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Pyruvate Orthophosphate Dikinase of C_3 Seeds and Leaves as Compared to the Enzyme from Maize[1]

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KAZUKO AOYAGI AND JAMES A. BASSHAM*
Laboratory of Chemical Biodynamics, Lawrence Berkeley Laboratory, University of California, Berkeley, California 94720

ABSTRACT

Pyruvate orthophosphate dikinase (PPDK) was found in various immature seeds of C_3 plants (wheat, pea, green bean, plum, and castor bean), in some C_3 leaves (tobacco, spinach, sunflower, and wheat), and in C_4 (maize) kernels. The enzyme in the C_3 plants cross-reacts with rabbit antiserum against maize PPDK. Based on protein blot analysis, the apparent subunit size of PPDK from wheat seeds and leaves and from sunflower leaves is about 94 kdaltons, the same as that of the enzyme from maize, but is slightly less (about 90 kdaltons) for the enzyme from spinach and tobacco leaves. The amount of this enzyme per mg of soluble protein in C_3 seeds and leaves is much less than in C_4 leaves. PPDK is present in kernels of the C_4 plant, *Zea mays* in amounts comparable to those in C_4 leaves.

Regulatory properties of the enzyme from C_3 tissues (wheat) are similar to those of the enzyme from C_4 leaves with respect to *in vivo* light activation and dark inactivation (in leaves) and *in vitro* cold lability (seeds and leaves).

Following incorporation of $^{14}CO_2$ by illuminated wheat pericarp and adjoining tissue for a few seconds, the labeled metabolites were predominantly products resulting from carboxylation of phosphoenolpyruvate, with lesser labeling of compounds formed by carboxylation of ribulose 1,5-bisphosphate and operation of the reductive pentose phosphate cycle of photosynthesis. PPDK may be involved in mechanisms of amino acid interconversions during seed development.

Pyruvate orthophosphate dikinase (EC 2.7.9.1) is an essential enzyme for photosynthetic carbon dioxide fixation in C_4 plants (12), and in some CAM plants (14). PPDK[2] activity is present also in seeds of barley (9) and wheat (18, 19), a C_3 plant. There have been conflicting reports about the presence of PPDK in some C_3 leaves (for references, see 1). Recently, the presence and amount of PPDK activity in wheat seeds and leaves were reported and compared with the amount of PPDK activity in maize, and the amounts of PPDK protein in these tissues were estimated by immunoprecipitation in the protein blot method (1).

The role of PPDK in the metabolism of C_3 plant tissues is not established, although a role in guard cell stomatal opening has been suggested (8, 25). The presence of PPDK in several additional C_3 seed and leaf tissues now has been seen. The characteristics of the enzyme from wheat tissues are compared with those from C_4 tissues. The pattern of incorporation of ^{14}C into metabolites during short periods of photosynthesis with $^{14}CO_2$ by wheat pericarp and associated tissue has been examined for evidence of possible roles of PPDK in the metabolism of these tissues.

MATERIALS AND METHODS

Plants. Wheat (*Triticum aestivum* L. cv Cheyenne, CI 8885), kidney beans (*Phaseolus vulgaris* L. cv late red kidney), pea (*Pisum sativum* L.), spinach (*Spinacia oleracea* L. cv Highpack or cv 5286 America), *Zea mays* L., and tobacco (*Nicotiana tabacum* L. cv Wisconsin) were grown in a greenhouse. Castor bean (*Ricinus communis* L.) and sunflower (*Helianthus annuus* L.) plants were grown outside in Berkeley. Immature fruit of plum (*Prunus tomentosa* L.) was obtained from a tree in the Botanical Garden at University of California, Berkeley.

Reagents. Radiochemicals were purchased from New England Nuclear; malic dehydrogenase (MDH) was purchased from Boeringer Mannheim Biochemicals; chemical reagents from Sigma Chemical Co. and from Biorad Laboratories.

Protein Blot. PPDK was isolated from maize and purified as previously described (29). Antiserum to PPDK was prepared by injecting New Zealand white rabbits with 150 μg of the purified enzyme and Freund's complete adjuvant, followed after 26 d by a booster containing the same amount of enzyme and Freund's incomplete adjuvant and a second such booster at 40 d. The rabbits were bled at 41 d. The crude antiserum was used for the protein blot. Nonimmunized serum showed no cross-reaction with PPDK, and PPDK antiserum cross-reacted only with PPDK (1). This antiserum was used as a probe for the presence of PPDK in the various tissues studied.

Each plant tissue studied (3 g) was ground in 6 ml of 0.1 M Tris buffer, pH 7.4, containing 10 mM $MgCl_2$, 18% w/v sucrose, and 1% β-mercaptoethanol. The homogenate was filtered and then centrifuged at 15,000*g* and 0°C for 20 min. Total soluble protein was determined by the method of Bradford (5). Cross-reacting protein subunits similar in size to that of maize PPDK were detected by the protein blot method (6, 21).

SDS-PAGE of each plant extract was carried out in a gradient gel (6.4 to 12.8%). Protein was transferred to cyanogen bromide paper prepared by the method of Clarke *et al.* (6). The transfer paper was probed with anti-PPDK serum and then with I^{125} protein A. An autoradiograph was prepared using Kodak AR5 x-ray film with an intensifying screen at −70°C overnight.

Densitometry. Relative levels of PPDK in preparations from different plant samples were estimated by densitometry of the x-ray film by comparison of the peak areas. Three or four known amounts of purified PPDK were loaded on the SDS gel adjacent to the plant tissue samples each time to calibrate the amount. Under the conditions chosen there was a linear relationship between the amount of protein loaded on the gel and the area of

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[2] Abbreviations: PPDK, pyruvate orthophosphate dikinase; PEPC, phosphoenolpyruvate carboxylase; RuBPC, ribulose 1,5-biphosphate carboxylase; MDH, malic dehydrogenase.

the densitometry peak.

Ouchterlony Two-Dimensional Diffusion (22). Small wells were cut in agar plates. One was filled with 20 μl of antiserum, a second with solution containing 4 μg of purified PPDK from maize, and the others with 20 μl of plant extract. The precipitin band was observed after 1 to 4 d of incubation at room temperature. The reaction was stopped by addition of 0.9% (w/v) NaCl solution, and the bands were stained with Coomassie Brilliant Blue. RuBPC antibody was kindly supplied by Dr. Mark Harpster, who prepared it by raising rabbit antibody to purified maize leaf RuBPC.

PPDK Activity Assay. PPDK activity was assayed as previously described (29). Dependence of the activity on ATP and on Pi were demonstrated by separate controls in which each was omitted from the reaction mixture. Protein content of tissue extracts was determined by the method of Bradford (5) and Chl content was measured according to Arnon's method (3).

Cold Lability. The crude extract was prepared under sunlight as described previously (1). The enzyme was then stored 30 and 60 min at 0°C, then transferred back to room temperature and assayed after 30, 60, and 90 min.

PEPC Activity Assay. PEPC was assayed by the reduction of oxaloacetate with MDH and NADH to malate and spectrophotometric observation of the change in absorption at 340 nm (17). Following 3 min preincubation of the PEPC at 22°C in 0.9 ml of assay mixture containing 0.1 M Tris HCl (pH 8), 10 mM $MgCl_2$, 2 mM DTT, 0.2 mM NADH, 10 mM $NaHCO_3$, and 4 units of MDH, the reaction was initiated by the addition of PEP (to give 2.5 mM).

Incorporation of Labeled Carbon Dioxide. Immature wheat seeds (25 d after flowering) were dissected. After removal of endosperm tissue, the pericarp with adjoining tissue was cut into small pieces (about 2 × 2 × 2 mm). For each sample, 80 mg green tissue was placed in 1 ml buffer (3 mM $NaHCO_3$, 20 mM Hepes, pH 7.8) in a glass homogenizing tube and preilluminated at about 650 μE $m^{-2}s^{-1}$ for 2 min. Then 0.2 ml $NaH^{14}CO_3$ solution was added to give a final concentration of 8.4 mM (25 μCi μmol^{-1}), and illumination was continued for 5, 10, 15, 30, and 60 s for the five samples. Liquid N_2 was poured into each tube at the end of this time to stop the reactions. Methanol was added to give 50% methanol and the mixture was ground as it thawed. An aliquot portion of this mixture was analyzed by two-dimensional paper chromatography and radioautography, and incorporation of ^{14}C into individual compounds was measured by liquid scintillation counting (23).

RESULTS

As previously reported, PPDK is present in wheat leaves and seeds, but at a much lower level than in maize leaves (1). The percentage of PPDK in soluble protein appears to be less than 0.05% in wheat leaves and even less in spinach and tobacco leaves while in contrast, PPDK can constitute 3 to 10% of the soluble protein in maize leaves. These estimates are based on densitometric measurements of the darkened areas of the radioautogram of protein blots (Figs. 1A, 3) and the assumption that PPDK polypeptide from wheat responds to maize PPDK antibody to the same extent as maize PPDK. The validity of this assumption is suggested by the fact that many Ouchterlony plates prepared with the maize PPDK antibody against crude protein extracts of maize and wheat leaf tissues showed no detectable 'spurs' such as were seen with plum immature fruit protein (Fig. 2B), where only partial identity is indicated. Such estimates of protein amount must be considered as lower limits. Ku *et al.* (16) however, found that RuBPC antibody to the enzyme from tobacco gave quantitative estimates for the enzyme from maize.

Based on the protein blot analysis, the subunit mol wt are similar at about 94kD (Fig. 1A). PPDK was also found in tobacco leaves but not in tobacco callus or in young leaves of one spinach cultivar. PPDK was detected in older spinach leaves from another cultivar (Fig. 1B), where the polypeptide apparent mol wt was about 90 kD. PPDK was found also in sunflower leaves, and the subunit size was about 94 kD (data not shown). The presence of PPDK in immature plum fruit was demonstrated by Ouchterlony two-dimensional diffusion (Fig. 2, A and B). Similar tests showed that PPDK was present in peas, green beans, and castor bean seed (data not shown). However, the mol wt of the polypeptide subunits of the enzyme from these species were not estimated.

The amount of PPDK in kernels from maize is quite high in the later stage of seed development (Fig. 3) about 20% of that found in maize leaves, amounting to about 2% of the total soluble protein. At an earlier stage of kernel development, the amount of PPDK was low. The activity of the kernel seed PPDK

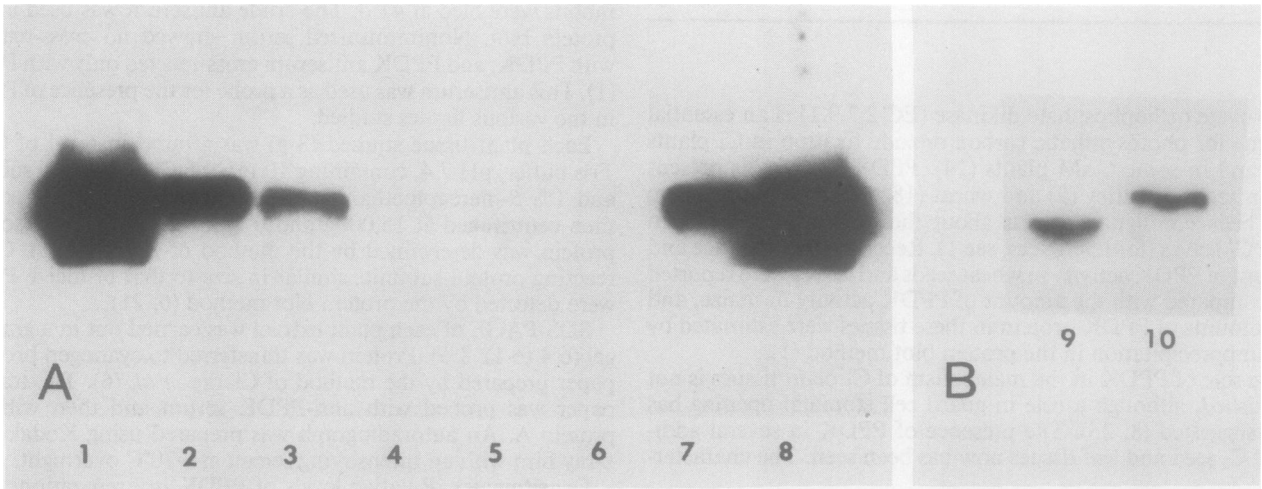

FIG. 1. Occurrence of PPDK in C_3 plant tissues. The total soluble protein was separated on SDS gradient gel (6.4–12.8%). The transfer paper was probed with antimaize-PPDK. A. Lanes 1 and 8, maize leaf (0.13 mg); lanes 2 and 7, purified maize leaf PPDK (0.5 μg); lane 3, wheat leaf (0.22 mg); lane 4, spinach leaf (cv 5286 America, 0.44 mg); lane 5, tobacco leaf (0.17 mg); lane 6, tobacco callus (0.02 mg). B. Lane 9, spinach leaf (cv Highpack, 0.38 mg); lane 10, purified maize leaf PPDK (0.25 μg). Although PPDK was not detected in the blot test in lane 4, other tests such as shown in lane 9 gave a positive result. The apparent mol wt of the spinach PPDK subunit seems to be slightly smaller (estimated 90 kD) than that of maize leaf PPDK.

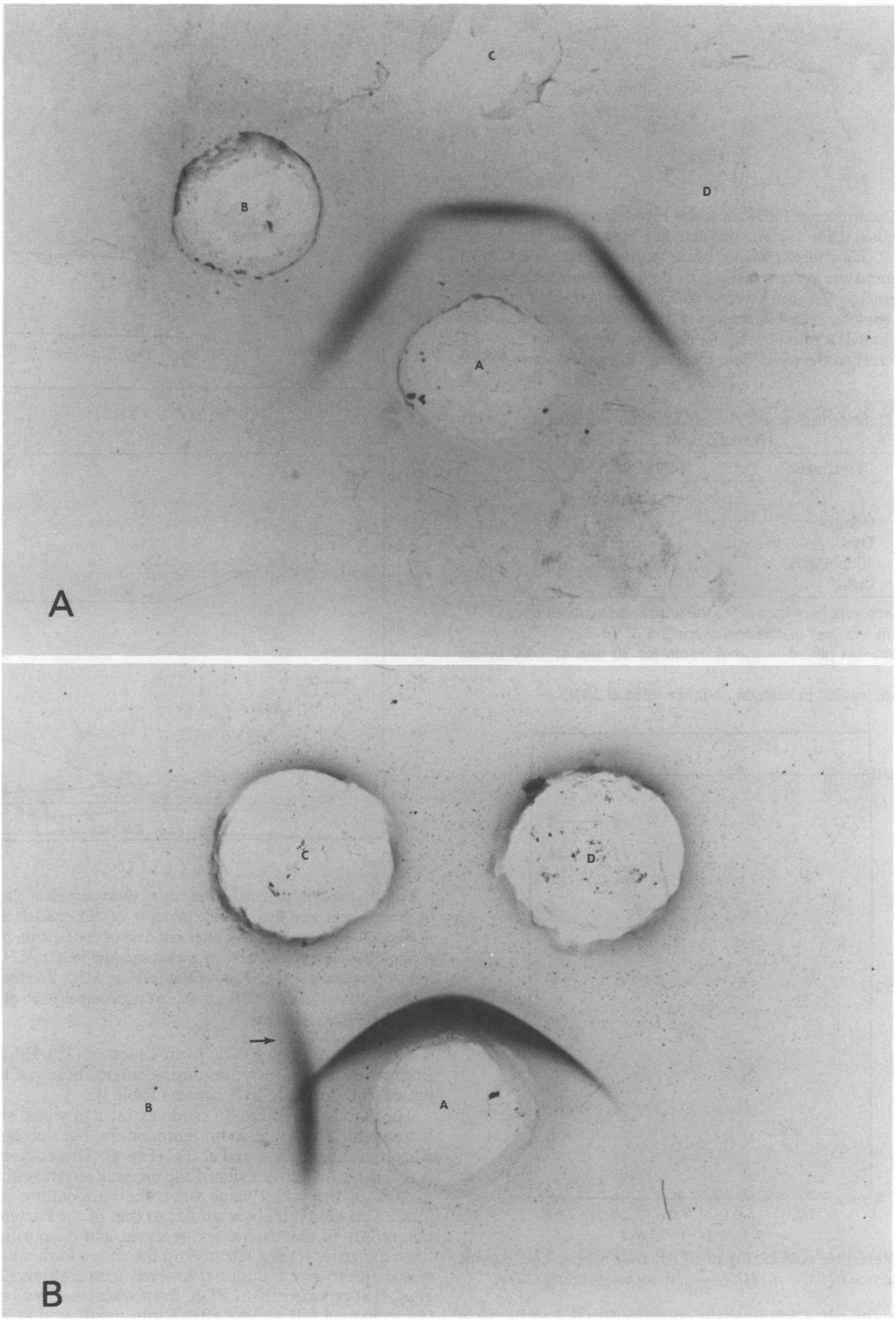

FIG. 2. Ouchterlony double diffusion of maize leaf and plum immature fruit PPDK. A. Well A, Anti-PPDK serum; well B, purified PPDK from maize leaf; wells C and D, crude enzyme extract from maize leaf. B. Well A, Anti-PPDK serum; well B, purified PPDK from maize leaf; wells C and D, the crude enzyme extract from immature plum fruit. The spur (arrow) indicates partial identity between plum PPDK and maize leaf PPDK.

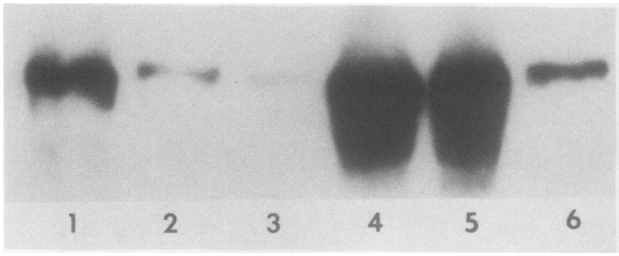

FIG. 3. Occurrence of PPDK in maize kernels according to the protein blot method. Lane 1, maize leaf obtained from mature plant about 70 d old (0.05 mg soluble protein); lane 2, purified maize PPDK (0.25 μg); lane 3, immature maize kernel at early stage (0.36 mg soluble protein) when the kernel is light green, average diameter of a kernel is 3 mm, and the ear is 5 cm long; lane 4, immature maize kernel at a later stage (0.5 mg soluble protein), when the kernel is yellow, average diameter of a kernel is 1 cm, and the ear is 7 cm long; lane 5, purified maize PPDK (0.5 μg).

Table 1. *Light Activation and Dark Inactivation of PPDK Activity in Wheat Leaves*

Treatment	PPDK Activity
	(units/g fresh wt)
Control[a]	0.22
Dark[b]	0.022
30 min dark[c]	0.043
Light[d]	0.62

[a] Flag leaves were harvested at 9 AM (sunrise was at about 6 AM).
[b] The plant was kept in darkness overnight.
[c] The plant was placed in a dark room for 30 min at 9 AM before harvesting.
[d] The plant was left in sunlight until harvested at 3 PM.

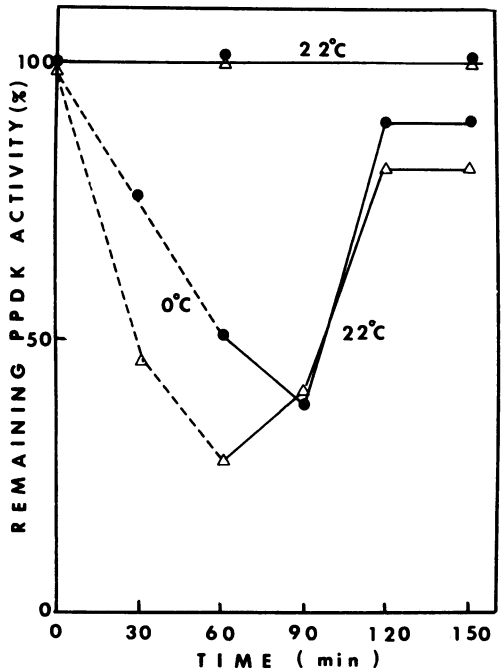

FIG. 4. Reversible cold lability of PPDK from wheat. Leaf enzyme (△); seed enzyme (●). (. . . .), 0°C; (——), room temperature (22°C).

was 0.26 units per g fresh weight compared to 1.7 units per g fresh weight for maize leaves. A smaller amount of PPDK was found in the maize husk, and none was found in the silk or root (data not shown).

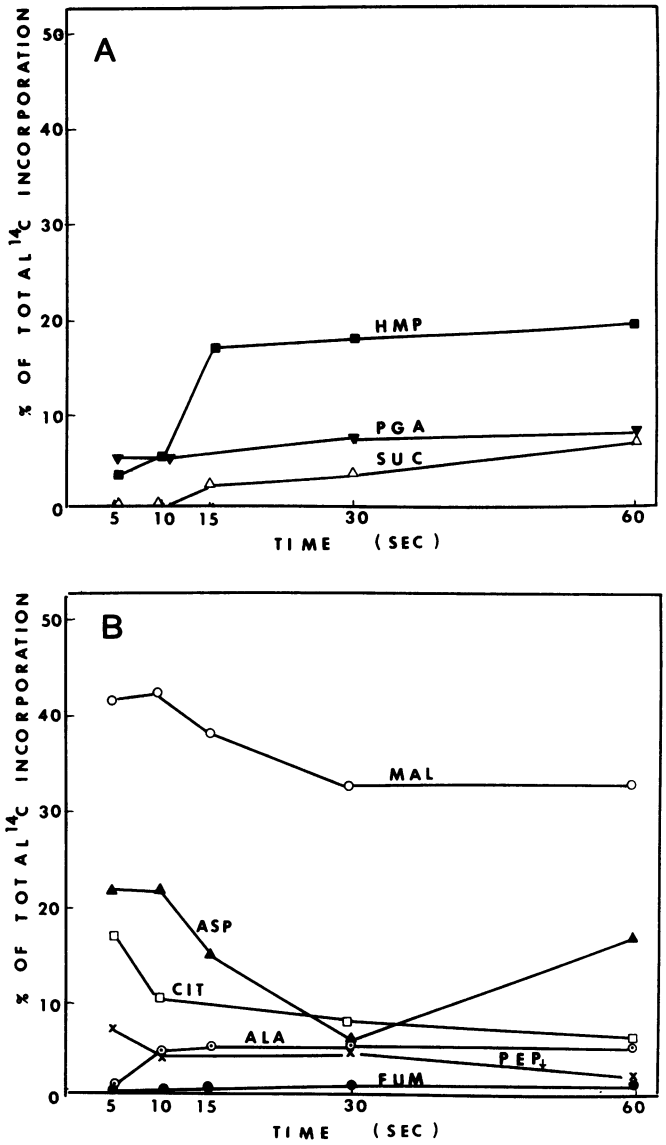

FIG. 5. Labeling of metabolites during short periods of photosynthesis by green wheat seed tissue in the presence of $^{14}CO_2$. Seeds at 25 d after flowering were dissected and, after removal of endosperm, pericarp and adjoining tissue was used (see "Materials and Methods"). A. HMP, hexose monophosphates; PGA, 3-phosphoglycerate; SUC, sucrose. B. MAL, malate; ASP, aspartate; CIT, citrate; ALA, alanine; PEP, phosphoenolpyruvate; FUM, fumarate.

As is the case with PPDK from C_4 leaves (29), PPDK activity from wheat leaves increases during several hours of leaf illumination and decreases in darkness (Table I).

The activity of PPDK in crude extracts of wheat seed or leaf is stable for 30 min at room temperature, but decreases rapidly when the enzyme is stored at 0°C (Fig. 4). This cold inactivation is reversible, as the activity of the enzyme recovers after 30 min to 90% of the initial value when the temperature is raised to 22°C. This cold lability is similar to that of the enzyme from C_4 plants (30). In that case, it was reported that the purified enzyme from maize leaves is a tetramer in the active form and reversibly dissociates to give a dimer at lowered temperatures (26). Meyer *et al.* (18) reported that PPDK from wheat seed was cold labile, but recovered full activity after 5 min incubation at 22°C. Since experimental conditions such as duration and temperature of cold treatment, and whether *in vivo* or *in vitro*, were not described we cannot evaluate differences between that result and ours,

although we note that a different cultivar was used. In a later report (19) the same authors failed to observe cold inactivation and light-mediated activation of wheat seed PPDK in preliminary experiments.

The pH optimum of the enzyme from wheat tissues is about 7.9. In wheat seeds 25 d after anthesis, when PPDK protein is at its highest level, its activity is about 0.1 unit per g fresh weight, while at that time the activity of PEPC is about ten times greater.

Analysis of photosynthetic fixation of ^{14}C-labeled bicarbonate by wheat seed green tissue (25 d after flowering) showed that the fixation at the shortest times is predominantly into compounds derived from oxaloacetate (malate, aspartate, fumarate, and citrate (Fig. 5). After 5 s labeling, malate accounted for 42% of radiocarbon found in compounds on the paper chromatogram. After longer periods of photosynthesis with labeled carbon, the normal pattern of C_3 assimilation (sugar phosphates, phosphoglycerate) becomes more prominent. Experiments with green seed tissue 18 d after flowering gave similar results (not shown), although labeling of C_4 compounds was slightly less dominant. Fixation of $^{14}CO_2$ was not linear, being at a somewhat greater rate during the first few seconds.

DISCUSSION

Although a limited number of C_3 species so far have been tested, PPDK appears to be more widely distributed among C_3 plants than had been generally realized. Negative results from past efforts to detect enzyme activity may be due to a variety of causes including variability in amount due to age of leaves and other physiological conditions, inactivation during extraction, and lack of purification (since there is so little activity, it often cannot be detected in crude extracts). The amount of PPDK is in fact very much less in C_3 tissues as compared with C_4 tissues. Moreover, PPDK thus far is found in C_3 plants only in some seeds at certain stages of development and in leaves. This limited occurrence, together with the regulatory properties of the enzyme which mandate special conditions for obtaining the active form, may explain the scarcity of previous reports (9, 19, 25) of its presence in such tissues.

There are many similarities between the C_3 PPDK (in wheat seeds and leaves) and the enzyme from C_4 plants. Mol wt (94 kD) of the subunit, *in vivo* light activation (in leaves; seeds not tested), cold instability, and pH profile of the wheat enzyme are all similar to these properties in the enzyme from maize, a C_4 plant. The polypeptide subunit from leaves of spinach and tobacco were slightly smaller (90 kD), while sunflower leaf PPDK subunit had an apparent mol wt of about 94 kD.

Based on the Ouchterlony double diffusion technique (22), wheat seed or leaf PPDK appeared identical with maize leaf PPDK, when tested with maize leaf PPDK antibody. Wheat leaf RuBPC, on the other hand, did not appear completely identical with maize leaf RuBPC, when tested with maize leaf RuBPC antibody (data not shown).

The role of PPDK in the metabolism of C_3 leaves is not well established. It has been proposed that there may be an involvement of PPDK in control of stomatal opening through a participation in exchange in ion transport into guard cells (8, 25). In seeds, PPDK may supply PEP for photosynthetic and dark CO_2 fixation. Photosynthesis by developing wheat grains can account for as much as 34% of the weight gain of the grains (10). Considerable additional photosynthesis by the grains may be involved in the refixation of respiratory CO_2 (15). Stomata were observed on wheat and barley grains toward the brush end on the flanks of the crease by Cochrane and Duffus (7) who examined the ultrastructure of immature grains and discussed it in relation to transport of gasses and metabolites and the relatively low rate of photosynthetic $^{14}CO_2$ fixation of intact seeds compared to the much larger rate seen upon removal of the outer pericarp (24).

The pattern of ^{14}C-labeled compounds formed during short periods of photosynthesis with ^{14}C-labeled bicarbonate in wheat seeds 25 d after flowering indicates a very high PEPC activity (Fig. 5). Seeds at this late development stage were chosen for the experiment since the highest level of PPDK is seen in such seeds (2). There is a preponderance of labeled products of PEP carboxylation such as aspartate, malate, citrate, and glutamate. The continued high percentage of labeling of these compounds to 1 min seems to suggest a relatively high rate of net synthesis of C_4 and C_5 compounds. The higher rate of fixation at the shortest time may indicate that accumulated PEP was rapidly used up when bicarbonate was administered to the exposed tissue.

Nutbeam and Duffus (20) reported predominant labeling of malic acid at 1 min of photosynthetic $^{14}CO_2$ fixation by barley pericarp tissue, with labeling moving to sucrose and hexose phosphates at longer times, and they considered this to be evidence for C_4 photosynthesis in that tissue. Similar results were said to have been obtained for wheat pericarp, but data were not presented. In any event, the time scale for the kinetics of those results with barley pericarp are quite different from those of the present study in which the labeling of malate and aspartate, although declining after the first 15 s, continued to be prominent. Wirth *et al.* (31) reported ^{14}C labeling in wheat pericarp at 10 s only, and found a predominance of labeled products characteristic of C_3 photosynthesis. Such differences in results from different studies may well be related to physiological differences in the seed tissue used, especially in stage of development and variety. In fact, in a preliminary experiment (data not shown) with another variety (Anza) 18 d after flowering we obtained a predominantly C_3 fixation pattern.

Given the greater activity of PEPC than of PPDK, some PEP for carboxylation may come from glycolysis. However, conversion of pyruvate to PEP, mediated by PPDK, may be an important source of the PEP substrate for carboxylation. In particular, PEP carboxylation and a preceding conversion of pyruvate to PEP mediated by PPDK may be required for the net conversion of the C_3 amino acid carbon skeleton of alanine to C_4 and C_5 amino acids in wheat grains. Amino acids present in major amounts in flag-leaf phloem are glutamate, aspartate, serine, alanine, and glycine (27). Conversion of this mixture of amino acids to that required for seed protein synthesis must occur in the seeds (13).

Wheat storage protein such as gliadin is very rich in glutamate and proline (11) which is made from glutamate. During the synthesis of such proteins, the utilization of other amino acids including alanine might lag, allowing them to accumulate in the endosperm during the first 3 weeks of seed development. Alanine is a major soluble amino acid of endosperm of wheat grains, and when the plants are not irrigated, alanine can constitute as much as 47% of total soluble amino acids (28). In this case, the peak in quantity of alanine occurs at about 25 d after flowering, at about the same time as the maximum in PPDK protein seen in other studies (2). Perhaps during the last 2 weeks, conversion of excess accumulated alanine to glutamate is needed for efficient utilization of this stored alanine. Another possibility, the conversion of alanine to aspartate (19) seems less important since a much smaller amount of C_4 amino acids than of C_5 amino acids are required in the storage protein (11). For similar reasons, other possibilities such as conversion of malate to pyruvate followed by reduction to sugars seem less likely.

In maize, alanine is the amino acid occurring in highest amount among the free amino acids in the developing kernel (4). There may, therefore, be a necessity in corn kernels for the conversion of alanine to C_4 and C_5 amino acids during the latter stages of kernel development. Both PPDK protein and activity were found in immature maize kernels. A previous study (19)

based only on activity assay did not find PPDK in maize kernels, despite finding PPDK activity in wheat grains, where we found less PPDK activity than in maize kernels. Possibly in the previous study the kernels were at an earlier stage of development when the level of PPDK is much lower (Fig. 3), or the enzyme may have been inactivated during harvesting and extraction.

The peak in PPDK activity in wheat seeds and in maize kernels occurs well after the maximum in Chl content and RuBPC content of the green seed tissue or ear (2). This timing also suggests that the role of PPDK in seed tissue is not primarily in photosynthetic CO_2 incorporation as it is in C_4 leaf tissue. It is interesting that nevertheless much more PPDK is expressed in maize kernels than in wheat seed. Thus the greater expression of this enzyme seen in C_4 leaves seems to be carried over into the seed, at least when maize and wheat are compared.

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