Intracellular Conversion of Malate and Localization of Enzymes Involved in the Metabolism of Malate in Photoautotrophic Cell Cultures of *Chenopodium rubrum*

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Cells from photoautotrophic cultures of *Chenopodium rubrum* were fractionated for the isolation of purified chloroplasts and mitochondria. The subcellular localization of the enzymatic activities involved in the metabolism of malic acid was investigated. Highly purified chloroplasts were obtained from the protoplasts, whereas peroxisomes were still present in the mitochondrial fraction. NAD- and NADP-dependent malate dehydrogenase and malic enzyme activities were found in the mitochondrial and chloroplast fractions, respectively. Exogenously supplied [^{14}C]labelled malate was metabolized by the photoautotrophic cell suspension, obviously to the greater part in mitochondria.

Introduction

Photoautotrophic cell suspension cultures of *Chenopodium rubrum* cells assimilate CO_2 predominantly by C 1→C 5-carboxylation mediated by the ribulosebisphosphate-carboxylase (RuBP-carboxylase), though phosphoenolpyruvate-carboxylase (PEP-carboxylase) also contributes to net CO_2 assimilation [1, 2]. Similar observations have been reported for tobacco cell cultures [3, 4]. The PEP-carboxylase-dependent formation of malate is activated by increasing the pH of the culture medium [5]. Besides its essential role in photosynthesis in C_4- and Crassulacean acid-metabolism plants, PEP-carboxylase seems to participate in the photosynthetic and non-photosynthetic metabolism of carbon through so-called anaplerotic reactions in C_3-plants, too [3, 6, 7]. The principal importance of such reactions is the supply of C_4-compounds to the tricarboxylic acid cycle as a source of carbon skeletons for various amino acids.

In the present report, the intracellular distribution of enzymes involved in malic acid metabolism in photoautotrophic cell cultures from *Chenopodium rubrum*, a C_3-plant, has been studied. In addition, I investigated the metabolism of exogenously supplied [^{14}C]labelled malate.

Some of the results presented in this paper were published in the proceedings of the VIIth International Congress of Plant Tissue Culture [8].

Materials and Methods

Plant material

Photoautotrophic cultures of *Chenopodium rubrum* cells were grown in a simple mineral salt medium according to Murashige and Skoog [9] in the presence of 2% CO_2 using two-tier culture flasks as previously described [10]. The cells were propagated on a gyratory shaker at 120 rpm and 25 °C under continuous illumination with white fluorescent light (100–110 μE m^{-2} sec^{-1}).

Isolation of protoplasts

The procedure was that of Bentrup *et al.* [11] with some modifications. Rapidly dividing cells (day 6 of the growth cycle; *ca.* 7 g fresh weight) were harvested by filtration and resuspended in 25 ml of medium I [20 mM Mes-KOH (pH 5.2), 300 mM mannitol, 2 mM $CaCl_2$, 10 mM KCl, 5 mM $MgCl_2$, 1 mM DTT and 0.5% (w/v) BSA] and allowed to settle for 15 min. Then, 25 ml of medium

Abbreviations: BSA, bovine serum albumin; DTT, dithiothreitol; GAPDH, D-glyceraldehyde-3-phosphate:NADP$^+$ oxidoreductase (phosphorylating) [EC 1.2.1.13]; NAD-MDH, L-malate:NAD$^+$ oxidoreductase [EC 1.1.1.37]; NADP-MDH, L-malate:NADP$^+$ oxidoreductase [EC 1.1.1.82]; NAD-ME, L-malate:NAD$^+$ oxidoreductase (decarboxylating) [EC 1.1.1.39]; NADP-ME, L-malate:NADP$^+$ oxidoreductase (decarboxylating) [EC 1.1.1.40]; PEP, phosphoenolpyruvate; RuBP, ribulose-1,5-bisphosphate.

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I, containing 1 g of cellulase TC (Serva, Heidelberg) and 1 g Pectinase 5S (Serva), was added. After digestion of cell walls for 120 min at 25 ± 1 °C on a shaker (120 rpm), protoplasts were collected by centrifugation at 4 °C and 100 × *g* for 15 min. The subsequent procedures were performed at 0–4 °C. The protoplasts were washed twice with medium II [20 mM Mes-KOH (pH 6.0), 300 mM mannitol, 25 mM KCl, 0.5 mM $MgCl_2$ and 0.1% (w/v) BSA]. For purification, the protoplast suspension was loaded on a discontinuous Ficoll gradient, 5% and 2.5% (w/w) Ficoll in medium II, and centrifuged at 100 × *g* for 15 min. The protoplasts which assembled at the interface of the 2.5% and 5% Ficoll were collected, washed and resuspended with medium II.

Isolation of chloroplasts

Solutions for isolation of chloroplasts were prepared with reference to the method of Leegood and Walker [12]. After sedimentation by centrifugation, the protoplasts were resuspended in ice-cold medium A [50 mM Mes-KOH (pH 6.1), 330 mM sorbitol, 2 mM KNO_3, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 0.5 mM KH_2PO_4, 20 mM NaCl, 2 mM Na_2EDTA, and 2 mM sodium-isoascorbate (added just before use)] and broken mechanically by passing twice through a 20 µm nylon mesh [13]. Released chloroplasts were collected by centrifugation at 1600 × *g* for 1 min, and resuspended in medium B [20 mM Hepes-KOH pH 7.6, 330 mM sorbitol, 1 mM Na_2EDTA, 0.2% (w/v) BSA] and centrifuged at 1600 × *g* for 2 min. The chloroplast pellet was resuspended in medium B and loaded on a 80% and 40% (v/v) Percoll gradient prepared in medium B and centrifuged at 1600 × *g* for 5 min. Chloroplasts banding at the interface between 80% and 40% Percoll were collected, washed and resuspended in medium B.

Evolution of oxygen from the chloroplast preparation was measured with four volumes of medium C [50 mM Hepes-KOH pH 7.6, 330 mM sorbitol, 2 mM Na_2 EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 5 mM sodiumpyrophosphate, 0.2 mM KH_2PO_4, 0.1 mM ATP] at 25 °C with a Clark-type oxygen-electrode (Bachofer, Tuttlingen, Germany).

Isolation of mitochondria

Protoplasts were suspended in medium D [25 mM Hepes-KOH pH 7.8, 330 mM sorbitol, 1 mM Na_2EDTA, 1 mM $MgCl_2$, 1 mM DTT, 2 mM sodium-ascorbate] and broken mechanically by two passages through 20 µm nylon mesh as described above. The cell debris and most chloroplasts were pelleted by centrifugation twice at 1600 × *g* for 4 min. The supernatant was centrifuged at 10,000 × *g* for 15 min (Sovall, SS-34 rotor). The pellet was resuspended in medium E (medium D without DTT and ascorbate), layered on 30 ml of 28% Percoll (v/v) in medium E in a Sovall SS-34 rotor tube, and centrifuged at 17,000 rpm for 40 min [14]. Mitochondria were found in a whitish band. This mitochondrial fraction was carefully removed and pelleted, after dilution with about five volumes of medium E, by centrifugating at 15,000 × *g* for 15 min. The pellet was resuspended with medium E and designated the mitochondrial preparation.

Assays of enzymatic activity

Enzymatic activities were extracted by mixing 200 µl of each preparation with 290 µl of extraction buffer and 10 µl of 20% (w/v) Triton X-100. The extraction buffer contained 25 mM Hepes-KOH pH 7.2, 2 mM $MnCl_2$, 5 mM DTT for assays of NADP-ME and NAD-ME; 25 mM Hepes-KOH at 7.5, 0.5 mM EDTA for assays of NADP-MDH, NAD-MDH and cytochrome c oxidase; and 50 mM Hepes-KOH pH 7.8, 1 mM DTT, 1 mM EDTA, 2.5 mM $MgCl_2$ for assays of other enzymes [15, 16].

PEP-carboxylase (EC 4.1.1.31) activity was assayed enzymatically by following the oxidation of NADH at 340 nm as described previously [15], except that 50 µl of the enzyme solution and 2 units of malate dehydrogenase were used.

RuBP-carboxylase (EC 4.1.1.39) activity was measured spectrophotometrically as described previously [15], while 1 mM RuBP as the substrate.

NADP-glyceraldehyde-phosphate dehydrogenase (GAPDH, EC 1.2.1.13) activity was determined by the decrease in absorbance at 340 nm, due to the oxidation of NADPH, following the method of Holtum and Winter [17] with modifications. The test solution (1 ml) contained 50 mM Tricin-Hepes-NaOH pH 9.0, 3 mM 3-phosphoglycerate, 2 mM $MgCl_2$, 0.1 mM NADPH, 2 mM DTT, 4 units of phosphoglyceratekinase, 1 mM ATP, 25–50 µl enzyme solution. The reaction was started by addition of ATP.

Fumarase (EC 4.2.1.2) was measured spectrophotometrically by following the production of fumarate from malate at 240 nm, as described by Hill and Bradshaw [18], in a total volume of 1 ml.

Cytochrome c oxidase (EC 1.9.3.1) activity was determined by following the decrease in levels of ferrocytochrome c at 550 nm and 30 °C [19]. The reaction mixture (1 ml) contained 10 mM potassium-phosphate buffer pH 7.0, 0.7% ferrocytochrome c.

Catalase (EC 1.11.1.6) activity was assayed spectrophotometrically according to Vigil [20], in a total volume of 1 ml.

Hydroxypyruvate reductase (EC 1.1.1.81) was assayed as described by Tolbert *et al.* [21].

NAD-malic enzyme (NAD-ME, EC 1.1.1.39) activity was determined as described by Hatch *et al.* [16]. The reaction mixture was preincubated for 30 min before the start of the reaction in order to reduce interference by NAD-MDH activity [16].

NADP-malic enzyme (NADP-ME, EC 1.1.1.40) activity was determined by the method of Raghavendra and Das [22]. The reaction mixture (1 ml) contained 25 mM Hepes-KOH, pH 8.0, 0.5 mM EDTA, 2.5 mM malate, 0.25 mM NADP, 2 mM DTT, 10 mM $MgCl_2$, and 100 µl of the enzyme solution. The reaction was started by addition of $MgCl_2$.

NAD-malate dehydrogenase (NAD-MDH, EC 1.1.1.37) and NADP-malate dehydrogenase (NADP-MDH, EC 1.1.1.82) activities were determined as described by Hatch *et al.* [16] with minor modifications. The reaction mixture contained 25 mM Hepes-KOH pH 7.5, 0.5 mM EDTA, 0.2 mM NADH or 0.4 mM NADPH, 3 mM (for assays of NAD-MDH) or 5 mM (for assays of NADP-MDH) oxaloacetic acid (pH 5.5), and 10–50 µl of the enzyme solution. The reaction was started by addition of oxaloacetate.

[^{14}C]Tracer experiments

[4-^{14}C]Malate was synthesized in reactions catalyzed by PEP-carboxylase and malate dehydrogenase. The reaction mixture (0.5 ml) contained 50 mM Hepes-KOH (pH 7.8), 10 mM $MgCl_2$, 2 mM DTT, 10 mM NADH, 10 mM PEP, 2 units PEP-carboxylase (Sigma), 2 units MDH (Sigma) and 3.7 MBq (about 2 µmol) $NaH^{14}CO_3$ (Amersham), and it was incubated at 30 °C for 30 min. All the solutions without $NaH^{14}CO_3$ were bubbled with N_2 before the incubation. The reaction was stopped by addition of 2 ml of a mixture of CH_3OH/HCOOH (1:1, v/v) and allowed to settle at 80 °C for 2 min. [4-^{14}C]Malate was isolated and purified by TLC [2] on cellulose plates. About 99% of the [^{14}C]radioactivity was located in C4-carbon of the malic acid molecule, as could be calculated by the decarboxylation reaction using commercial NADP-ME (Sigma) in the mixture used for the assay of the NADP-ME described above.

Tracer experiments were performed as described by Hüsemann *et al.* [2] with modifications. Cells were harvested by filtration and 0.5 g (fresh weight) of cells were resuspended in 1.0 ml of the same culture medium in which they had been grown before. The cell suspension was allowed to settle into a Warburg flask constructed with two small chambers and preincubated for 1 h at 25 °C under 2% CO_2 in the light (100 µE m^{-2} sec^{-1}), as described previously [2]. After the preincubation, 370 kBq of [U-^{14}C]malate or [4-^{14}C]malate (final concentration 2 mM) were added to the cells and they were further incubated in the light or in darkness. The uptake of malate by the cells was determined by measuring the radioactivity left in the medium. After incubation for 60 min, 4 ml of a boiling solution of HCOOH and CH_3OH (1:1, v/v) was added to the cell suspension.

The metabolic flow of malate was determined as follows. The metabolically produced $^{14}CO_2$ was absorbed by 2 M NaOH which was injected into the second chamber of the Warburg flask. The cells were homogenized with a glass homogenizer. The homogenate was centrifugated and then extracted twice with 70% (v/v) methanol. The resultant pellet was designated as the insoluble fraction, and the combined extracts as the soluble fraction. The soluble fraction was further analyzed by thin-layer chromatography (TLC) [2, 15]. The radioactivity in each fraction or in each spot on the TLC plates was determined by liquid scintillation counting.

Chlorophyll content

The amounts of chlorophylls a and b were determined in 80% (v/v) acetone extracts as described by Ziegler and Egle [23].

Results

Chloroplast preparation and enzymatic activities

Table I shows the activities of various marker enzymes, malic enzymes and malate dehydrogenases in the preparations of protoplasts and chloroplasts. In the chloroplast preparation there was little or no activity of fumarase, PEP-carboxylase and catalase, indicating that the preparation was almost free of mitochondria, cytoplasm and peroxisomes. The rate of oxygen evolution from the preparation was 30–50 μmol mg^{-1} chl h^{-1} in the presence of 3 mM 3-phosphoglycerate, although the rate of $^{14}CO_2$-assimilation was only 3–8 μmol mg^{-1} chl h^{-1}. These values are not so low if compared with the photosynthetic activity of cultured cells previously determined with $NaH^{14}CO_3$ [5]. Moreover, the intactness of the chloroplasts in the preparation, determined by ferricyanide test [12], was at least 96%. Therefore, the chloroplast envelope appeared to be intact. The intactness of the chloroplasts was also examined with a phase-contrast microscope.

With respect to malic enzymes and malate dehydrogenases, similarly high levels of NADP-ME and NADP-MDH activities to those in the protoplast preparation, a low level of NAD-MDH activity, and no activity of NAD-ME were detected in the preparation of chloroplasts (Table I).

Mitochondrial preparation and enzymatic activities

Enzymatic activities determined in one experiment are shown in Table II. The recovery of enzymatic activities in the mitochondrial preparation from the protoplast preparation was different in each experiment. Thus, the recoveries of enzymatic activities calculated with reference to the recovery of fumarase are shown in Fig. 1. There was little or no evidence of markers of the cytoplasm and chloroplasts in the mitochondrial preparation, and the contamination by these components of the cell seemed to be negligible. However, the recovery rate of catalase and hydroxypyruvate reductase (HPR) activity was 20–40% corresponding to fumarase activity. This might be due to an incomplete separation of the mitochondria from peroxisomes. In fact, the distribution of catalase and fumarase activities in Percoll gradients overlapped (Fig. 2). In comparison to the recovery of fumarase, about the same level of recovery of NAD-ME was observed, while recoveries of NAD-MDH and NADP-MDH activities were about 50% and

Table II. Activities of enzymes in the preparations of protoplasts and mitochondria in the experiment I (Fig. 1). Each value is expressed in nmol min^{-1} ml^{-1}.

Enzyme	Protoplasts	Mitochondria
NAD-MDH	5991	1387
NADP-MDH	172.5	63.0
NAD-ME	92.7	44.4
NADP-ME	16.8	n.d.
Fumarase	211.1	96.7
Cytochrome *c* oxidase	21.3	10.2
GAPDH	895.2	n.d.
PEP-carboxylase	87.9	n.d.
Catalase	92010	15070
HPR	282.3	50.4

n.d., not detected; HPR, hydroxypyruvate reductase.

Table I. Activities of enzymes in the preparations of chloroplasts and protoplasts (μmol mg^{-1} chl · h^{-1}).

Enzyme	Protoplasts (A)	Chloroplasts (B)	(B)/(A) %
NAD-MDH	10060 ± 1590	276 ± 61	2.6 ± 0.3
NADP-MDH	100.4 ± 33.9	72.4 ± 18.5	72.9 ± 10.3
NAD-ME	66.9 ± 8.9	1.3**	1.7**
NADP-ME	27.0 ± 3.3	19.1 ± 3.3	71.3 ± 8.5
RuBP-carboxylase	84.6 ± 9.8	74.4 ± 4.1	91.7 ± 7.7
GAPDH	253.7 ± 36.2	219.3 ± 26.4	89.7 ± 8.9
PEP-carboxylase	80.6 ± 3.9	n.d.	0
Fumarase	133.5 ± 18.3	0.3*	0.2*
Catalase	46570 ± 6350	n.d.	0

n.d., not detected.
*, ** Only once (*) or twice (**) in five experiments was the activity detected.

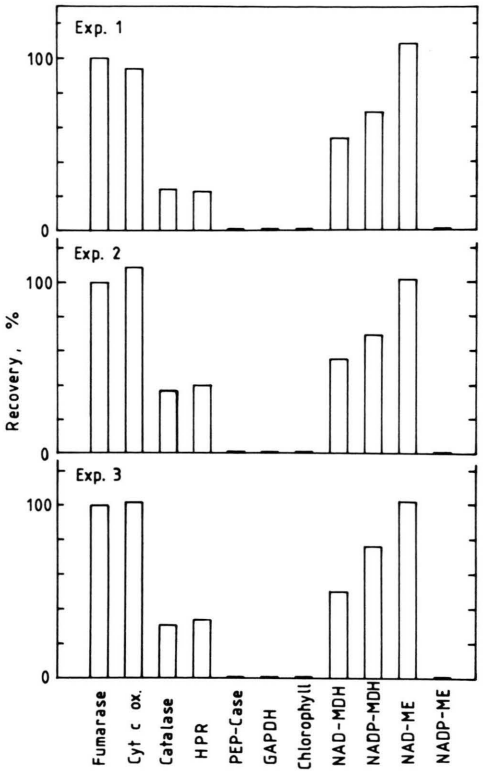

Fig. 1. Recovery of enzymatic activities in mitochondria isolated from protoplasts. Each value was calculated with reference to the recovery of fumarase activity (100%). Abbreviations: Cyt c ox., cytochrome *c* oxidase; HPR, hydroxypyruvate reductase; PEP-Case, PEP-carboxylase.

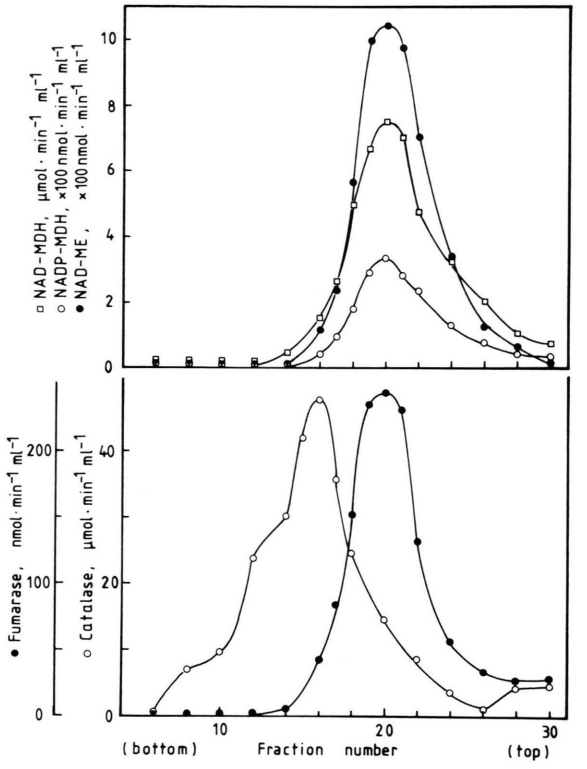

Fig. 2. Distribution of enzymatic activities in a Percoll gradient.

70% of that of fumarase, respectively. No activity of NADP-ME was detected.

Metabolism of malate

Table III shows the uptake of exogenously supplied ^{14}C-labelled malate and the distribution of the radioactivity after incubation of 60 min. About 95% of the added malate was absorbed by the cells in 60 min. The uptake after 30 min of incubation was about 85% and there was no significant difference in the rate of uptake between [4-^{14}C]malate and [U-^{14}C]malate. After the incubation, only 4–5% of the absorbed radioactivity was found in malate, in the light and in darkness (Table III).

In the light, a large proportion of the radioactivity from absorbed malate was found in the 70% methanol-soluble fraction, and the major ^{14}C-labelled compounds were aspartate and alanine. In the dark, a large amount of $^{14}CO_2$ was released from exogenously supplied malate, and in particular from [4-^{14}C]malate. Less radioactivity was transferred to sugars and to the 70% MeOH-insoluble fraction in darkness than in the light. No radioactive succinate was found after [4-^{14}C]malate had been added (Table III).

Discussion

From the patterns of distribution of the activities of marker enzymes (Table I) as well as from the measurements of oxygen evolution, it can be concluded that the chloroplasts were highly purified and intact. On the other hand, the mitochondrial preparation still contained peroxisomes. The

Table III. Uptake of exogenously added [U-^{14}C]malate or [4-^{14}C]malate by *Chenopodium* cells and distribution of the radioactivity after incubation for 1 h in the light or in darkness. The average chlorophyll content (in 1.5 ml cell suspension) was 95.5 ± 3.4 µg.

Position of radioactive C atom in malate	Light		Dark	
	[U-^{14}C]	[4-^{14}C]	[U-^{14}C]	[4-^{14}C]
Uptake (%)	96.5 ± 0.7	95.9 ± 0.8	95.1 ± 2.2	95.0 ± 2.5
Metabolized (% of uptake)	95.9 ± 0.2	95.2 ± 0.3	96.4 ± 1.4	96.3 ± 0.8
Distribution of radioactivity metabolized (%)				
$^{14}CO_2$	4.0 ± 0.1	7.1 ± 1.0	38.8 ± 2.0	66.1 ± 3.8
70% MeOH-insoluble**	17.1 ± 1.4	22.5 ± 1.1	5.4 ± 0.3	4.2 ± 0.2
70% MeOH-soluble**	78.9 ± 1.5	70.4 ± 2.0	55.8 ± 2.2	29.7 ± 4.0
Sugars	8.5 ± 1.5	18.0 ± 3.0	0.3*	0.2*
Asp	27.3 ± 3.0	17.4 ± 1.9	27.2 ± 0.4	16.9 ± 1.9
Ala	27.6 ± 1.2	25.3 ± 1.0	15.4 ± 1.6	6.0 ± 2.3
Gly/Ser/Gln	4.5 ± 1.2	4.2 ± 1.7	5.6 ± 1.6	3.0 ± 0.3
Fumarate	1.3 ± 0.5	1.3 ± 0.4	1.4 ± 0.3	1.2 ± 0.2
Succinate	2.5 ± 0.2		2.5 ± 0.6	
Citrate	3.0 ± 2.0	1.7 ± 1.3	2.4 ± 1.0	2.2 ± 0.3
N.i.***	4.2 ± 1.4	2.5 ± 1.4	1.0 ± 0.2	0.2 ± 0.1

Each values is expressed as a mean ± S.D. (n = 3).
* Detected only twice in the three experiments.
** 70% methanol-soluble and -insoluble fractions.
*** Not identified.

contamination of isolated mitochondria by peroxisome enzymes has been observed in many other cases (*e.g.* [14, 24]). NAD-MDH activity was detected in the mitochondrial preparation but very little was found in the chloroplasts. In C_3 plants, three different isozymes of NAD-MDH have been reported in general, being in the cytosol, in the mitochondria and in the microbody, respectively (*e.g.* [25]). In the present experiment, the recovery of NAD-MDH activity in mitochondria was about half of that of fumarase activity (Table II, Fig. 1). This might result from the cytosolic isozymes. However, the recovery of NAD-MDH activity seems to be independent of the rate of contamination by catalase or hydroxypyruvate reductase activities. As can be deduced from the pattern of distribution of enzymatic activities in a Percoll gradient (Fig. 2), the NAD-MDH activity observed in the mitochondrial preparation could be due to the mitochondria themselves and not to contamination by peroxisomes.

NAD-MDH activity in peroxisomes in photoautotrophic tissues is thought to be involved in supplying the reducing equivalent that is coupled with the hydroxypyruvate reductase reaction in the photorespiration pathway [26]. It is possible that photorespiration activity is not high in photoautotrophically cultured cells because they are growing under 2% CO_2 and, furthermore, that there is little or no NAD-MDH activity in peroxisomes.

NAD-ME was also detected in the mitochondrial preparation and the extent of its recovery was about the same as that of fumarase (Fig. 1). This result might indicate that the activity of NAD-ME is located exclusively in mitochondria. This conclusion corresponds to the distribution of the activity of NAD-ME in a Percoll gradient (Fig. 2).

NADP-MDH activity was present in the preparations of both chloroplasts and mitochondria. NADP-MDH has been suggested to be a chloroplast enzyme (*e.g.* [27]). The recovery of the activity per chlorophyll was 72.9% on average and the result does not exclude the possible presence of isozymes in other cellular compartments. The activation by light of NADP-MDH is well known [28], and there may be various inhibitory or stimulatory factors in the crude preparation of enzyme. Thus, it is difficult to assess the possibility of isozymes in this system.

The presence of the activity of NADP-MDH activity in the mitochondrial preparation seemed to be mainly due to insufficient specificity of the NAD-MDH [29].

It has been reported [30, 31] that NADP-ME is located in the chloroplasts of the bundle sheath cells in C_4 plants, while this enzymatic activity has also been found in the cytoplasm in Crassulacean acid metabolism plants [32, 33], in *Pisum* roots [34] and in tomato fruits [35]. The present results indicate that NADP-ME seems to be present in chloroplasts (Table I), although the possibility of isozymes in other cellular compartments cannot be completely excluded.

In summary, NAD-dependent malate dehydrogenase and malic enzyme activities are distributed in mitochondria and NADP-dependent activities are distributed in chloroplasts, although isozymes in other cellular compartments might possibly exist.

When malate was supplied exogenously to the cells, the cells absorbed and metabolized it (Table III). In the dark, a large amount of $^{14}CO_2$ was released, suggesting that the absorbed malate was predominantly metabolized through the malic enzyme (decarboxylating) reaction and/or through the tricarboxylic acid (TCA) cycle. This hypothesis coincides with the result that more radioactivity was released from [4-^{14}C]malate. The radioactive aspartate and alanine formed may have been synthesized *via* transamination from oxaloacetate and pyruvate, respectively. The citrate formed may be produced *via* reactions of the TCA cycle, and fumarate through equilibrium of the fumarase reaction. In this equilibrium, the C4 atom of malate could be replaced by a C1 atom, and radioactive alanine could also be formed from [4-^{14}C]malate. The C1 or C4 atom of malate was released as CO_2 through the TCA cycle, it is, therefore, reasonable that no radioactive succinate was found when [4-^{14}C]malate was supplied.

In the light, less radioactivity was found as $^{14}CO_2$, while more radioactivity was recovered in sugars and in the 70% methanol-insoluble fraction. The most possible interpretation of the differences between events in the light and in darkness is that $^{14}CO_2$ is released at about the same rate in the dark and in the light and that released $^{14}CO_2$ is reincorporated through photosynthetic reactions.

There remains the problem of the cellular compartment in which malate is metabolized. Aoyagi and Bassham [36] suggested that CO_2 is assimilated through the C_4 pathway (NADP-ME type), even in C_3 plant cells when they are cultured *in vitro*. The present results showed that mitochondria participate in the metabolism of malate, for instance, [^{14}C]alanine was formed from [4-^{14}C]-malate in the dark. Moreover, the radioactivity was recovered in aspartate and the intermediates of the TCA cycle to a similar extent in the dark and in the light. Thus, it is likely that malate is metabolized preferentially in mitochondria, and that the PEP carboxylase in photoautotrophic cultures of *Chenopodium* cells may participate in the so-called anaplerotic reactions.

The present results show that highly purified and intact organelles can be isolated from photoautotrophic cultures of *Chenopodium rubrum* cells, and the roles of PEP-carboxylase and related reactions seem to have been clarified to some extent.

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