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INTRACELLULAR LOCALIZATION OF CO_2 METABOLISM ENZYMES IN CACTUS PHYLLOCLADES*

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Abstract—Chloroplasts were prepared from cactus, *Opuntia Ficus-indica* Mill., phylloclades by aqueous and nonaqueous techniques in order to localize CO_2 metabolism enzymes. Phosphoenolpyruvate carboxylase, malate dehydrogenase, malic enzyme, glutamic-oxaloacetic transaminase, and glucose-6-phosphate dehydrogenase were shown to be associated with the purified chloroplasts. Malic dehydrogenase, malic enzyme, glutamic-oxaloacetic transaminase and glucose-6-phosphate dehydrogenase were associated with mitochondria purified by sucrose density gradients. Recovery of enzymic activity from chloroplasts prepared by carbon tetrachloride-hexane density gradient centrifugation was significantly better than plastids prepared by sucrose density gradient centrifugation. Because P-enolpyruvate carboxylase, malic dehydrogenase, and malic enzyme activities were associated with chloroplasts, CO_2 metabolism mediated by these enzymes must be associated with chloroplast metabolism.

INTRODUCTION

IN GREEN tissues of succulent plants, it has long been known that in the dark CO_2 is incorporated into organic acids. This observation has been made both for cactus stem and root tissue.[1,2] In green tissue phosphoenolpyruvate carboxylase is implicated as the main carboxylating enzyme while the malic enzyme is suggested to function in either carboxylation or decarboxylation. The same enzymes are implicated in CO_2 metabolism in non-green tissues.[3] In this laboratory, both P-enolpyruvate carboxylase and the malic enzyme have been demonstrated in cactus phylloclades. The exact significance of dark CO_2 metabolism is not completely understood and for this reason information concerning the intracellular location of enzymes implicated in CO_2 fixation is important. In the present report we present data indicating that P-enolpyruvate carboxylase, malic dehydrogenase, the malic enzyme, glucose-6-phosphate dehydrogenase, and glutamic-oxaloacetic transaminase are present in chloroplasts of cactus.

RESULTS

Nonaqueously Prepared Chloroplasts

Three distinct chloroplast containing bands were separated by nonaqueous density gradient centrifugation (Table 1). The lightest band (fraction 1, density = 1·420 to 1·452) contained the least amount of chlorophyll and protein, but the best appearing chloroplasts as viewed with the electron microscope. Fraction 2 (density = 1·452 to 1·479) contained the

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[1] I. P. TING and W. M. DUGGER, *Plant Physiol.* **40**, 68 (1965).
[2] I. P. TING and W. M. DUGGER, *Plant Physiol.* **41**, 500 (1966).
[3] D. A. WALKER, *Biol. Rev.* **37**, 215 (1962).

TABLE 1. THE INTRACELLULAR LOCALIZATION OF MALATE DEHYDROGENASE, P-ENOLPYRUVATE CARBOXYLASE, MALIC ENZYME, GLUCOSE-6-PHOSPHATE DEHYDROGENASE, GLUTAMIC-OXALOACETIC TRANSAMINASE AND NADP-DEPENDENT GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE OF CACTUS PHYLLOCLADES AS DETERMINED BY NONAQUEOUS ISOLATION OF CHLOROPLASTS

Experiment	Fraction	Total chlorophyll (mg)	Malic dehydrogenase		P-enol-pyruvate carboxylase		Malic enzyme		Glucose-6-phosphate dehydrogenase		Glutamic-oxaloacetic transaminase		NADP-dependent glyceraldehyde-3-phosphate dehydrogenase	
			Activity*	Ratio†	Activity	Ratio	Activity	Ratio	Activity	Ratio	Activity	Ratio	Activity	Ratio
1	I	0·07	2442	1·0	2·9	1·0	7·5	1·0	12·9	1·0	9·6	1·0	0·5	1·0
	II	0·24	2532	1·0	0·9	0·3	7·2	1·0	4·5	0·4	8·8	0·9	0·4	0·8
	III	0·15	2528	1·0	0·6	0·2	7·9	1·0	4·0	0·3	6·8	0·7	0·4	0·8
2	I	0·03	2586	1·0	3·1	1·0	6·8	1·0	12·1	1·0	7·3	1·0	0·4	1·0
	II	0·25	2006	0·8	1·5	0·5	6·6	1·0	3·5	0·3	6·2	0·9	0·5	1·2
	III	0·05	2688	1·0	0·3	0·1	6·9	1·0	1·4	0·1	5·6	0·8	0·4	1·0

* Activity expressed in terms of change in optical density at 340 nm/min/mg chlorophyll.
† The enzymic activities have been converted to ratio values with fraction I values being established at unity.

most chlorophyll and protein as well as the most enzymic activity. Fraction 3 (density > 1·50) contained an intermediate amount of chlorophyll, protein, and enzymic activity. The latter two fractions had intact and broken chloroplasts as well as other cellular debris. Although all six of the enzymes investigated were associated with the nonaqueous chloroplast fractions, the specific activities (chlorophyll basis) of malic dehydrogenase, the malic enzyme, glutamic-oxaloacetic transaminase, and NADP-glyceraldehyde-3-phosphate dehydrogenase were constant in all three fractions. These data indicate a direct association with the chloroplasts. The specific activities of P-enolpyruvate carboxylase and glucose-6-phosphate dehydrogenase, however, decreased with an increase in the density of the chloroplast preparation. Considering total activity, approximately 40 per cent of the latter enzymes were associated with fraction 2 while nearly 50 per cent of the others were associated with this fraction. Fraction 1 contained about 40 per cent of the P-enolpyruvate carboxylase and glucose-6-phosphate dehydrogenase, but only 15 to 18 per cent of the others. Fraction 3 contained less P-enolpyruvate carboxylase and glucose-6-phosphate dehydrogenase (18–23 per cent) than the others (26–35 per cent).

Aqueous Preparation of Chloroplast and Mitochondrial Fractions

Low enzymic activity was recovered in particles isolated by standard aqueous techniques. Recovery of enzymic activity in nonaqueously prepared chloroplasts was significantly better than aqueously prepared chloroplasts, however, the recovery of chlorophyll was lower in nonaqueous preparations (Table 2). Leaching, rather than differential inactivation, probably accounted for low aqueous recoveries. Because of the nature of the cactus phylloclade (i.e. thick, fibrous epidermal tissue and abundant mucilage), many chloroplasts were certainly destroyed during manipulation. Except for the lower recovery, the aqueous data agree with the nonaqueous data.

TABLE 2. RECOVERY OF ENZYMIC ACTIVITY IN CHLOROPLASTS ISOLATED BY AQUEOUS AND NONAQUEOUS METHODS

	Nonaqueous	Aqueous	Ratio, nonaqueous/aqueous
Protein*	425·0	32·0	13·3
Chlorophyll	4·71	9·1	0·52
Malic dehydrogenase	10,970	5,200	2·2
P-enolpyruvate carboxylase	6·8	1·3	5·2
Malic enzyme	33	1·7	19·4
Glucose-6-phosphate dehydrogenase	22·7	4·7	4·82
Glutamic-oxaloacetic transaminase	33·7	5·0	6·8

* Protein and chlorophyll expressed as mg per g fresh weight × 10^3. Enzymes expressed as units per g fresh weight × 10^3.

Distribution of Protein, Chlorophyll, and Enzymes in Sucrose Density Gradients of Chloroplast Preparations

When aqueously prepared chloroplasts were purified by sucrose density gradient centrifugation, the distribution of both protein and chlorophyll followed a similar pattern (Fig. 1).

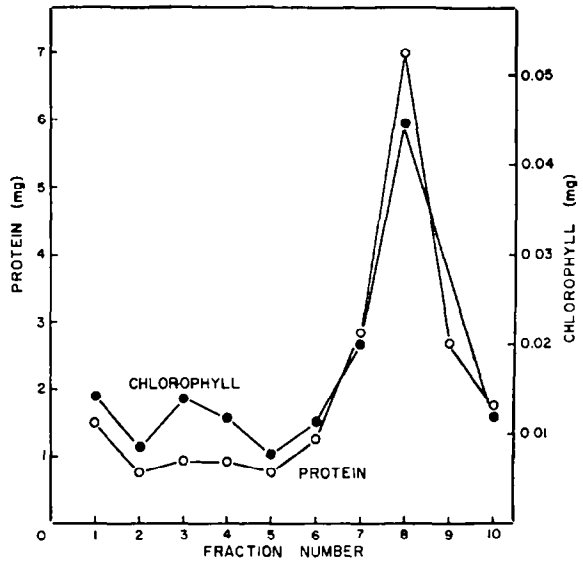

FIG. 1. DISTRIBUTION OF PROTEIN AND CHLOROPHYLL AFTER SUCROSE DENSITY GRADIENT CENTRIFUGATION OF THE PARTICULATE PREPARATION OBTAINED AT 1000 × *g* (CHLOROPLASTS) FROM CACTUS PHYLLOCLADES.

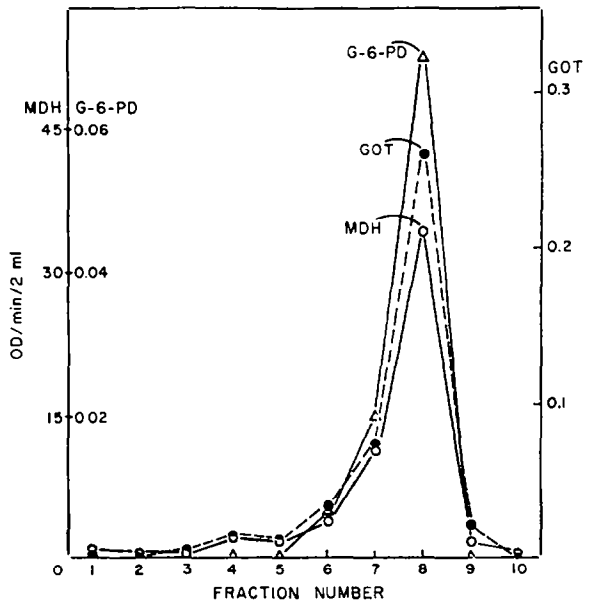

FIG. 2. DISTRIBUTION OF MALATE DEHYDROGENASE (MDH), GLUCOSE-6-PHOSPHATE DEHYDROGENASE (G-6-PD) AND GLUTAMIC-OXALOACETIC TRANSAMINASE (GOT) ACTIVITIES AFTER SUCROSE DENSITY GRADIENT CENTRIFUGATION OF THE PARTICULATE PREPARATION OBTAINED AT 1000 × *g* (CHLOROPLASTS) FROM CACTUS PHYLLOCLADES. P-ENOLPYRUVATE CARBOXYLASE AND MALIC ENZYME ACTIVITIES WERE DETECTABLE IN FRACTION 8.

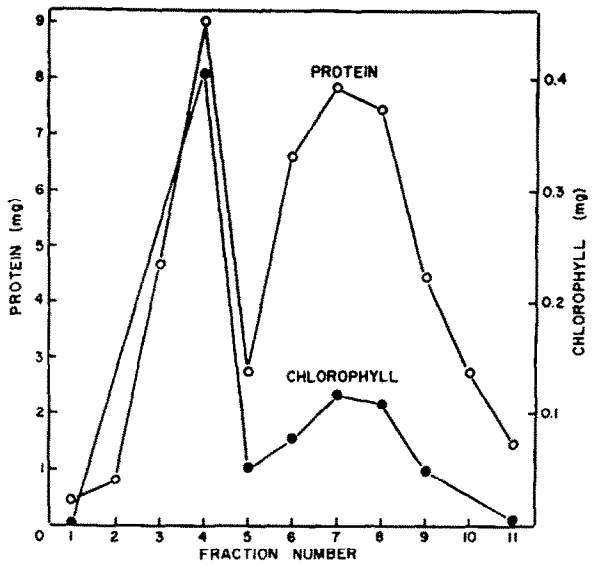

FIG. 3. DISTRIBUTION OF PROTEIN AND CHLOROPHYLL AFTER SUCROSE DENSITY GRADIENT CENTRIFUGATION OF THE PARTICULATE PREPARATION OBTAINED AT 10,000 × g (MITOCHONDRIA) FROM CACTUS PHYLLOCLADES.

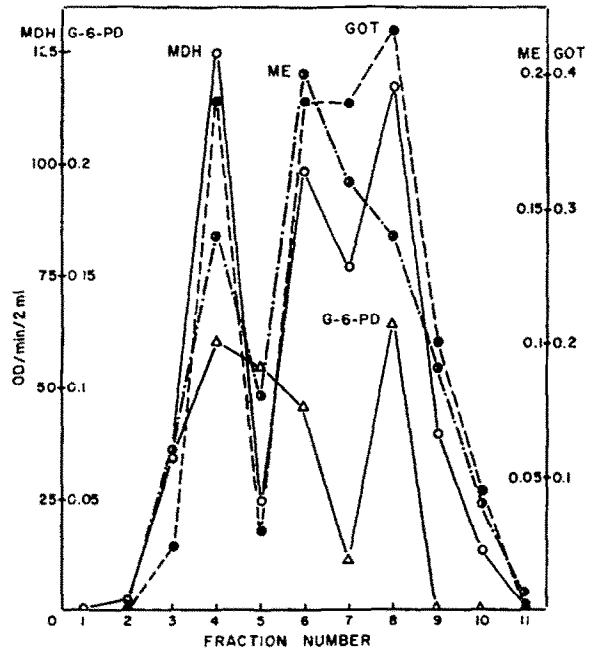

FIG. 4. DISTRIBUTION OF MALATE DEHYDROGENASE (MDH), MALIC ENZYME (ME), GLUCOSE-6-PHOSPHATE DEHYDROGENASE (G-6-PD), AND GLUTAMIC-OXALOACETIC TRANSAMINASE (GOT) ACTIVITIES AFTER SUCROSE DENSITY GRADIENT CENTRIFUGATION OF THE PARTICULATE PREPARATION OBTAINED AT 10,000 × g (MITOCHONDRIA) FROM CACTUS PHYLLOCLADES.

Maximum chlorophyll, protein, and enzymic activity occurred at a density of 1·102 (fraction 8). The distribution of malic dehydrogenase, glutamic-oxaloacetic transaminase, and glucose-6-phosphate dehydrogenase corresponded exactly with that of chlorophyll and protein indicating association with chloroplasts (Fig. 2). P-enolpyruvate carboxylase and malic enzyme activities were detected in fraction 8 (see Fig. 2), thus suggesting a similar correlation.

Distribution of Protein, Chlorophyll, and Enzymes in Sucrose Density Gradients of Mitochondrial Preparations

Sucrose density gradient centrifugation of mitochondrial preparations resulted in two protein and chlorophyll peaks (Fig. 3). The second peak (density = 1·102 to 1·134) contained less chlorophyll than the more dense peak (density = 1·20). Electron micrographs indicate that the lighter band contained good, intact mitochondria. Both malic dehydrogenase and glutamic-oxaloacetic transaminase were distributed in three peaks, two near the mitochondrial band (density = 1·102 and 1·134) and one in the chlorophyll dominated band (Fig. 4). Malic enzyme had maximal activities in two peaks (density = 1·20 and 1·134). Glucose-6-phosphate dehydrogenase also had two peaks occurring in fractions with densities of 1·20 and 1·102. These data suggest different particulate distributions of these enzymes.

DISCUSSION

The data presented in this report strongly suggest that in cactus, P-enolpyruvate carboxylase, malic dehydrogenase, glucose-6-phosphate dehydrogenase, glutamic-oxaloacetic transaminase, and the malic enzyme are localized, at least in part, in chloroplasts. Both aqueously and nonaqueously isolated chloroplasts, after washing and purifying by gradient centrifugation, gave positive assays for the above enzymes (Table 1 and Fig. 2). Although aqueously prepared chloroplasts contained activity, after sucrose density gradient fractionation, P-enolpyruvate carboxylase and malic enzyme activity were too low for reproducible assays. Prior to purification by density gradient centrifugation, however, activity was present. Chloroplasts prepared in nonaqueous solvents and purified on hexane-carbon tetrachloride gradients resulted in three distinct chloroplast bands. The activities of malic dehydrogenase, the malic enzyme, glutamic-oxaloacetic transaminase, and NADP-glyceraldehyde-3-phosphate dehydrogenase were constant when expressed on a chlorophyll basis. P-enolpyruvate carboxylase and glucose-6-phosphate dehydrogenase, however, were not distributed as a function of chlorophyll. Relatively more activity was present in the less dense chloroplasts. Although an explanation is not readily apparent, these results may indicate differences in localization within the chloroplasts.

Sucrose density gradient fractionation of aqueously isolated mitochondria did not result in a direct correlation between protein and enzymic activities (Figs. 3 and 4). Malic dehydrogenase and glutamic-oxaloacetic transaminase were distributed in three distinct bands, while malic enzyme and glucose-6-phosphate dehydrogenase occurred in two bands. P-enolpyruvate carboxylase was not detectable after layering. In general, these data suggest that glucose-6-phosphate dehydrogenase, the malic enzyme, malic dehydrogenase, and glutamic-oxaloacetic transaminase are present in both chloroplasts and mitochondria. P-enolpyruvate carboxylase activity was shown in chloroplasts, but insufficient evidence was obtained to demonstrate mitochondrial activity. When aqueous particles were prepared, most activity was found in the high-speed supernatant fraction. Undoubtedly, much of the supernatant

activity was leached from the particles. An interesting question which remains is whether or not these different enzymic activities, i.e. in chloroplasts, mitochondria, and perhaps in the high-speed supernatant, reflect different isozymes.

The intracellular location of CO_2 metabolism enzymes has been of interest in recent years. In 1957, Mazelis and Vennesland[4] and later Rosenberg *et al.*[5] demonstrated P-enolpyruvate carboxylase in chloroplasts of spinach. Garnier-Dardart[6] reported P-enolpyruvate carboxylase, malic dehydrogenase, malic enzyme, glucose-6-phosphate dehydrogenase, and NADP-oxidoreductase in chloroplasts of the succulent *Bryophyllum daigremontianum*, and Brandon[7] presented evidence for CO_2 metabolism enzymes in mitochondria of *B. tubiflorum*. Also, Mazelis and Vennesland[4] reported P-enolpyruvate carboxylase in mitochondrial fractions from cauliflower and pea seedlings. In corn-root tissue, in this laboratory, we were not able to find P-enolpyruvate carboxylase nor malic enzyme activities in mitochondria.[8] Slack and Hatch[9] reported P-enolpyruvate carboxylase, malic dehydrogenase, glucose-6-phosphate dehydrogenase, malic enzyme, as well as others in nonaqueously prepared chloroplasts of maize leaves. In crassulacean succulents[6] and in the cactus reported here, P-enolpyruvate carboxylase and associated enzymes were present in chloroplast preparations.

Important relative to the significance of CO_2 metabolism is the observation that the first stable products of photosynthesis in sugarcane were malic and aspartic acids;[10] the main products of dark CO_2 fixation. Later it was shown that sugarcane leaves contain P-enolpyruvate carboxylase activity.[9] Significantly, malate is a dominant product of photosynthesis in cactus.[11]

MATERIALS AND METHODS

Plant Material

Young phylloclades of the cactus, *Opuntia Ficus-indica* Mill., were freshly collected. The stem tissue was washed, cut into pieces, and vacuum infiltrated with 1% NH_3 for 2–3 hr in the cold (0–5°). The latter was used for aqueous extractions. For nonaqueous extractions, the tissue was lyophilized while frozen at approximately $-10°$ in a salt–ice mixture. The dried samples were stored under vacuum at $-10°$ until used.

Nonaqueous Chloroplasts

Chloroplasts were prepared essentially by the method of Stocking[12] and Bird *et al.*[13] Generally, duplicate 2·5 g samples of the lyophilized phylloclade material were ground at low speed in a Waring Blendor for 30 sec with about 75 ml of an ice-cold hexane–CCl_4 mixture (density = 1·50). The homogenate was filtered through four layers of cheesecloth and the filtrate centrifuged for 30 min at 12,000 × *g*. The green pellet deposited on the wall of the centrifuge tube near the surface together with the green supernatant solution were separated by decantation from the heavy sedimented material. The light supernatant fraction was

[4] M. MAZELIS and B. VENNESLAND, *Plant Physiol.* **32**, 591 (1957).
[5] L. L. ROSENBERG, J. B. CAPINDALE and F. R. WHATLEY, *Nature* **181**, 632 (1958).
[6] J. GARNIER-DARDART, *Physiol. Veg.* **3**, 215 (1965).
[7] P. C. BRANDON, *Plant Physiol.* **42**, 977 (1967).
[8] J. DANNER and I. P. TING, *Plant Physiol.* **42**, 719 (1967).
[9] C. R. SLACK and M. D. HATCH, *Biochem. J.* **103**, 660 (1967).
[10] H. P. KORTSCHAK, C. E. HARTT and G. O. BURR, *Plant Physiol.* **40**, 209 (1965).
[11] I. P. TING and W. M. DUGGER, *Botan. Gaz.*, in press.
[12] C. R. STOCKING, *Plant Physiol.* **34**, 56 (1959).
[13] I. F. BIRD, H. K. PORTER and C. R. STOCKING, *Biochim. Biophys. Acta* **100**, 366 (1965).

mixed with an equal volume of hexane. After recentrifugation of the latter at 4500 × *g* for 15 min, the supernatant liquid was discarded, and the sedimented material was suspended in 4 ml of a cold hexane and CCl_4 mixture (density = 1·42). The suspension was layered on a density gradient of hexane and CCl_4 from 1·420 to 1·538 and centrifuged at 1000 × *g* in a swing-out head (International Refrigerated Centrifuge, PR-2).

The material sedimented by the initial centrifugation (density greater than 1·50) was suspended in 4 ml of hexane and CCl_4 (density = 1·50) and layered on a density gradient ranging from 1·502 to 1·590 and centrifuged at 1000 × *g*.

Density layers were removed with a pipette and diluted with an equal volume of hexane. The particles were sedimented by centrifuging at 4500 × *g* for 15 min and the solvent was decanted. The preparation was dried by vacuum evaporation in the cold and the pellet was resuspended in 4 ml of 0·1 M tris, pH 7·4. Solubilization of the pellet was conducted by sonic irradiation at 60 W for 60 sec. Aliquots were removed for chlorophyll and protein determinations. After centrifuging for 10 min at 2000 × *g*, supernatants were assayed for enzymatic activity. All operations were completed in the cold (0–5°).

Aqueous Chloroplasts and Mitochondria

Chloroplasts and mitochondria were prepared according to Pierpoint.[14] After infiltration with 1 % NH_3, approximately 100 g (fresh weight) of tissue was homogenized in 4 to 5 volumes (wt./vol.) of sucrose–tris buffer (sucrose, 0·4 M; tris 0·2 M; EDTA, 5 mM; Na_3 citrate, 0·02 M; final pH = 7·8). Homogenization was conducted by grinding at low speed in a Waring Blendor for a total of 30 sec, in 5 sec increments. The resulting homogenate was squeezed through four layers of cheesecloth and centrifuged at 500 × *g* for 5 min, and the supernatant was centrifuged at 1000 × *g* for 20 min. The pellet was resuspended in 5 ml of the extracting medium and designated as chloroplasts. The 1000 × *g* supernatant was recentrifuged for 30 min at 10,000 × *g* to obtain a mitochondrial preparation. The latter was suspended in 5 ml of extracting medium. The 10,000 × *g* supernatant was designated as a soluble fraction. All operations were carried out at 0–5°. Enzyme activity, protein, and chlorophyll were determined in all three fractions. Sucrose gradients were made by layering sucrose solutions of decreasing densities in 25-ml cellulose nitrate tubes (solutions of sucrose used were 1·75 M (density = 1·228), 1·5 M (density = 1·200), 1·0 M (density = 1·134), 0·75 M (density = 1·102), 0·5 M (density = 1·070), and 0·25 M (density = 1·032) in 0·05 M tris buffer (pH 7·4). Gradients were established with 4 ml of sucrose of each solution except 2 ml of the lightest solution was placed at the top of the centrifuge tubes. The chloroplast or mitochondrial preparations were layered on the top after the interfaces of the sucrose layers were allowed to smooth by standing for at least 4–5 hr in the cold. The tubes were centrifuged at 1000 × *g* for 30 min in a swing-out head.

After centrifugation, a pin hole was made at the bottom of the tube and 10 to 11 fractions of approximately equal volume in decreasing densities were collected by applying a mild air pressure from above. All isolation steps were carried out at 0 to 5°. Solubilization of the pellet for enzymic activity was conducted by sonic irradiation in the same way as for the nonaqueous procedure.

Enzyme Assays

All enzyme assays were conducted in a dual beam spectrophotometer equipped with a recorder and automatic sample changer, by measuring the change in absorptivity of pyridine

[14] W. S. PIERPOINT, *Biochem. J.* **82**, 143 (1962).

nucleotide cofactors at 340 nm. Unless otherwise indicated, all reagents were prepared in the buffer used in the assays. Preliminary experiments were conducted to determine the pH optimum of each enzyme under assay conditions.

Malic dehydrogenase (L-malate; NAD oxidoreductase, EC 1.1.1.37) was assayed in the direction of oxaloacetate reduction with oxaloacetate, 1·2 μmoles; NADH, 0·4 μmole; tris 0·1 M, pH 7·4 and 20–50 times diluted enzyme; total volume = 3·0 ml.

P-enolpyruvate carboxylase (orthophosphate: oxaloacetate carboxy-lyase (phosphorylating) EC 4.1.1.31) activity was determined according to Danner and Ting[8] by coupling the reaction with endogenous malic dehydrogenase. The reaction mixture consisted (in μmoles) of P-enolpyruvate, 3; $NaHCO_3$, 2; $MgCl_2$, 20; NADH, 0·4; tris, 0·1 M, pH 7·4 and enzyme; total volume = 3·0 ml.

Malic enzyme (L-malate: NADP oxidoreductase (decarboxylating) EC 1.1.1.40) assay mixtures contained the following components in μmoles: L-malate, 1·5; $MnCl_2$, 3; NADP, 0·6; tris, 0·1 M, pH 7·2 and enzyme; total volume = 3·0 ml.

Glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate: NADP oxidoreductase, EC 1.1.1.49) assay mixtures contained the following components in μmoles in a final volume of 3·0 ml; glucose-6-phosphate, 5; $MgCl_2$, 10; NADP, 0·6; tris buffer, 0·1 M, pH 7·2 and enzyme.

Glutamic-oxaloacetic transaminase (L-aspartate: 2-oxoglutarate aminotransferase, EC 2.6.1.1) was assayed by coupling the reaction with endogenous malate dehydrogenase. The composition of the reaction mixture in μmoles was: α-ketoglutarate, 10; DL-aspartate, 10; NADH, 0·4; tris buffer, 0·1 M, pH 8·0 and enzyme; total volume = 3·0 ml.

NADP-dependent 3-phosphoglyceraldehyde dehydrogenase (D-glyceraldehyde-3-phosphate: NADP oxidoreductase, EC 1.2.1.9) was assayed according to Arnon.[15] The reaction mixture contained in μmoles of DL-glyceraldehyde-3-phosphoric acid, 3; $NaAsO_3$, 15; NaF, 10; NADP 0·6; tris buffer, 0·1 M, pH 8·5 and enzyme in a total volume of 3·0 ml.

Specific activity was expressed as change in optical density per min per mg protein, per mg chlorophyll or per ml of enzyme preparation. Protein was estimated by the method of Lowry *et al.*[16] as described earlier[17] and chlorophyll by the method of Arnon.[18]

Acknowledgement—We thank Dr. W. W. Thomson for the electron microscopy associated with this work.

[15] D. I. Arnon, *Science* **116**, 635 (1952).
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[18] D. I. Arnon, *Plant Physiol.* **24**, 1 (1949).