Plant Physiol. (1979) 64, 1099–1103
0032-0889/79/64/1099/05/$00.50/0

Pyruvate Dehydrogenase Complex from Chloroplasts of *Pisum sativum* L.[1]

Received for publication April 2, 1979 and in revised form July 30, 1979

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ABSTRACT

Pyruvate dehydrogenase complex is associated with intact chloroplasts and mitochondria of 9-day-old *Pisum sativum* L. seedlings. The ratio of the mitochondrial complex to the chloroplast complex activities is about 3 to 1. Maximal rates observed for chloroplast pyruvate dehydrogenase complex activity ranged from 6 to 9 micromoles of NADH produced per milligram of chlorophyll per hour. Osmotic rupture of pea chloroplasts released 88% of the complex activity, indicating that chloroplast pyruvate dehydrogenase complex is a stromal complex. The pH optimum for chloroplast pyruvate dehydrogenase complex was between 7.8 and 8.2, whereas the mitochondrial pyruvate dehydrogenase complex had a pH optimum between 7.3 and 7.7. Chloroplast pyruvate dehydrogenase complex activity was specific for pyruvate, dependent upon coenzyme A and NAD and partially dependent upon Mg^{2+} and thiamine pyrophosphate.

Chloroplast-associated pyruvate dehydrogenase complex provides a direct link between pyruvate metabolism and chloroplast fatty acid biosynthesis by providing the substrate, acetyl-CoA, necessary for membrane development in young plants.

PDC[3] catalyzes the oxidative decarboxylation of pyruvate to yield acetyl-CoA, CO_2 and NADH according to the equation:

$$\text{Pyruvate} + \text{CoA} + \text{NAD}^+ \rightarrow \text{Acetyl-CoA} + CO_2 + \text{NADH} + H^+$$

The complex is composed of three enzymes acting sequentially: pyruvate dehydrogenase (decarboxylating), dihydrolipoyl transacetylase, and dihydrolipoyl dehydrogenase (8, 23). The first enzyme, pyruvate dehydrogenase, acts irreversibly and is regulated by covalent modification by a phosphorylation (inactivation)-dephosphorylation (activation) mechanism (13, 19, 20, 23). In addition, the complex is regulated by feedback-product inhibition (NADH, acetyl-CoA) (19, 20, 26, 28, 29) and these products also affect phosphorylation of pyruvate dehydrogenase (8, 10).

In eucaryotic tissues, PDC is usually associated with mitochondria (8, 23). Reports describing PDC in higher plants are limited, but the mitochondrial complex has been prepared from floral buds of cauliflower and broccoli (20, 26), etiolated pea epicotyls (25, 28), potato tubers (3), spinach leaves (21), and germinating and developing castor bean endosperm (22, 24, 25). The only reported extramitochondrial locations of PDC in eucaryotic tissues are proplastids of developing (24, 25, 28), but not germinating (22), castor bean endosperm and crude pea chloroplast preparations (5).

Chloroplast fatty acid biosynthesis is essential to photosynthesis and growth of plants. Indeed, the site of *de novo* synthesis of C_{16} and C_{18} fatty acids may be only in the chloroplast (17). However, the source of acetyl-CoA necessary for fatty acid synthesis in developing chloroplasts has never been established (27). Acetate is the conventionally used substrate for chloroplast fatty acid biosynthesis (27, and references therein) and an activating enzyme, acetyl-CoA synthetase, has been described from potato tubers (27). The origin of acetate and subsequent activation to acetyl-CoA in green leaf tissues is obscure (27) and may be nonphysiological (16).

Spinach chloroplasts support ^{14}C-fatty acid synthesis from [2-^{14}C]pyruvate with predicted product label distributions and rates comparable to [1-^{14}C]acetate (15, 16, 27). Label from [1-^{14}C]pyruvate is released as $^{14}CO_2$ (27). Pyruvate apparently enters and exits the chloroplast readily and can be synthesized in the cytosol or chloroplast via glycolysis from hexose- or triose-P photosynthate. A pathway for chloroplast fatty acid synthesis from CO_2 via pyruvate and acetyl-CoA has been proposed (16, 31), but evidence for chloroplast PDC is limited. This report confirms the presence of PDC in pea chloroplasts as well as in mitochondria of green pea leaves and provides a direct link between pyruvate metabolism and acetyl-CoA synthesis for chloroplast fatty acid production.

MATERIALS AND METHODS

Plant Tissue. Pea seeds (*Pisum sativum* L. var. Little Marvel) were grown in moist Vermiculite. Seedlings were grown at 70 $\mu E/m^2 \cdot s$ (400–700 nm) in a growth chamber with a 12-h photoperiod (22 C light/18 C dark) provided by fluorescent bulbs supplemented with far red light. Green shoots (3–5 cm tall) were harvested 9 days after planting following a 3-h exposure to light.

Isolation of Chloroplasts. All extractions were performed at 0 to 4 C. Chloroplasts were isolated by a modified procedure of Murphy and Leech (15, 16). Shoot material was homogenized in 100-g batches with 150 ml buffer A (0.3 M D-sorbitol, 50 mM Tricine [pH 8.0], 4.4 mM sodium pyrophosphate, 1 mM EDTA, 3.5 mM $MgCl_2$, and 13.5 mM 2-mercaptoethanol) with short (1-s) high speed bursts in a chilled Braun homogenizer. The crude extract was squeezed through eight layers of cheesecloth, gravity-filtered through two layers of Miracloth (Chicopee Mills, Inc.), and centrifuged at 3,020*g* (90 s, rest to top speed to rest). Chloroplast pellets were washed twice by gentle resuspension using a cotton swab in 5 ml buffer A, filtered through a fine-mesh nylon screen and centrifuged at 3,020*g* as above. Twice-washed chloroplasts were resuspended in 10 ml buffer A, layered on 10 ml buffer B (0.6 M D-sorbitol, 50 mM Tricine [pH 8.0], 4.4 mM sodium pyrophosphate, 1 mM EDTA, 3.5 mM $MgCl_2$, and 13.5 mM 2-mercaptoethanol) and pelleted by centrifugation at 2,000 rpm (5 min) in a Beckman model L2-65B centrifuge (SW 27 rotor). Pelleted chloroplasts were resuspended in a minimal volume of buffer B

[1] This work was supported by National Science Foundation Grant PCM-77-11390. Missouri Agricultural Experiment Station Series No. 8338.
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[3] Abbreviations: PDC: pyruvate dehydrogenase complex; NAD-IDH: NAD-specific isocitrate dehydrogenase; RuBP: ribulose bisphosphate; MOPS: morpholinopropanesulfonic acid; α-KG: α-ketoglutarate; TPP: thiamine pyrophosphate.

containing 1 mg DTT/ml and stored on ice unless otherwise noted.

Isolation of Mitochondria. Mitochondria were purified from the crude extract supernatant by centrifugation at 2,000*g* (15 min) followed by centrifugation of the supernatant at 14,500*g* (30 min). Mitochondria-enriched pellets were washed twice by resuspending in buffer C (0.3 M sucrose, 50 mM K-phosphate [pH 7.8], 10 mM KCl, 0.1% [w/v] BSA [fraction V], and 13.5 mM 2-mercaptoethanol) followed by centrifugation at 10,800*g* for 15 min. Mitochondria were resuspended in a minimal volume of buffer C containing 1 mg DTT/ml and stored on ice.

Assays. Protein was determined as in reference 14 using crystalline BSA as the standard. Standard procedures were used to assay Chl (1), PDC (20, and Table III), fumarase (7), NAD-IDH (2), and ferricyanide-dependent O_2 evolution (12). One unit of PDC and NAD-IDH activity was defined as 1 μmol NADH produced/min. Relative amounts of pea RuBP carboxylase were determined by a rocket immunoelectrophoresis assay (9) using a carboxylase standard purified from tall fescue (*Festuca arundinacea* Schreb.) and rabbit anti-RuBP carboxylase (tall fescue).

RESULTS

Preparation of Chloroplasts. Preliminary experiments showed that substantial PDC activity was associated with seedling mitochondria. Therefore, pea seedling chloroplasts were prepared by a rigorous technique involving several washes and pelleting steps to minimize mitochondrial contamination. Although this procedure gave low yields of intact chloroplasts, as measured by recovery of Chl (6–11%) and RuBP carboxylase (8–10%) (Table I), these purified chloroplasts were estimated to be at least 90% intact (class I) by ferricyanide-dependent O_2 evolution and phase contrast and electron microscopy (Fig. 1). Most of the chloroplasts appeared mature with well developed grana, but about 10% of the population contained extensive networks of prolamellar bodies. Nominal mitochondrial contamination was observed in electron micrographs (Fig. 1) and activity of NAD-IDH, a mitochondrial enzyme, was barely detectable. Fumarase activity was absent.

Chloroplast PDC. Table I shows the distribution of PDC activity and organelle markers in a typical preparation of chloroplasts and mitochondria from 9-day-old pea seedlings. About 50% of the crude extract PDC was routinely isolated with the mitochondrial fraction. A smaller, consistent level of PDC activity was always associated with mitochondria-free chloroplast preparations. This activity could not be reduced or abolished by washing nor was it associated with chloroplast envelopes via nonspecific protein binding (see below). PDC to Chl ratios in several chloroplast preparations ranged from 0.1 to 0.15 units/mg Chl, but this ratio was reduced by about 50% in preparations from older tissues (14–16 days old) (data not shown). Lower PDC to Chl ratios in older tissues was partly due to increased Chl content, but the total activity of PDC isolated per g fresh weight of seedlings was also reduced. PDC to NAD-IDH ratios in several chloroplast preparations were 13 or greater while in mitochondria the ratio was about 1.3. Fumarase activity was absent in all chloroplast preparations.

Table I. *Distribution of PDC and Marker Activities in Chloroplasts and Mitochondria of 9-Day-Old Pea Seedlings*

Two hundred g of pea seedlings were extracted.

Assay	Crude Extract	Chloroplasts	Mitochondria
Protein (mg)	6800	190	440
Chl (mg)	128	11 (8.6)[a]	
RuBP carboxylase (relative units)	100	8.2	
NAD-IDH (units recovered)	37	0.1	16 (43)[b]
PDC (units recovered)	41	1.3	20
PDC (total units associated with organelle)		15 (24)[c]	47 (76)[c]
PDC/Chl (units/mg)		0.12	
PDC/NAD-IDH		13	1.3

[a] Per cent of crude extract Chl.

[b] Per cent of crude extract NAD-IDH.

[c] Per cent contribution of organelle PDC to total plant PDC, average of three experiments, SD = 2.5. See calculations under "Results."

The activity of organelle markers (chloroplasts: Chl and RuBP carboxylase; mitochondria: NAD-IDH) was used to calculate relative contributions of chloroplast and mitochondrial PDC to crude extract PDC. Assuming constant PDC to Chl and PDC to NAD-IDH ratios for chloroplasts and mitochondria, respectively, throughout the isolation procedure, the formulas for calculating per cent contribution were:

$$\text{Total chloroplast PDC (units)} = \frac{\text{Recovered chloroplast PDC (units)}}{\text{\% Yield Chl or RuBP carboxylase}} \times 100$$

$$\text{Total mitochondrial PDC (units)} = \frac{\text{Recovered mitochondrial PDC (units)}}{\text{\% Yield NAD-IDH}} \times 100$$

Substitution of appropriate Table I values into these equations gave 15 units chloroplast PDC and 47 units mitochondrial PDC, or 24 and 76% (SD = 2.5 for three independent experiments) of the seedling PDC, respectively (Table I).

The extent of nonspecific association of PDC with the chloroplast envelope during preparation of the chloroplast fraction was evaluated by preparation of chloroplast envelope, thylakoid (stroma lamellae and grana), and stroma fractions (4). Each component was assayed for PDC activity. This method takes advantage of the fact that gentle osmotic rupture of intact chloroplasts results in complete detachment of the envelope and release of stroma contents. Of the recovered PDC, 88% was found in the stroma fraction and 12% remained associated with the thylakoid fraction. These results do not support nonspecific association of PDC with the chloroplasts during preparation and indicate that PDC is a soluble, stromal enzyme (see below).

Extraction of Chloroplast PDC. The association of PDC with chloroplasts and its distribution within the organelle were further characterized by a comparison of the extraction properties of PDC with RuBP carboxylase, a known stromal enzyme, and total stromal protein. Chloroplasts were repeatedly extracted by osmotic rupture and separated into stromal and membrane fractions which were assayed for PDC, RuBP carboxylase, and protein. If PDC were a chloroplast stromal enzyme, extraction of the complex should parallel the distributions of RuBP carboxylase and stromal protein in sequential extracts of chloroplasts. The results presented in Table II clearly bear out these predictions. The absence of RuBP carboxylase in the twice-extracted membrane fraction signifies complete extraction of stromal protein. Of the total soluble PDC, RuBP carboxylase, and protein, 89, 95, and 91%, respectively, were recovered in the first extract. As observed in the previous experiment, about 13% of the recovered PDC remained with the membrane fraction.

Effect of pH on Chloroplast and Mitochondria PDC Activity. The effect of pH on chloroplast and mitochondrial PDC is illustrated in Figure 2. The optimal pH for chloroplast PDC was between 7.8 and 8.2, with the activity falling off rapidly at more alkaline values. The optimum pH for mitochondrial PDC was 7.3 to 7.7. Mitochondrial PDC activity, but not chloroplast PDC activity, was markedly reduced (about 60%) at all pH values when the assay buffer concentration was increased from 50 mM to 83.3 mM MOPS-glycylglycine. The points shown in Figure 2 are the pH values of the reaction mixture after assay. The final pH of the

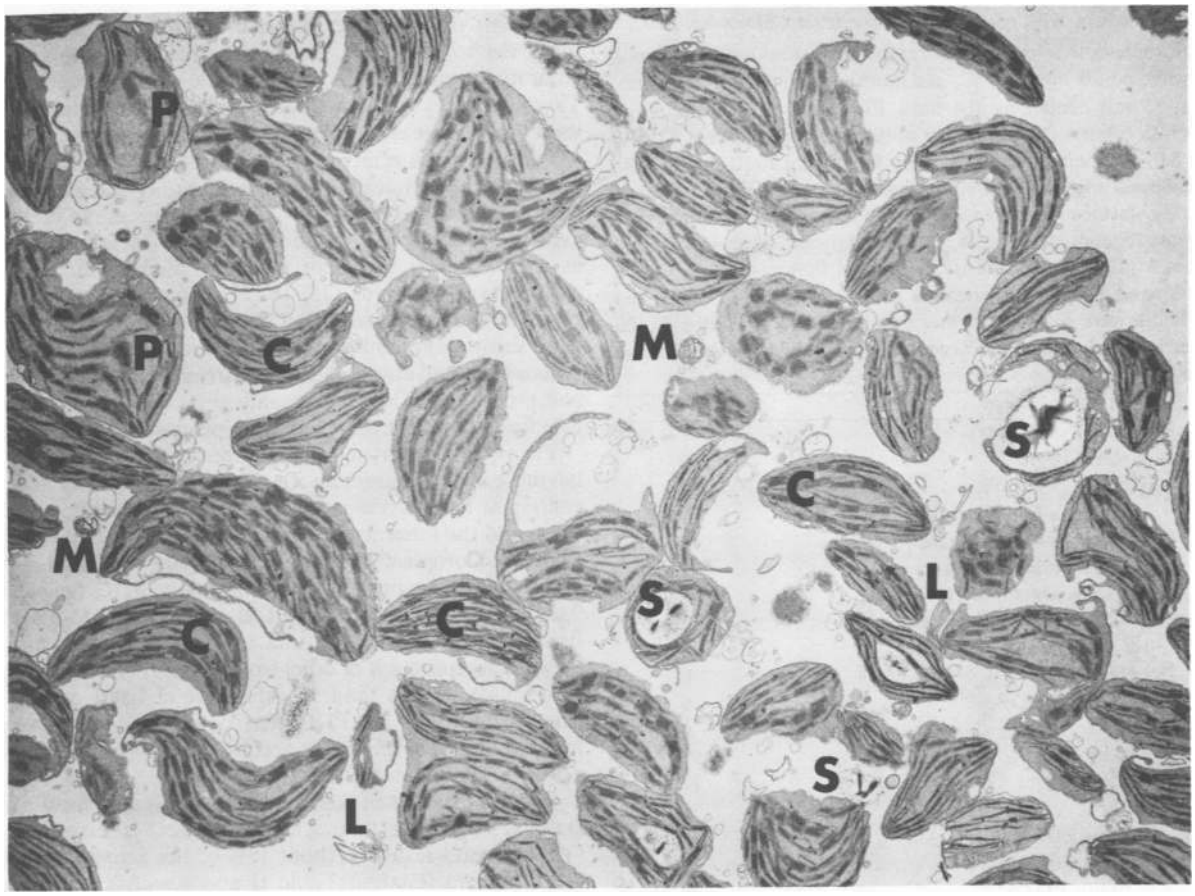

FIG. 1. Electron micrograph of pea chloroplasts. Chloroplasts suspended in buffer B were fixed in 3% (v/v) glutaraldehyde for 3 h at 2 C, centrifuged (10,000*g*, 15 min) and embedded in 2% (w/v) agar. Agar cubes (1 mm^3) were postfixed in buffered 1% (w/v) OsO_4 (50 mM Na-phosphate [pH 7.0]), dehydrated in a graded series of aqueous acetone, and embedded in Epon 812. Thin sections were stained with 0.5% (w/v) uranyl acetate and 0.4% (w/v) lead citrate and viewed and photographed with a JEOL 100B at 100 kv. (×4,250). Intact chloroplasts (C), lamellae fragments (L), mitochondrion (M), starch grain (S), prolamellar body (P).

Table II. *Extraction of Pea Seedling Chloroplasts*

Intact chloroplasts were osmotically ruptured in 25 ml buffer A minus sorbitol, incubated 30 min at 4 C, and centrifuged (48,200*g*, 30 min). The pellet was reextracted in the same manner. Values in parentheses are the per cent soluble protein or PDC found in each extract.

Fraction	Assay			
	Protein	PDC	RuBP carboxylase	Chl
	mg	*units*	*relative units*	*mg*
Chloroplasts	188	0.93	100	16
First extract	100 (91)	0.72 (89)	95	0
Second extract	10 (9)	0.09 (11)	5	0
Membranes	78	0.12	0	16

reaction mixture decreased 0.1 to 0.2 pH units from the initial pH.

Substrate and Cofactor Requirements for Chloroplast PDC. Table III shows the components necessary for the over-all chloroplast PDC reaction. The impure complex was reasonably specific for pyruvate, with α-ketobutyrate and hydroxypyruvate giving about one-tenth the rate with pyruvate. No activity was observed with α-KG as substrate, suggesting the absence of the α-KG dehydrogenase complex. The complex required added CoA and NAD for activity. Reduction of NADP was one-tenth the rate with NAD. This observation is being investigated further with purified complex. A partial dependence upon added TPP and Mg^{2+} was observed after the complex was treated by Sephadex G-25 gel filtration. Addition of 5 or 10 mM EDTA to the treated

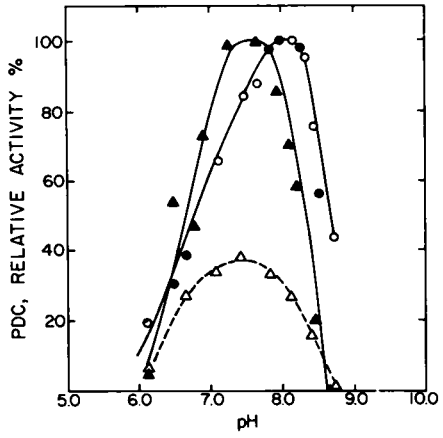

FIG. 2. Effect of pH on activity of pea chloroplast PDC (50 mM buffer, ●——●; 83.3 mM buffer, ○——○) and pea mitochondria PDC (50 mM buffer, ▲——▲; 83.3 mM buffer, Δ--Δ). The reaction buffer was MOPS-glycylglycine and the concentration of other reaction components were as given in Table III. Intact chloroplasts were osmotically ruptured in buffer A minus sorbitol, incubated 30 min at 4 C, and centrifuged (48,200*g*, 30 min). The supernatant was used for determination of pH activity curves. Mitochondria prepared as described under "Materials and Methods" were ruptured during assay by including 0.1% Triton X-100 in the reaction mixture. The pH values were determined from final reaction mixtures following assay of PDC.

Table III. *Substrate and Cofactor Requirements for Chloroplast PDC*

Intact chloroplasts were osmotically ruptured in 25 ml buffer A minus sorbitol, incubated 30 min at 4 C, and centrifuged (48,200*g*, 30 min). Supernatant (5 ml) containing 0.4 units PDC was passed through a Sephadex G-25 column (1.5 × 20 cm) equilibrated with 50 mM Tricine buffer (pH 8.0) containing 13.5 mM 2-mercaptoethanol and 1 mM EDTA. Fractions collected with the void volume were used for determination of substrate and cofactor requirements. The complete reaction mixture contained enzyme, 25 μmol MOPS + 25 μmol glycylglycine (pH 8.1), 0.2 μmol TPP, 1 μmol $MgCl_2$, 2.3 μmol NAD, 0.12 μmol lithium-CoA, 2.6 μmol cysteine·HCl, and 1.5 μmol pyruvate in a total volume of 1 ml. When intact organelles were assayed in other experiments, 0.1% Triton X-100 was included in the assay mixture. Reactions were initiated by the addition of pyruvate.

Assay Mixture	Relative Activity
	%
Complete	100
−Pyruvate	0
+ 3.0 mM α-ketoglutarate	0
+ 1.5 mM α-ketoglutarate	0
+ 3.0 mM α-ketobutyrate	9
+ 1.5 mM α-ketobutyrate	6
+ 3.0 mM hydroxypyruvate	11
+ 1.5 mM hydroxypyruvate	6
−NAD	0
+ 2.3 mM NADP	9
−CoA	0
−Cysteine·HCl	97
−TPP	77
$-MgCl_2$	31
+ 5 mM EDTA[a]	33
+ 5 mM EDTA[b]	27
+ 10 mM EDTA[b]	29
+ 5 mM $MgCl_2$	140
+ 2.5 mM	131
+ 1.0 mM	100
+ 0.5 mM	64
+ 1.0 mM $MnCl_2$	95
+ 0.5 mM	88
+ 0.25 mM	69
+ 5.0 mM $CaCl_2$	116
+ 2.5 mM	118
+ 1.0 mM	80
+ 0.5 mM	58

[a] Enzyme was incubated with 5 mM EDTA for 10 min at 4 C, then added to the assay mixture. Final concentration of EDTA in the assay was 0.1 mM.

[b] Five mM or 10 mM EDTA was included directly in the assay mixture.

enzyme had no effect upon the remaining rate. Mn^{2+} and Ca^{2+} could substitute for Mg^{2+} in restoration of G-25 Sephadex-treated complex activity, but Mg^{2+} was more effective at the same concentration.

DISCUSSION

Chloroplast fatty acid synthesis is essential to membrane development in young plants and provides a physical and biochemical framework for photosynthesis and growth. Chloroplast stroma contains all of the enzymes necessary for *de novo* synthesis of chloroplast fatty acids while the necessary energy (ATP) and reducing potential (NADPH) are provided by the light reactions of photosynthesis (17, 27). The conventionally used substrate for study of chloroplast fatty acid synthesis in intact or ruptured chloroplasts is acetate (16, 27), but there are no reports confirming the synthesis of acetate in higher plants. Activation of exogenously supplied acetate to acetyl-CoA does occur, but this may be due to nonspecific thiokinase activities in green plants. Both acetyl-CoA and malonyl-CoA are effective precursors to fatty acids in ruptured chloroplasts (27), but not in intact chloroplasts since the chloroplast envelope is impermeable to CoA derivatives (27). Thus, acetyl-CoA synthesized outside the chloroplast cannot be used directly for chloroplast fatty acid synthesis.

Pyruvate is an effective precursor to fatty acids in both intact and ruptured chloroplasts and is a readily available subtrate in this organelle from a variety of carbon sources (16, 27). Available evidence suggests that C2 and C3 of pyruvate are preserved intact as a two-carbon fragment which is directly incorporated into chloroplast fatty acids (15, 16, 27). The carboxyl group (C1) of pyruvate is released as CO_2 (27). Since acetyl-CoA is the immediate substrate for chloroplast fatty acid synthesis (via acetyl-CoA carboxylase and chloroplast acyl carrier protein) and the substrate and product of PDC are pyruvate and acetyl-CoA, respectively, a role for chloroplast PDC has been proposed (15, 16, 31).

The present report shows that green cells of young pea seedlings having a high requirement for membrane and fatty acid synthesis contain at least two separate pools of PDC, one in the mitochondrion and the other in the chloroplast. The ratio of mitochondrial PDC to chloroplast PDC in 9-day-old pea seedlings is about 3 to 1. Maximal rates observed for chloroplast PDC ranged from 6 to 9 μmol NADH produced/mg Chl·h or about 2 to 3% of the CO_2 fixation rates observed for intact, isolated chloroplasts and intact leaves. Incorporation of label from $H^{14}CO_3^-$ into lipid by isolated chloroplasts is less than 1% of the total fixed carbon (15, 16). Therefore, chloroplast PDC activity observed in young pea seedlings would be sufficient to account for rates of chloroplast fatty acid synthesis from CO_2.

Osmotic rupture of pea chloroplasts released most of the PDC activity, indicating that chloroplast PDC is a stromal complex (Table II and results). About 13% of the activity remained with the thylakoid fraction (Table II and Results), but there was no PDC activity associated with envelope membranes. Thylakoid PDC activity may reflect a small pool of membrane-bound PDC within the chloroplast. It is difficult to measure accurately PDC activity in the green, highly concentrated thylakoid fraction due to high background *A* at 340 nm, even in the presence of 0.1% Triton X-100. In addition, the presence of unbroken chloroplasts in this fraction cannot be discounted. Finally, PDC in the stromal fraction can be pelleted by centrifugation at 184,000*g* for 3 h (data not shown). Centrifugation procedures separating stroma and thylakoid fractions may have pelleted some PDC with the latter fraction.

Chloroplast PDC activity, but not mitochondrial PDC activity (data not shown), was increased by 40% when the Mg^{2+} concentration in the assay was increased from 1 to 5 mM (Table III). This also contrasts with mitochondrial PDC from other sources in which the activity was unchanged (20, 26) or inhibited (unpublished results) by Mg^{2+} concentrations greater than 1 mM. Also, like proplastid PDC from developing castor bean endosperm (25), pea chloroplast PDC is most active at a slightly alkaline pH (7.8–8.2, Fig. 2). At pH 7.0, PDC activity is reduced 40%. These results suggest a possible regulatory mechanism for chloroplast PDC since, during photosynthesis, the chloroplast stroma becomes more alkaline (pH 7–8) (6, 30) and Mg^{2+} concentration increases (1–3 mM) (11, 18). This would further support the involvement of PDC with chloroplast fatty acid biosynthesis since there is substantial evidence suggesting chloroplast fatty acid biosynthesis is light-dependent (27).

Ruptured, immature spinach chloroplasts (poorly developed grana, numerous prolamellar bodies) are more effective in incorporating acetate, acetyl-CoA, malonate, and malonyl-CoA into chloroplast fatty acids than ruptured, mature chloroplasts (27), which probably reflects a decreased requirement for membrane development in the more mature organelle. Similarly we have observed a loss of pea seedling chloroplast PDC with increasing age of the plant (data not shown).

Virtually nothing is known about regulation of carbon flow into

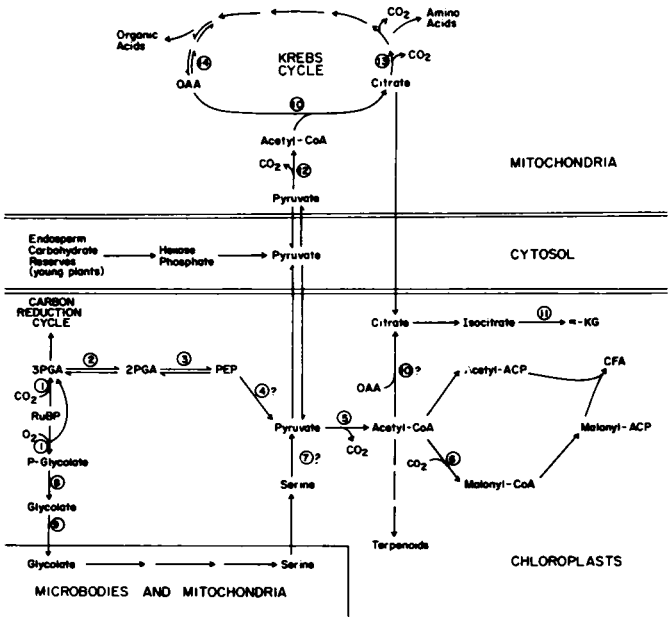

FIG. 3. Enzymes: (1) RuBP carboxylase/oxygenase; (2) phosphoglyceromutase; (3) enolase; (4) pyruvate kinase; (5) chloroplast PDC; (6) acetyl-CoA carboxylase; (7) serine dehydratase; (8) phosphoglycolate phosphatase; (9) glycolate-glyoxylate shuttle; (10) citrate synthase; (11) NADP-specific isocitrate dehydrogenase; (12) mitochondrial PDC; (13) NAD-IDH; (14) malate dehydrogenase.

chloroplast fatty acids, but chloroplast PDC may be a control point for directing pyruvate to chloroplast fatty acids and terpenoids (Fig. 3). Mitochondrial PDC may control entry of carbon into the Krebs cycle for energy production and organic acid and amino acid synthesis. Pyruvate is found in at least three subcellular pools (Fig. 3). The magnitude and turnover rate of each will be dependent upon levels of regulatory metabolites and conditions effecting pyruvate utilization and production. Regulatory aspects of both chloroplast PDC and mitochondrial PDC are presently under investigation. In addition, the interrelationship between photosynthesis and dark respiration remains obscure, but regulation of both mitochondrial PDC and chloroplast PDC by common intermediate exchange *in vivo* may partly determine the direction and fate of carbon flow.

Acknowledgment—The authors gratefully acknowledge the excellent technical assistance provided by the Missouri Agricultural Experiment Station-Electron Microscope Facility.

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