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# Intracellular Localization of Enzymes of Carbon Metabolism in *Mesembryanthemum crystallinum* Exhibiting $C_3$ Photosynthetic Characteristics or Performing Crassulacean Acid Metabolism[1]

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KLAUS WINTER, JOYCE G. FOSTER, GERALD E. EDWARDS[2], AND JOSEPH A. M. HOLTUM
*Department of Horticulture (K. W., J. G. F., G. E. E.) and Department of Chemistry (J. A. M. H.), University of Wisconsin, Madison, Wisconsin 53706*

## ABSTRACT

***Mesembryanthemum crystallinum*, a halophilic, inducible Crassulacean acid metabolism (CAM) species, was grown at NaCl concentrations of 20 and 400 millimolar in the rooting medium. Plants from the low salinity treatment showed exclusively $C_3$-photosynthetic net $CO_2$ fixation, whereas plants exposed to the high salinity level exhibited net $CO_2$ dark fixation involving CAM. Mesophyll protoplasts, isolated from both tissues, were gently ruptured, and the intracellular localization of enzymes was studied following differential centrifugation and Percoll density gradient centrifugation of protoplast extracts. Both centrifugation techniques resulted in the separation of intact chloroplasts, with up to 90% yield, from other organelles and the nonparticulate fraction of cells. Enzymes were identified by determination of activity and by sodium dodecyl sulfate gel electrophoresis of enzyme protein.**

**Experiments established the extraorganellar (cytoplasmic) location of phosphoenolpyruvate carboxylase, enolase, phosphoglyceromutase, and NADP-malic enzyme; the mitochondrial location of NAD-malic enzyme; and the chloroplastic location of pyruvate, Pi dikinase. NAD-glyceraldehyde-3-phosphate dehydrogenase, phosphohexose isomerase, and phosphoglycerate kinase were associated with both cytoplasm and chloroplasts. NADP-dependent malate dehydrogenase activity was found in both the chloroplastic and extrachloroplastic fractions; the activity in the chloroplast showed an optimum at pH 8.0 and was dependent upon preincubation of enzyme with dithiothreitol. The extrachloroplastic activity showed an optimum at pH 6.5 and was independent of pretreatment with dithiothreitol. Protoplast extracts of *M. crystallinum* performing CAM exhibited higher activities (expressed per mg chlorophyll per min) of phosphoenolpyruvate carboxylase, pyruvate,Pi dikinase, NADP-malic enzyme, NAD-malic enzyme, NADP-malate dehydrogenase, enolase, phosphoglyceromutase, NAD-glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase, and phosphohexose isomerase than protoplast extracts from *M. crystallinum* not exhibiting CAM. The increase in total activity of the latter three enzymes following exposure of plants to 400 millimolar NaCl and the development of CAM was due to specific increases in the levels of activity in the cytoplasm.**

Intracellular localization of enzymes from CAM plants has been the focus of several recent research efforts, but conflicting results have been obtained, and completely opposing schemes on the flow of carbon during CAM have been proposed. One major point of discrepancy concerns the compartmentation of PEP[3] carboxylase which has been considered as either a chloroplastic (20) or a cytoplasmic enzyme (4, 12, 13, 21). To some extent, these conflicting results are a consequence of the generally lamented difficulties in isolation of intact organelles from CAM tissues. For example, the methods and plant material used for localization of enzymes in the investigations by Schnarrenberger *et al.* (20) and Spalding *et al.* (21) resulted in breakage of 50 to 80% of all chloroplasts.

In the paper presented here, the intracellular distribution of 16 enzymes was studied in the halophilic, inducible CAM plant *Mesembryanthemum crystallinum* (26). Leaves of *M. crystallinum* are apparently low in phenolic compounds, and high extractable activities of several enzymes have been reported for this species (9). Experimental plants were manipulated to show either exclusively $C_3$-photosynthetic net $CO_2$ fixation during the light (growth at low salinity levels in the rooting medium) or to exhibit nocturnal net $CO_2$ uptake involving CAM (growth at high salinity levels). Mesophyll extracts with almost all chloroplasts intact were routinely obtained from plants of both salinity treatments by gentle rupture of enzymically isolated protoplasts. Two techniques, differential centrifugation and Percoll density gradient centrifugation, were used to study the intracellular localization of enzymes. Chloroplast integrity was preserved during these procedures, and the two methods gave identical results.

## MATERIALS AND METHODS

**Growth of Plants.** The *M. crystallinum* L. plants used in the experiments were about 8 weeks old. One set was cultivated in soil, and plants were watered from the 6th to the 8th week, at 2-day intervals, with modified half-strength Johnson nutrient solution (24) containing 20 mM NaCl. These plants did not show nocturnal accumulation of malic acid in the leaf tissue and are referred to as $C_3$-*M. crystallinum*. The second set of plants was grown in half-strength Johnson nutrient solution containing 400 mM NaCl as described earlier (25). These plants performed pronounced CAM and are designated CAM-*M. crystallinum*. Plants were maintained in a growth cabinet under a 12-h light (25°C; 40–50% RH)/12-h dark (15°C; 70% RH) cycle. Irradiance was 200 $\mu E\ m^{-2}\ s^{-1}$ for the first 3 h of the light period, 430 $\mu E\ m^{-2}\ s^{-1}$ for the next 6 h, and 200 $\mu E\ m^{-2}\ s^{-1}$ for the last 3 h of the light period. The third foliar leaves (expanded) were used in the experiments.

**Protoplast Isolation for Differential Centrifugation.**

*Protoplasts from $C_3$-M. crystallinum.* Four to five leaves were harvested during the light period. The midrib was excised, and

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[2] To whom requests for reprints should be sent, at Department of Botany, Washington State University, Pullman, WA 99164.

[3] Abbreviations: PEP, P-enolpyruvate; RuBP, ribulose 1,5-bisP.

the upper epidermis was removed. Leaves were cut into 2-mm-thick slices with a razor blade. The leaf slices were washed in an ice-cold solution containing 400 mM sorbitol and 0.5 mM $CaSO_4$ and were transferred into 100 ml of isolation medium containing 400 mM sorbitol, 20 mM Mes/HCl, 0.5 mM $CaCl_2$, 2 g cellulase Onozuka 4S (Yakult Biochemicals), and 0.5 g Macerase (Calbiochem), pH 5.2. Following vacuum infiltration, leaf slices were incubated for 3 h at 25 to 27°C with very gentle agitation. After filtration to remove undigested leaf material, the enzyme medium containing protoplasts was diluted with 40 ml of a 400 mM sucrose solution, and the suspension was transferred into 50-ml Babcock bottles. Solutions of the following composition were layered on top (1 to 2 ml each): first, 400 mM sorbitol, 50 mM Hepes/KOH, pH 7.6; and second, 200 mM sorbitol, 200 mM Hepes/KOH, pH 8.0. During centrifugation at 80*g* for 5 min, most intact protoplasts floated to the surface and were collected with a Pasteur pipette.

*Protoplasts from CAM-M. crystallinum.* Procedures were as above, but the isolation medium contained 800 mM sorbitol, and a solution of 800 mM sucrose was added to the protoplast suspension before transfer into Babcock bottles. The protoplast suspension was first overlayered with a solution containing 800 mM sorbitol, 50 mM Hepes/KOH, pH 8.0, which in turn was overlayered with a solution containing 400 mM sorbitol, 200 mM Hepes/KOH, pH 8.0. This higher level of osmoticum was used since the CAM tissue has a higher concentration of solutes, *i.e.* $Na^+$ and $Cl^-$.

**Protoplast Isolation for Percoll Density Gradient Centrifugation.**

*Protoplasts from $C_3$-M. crystallinum.* The method was identical to the protoplast isolation procedure for differential centrifugation, except that a third overlayering solution containing 100 mM sorbitol, 50 mM NaCl, 100 mM Hepes/KOH, pH 8.0, was used. After centrifugation, purified intact protoplasts were present at the interface between the second and third layers.

*Protoplasts from CAM-M. crystallinum.* The method was identical to the protoplast isolation procedure for differential centrifugation, except that a third overlayering solution containing 100 mM sorbitol, 150 mM NaCl, 100 mM Hepes/KOH, pH 8.0, was used. Purified intact protoplasts partitioned mainly at the interface between the second and third layers.

**Differential Centrifugation.** Protoplasts from $C_3$- and CAM-*M. crystallinum* were diluted with solutions containing 200 mM sorbitol, 200 mM Hepes/KOH, pH 8.0, and 600 mM sorbitol, 200 mM Hepes/KOH, pH 8.0, respectively, to a Chl concentration of 0.09 to 0.15 mg $ml^{-1}$ and were ruptured by one pass through a 25-gauge needle. Ruptured protoplast preparations were centrifuged at 820*g* for 2 min in 1.5-ml tubes using a Beckman microfuge B. After removal of the supernatant, the chloroplast-containing pellet was resuspended in 50 mM Hepes/KOH, pH 8.0.

**Percoll Density Gradient Centrifugation.**

*Protoplast Extracts from $C_3$-M. crystallinum.* To generate the Percoll gradient, 12.25 ml Percoll, 1.75 ml $H_2O$, and 21 ml of 200 mM Hepes/KOH, pH 8.0, containing 600 mM sorbitol and 0.2% (w/v) BSA were mixed in a 50-ml polycarbonate tube and centrifuged at 48,000*g* for 1 h at 4°C in a Beckman JA-20 rotor. Intact protoplasts (0.23 to 0.35 mg Chl $ml^{-1}$) were diluted with an equal volume of a solution containing 100 mM Hepes/KOH, 100 mM sorbitol, 50 mM NaCl, 7.8 mM $Na_2$-EDTA, 2% (w/v) PVP-40, 1% (w/v) BSA (defatted), pH 8.0. Protoplasts were ruptured, and 3 ml of the protoplast extract were layered on top of the Percoll gradient. Organelles were separated by centrifugation at 1000*g* for 1 h at 4°C in a Beckman JS-13 rotor. Thirty to 32 fractions were collected per gradient, starting from the bottom of the centrifuge tube, using a fractionation system composed of a glass capillary, peristaltic pump, and fraction collector.

*Protoplast Extracts from CAM-M. crystallinum.* Intact protoplasts were diluted with an equal volume of 100 mM Hepes/KOH, pH 8.0, containing 100 mM sorbitol, 150 mM NaCl, and 1% (w/v) BSA (defatted). Protoplasts were ruptured, and 3 ml of protoplast extract were layered on top of the Percoll gradient. The composition of the gradient and the method for separation of organelles were as described above.

**Enzyme Assays.** The following enzymes were measured at 25 to 27°C by following the change in *A* of a pyridine nucleotide at 340 nm (unless otherwise stated) in a 1.2 ml reaction mixture as described below. Reactions were initiated by the compound listed last. Assays were conducted in the range where there was a linear relationship between amount of enzyme added and activity detected.

*PEP Carboxylase (EC 4.1.1.31).* Fifty mM Hepes/KOH, pH 8.0, 2 mM $KHCO_3$, 5 mM $MgCl_2$, 3 IU NAD-malate dehydrogenase, 0.08 mM NADH, 40 μl enzyme extract, 2 mM PEP.

*Enolase (EC 4.2.1.11).* Fifty mM Mes/KOH, pH 7.0, 2 mM $MgCl_2$, 0.08 mM NADH, 2 mM $KHCO_3$, 3 IU NAD-malate dehydrogenase, 0.5 IU PEP carboxylase, 40 μl enzyme extract, 2 mM 2-P-glyceric acid.

*P-glyceromutase (EC 2.7.5.3).* Fifty mM Mes/KOH, pH 7.0, 2 mM $MgCl_2$, 0.08 mM NADH, 6 IU enolase, 3 IU NAD-malate dehydrogenase, 0.5 IU PEP carboxylase, 0.2 mM 2,3-bisP-glyceric acid, 40 μl enzyme extract.

*P-glycerate Kinase (EC 2.7.2.3).* Fifty mM Mes/KOH, pH 7.0, 2 mM $MgCl_2$, 0.08 mM NADH, 3 IU glyceraldehyde-3-P dehydrogenase, 20 μl enzyme extract, 3 mM 3-P-glyceric acid, 1 mM ATP.

*P-hexose Isomerase (EC 5.3.1.9).* Fifty mM Hepes/KOH, pH 8.0, 10 mM $MgCl_2$, 2 mM $Na_2$-EDTA, 0.2 mM NADP, 3 IU glucose-6-P dehydrogenase, 40 μl enzyme extract, 2 mM fructose-6-P.

*NADP-glyceraldehyde-3-P Dehydrogenase (EC 1.2.1.13).* Fifty mM Hepes/KOH, pH 8.0, 10 mM $MgCl_2$, 5 mM GSH, 5 mM ATP, 0.2 mM NADPH, 0.6 IU P-glycerate kinase, 40 μl enzyme extract, 2 mM 3-P-glyceric acid.

*NAD-glyceraldehyde-3-P Dehydrogenase (EC 1.2.1.12).* As for determination of NADP-linked activity, but 0.08 mM NADH was used instead of NADPH, and 20 μl enzyme extract instead of 40 μl.

*NAD-malate Dehydrogenase (EC 1.1.1.37).* Fifty mM Hepes/KOH, pH 8.0, 0.08 mM NADH, 10 μl enzyme extract, 1 mM oxalacetate.

*NADP-malic Enzyme (EC 1.1.1.40).* Fifty mM Hepes/KOH, pH 7.5, 3 mM $MgCl_2$, 0.25 mM NADP, 40 to 120 μl enzyme extract, 3 mM malate.

*Hydroxypyruvate Reductase (EC 1.1.1.26).* Fifty mM Mes/KOH, pH 6.5, 0.08 mM NADH, 40 μl enzyme extract, 1 mM hydroxypyruvate.

*Fumarase (EC 4.2.1.2).* Fifty mM Hepes/KOH, pH 7.5, 4 mM $MgCl_2$, 5 mM $KH_2PO_4$, 0.6 IU NADP-malic enzyme, 0.4 mM NADP, 40 μl enzyme extract, 5 mM fumarate.

*NAD-malic Enzyme (EC 1.1.1.38).* Total protoplast extracts and the supernatant and pellet fractions obtained after differential centrifugation were incubated with digitonin (5 mg $ml^{-1}$) for 5 min at room temperature. Extracts were desalted at room temperature using a Sephadex G-25 (medium) column which was equilibrated with 25 mM Hepes/KOH, pH 7.5, containing 5 mM DTT and 1 mM $MnCl_2$. Desalted extracts were maintained at room temperature for 30 min prior to enzyme assays. Enzyme activity in fractions of the Percoll gradient was determined without the above pretreatment of samples. Assay mixtures contained 50 mM Hepes/KOH, pH 7.2, 0.2 mM $Na_2$-EDTA, 5 mM DTT, 2 mM NAD, 40 μl enzyme extract, 5 mM malate, 5 mM $MnCl_2$, and 75 μM coenzyme A. Coenzyme A-dependent rates, which were 7- to 10-times higher than the $Mn^{2+}$-dependent rates, are reported (see also Ref. 8).

*NADP-malate Dehydrogenase (EC 1.1.1.82).* Total protoplast extracts and the supernatant and pellet fractions obtained after differential centrifugation were desalted at 4°C using a Sephadex

G-25 (medium) column equilibrated with 50 mM Hepes/KOH, pH 7.6. Desalted extracts were either kept at 0°C or were incubated with 20 mM DTT at room temperature for 30 min. Enzyme activity in fractions from the Percoll gradient was determined without incubating samples with DTT. Assay mixtures contained 50 mM Mes-50 mM Bicine/KOH (pH 6.0–9.0), 1 mM $Na_2$-EDTA, 0.2 mM NADPH, 40 μl enzyme extract, 1 mM oxalacetate.

*Pyruvate,Pi Dikinase (EC 2.7.9.1).* Protoplasts were purified as described earlier except that the overlayering solutions in which protoplasts were collected were prepared at pH 7.6 and contained 10 mM $MgCl_2$, 2.5 mM pyruvate, 2 mM $K_2HPO_4$, 1 mM $Na_2$-EDTA, 0.5% (w/v) ascorbate, and 5 mM DTT in addition to the specified concentrations of sorbitol and Hepes. After differential centrifugation, chloroplast pellets were resuspended in 50 mM Hepes/KOH, pH 7.6, containing 10 mM $MgCl_2$, 2.5 mM pyruvate, 2 mM $K_2HPO_4$, 1 mM $Na_2$-EDTA, 0.5% (w/v) ascorbate, and 5 mM DTT. Total protoplast extracts and the supernatant and pellet fractions were incubated at room temperature for 60 min. They were used for enzyme assays immediately following desalting on a Sephadex G-25 (medium) column equilibrated with 50 mM Hepes/KOH, pH 7.6, containing 5 mM $MgCl_2$, 0.1 mM $Na_2$-EDTA, and 1 mM DTT. Assay mixtures contained: 50 mM Hepes/KOH, pH 8.0, 10 mM $MgCl_2$, 0.1 mM $Na_2$-EDTA, 2 mM $KHCO_3$, 0.08 mM NADH, 1 IU PEP carboxylase, 3 IU NAD-malate dehydrogenase, 40 μl enzyme extract, 1.25 mM pyruvate, 1.25 mM ATP, 2.5 mM $K_2HPO_4$. Activities reported are those dependent on Pi.

*Cytochrome c Oxidase (EC 1.9.3.1).* Eighty-eight mM K-phosphate, pH 7.0, 50 μl enzyme extract, 23 mM cytochrome *c* reduced with sodium dithionite immediately prior to use in the assay. Enzyme activity was measured by following the decrease in *A* at 550 nm.

*RuBP Carboxylase (EC 4.1.1.39).* RuBP carboxylase was assayed using a radiochemical method (16). Reactions were performed in scintillation vials. The total assay volume was 0.5 ml. Fifty μl of enzyme extract were added to 100 mM Tris/HCl, pH 8.1, containing 20 mM $MgCl_2$ and 20 mM $NaH^{14}CO_3$. After 1 min incubation at 25°C, the reaction was initiated with 1 mM RuBP and stopped after 1 min with 100 μl of 5 N HCl. The reaction mixture was dried under a stream of air, and the acid-stable $^{14}C$ radioactivity was determined using liquid scintillation counting.

In addition to the ingredients listed above, 0.03% (v/v) Triton X-100 was present in reaction mixtures for assay of all enzymes in fractions from Percoll gradients. After differential centrifugation, Triton X-100 was added to reaction mixtures only for determination of activities of cytochrome *c* oxidase and hydroxypyruvate reductase.

**Chlorophyll Determination.** Chl was determined after Arnon (1).

**Protein Determination.** Protein was precipitated with 10% (w/v) TCA. Pellets were resuspended in 10% TCA, centrifuged, resuspended in 5% TCA, and centrifuged again. Protein was determined after Lowry *et al.* (17) using crystalline BSA as the standard.

**SDS Gel Electrophoresis.** Electrophoretic analysis of samples was conducted using the alkaline SDS-discontinuous buffer system of Laemmli (14). Gels, polymerized between two glass plates separated by 0.75-mm-thick spacers, consisted of a 1-cm, 4.5% (w/v) acrylamide stacking gel (4.6% [w/v] T; 2.6% [w/w] $C_{bis}$) and a 9.5-cm resolving gel containing a 7.5 to 15% (w/v) linear acrylamide gradient (7.7–15.4% [w/v] T; 2.6% [w/w] $C_{bis}$) stabilized by a 5 to 17.5% linear sucrose gradient (2). Total protoplast extracts and pellet and supernatant fractions from differential centrifugation studies were prepared for electrophoresis by heating for 1 min in the sample buffer described by Laemmli (14). Immediate heat treatment following addition of the sample buffer was absolutely essential to prevent the extremely rapid and complete proteolytic degradation characteristic of these extracts under dissociating conditions. Mass markers, rabbit muscle phosphorylase b (92,500 daltons), bovine serum albumin (68,000 daltons), bovine liver catalase (60,000 daltons), porcine heart fumarase (49,000 daltons), rabbit muscle aldolase (40,000 daltons), porcine heart mitochondrial NAD-malate dehydrogenase (34,000 daltons), bovine erythrocyte carbonic anhydrase (29,000 daltons), soybean trypsin inhibitor (21,500 daltons), and chicken egg white lysozyme (14,000 daltons), all from Sigma Chemical Company, were electrophoresed in a separate lane for mass calibration. Electrophoresis was performed in the direction cathode to anode for 4 h at 25°C using a constant current of 7.5 mamp/gel for the 1st h and 15 mamp/gel for the remaining time. Gels were stained for 1 h at 40°C or overnight at room temperature in a methanol:water:acetic acid (5:5:1, v/v/v) solution containing 0.1% (w/v) Coomassie brilliant blue R-250, then destained for 24 to 48 h in 10% (v/v) acetic acid. For densitometric analysis, destained gels were soaked for 30 min in destaining solution containing 2% (v/v) glycerol, dried between sheets of porous cellophane (dialysis membrane), and scanned using a Joyce-Loebel double-beam recording microdensitometer with a 0.39 density wedge.

## RESULTS

**Differential Centrifugation.** Following centrifugation of protoplast extracts from $C_3$- and CAM-*M. crystallinum* at 820*g*, more than 97% of the Chl sedimented with the pellet (Table I). The pellet also contained about 90% of the activity of NADP-glyceraldehyde-3-P dehydrogenase which was used as a chloroplastic marker. Pyruvate,Pi dikinase activity was detected in CAM protoplasts only and was restricted to the chloroplast pellet.

PEP carboxylase, enolase, P-glyceromutase, and NADP-malic enzyme were nonchloroplastic. Up to 100% of the activity of these enzymes was observed in the supernatant fraction.

Hydroxypyruvate reductase and cytochrome *c* oxidase were used as peroxisomal and mitochondrial markers, respectively. In protoplast extracts of $C_3$-*M. crystallinum*, about 20% of the activity of both enzymes was found in the pellet. In protoplast extracts of CAM-*M. crystallinum*, only 3 to 5% of the activity of these enzymes was associated with the pellet. NAD-malic enzyme showed a similar distribution as hydroxypyruvate reductase and cytochrome *c* oxidase, suggesting that the enzyme is neither chloroplastic nor cytoplasmic.

NAD-glyceraldehyde-3-P dehydrogenase, P-hexose isomerase, and P-glycerate kinase activities were found to various degrees both in the supernatant and pellet fractions; the activity of these enzymes in supernatant fractions from protoplasts of CAM-*M. crystallinum* were markedly higher compared to the activity in supernatant fractions from $C_3$-*M. crystallinum*.

Results with NADP-dependent malate dehydrogenase, a light-stimulated enzyme (7), were complex (Figs. 1 and 2). The activity in the supernatant fractions of protoplast extracts of $C_3$- and CAM-*M. crystallinum* had an optimum around pH 6.5, and there was no effect of pretreatments such as storage of the supernatant fractions at 0°C in the absence of DTT or at 25°C in the presence of DTT. In the pellet fractions, high activity was obtained at pH 8.0 and was dependent upon preincubation of extracts at 25°C with DTT. Activity in the pellet fractions was low at pH 6.5 following either pretreatment. Enzyme activities per mg Chl per min observed in the supernatant (measured at pH 6.5) and pellet fractions (measured at pH 8.0) were 2- and 3.2-times higher, respectively, in preparations from CAM- than from C3-*M. crystallinum*.

For most enzymes, the sum of activities recovered in supernatant and pellet fractions was in close agreement with the activity in the total protoplast extract (Table I). For NADP-glyceraldehyde-3-P dehydrogenase, NAD-glyceraldehyde-3-P dehydrogenase, and P-glycerate kinase, activities of the total protoplast preparations were 12 to 22% less than the sum of activities in the supernatant

Table I. *Distribution of Chl and of Enzyme Activities following Centrifugation of Protoplast Extracts from C₃- and CAM-M. crystallinum at 820g for 2 Minutes*

| Measurement | Enzyme Activity | | | | Distribution of Activity | |
|---|---|---|---|---|---|---|
| | Pellet | Supernatant | Pellet + supernatant | Protoplast extract | Pellet | Supernatant |
| | *μmol min⁻¹ mg Chl⁻¹* | | | | % | |
| C₃-*M. crystallinum* | | | | | | |
| Chl | | | | | 97.3 | 2.7 |
| NADP-glyceraldehyde-3-P dehydrogenase | 6.06 | 0.60 | 6.66 | 5.89 | 91.0 | 9.0 |
| Pyruvate, Pi dikinase | ND[a] | ND | | ND | | |
| PEP carboxylase | ND | 0.51 | 0.51 | 0.38 | 0.0 | 100.0 |
| Enolase | 0.04 | 1.41 | 1.45 | 1.41 | 2.8 | 97.2 |
| P-Glyceromutase | ND | 1.11 | 1.11 | 1.24 | 0.0 | 100.0 |
| NADP-malic enzyme | ND | 0.04 | 0.04 | 0.04 | 0.0 | 100.0 |
| NAD-malic enzyme | 0.04 | 0.14 | 0.18 | 0.11 | 22.2 | 77.8 |
| Hydroxypyruvate reductase | 2.38 | 10.17 | 12.55 | 12.13 | 19.0 | 81.0 |
| Cytochrome *c* oxidase | 0.15 | 0.63 | 0.78 | 0.82 | 23.2 | 76.8 |
| NAD-glyceraldehyde-3-P dehydrogenase | 6.65 | 5.38 | 12.03 | 9.65 | 55.3 | 44.7 |
| P-Hexose isomerase | 0.88 | 1.40 | 2.28 | 2.15 | 38.6 | 61.4 |
| P-Glycerate kinase | 28.27 | 11.94 | 40.21 | 30.91 | 70.3 | 29.7 |
| CAM-*M. crystallinum* | | | | | | |
| Chl | | | | | 98.2 | 1.8 |
| NADP-glyceraldehyde-3-P dehydrogenase | 7.87 | 1.16 | 9.03 | 7.08 | 87.2 | 12.8 |
| Pyruvate, Pi dikinase | 0.73 | 0.0 | 0.73 | 0.45 | 100.0 | 0.0 |
| PEP carboxylase | 0.21 | 17.10 | 17.31 | 16.58 | 1.2 | 98.8 |
| Enolase | 0.32 | 13.70 | 14.02 | 14.57 | 2.3 | 97.7 |
| P-Glyceromutase | 0.11 | 8.55 | 8.66 | 9.50 | 1.3 | 98.7 |
| NADP-malic enzyme | 0.05 | 0.98 | 1.03 | 1.08 | 4.9 | 95.1 |
| NAD-malic enzyme | 0.06 | 1.81 | 1.87 | | 3.4 | 96.6 |
| Hydroxypyruvate reductase | 0.55 | 9.60 | 10.15 | 9.65 | 5.4 | 94.6 |
| Cytochrome *c* oxidase | 0.05 | 1.65 | 1.70 | 1.71 | 2.9 | 97.1 |
| NAD-glyceraldehyde-3-P dehydrogenase | 6.42 | 32.46 | 38.88 | 32.90 | 16.5 | 83.5 |
| P-Hexose isomerase | 1.76 | 17.33 | 19.09 | 18.76 | 9.2 | 90.8 |
| P-Glycerate kinase | 30.16 | 36.70 | 66.86 | 53.92 | 45.1 | 54.9 |

[a] ND, not detected.

and pellet fractions. This difference was almost 40% for pyruvate,Pi dikinase which was very unstable in total protoplast extracts compared to chloroplast fractions.

Enzyme activities from protoplast preparations of C₃- and CAM-*M. crystallinum* were similar to those reported earlier for crude and desalted whole-leaf extracts (9). As in these earlier studies, activity of pyruvate,Pi dikinase was detected in CAM-*M. crystallinum* only, and there was an increase in the activities of PEP carboxylase, NADP-malic enzyme, NAD-malic enzyme, enolase, P-glyceromutase mutase, NAD-glyceraldehyde-3-P dehydrogenase, P-glycerate kinase, and P-hexose isomerase in response to growth of plants at 400 mM NaCl and the induction of CAM. The increased activity of the latter three enzymes, all of which were found in both the supernatant and pellet fractions, was due to an increase in activity in the supernatant fraction.

**Gel Electrophoresis.** Following differential centrifugation of protoplast extracts of C₃- and CAM-*M. crystallinum*, the supernatant and pellet fractions were subjected to electrophoresis under dissociating conditions (Fig. 3). As judged from the distribution of the large subunit of RuBP carboxylase in the supernatant and pellet fractions, recovery of chloroplasts in the pellet fractions from C₃- and CAM-*M. crystallinum* was 81% and 72%, respectively, for the experiments depicted in Figure 3. The subunit of PEP carboxylase (98,000 daltons) which was identified by SDS gel electrophoresis of partially purified PEP carboxylase from *M. crystallinum* (J. G. Foster, G. E. Edwards, and K. Winter, manuscript in preparation) was restricted to the supernatant fractions. The staining intensity of the polypeptide attributed to PEP carboxylase was several-fold higher in total protoplast extracts and supernatant fractions of CAM-*M. crystallinum* in comparison with corresponding samples from C₃-*M. crystallinum*.

**Percoll Density Gradient Centrifugation.** Data from typical Percoll gradient centrifugations using protoplast extracts of CAM-*M. crystallinum* are presented in Figures 4 and 5. As indicated by the distribution of Chl (Fig. 4A) and the chloroplast marker enzymes, NADP-glyceraldehyde-3-P dehydrogenase and RuBP carboxylase (Fig. 4B), minimal breakage of chloroplasts occurred, and intact chloroplasts formed a distinct band in the lower part of the centrifuge tube. This band contained more than 80% of the activities of NADP-glyceraldehyde-3-P dehydrogenase and RuBP

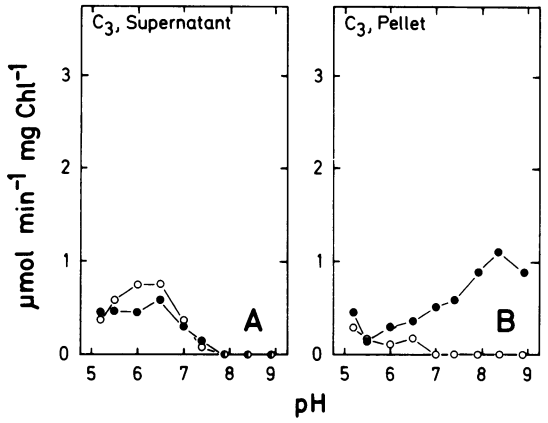


FIG. 1. pH response curves of NADP-dependent malate dehydrogenase activity in supernatant (A) and pellet fractions (B) following centrifugation of protoplast extracts from $C_3$-*M. crystallinum* at 820*g* for 2 min. ●, extracts were incubated with 20 mM DTT at room temperature for 30 min prior to enzyme assays; ○, extracts were kept at 0°C in the absence of DTT prior to assays.

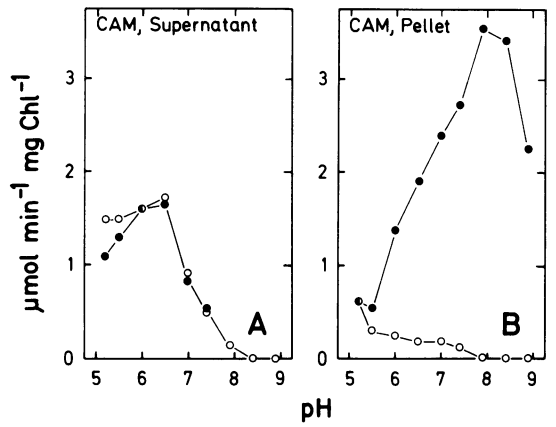


FIG. 2. As in Fig. 1, but extracts were obtained from CAM-*M. crystallinum*.

carboxylase. Hydroxypyruvate reductase activity was found to peak in fraction 10, but was also observed at the top of the gradient, probably indicating some breakage of peroxisomes (Fig. 4C). No activity of the mitochondrial markers fumarase and cytochrome *c* oxidase was found at the top of the gradient (Figs. 4D1 and 4D2). There was a peak of activity of these enzymes around fraction 9, and some activity was associated with the chloroplasts. Evidently, there was no breakage of mitochondria, but some of these organelles comigrated with the chloroplasts during centrifugation. The distribution of NAD-malic enzyme on the gradient (Fig. 4D3) was identical with that of the mitochondrial marker enzymes.

Activities of PEP carboxylase (Fig. 4E), P-glyceromutase (Fig. 4F), enolase (Fig. 4G), and NADP-malic enzyme (Fig. 4H) were restricted to the top of the gradient, suggesting cytoplasmic localization of these enzymes. Most of the activity of NAD-glyceraldehyde-3-P dehydrogenase (Fig. 4I), P-glycerate kinase (Fig. 4J), and P-hexose isomerase (Fig. 5B) occurred at the top of the gradient, but some activity was found with the chloroplasts. NAD-malate dehydrogenase activity was observed at the top of the gradient and was also associated with the peroxisomal-mitochondrial peak as well as with the chloroplasts (Fig. 4K). NADP-malate dehydrogenase, measured at pH 6.0, showed activity at the top of the gradient and was associated with the chloroplasts (Fig. 4L1); however, activity at pH 8.0 was restricted to the chloroplast peak (Fig. 4L2).

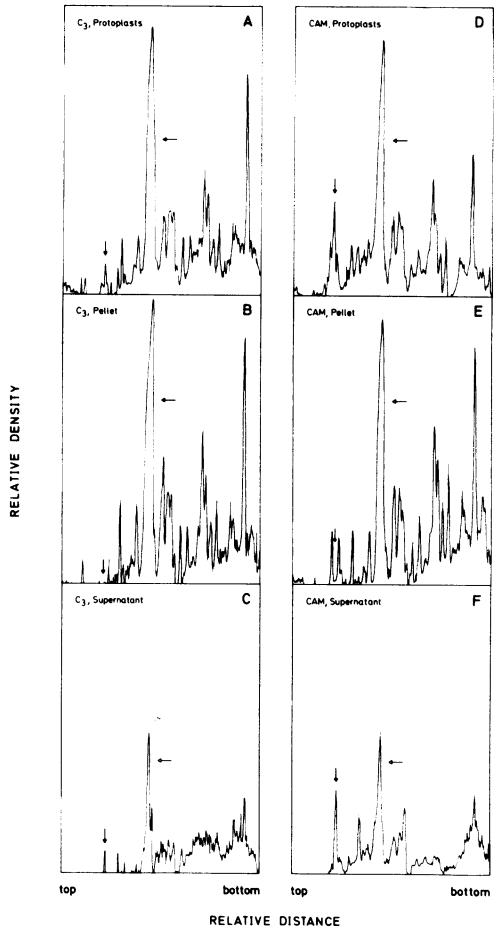


FIG. 3. Densitometric scans of SDS polyacrylamide gradient slab gels of total protoplast extracts from $C_3$- and CAM-*M. crystallinum* (A and D) as well as of pellet (B and E) and supernatant fractions (C and F) after centrifugation of protoplast extracts at 820*g* for 2 min. Protein content (mg/ml) in the protoplast extract and in pellet and supernatant fractions was 1.56, 0.53, and 1.20 for $C_3$-*M. crystallinum*, and 1.63, 0.75, and 1.07 for CAM-*M. crystallinum*, respectively. One hundred µl of extract were diluted with an equal volume of sample buffer containing 0.125 M Tris/HCl, pH 6.8, 20% (v/v) glycerol, 10% (v/v) β-mercaptoethanol, 4% (w/v) SDS, and 0.02% (w/v) bromphenol blue. After a 1-min heat treatment at 100°C, 50 µl/sample were applied to the gels. The open arrow indicates the large subunit of RuBP carboxylase; the closed arrow indicates the subunit (98,000 daltons) of PEP carboxylase.

The distribution of enzymes in $C_3$-*M. crystallinum* protoplast extracts on a Percoll gradient is shown in Figure 6. Chloroplast integrity (Fig. 6, A and B) was comparable to that observed in experiments with protoplasts of CAM-*M. crystallinum* (Figs. 4, A and B, and 5). There was, however, considerable association of hydroxypyruvate reductase and cytochrome *c* oxidase with the chloroplasts (Fig. 6, C and D), phenomena which could not be avoided by the inclusion of Ficoll (*e.g.* Ref. 20) or by variations in the concentrations of PVP-40 and EDTA in the protoplast extract. Furthermore, the study of several enzymes from $C_3$-*M. crystallinum* on the Percoll gradient, *e.g.* PEP carboxylase and enolase, was complicated by the low activity present in this tissue (Table I; Ref. 9). Even so, PEP carboxylase and enolase activities were found exclusively on top of the gradient (Fig. 6, E and F). NAD-glyceraldehyde-3-P dehydrogenase (Fig. 6G) and P-hexose isomerase (Fig. 6H), also low in total activity, occurred in equal quantities at the top of the gradient and in the chloroplast fraction,

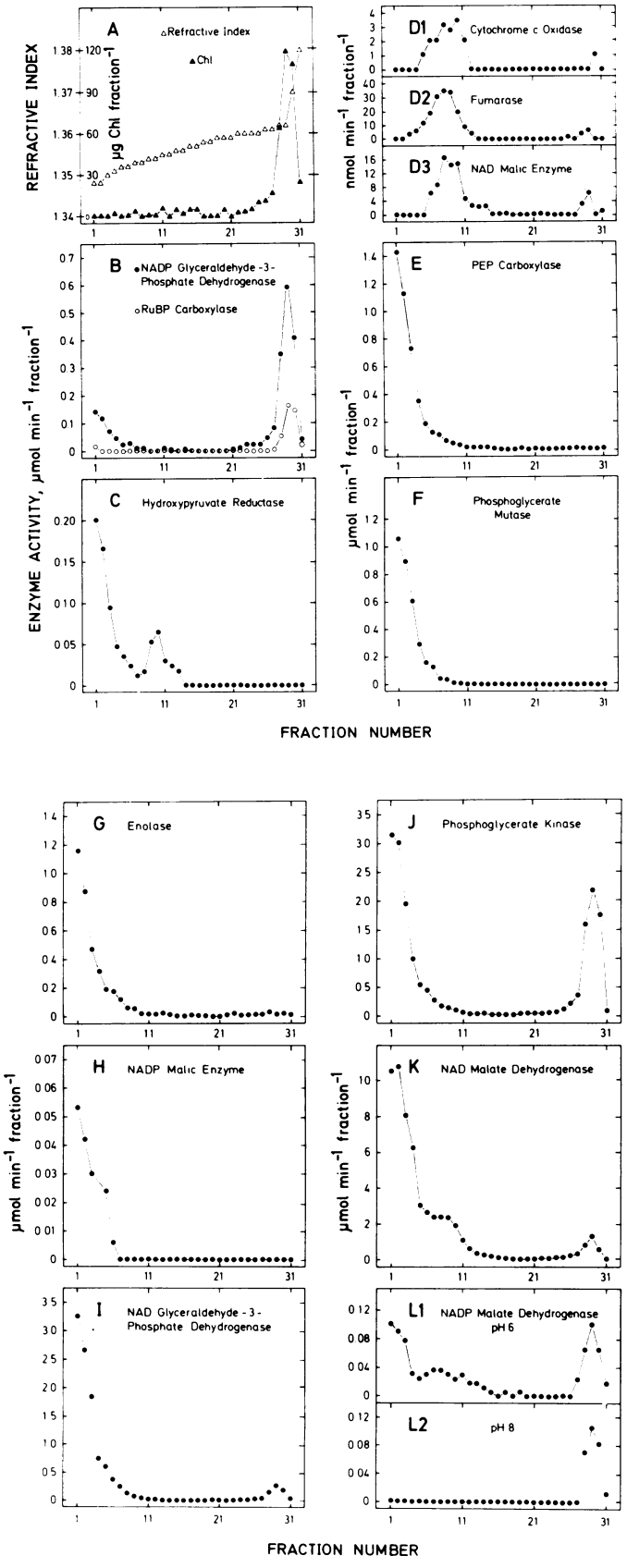


FIG. 4. Distribution of Chl (A) and activity of several enzymes (B–L) following centrifugation of a protoplast extract from CAM-*M. crystallinum* on a Percoll density gradient. Fractions are numbered from top to bottom of the gradient.

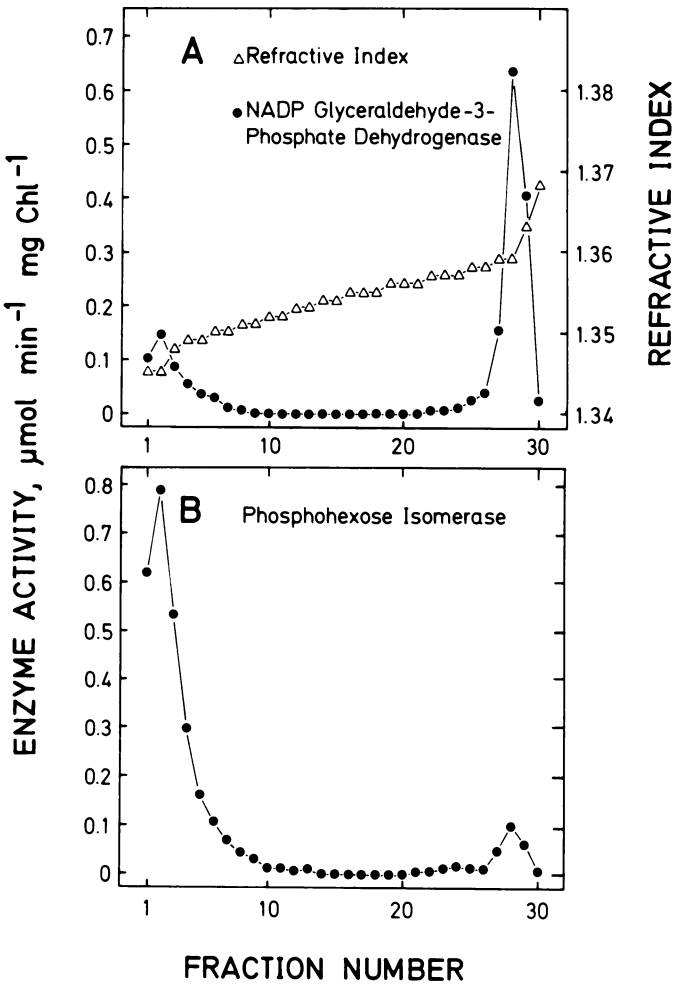


FIG. 5. Distribution of NADP-glyceraldehyde-3-P dehydrogenase (A) and P-hexose isomerase activity (B) following centrifugation of a protoplast extract from CAM-*M. crystallinum* on a Percoll density gradient. Fractions are numbered from top to bottom of the gradient.

whereas P-glycerate kinase activity was higher in the chloroplast fraction than at the top (Fig. 6I).

## DISCUSSION

Surprisingly few difficulties were encountered in the isolation of protoplasts and chloroplasts from *M. crystallinum*, despite the high NaCl content of leaves of plants grown at 400 mM NaCl (around 500 μeq/g fresh weight). Following rapid centrifugation of protoplast extracts from $C_3$- and CAM-*M. crystallinum*, pellets contained more than 95% of the total Chl and retained up to 90% of the total activity of the chloroplast marker enzyme NADP-glyceraldehyde-3-P dehydrogenase (Table I). Chloroplast integrity was similarly high after Percoll density gradient centrifugation (Figs. 4, A and B; 5, A and B; 6, A and B).

More than 95% of the activity of PEP carboxylase, enolase, P-glyceromutase, and NADP-malic enzyme was found in the supernatant fraction after differential centrifugation or in the top fractions following Percoll density gradient centrifugation. These enzymes are considered cytoplasmic, tacitly presupposing that they are not of vacuolar nature. The extrachloroplastic location of PEP carboxylase was also apparent from the identification of PEP carboxylase protein in the supernatant fractions using SDS gel electrophoresis (Fig. 3). The increased intensity of staining of PEP carboxylase protein in extracts of CAM-*M. crystallinum* as compared to extracts of $C_3$-*M. crystallinum* suggests that the increase

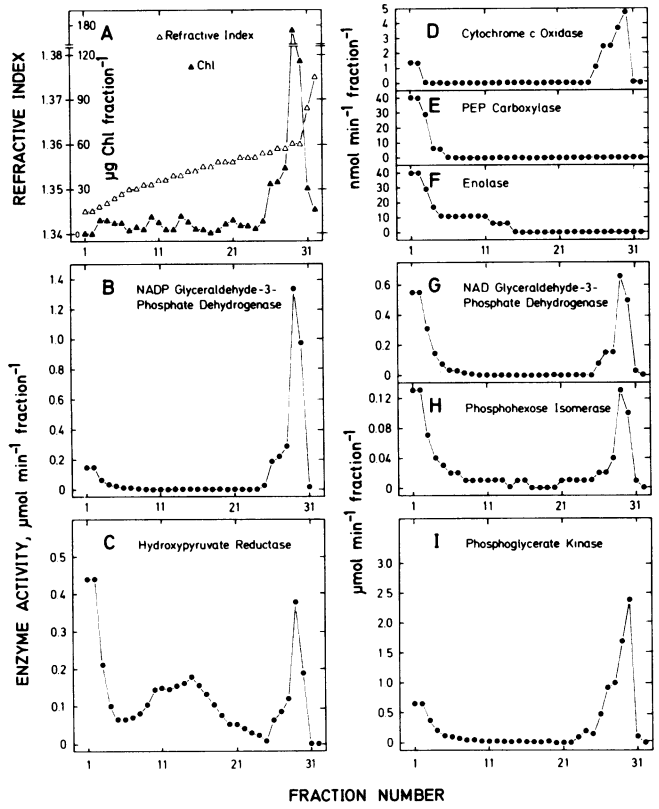


FIG. 6. Distribution of Chl (A) and activity of several enzymes (B–I) following centrifugation of a protoplast extract from $C_3$-*M. crystallinum* on a Percoll density gradient. Fractions are numbered from top to bottom of the gradient. In this experiment, fraction 1, which was collected last, had a greater volume (2.38 ml) than all other fractions (1.19 ml each); enzyme activities given for fractions 1 and 2 represent the mean of the sum of activities in both fractions.

in PEP carboxylase activity during the induction of CAM is due to net synthesis of enzyme protein. This aspect will be considered in more detail elsewhere (J. G. Foster, G. E. Edwards, and K. Winter, manuscript in preparation).

Pyruvate,Pi dikinase activity was restricted to the chloroplast pellet after differential centrifugation (Table I), an observation which is consistent with the chloroplastic occurrence of this enzyme in other tissues (5, 21). NAD-malic enzyme can be assigned to the mitochondria since its distribution on the Percoll density gradient was identical with that of the mitochondrial marker enzymes cytochrome *c* oxidase and fumarase (Fig. 4D). Previous reports of mitochondrial localization of NAD-malic enzyme in $C_3$ (18, 23), $C_4$ (5, 6), and constitutive CAM (3) species support our finding with the inducible CAM plant *M. crystallinum*.

NAD-glyceraldehyde-3-P dehydrogenase, P-hexose isomerase, and P-glycerate kinase occurred in both cytoplasm and chloroplasts. The ratio of activities in supernatant *versus* pellet fractions after differential centrifugation of $C_3$ protoplast extracts was 0.8:1, 1.6:1, and 0.4:1 for NAD-glyceraldehyde-3-P dehydrogenase, P-hexose isomerase, and P-glycerate kinase, respectively (Table I). The activity of these enzymes was increased 3-, 10-, and 1.5-fold, respectively, in protoplast extracts of CAM-*M. crystallinum*. This increase was almost entirely due to the increase in activity in the supernatant fractions (Table I). For both NAD-glyceraldehyde-3-P dehydrogenase and P-glycerate kinase, the sum of the activities in the supernatant and pellet fractions after differential centrifugation was higher than the corresponding activities in the unfractionated protoplast extracts for both $C_3$- and CAM-*M. crystallinum*. However, the increases are, by far, too small to account for the higher cytoplasmic activity of these enzymes in CAM-*M. crystallinum* compared to $C_3$-*M. crystallinum*. Furthermore, activity and distribution of NAD-glyceraldehyde-3-P dehydrogenase, P-hexose isomerase, and P-glycerate kinase from $C_3$- and CAM-*M. crystallinum* on Percoll gradients also suggest a specific increase in the cytoplasmic activity of these enzymes following the induction of CAM (Figs. 4, I and J; 5B; 6, G, H, and I). The physiological significance of this increase is not clear. Since all three enzymes catalyze freely reversible reactions, and because the cytoplasmic activities of at least NAD-glyceraldehyde-3-P dehydrogenase and phosphoglycerate kinase from $C_3$-*M. crystallinum* are in considerable excess of the sustained rates of carbon flow *in vivo*, these enzymes are not thought to be regulatory. Nevertheless, it is intriguing that changes in environmental or internal conditions (increased soil salinity, change from $C_3$ photosynthesis to CAM) can differentially influence the activity of an enzyme present in different cellular compartments. Future research on other glycolytic and gluconeogenic enzymes not studied here, including regulatory enzymes such as P-fructokinase and fructose-1,6-bisphosphatase, may reveal whether the cytoplasmic route (or part of it) of either glycolysis or gluconeogenesis, or both, acquires increased significance in response to high salinity and/or the development of CAM in *M. crystallinum*. The observation that activities of both NADP-glyceraldehyde-3-P dehydrogenase and the chloroplastic NAD-glyceraldehyde-3-P dehydrogenase were similar in $C_3$- and CAM-*M. crystallinum* (Table I) is consistent with the notion that the NADP- and NAD-linked activities in the chloroplast may reflect the operation of the same enzyme protein (15).

NADP-dependent activity of malate dehydrogenase showed two pH optima (Figs. 1 and 2). One optimum occurs at pH 6.5 and is attributed to an extrachloroplastic enzyme. The second optimum at pH 8.0 was associated with a chloroplastic enzyme which required DTT for activation. Similar results were obtained for NADP-malate dehydrogenase extracted from leaves of *Phaseolus vulgaris* (22). These authors showed that the activity at the lower pH was due to an NAD-malate dehydrogenase which also exhibited NADP-dependent activity.

NADP-dependent malate dehydrogenase activity at both pH 6.5 and pH 8.0 was higher in protoplast preparations from CAM- than from $C_3$-*M crystallinum* (Figs. 1 and 2). Previous experiments using total leaf extracts (9) revealed about a 2-fold increase in the activity of NAD-malate dehydrogenase following the induction of CAM in *M. crystallinum* by high salinity. This increase could explain the increase in the NADP-dependent activity at pH 6.5. NADP-malate dehydrogenase activities of 3.5 µmol per mg Chl per min, obtained at pH 8.0 for chloroplast preparations of CAM-*M. crystallinum*, are intermediate between those reported for $C_3$ plants (0.5–3 µmol per mg Chl per min; Refs. 10 and 19) and $C_4$ plants (1–12 µmol per mg Chl per min; Ref. 11). The increased activity at pH 8.0 in CAM-*M. crystallinum* as compared to the $C_3$ tissue suggests participation of this enzyme in CAM, but its specific role in CAM is not clear. NADP-dependent malate dehydrogenase in mesophyll chloroplasts of $C_4$ plants seems to be responsible for the reduction of oxalacetate, the product of $\beta$-carboxylation in $C_4$ photosynthesis. In chloroplasts of $C_3$ plants, NADP-dependent malate dehydrogenase may be involved in a malate/oxalacetate shuttle, allowing for export of reducing equivalents from the chloroplasts.

In the Percoll gradients with protoplast extracts of CAM-*M. crystallinum*, low activities of mitochondrial marker enzymes were associated with the chloroplast band, and some breakage of peroxisomes occurred as indicated by the presence of hydroxypyruvate reductase activity in the top fractions (Fig. 4, C and D). These results do not complicate interpretation of data because the distributions of mitochondrial and peroxisomal marker enzymes are clearly distinct from each other and from those enzymes we have proposed to occur in either cytoplasm or chloroplasts or both. For

instance, enzyme activities found exclusively at the top of the gradient and considered cytoplasmic cannot be attributed to enzymes released during breakage of peroxisomes because corresponding peaks of activity do not also appear in fraction 10 where hydroxypyruvate reductase occurs. Similarly, enzymes proposed to occur in the chloroplasts cannot be attributed to mitochondria attached to chloroplasts because these enzymes should then also show a peak in activity around fraction 9, similar to cytochrome *c* oxidase and fumarase (Fig. 4). Only the chloroplastic activity of NAD-malate dehydrogenase could be due to mitochondrial contamination of chloroplasts (Fig. 4K), contamination which could also explain the association of some NADP-linked malate dehydrogenase activity at pH 6.0 with the chloroplasts (Fig. 4L1).

Binding of peroxisomal and mitochondrial marker enzymes to chloroplasts was much greater in Percoll gradients with protoplast extracts from $C_3$- than from CAM-*M. crystallinum* (Fig. 6, C and D). Even so, the above arguments can also be applied to these experiments. Furthermore, the association of mitochondrial and peroxisomal marker enzymes with chloroplasts was much less pronounced after differential centrifugation of $C_3$ protoplast extracts (Table I).

Our findings on the intracellular distribution of enzymes in the inducible CAM plant *M. crystallinum* are consistent with suggestions by Spalding *et al.* (21) on the compartmentation of enzymes in *Sedum praealtum*, a malic enzyme-type CAM plant, and support the schemes of carbon flow during CAM proposed by these authors. The suggestion of nonchloroplastic localization of PEP carboxylase in *Opuntia ficus-indica*, based on nonaqueous techniques for plastid isolation (12), also agrees with our results for *M. crystallinum* using protoplast extracts. The results presented here, however, contrast conclusions by Schnarrenberger *et al.* (20) that PEP carboxylase and NADP-malic enzyme from CAM plants are chloroplastic. Although different species were investigated in all three studies, it is noteworthy that, in the sucrose density gradients reported by Schnarrenberger *et al.* (20), no single enzyme was found exclusively at the top of the gradient, *i.e.* all enzymes were associated to varying degrees with organelles. It is possible that enzymes such as PEP carboxylase and NADP-malic enzyme may have adsorbed to organelles during isolation, leading to erroneous conclusions regarding intracellular location *in vivo*. Furthermore, the schemes of carbon flow during CAM proposed by Schnarrenberger *et al.* (20) assume a chloroplastic location of enolase and P-glyceromutase. Evidence presented here and elsewhere (21) points to an exclusively cytoplasmic occurrence of these enzymes.

The chloroplastic location of pyruvate,Pi dikinase and the restriction of enolase and P-glyceromutase to the cytoplasm suggest a rather complicated flow of carbon during deacidification and gluconeogenesis in the light period. Pyruvate formed upon decarboxylation of malate would enter the chloroplasts for conversion into PEP by pyruvate,Pi dikinase. PEP would then leave the chloroplasts to allow for conversion into 2-P-glycerate and 3-P-glycerate via enolase and P-glyceromutase. Carbon in the form of 3-P-glycerate or glyceraldehyde-3-P could then reenter the chloroplasts for incorporation into starch. Studies on transport and metabolism of pyruvate using chloroplasts isolated from $C_3$- and CAM-*M. crystallinum* are in progress.

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