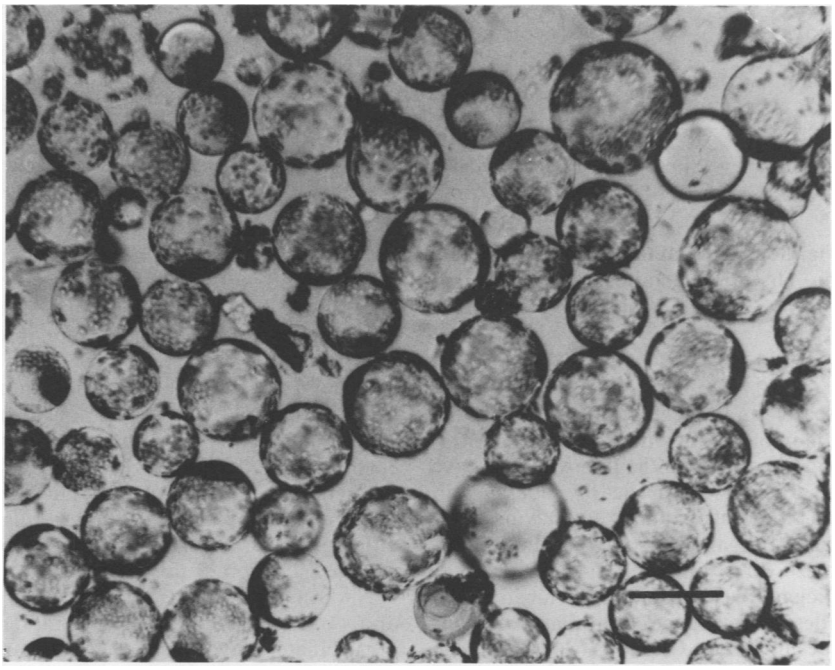

FIG. 1. Light micrograph of enzymically isolated mesophyll protoplasts of *S. praealtum* after purification. Bar indicates 100 μm.

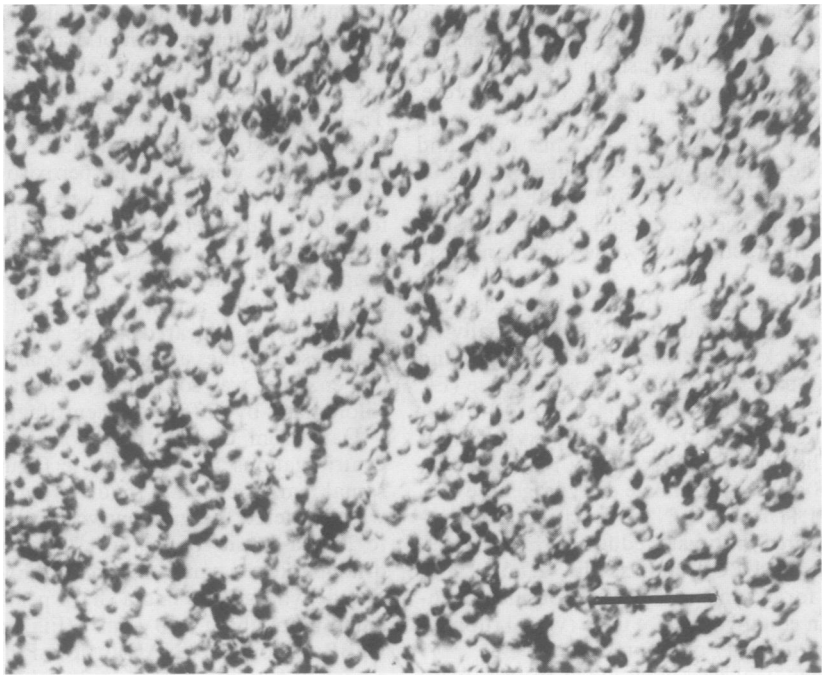

FIG. 2. Light micrograph of an extract prepared from mesophyll protoplasts of *S. praealtum*. Bar indicates 50 μm.

TABLE I. Distribution of enzyme activities after sedimenting chloroplasts by centrifugation of *Sedum praealtum* protoplast extracts

Measurement	Total Activity[1] in Protoplast Extract	Activity in 240 g Pellet	Activity in 240 g Supernatant
	μmoles·mg chl^{-1}·hr^{-1}	distribution (%)	
chlorophyll		51	49
NADP-triose-P dehydrogenase	591	56	44
hydroxypyruvate reductase	450	20	80
catalase	42,000	22	78
NAD-malate dehydrogenase	17,150	7	93
NADP-malic enzyme	144	2	98
PEP carboxylase	549	11	89

[1] Total activity was calculated from the sum of the activities in the pellet and the supernatant.

the chloroplast markers (Fig. 3e). NAD-malic enzyme showed a peak of activity in fraction 11 with the mitochondrial marker enzymes (Fig. 3f).

DISCUSSION

A major limitation in research on the regulation of CAM at the cellular level has been the lack of good evidence as to the intracellular localization of enzymes involved in the pathway. We have prepared a sucrose density gradient which clearly separates all major organelles as demonstrated using marker enzymes. Although considerable breakage of organelles occurred, the proportion recovered (chloroplasts, 20–30%; mitochondria, 25–30%; peroxisomes, 15–20%) was sufficient to establish enzyme localization. The densities of all organelles except intact chloroplasts are consistent with established values in C_3 and C_4 plants (8, 21, 23). The very high density of intact chloroplasts probably reflects the large amount of starch which we observed by iodine staining (unpublished). In this report, data have been presented which demonstrate the distribution of six key enzymes in the CAM pathway.

Pyruvate Pi dikinase activity is clearly associated with intact chloroplasts, with most of the remaining activity at the top of the gradient. At a density of 1.17 g cm^{-3}, Chl was found without associated activities of chloroplast marker enzymes, indicative of broken chloroplasts (23), and considerable activity for both chloroplast marker enzymes was found at the top of the gradient. Therefore, the pyruvate Pi dikinase activity at the top of the gradient was very likely due to release of the enzyme from ruptured chloroplasts. Location of this enzyme in the chloroplast, although never reported for a CAM plant, is consistent with the reported location of this enzyme in C_4 plants (8).

NAD-malic enzyme activity is associated with the mitochondria. Activity at the top of the gradient follows a similar pattern of succinate dehydrogenase activity and probably reflects release of enzymes from broken mitochondria. The lack of activity of NAD-isocitrate dehydrogenase at the top of the gradient may be

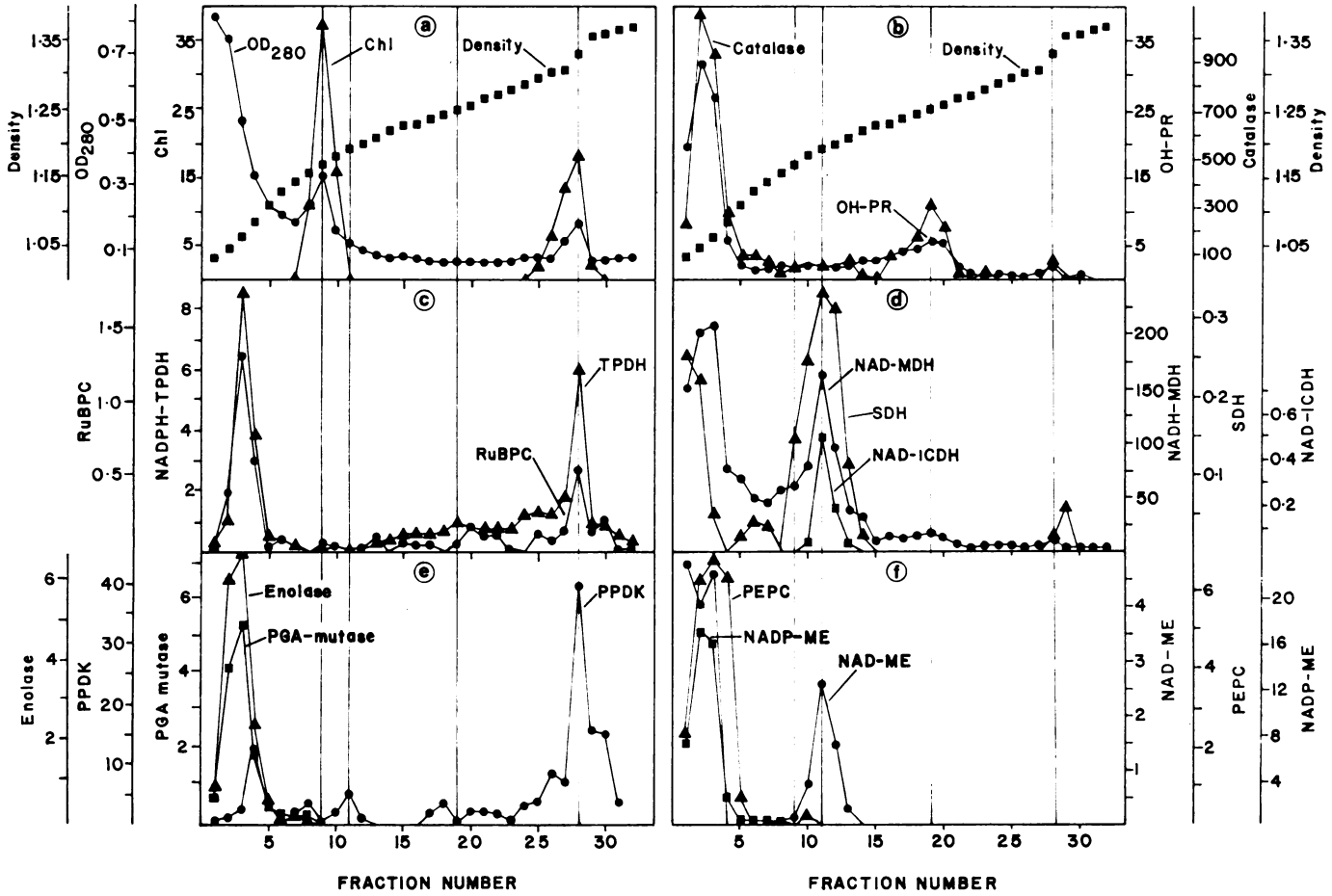

FIG. 3. Isopycnic sucrose density gradient profile of extracts from mesophyll protoplasts of *S. praealtum*. Fractions are numbered beginning with the top of the gradient. All fractions were assayed for each enzyme. Absence of a particular symbol indicates no detectable activity of that enzyme in the fraction. Abbreviations are: a. Chl, chlorophyll; b. OH-PR, hydroxypyruvate reductase; c. RuBPC, ribulose-bisP carboxylase; TPDH, NADP-linked triose-P dehydrogenase; d. NAD-MDH, NAD-linked malate dehydrogenase; SDH, succinate dehydrogenase; NAD-ICDH, NAD-linked isocitrate dehydrogenase; e. PGA mutase, P-glycerate mutase; PPDK, pyruvate Pi dikinase; F. PEPC, P-enolpyruvate carboxylase; NADP-ME, NADP-linked malic enzyme; NAD-ME, NAD-linked malic enzyme. Chl is given as μg fraction^{-1}. Density is given in g cm^{-3}. All enzyme activities are given as μmol substrate converted h^{-1} fraction^{-1}. Recovery of enzyme activities from the gradient as compared to the protoplast extract applied was greater than 90% in all cases except catalase (79%), enolase (79%), and NAD-malic enzyme (85%).

attributed to the extreme lability of this enzyme, since no activity could be found in the protoplast extract either under the assay conditions employed. Location of NAD-malic enzyme primarily in the mitochondria is consistent with data reported by Dittrich (5) on the distribution of this enzyme in *Kalanchoë daigremontiana* and its reported intracellular distribution in C_4 plants (21).

PEP carboxylase, NADP-malic enzyme, enolase, and PGA mutase activities were found only at the top of the gradient and not associated with any organelles. Extrachloroplastic locations of PEP carboxylase and NADP-malic enzyme are both contrary to the chloroplastic location of these two enzymes reported by Mukerji and Ting in *Opuntia ficus-indica* (18) and by Garnier-Dardart in *Bryophyllum* sp. (7). In both of these localization studies, nonaqueously isolated chloroplasts were used which have been demonstrated to contain cytoplasmic contamination often (1). This, combined with the facts that neither report indicates any determination of contamination by cytoplasm or other organelles or what proportion of the total NADP-malic enzyme or PEP carboxylase activity was found in the chloroplast fraction, makes interpretation of their results difficult. Inasmuch as in both studies all enzymes assayed were found to be particulate, this may reflect some nonspecific binding of enzymes similar to problems we encountered under certain conditions. Mukerji and Ting (19) have also reported the identification of three isoenzymes of NADP-malic enzyme in *O. ficus-indica* associated with chloroplasts, mitochondria, and the cytoplasm. It is possible that the reports mentioned above indicate that intracellular distribution of at least some enzymes of the CAM pathway is not the same in all CAM species. Location of PEP carboxylase outside the chloroplast, mitochondrion, and peroxisome is consistent with the intracellular distribution in C_4 plants (8), but an extrachloroplastic location of NADP-malic enzyme is at variance with the reported location of this enzyme in C_4 plants (21). This represents the first well documented demonstration of a difference in intracellular location of an enzyme associated with C_4 dicarboxylic acid metabolism in CAM *versus* C_4 plants.

With this demonstration of the compartmentalization of key enzymes in the CAM pathway, we are able to propose a tentative scheme for intracellular photosynthetic carbon flow in *S. praealtum* (Fig. 4). The pathway for nighttime acidification is illustrated in Figure 4A. Based on enzyme localization, the steps from 3-PGA to oxaloacetic acid are proposed to be located in the cytoplasm. The conversion of oxaloacetic acid to malate in the cytoplasm is also consistent with a major portion of NAD-malate dehydrogenase activity being apparently nonparticulate. The compartmentation of the pathway prior to the PGA mutase step is yet to be demonstrated, based on transport capabilities of chloroplasts from C_3 plants (11), the metabolites from starch breakdown most

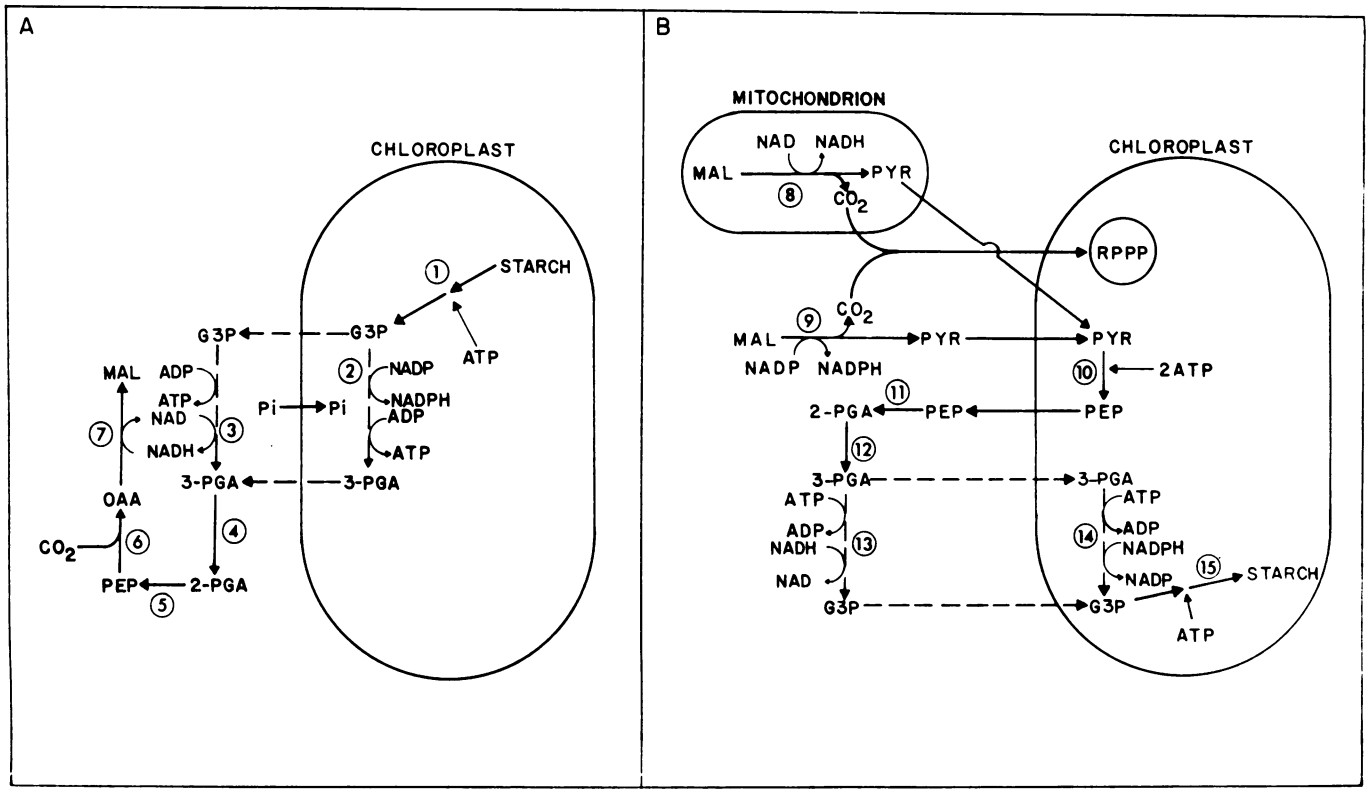

FIG. 4. Proposed schemes for carbon flow in CAM during: A, nighttime acidification; B, daytime decarboxylation and refixation. Malate formed during the night would be stored in the vacuole and released during the day for decarboxylation. Broken lines are used to indicate possible alternate routes of carbon flow. Portions of the pathway illustrated as occurring outside the chloroplast (and mitochondrion in B) are not necessarily occurring in the cytoplasm but are extrachloroplastic, extramitochondrial, and extraperoxisomal. Circled numbers refer to enzymes catalyzing the steps indicated as follows: 1, enzymes for starch degradation to G3P; 2, NADP-triose-P dehydrogenase plus P-glycerate kinase; 3, either irreversible NADP-triose-P dehydrogenase or NAD-triose-P dehydrogenase plus P-glycerate kinase; 4, P-glycerate mutase; 5, enolase; 6, P-enolpyruvate carboxylase; 7, NAD-malate dehydrogenase; 8, NAD-malic enzyme; 9, NADP-malic enzyme; 10, pyruvate Pi dikinase; 11, enolase; 12, P-glycerate mutase; 13, P-glycerate kinase plus NAD-triose-P dehydrogenase; 14, P-glycerate kinase plus NADP-triose-P dehydrogenase; 15, enzymes for starch synthesis from G3P. RPPP: reductive pentose-P pathway.

likely to be transported out of the chloroplast would be either 3-PGA or G3P.

The proposed scheme for carbon flow during the decarboxylation and refixation phase is illustrated in Figure 4B. If pyruvate formed upon decarboxylation of malate by either NAD-malic enzyme or NADP-malic enzyme is to be incorporated into starch, the compartmentation of enzymes catalyzing steps from malate to 2-PGA requires that pyruvate, PEP, and probably either 3-PGA or G3P are transported across the chloroplast envelope. In addition, pyruvate formed from decarboxylation of malate via NAD-malic enzyme would need to be transported out of the mitochondrion.

The proposed transport is not unprecedented. Transport of pyruvate into and PEP out of NADP-malic enzyme-type C_4 mesophyll chloroplasts has been demonstrated (12, 13), and transport of pyruvate out of bundle sheath mitochondria is required for the currently accepted C_4 cycle of NAD-malic enzyme type C_4 plants (21). Verification of the pathway awaits isolation of organelles involved and demonstration of their ability to perform the proposed transport.

Demonstration of enzyme compartmentation in *S. praealtum* brings to light an important area of control, since both PEP carboxylase and NADP-malic enzyme appear to be located in the same compartment and operate in opposing directions. This becomes especially critical if the proposed scheme of carbon flow is correct, since substrates for both enzymes (PEP and malate) would be available in the cytoplasm during both the acidification and the decarboxylation and refixation phases. An important area of investigation in control of the CAM pathway will be regulation of PEP carboxylase and NADP-malic enzyme.

LITERATURE CITED

1. BIRD IF, MJ CORNELIUS, TA DYER, AJ KEYS 1973 The purity of chloroplasts isolated in non-aqueous media. J Exp Bot 24: 211-215
2. BRANDON PC 1967 Temperature features of enzymes affecting Crassulacean acid metabolism. Plant Physiol 42: 977-984
3. COX GF 1969 Isocitrate dehydrogenase (NAD specific) from pea mitochondria. Methods Enzymol 13: 47-51
4. DAVIS B, MJ MERRETT 1973 Malate dehydrogenase isoenzymes in division synchronized cultures of *Euglena*. Plant Physiol 51: 1127-1132
5. DITTRICH P 1976 Nicotinamide adenine dinucleotide-specific "malic" enzyme in *Kalanchoë daigremontiana* and other plants exhibiting Crassulacean acid metabolism. Plant Physiol 57: 310-314
6. EDWARDS GE, M GUTIERREZ 1972 Metabolic activities in extracts of mesophyll and bundle sheath cells of *Panicum mileaceum* (L.) in relation to the C_4 dicarboxylic acid pathway of photosynthesis. Plant Physiol 50: 728-732
7. GARNIER-DARDART J 1965 Activities enzymatiques des chloroplastes isoles de feuilled de *Bryophyllum daigremontianum* Berger: oxydation des hexoses, formation et degradation d'acide malique. Physiol Vég 3: 215-217
8. GUTIERREZ M, SC HUBER, SB KU, R KANAI, GE EDWARDS 1974 Intracellular localization of carbon metabolism in mesophyll cells of C_4 plants. *In* M Avron, ed, Proc Third Int Congress on Photosynthesis, Vol 2. Elsevier, Amsterdam, pp 1219-1230
9. HATCH MD, T KAGAWA 1974 NAD malic enzyme in leaves with C_4-pathway photosynthesis and its role in C_4 acid decarboxylation. Arch Biochem Biophys 160: 346-349
10. HATCH MD, CR SLACK 1975 Pyruvate, Pi dikinase from leaves. Methods Enzymol 42: 212-219
11. HELDT HW 1976 Metabolite transport in intact spinach chloroplasts. *In* J Barber, ed, The Intact Chloroplast. Elsevier, Amsterdam, pp 215-234
12. HUBER SC, GE EDWARDS 1977 Transport in C_4 mesophyll chloroplasts. Characterization of the pyruvate carrier. Biochim Biophys Acta 462: 583-602
13. HUBER SC, GE EDWARDS 1977 Transport in C_4 mesophyll chloroplasts. Evidence for an exchange of inorganic phosphate and phosphoenol pyruvate. Biochim Biophys Acta 462: 603-612

14. KU SB, GE EDWARDS 1975 Photosynthesis in mesophyll protoplasts and bundle sheath cells of various types of C_4 plants. IV. Enzymes of respiratory metabolism and energy utilizing enzymes of photosynthetic pathways. Z Pflanzenphysiol 77: 16–32
15. LATZKO E, M GIBBS 1969 Enzyme activities of the carbon reduction cycle in some photosynthetic organisms. Plant Physiol 44: 295–300
16. LORIMER GH, MR BADGER, TJ ANDREWS 1977 D-Ribulose-1,5-bisphosphate carboxylase-oxygenase. Improved methods for the activation and assay of catalytic activities. Anal Biochem 78: 66–75
17. MALHY AC, B CHANCE 1954 The assay of catalase and peroxidase. Methods Biochem Anal 1: 357–424
18. MUKERJI SK, IP TING 1968 Intracellular localization of CO_2 metabolism enzymes in cactus phylloclades. Phytochemistry 7: 903–911
19. MUKERJI SK, IP TING 1968 Malate dehydrogenase (decarboxylating) isoenzymes of *Opuntia* stem tissue. Biochim Biophys Acta 167: 239–249
20. OSMOND CB 1978 Crassulacean acid metabolism: a curiosity in context. Annu Rev Plant Physiol 29: 379–414
21. RATHNAM CKM, GE EDWARDS 1975 Intracellular localization of certain photosynthetic enzymes in bundle sheath cells of plants possessing the C_4 pathway of photosynthesis. Arch Biochem Biophys 171: 214–225
22. RATHNAM CKM, GE EDWARDS 1976 Protoplasts as a tool for isolating functional chloroplasts from leaves. Plant Cell Physiol 17: 177–186
23. ROCHA V, IP TING 1970 Preparation of cellular plant organelles from spinach leaves. Arch Biochem Biophys 140: 398–407
24. SPALDING MH, GE EDWARDS 1978 Photosynthesis in enzymatically isolated leaf cells from the CAM plant *Sedum telephium* L. Planta 141: 59–63
25. WINTERMANS JFGM, A DE MOTS 1965 Spectrophotometric characteristics of chlorophyll and their pheophytins in ethanol. Biochim Biophys Acta 109: 448–453
26. ZELITCH I 1955 The isolation and action of crystalline glyoxylic acid reductase from tobacco leaves. J Biol Chem 216: 553–575