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Inhibition of 3-Phosphoglycerate-Dependent O_2 Evolution by Phosphoenolpyruvate in C_4 Mesophyll Chloroplasts of *Digitaria sanguinalis* (L.) Scop.[1]

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MARY E. RUMPHO* AND GERALD E. EDWARDS
Department of Botany (M. E. R., G. E. E.) and Institute of Biological Chemistry (G. E. E.), Washington State University, Pullman, Washington 99164-4230

ABSTRACT

The effects of phosphoenolpyruvate (PEP), inorganic phosphate (Pi), and ATP on 3-phosphoglycerate (PGA)-dependent O_2 evolution by chloroplasts of *Digitaria sanguinalis* (L.) Scop. (crabgrass) were evaluated relative to possible mechanisms of PEP transport by the C_4 mesophyll chloroplast. Crude and Percoll purified chloroplast preparations exhibited rates of PGA-dependent O_2 evolution in the range of 90 to 135 micromoles O_2 per milligram chlorophyll per hour, and up to 180 micromoles O_2 per milligram chlorophyll per hour at optimal Pi concentrations (approximately 0.2 millimolar at 9 millimolar PGA). Higher concentrations of Pi were inhibitory. PEP inhibited O_2 evolution (up to 70%) in both chloroplast preparations when the PEP to PGA ratio was high (*i.e.* 9 millimolar PEP to 0.36 millimolar PGA). Usually no inhibition was seen when the PEP to PGA ratio was less than 2. PEP acted as a competitive inhibitor and, at a concentration of 9 millimolar, increased the apparent K_m (PGA) from 0.15 to 0.53 millimolar in Percoll purified chloroplasts. A low concentration of PGA and high ratio of PEP to PGA, which are considered unphysiological, were required to detect any inhibition of O_2 evolution by PEP. Similar results were obtained from crude *versus* Percoll purified preparations. Neither the addition of Pi nor ATP could overcome PEP inhibition. As PEP inhibition was competitive with respect to PGA concentration, and as addition of ATP or Pi could not prevent PEP inhibition of PGA-dependent O_2 evolution, the inhibition was not due to PEP exchange of adenylates or Pi out of the chloroplast. Analysis of the effect of Pi and PEP, separately and in combination, on PGA-dependent O_2 evolution suggests interactions between PEP, Pi, and PGA on the same translocator in the C_4 mesophyll chloroplast. C_3 spinach chloroplasts were also found to be sensitive to PEP, but to a lesser extent than crabgrass chloroplasts. The apparent K_i values (PEP) were 3 and 21 millimolar for crabgrass and spinach, respectively.

Phosphoenolpyruvate plays a central and perhaps regulatory role in the photosynthetic processes of C_4 plants. In NADP-malic enzyme type C_4 species such as *Digitaria sanguinalis* (crabgrass), PEP[2] is formed in the mesophyll chloroplast from pyruvate (plus ATP and Pi) via pyruvate,Pi dikinase (10). PEP is then transported across the chloroplast envelope to the cytoplasm where carboxylation by PEP carboxylase produces OAA. Pi is released in the PEP carboxylation reaction and returns to the chloroplast and is used in photophosphorylation to yield ATP. OAA in turn enters the chloroplast and is reduced to malate consuming NADPH. Malate leaves the mesophyll chloroplast and is decarboxylated in the bundle sheath chloroplast by NADP-malic enzyme (4, 10). The pyruvate generated by this reaction is transported back to the mesophyll chloroplast and used in PEP synthesis to continue the C_4 cycle. The CO_2 produced in the bundle sheath is fixed by RuBP carboxylase, yielding PGA which is metabolized in the reductive pentose phosphate pathway. Part of the PGA produced is thought to be transported to the mesophyll cell, taken up by the chloroplast, and reduced to DHAP with the consumption of NADPH and ATP (7, 19).

To maintain a continuous C_4 cycle, energy must be generated photochemically and a coordinated and rapid transport of several metabolites must take place. Several translocators have been identified in C_4 mesophyll chloroplasts including: a pyruvate translocator in crabgrass (14), a dicarboxylate translocator (1, 7), a phosphate translocator (2, 15), and an adenine nucleotide translocator in crabgrass and maize (13, 26). In mesophyll chloroplasts of both C_3 and C_4 plants the phosphate translocator catalyzes the exchange of PGA and triose-P (2, 11). However, the C_4 chloroplast phosphate translocator will also exchange PEP and Pi at rapid rates (2, 15). It has been suggested that PEP, PGA, and Pi are transported on a common phosphate translocator in C_4 mesophyll chloroplasts (2). Recently it was shown that PEP can also be transported in exchange for ATP or ADP in pea and maize mesophyll chloroplasts, apparently on the adenine nucleotide translocator (26). While the authors concluded this exchange occurred at a rapid rate, rates of transport were not reported.

Studies on metabolite transport with C_4 mesophyll chloroplasts have indicated a competitive inhibition of PGA uptake by Pi and PEP (2). Until recently (9), however, inhibition of PGA-dependent O_2 evolution by PEP could not be demonstrated (3, 5). Hallberg and Larsson (9) reported inhibition of PGA-dependent O_2 evolution in crabgrass mesophyll chloroplasts by both PEP and Pi, citing the lack of purity of chloroplast preparations for the discrepancies in the past.

In this study we have evaluated the effect of PEP, Pi, and ATP on PGA-dependent O_2 evolution in C_4 *versus* C_3 chloroplasts under various conditions. PEP inhibition of O_2 evolution in crabgrass chloroplasts was demonstrated regardless of the purity of the preparations (crude chloroplast pellets *versus* Percoll gradient purification). Experiments were performed to assess whether PEP may inhibit PGA-dependent O_2 evolution by com-

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[2] Abbreviations: PEP, phosphoenolypyruvate; OAA, oxaloacetate; RuBP, ribulose 1,5-bisphosphate; PGA, 3-phosphoglycerate; DHAP, dihydroxyacetone phosphate; triose-P, triose-phosphate; PPFD, photosynthetic photon flux density; K_i, inhibition constant; I, inhibitor.

peting for PGA uptake, or by exchanging Pi or adenylates out of the chloroplast. Furthermore, PEP was found to inhibit PGA-dependent O_2 evolution in the C_3 chloroplasts of spinach, but less effectively than in the C_4 mesophyll chloroplasts.

MATERIALS AND METHODS

Plant Material. *Digitaria sanguinalis* (L.) Scop. (crabgrass) was grown in soil in a growth chamber with a 14-h photoperiod and a 28/23°C day/night temperature regime. A PPFD of 700 μmol m^{-2} s^{-1} was provided and plants were fertilized with a modified Hoagland nutrient solution twice a week. Leaves (4–6 cm in length) were used from seedlings 14 to 16 d old. *Spinacia oleracea* L. was purchased from a local supermarket and used immediately.

Reagents. Pectinase (Macerozyme R-10) and cellulase R-10 were obtained from Yakult Biochemical Co., Ltd., Nishinomiya, Japan. Dextran (mol wt 15,000–20,000) was supplied from U.S. Biochemical Corp. PEP was obtained as the monopotassium salt from Sigma. All other reagents were purchased from either Sigma or Calbiochem and were of analytical grade.

Protoplast Isolation. Mesophyll protoplasts were isolated and purified from crabgrass leaves based on the procedures of Edwards *et al.* (6) and Day *et al.* (3). Ten leaves at a time were stacked and cut into segments about 1 mm in width, placed into digestion media (approximately 5 g/100 ml) and subjected to a light vacuum infiltration. The medium consisted of: 500 mM sorbitol, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 0.05% BSA, 2% cellulase R-10, 0.5% Macerozyme R-10, 0.1% Pectolyase and 5 mM Mes buffer (pH 5.5). Digestion was at 28°C for 2.5 h with a PPFD of 200 μmol m^{-2} s^{-1} and slight agitation throughout (21). After digestion, the mixture was swirled and filtered through a 1-mm tea strainer and 80 μm net. The filtrate was centrifuged 5 min at 100*g* and the pellet resuspended in a medium containing 500 mM sucrose, 15% (w/v) dextran T_{20}, 1 mM $CaCl_2$, 0.2% BSA, and 5 mM Hepes (pH 7.8). This suspension was placed in a Babcock bottle and brought to a volume of about 45 ml and then overlayered with about 4 ml of a medium containing 500 mM sorbitol, 1 mM $CaCl_2$, 0.2% BSA, and 5 mM Hepes (pH 7.8). The gradient was centrifuged at 250*g* for 7 min and the protoplasts collected at the interphase of the two solutions.

Isolation of Chloroplasts from Protoplasts. To the crabgrass protoplast suspension an equal volume of breaking medium (333 mM sorbitol, 10 mM EDTA, and 50 mM Hepes [pH 7.8]) was added. The protoplasts were ruptured by passing through a Yeda press under N_2 at 70 p.s.i. The protoplast extracts were then either centrifuged at 250*g* for 2 min or further purified by Percoll centrifugation as described later. The pellet was resuspended in breaking media, centrifuged again at 250*g* for 2 min, and the resulting pellet washed once and then resuspended in the breaking medium plus 0.2% BSA and placed on ice for immediate use.

Mechanical Isolation of Spinach Chloroplasts. Chloroplasts were isolated from spinach leaves essentially according to Leegood and Walker (17). Leaves were floated on cold water and illuminated for 20 min with a PPFD of 800 to 1000 μmol m^{-2} s^{-1} before homogenization with a Polytron for 2 to 3 s. The isolation medium contained: 330 mM sorbitol, 5 mM $MgCl_2$, 2 mM isoascorbate, and 10 mM Mes (pH 6.5). After filtration and centrifugation, the chloroplast pellet was resuspended in the same medium used for crabgrass chloroplasts (333 mM sorbitol, 10 mM EDTA, 0.2% BSA, and 50 mM Hepes [pH 7.8]). Only Percoll-purified chloroplasts (described below) were used in these experiments.

Percoll Purification of Chloroplasts. Chloroplasts of both crabgrass and spinach were further purified by centrifuging through 20% (v/v) Percoll (20) in a solution identical to the resuspending medium. The centrifugation was at 680*g* for 5 min and the intact chloroplasts pelleted at the bottom of the tube. The pellets were resuspended, combined, and centrifuged at 250*g* for 2 min. The resulting pellet was washed once, resuspended again (in 330 mM sorbitol, 10 mM EDTA, 0.2% BSA, and 50 mM Hepes [pH 7.8]) and placed on ice for use. Spinach chloroplasts maintained the same rate of O_2 evolution for several hours. Crabgrass chloroplasts began to decline in activity after 2 to 3 h; therefore, new chloroplasts were prepared from intact protoplast suspensions every 2 h or as needed.

Oxygen Evolution. PGA-dependent O_2 evolution was measured polarographically at 30°C (crabgrass) or 25°C (spinach) with Rank Brothers (Cambridge, U.K.) O_2 electrodes. Two O_2 electrode setups were run simultaneously. The light sources were two 150-w low temperature flood lamps. The PPFD at the chamber surface was 800 to 1000 μmol m^{-2} s^{-1} at 400 to 700 nm. Approximately 12 to 16 μg of Chl was added to 1.4 ml of reaction medium (the basal medium contained 333 mM sorbitol, 10 mM EDTA, 50 mM Hepes [pH 7.8], and 200 units/ml of catalase). In addition, 3.6 mM glycolaldehyde was added to the reaction medium for spinach chloroplasts to prevent any CO_2-dependent O_2 evolution (23). The chloroplasts and substrates/inhibitors (all at pH 7.8) were added in the dark 2 min prior to illumination unless otherwise indicated. Percoll-purified chloroplasts were used in all the experiments shown except Figure 4, A and B. All rates of O_2 evolution refer to the initial maximum linear rate. Chl was determined according to Wintermans and De Mots (25). Intactness was measured by ferricyanide-dependent O_2 evolution before and after osmotic shock (18).

PEP Carboxylase Assay. PEP carboxylase (EC 4.1.1.31) was assayed spectrophotometrically as previously described (24) to assess the degree of cytoplasmic contamination. An aliquot of chloroplasts (2 to 7 μg Chl) was added to the assay (total volume of 1 ml) and the oxidation of NADH followed at 340 nm after addition of 5 mM PEP. The enzyme was assayed with the cofactor $MgCl_2$ in excess (5 mM $MgCl_2$ and 2 mM EDTA) in order to measure the maximum activity. The potential activity of PEP carboxylase in the photosynthetic measurements would be even lower since the assay media contained 10 mM EDTA and no $MgCl_2$.

RESULTS

Chloroplast Preparation and Purification. When protoplasts of crabgrass were treated with the Yeda press they were completely broken and the cellular contents were dispersed. Crude chloroplast preparations could be obtained within 15 min after breakage of the protoplasts and Percoll-purified chloroplasts within about 30 min. Based on the ferricyanide test for intactness (18), the chloroplasts were at least 80% intact for all experiments reported. The activity of PEP carboxylase was measured in both chloroplast preparations to estimate the extent of cytoplasmic contamination. In the crude preparations, PEP carboxylase activity varied from 25 to 40 μmol mg^{-1} Chl h^{-1} (only about 3% of the activity in protoplast extracts) compared to 2 to 8 μmol mg^{-1} Chl h^{-1} (only about 0.6% of the activity in protoplast extracts) in the Percoll purified chloroplasts.

PGA-Dependent O_2 Evolution. High rates of PGA-dependent O_2 evolution were observed in isolated mesophyll chloroplasts from crabgrass whether they were crude or purified preparations. Maximum rates without Pi present varied from 90 to 135 μmol O_2 mg^{-1} Chl h^{-1} (Figs. 1–5), and with optimal Pi, rates up to 180 μmol mg^{-1} Chl h^{-1} were achieved (Fig. 5). In all cases (unless specifically stated), the chloroplasts were incubated for 2 min in the dark with PGA and any other compounds indicated (*i.e.* PEP and Pi). Upon illumination there was a very short lag time before maximum O_2 evolution was achieved (Fig. 1). This lag time could be shortened by the addition of Pi or by prolonged storage of the chloroplasts on ice (data not shown). Linearity was main-

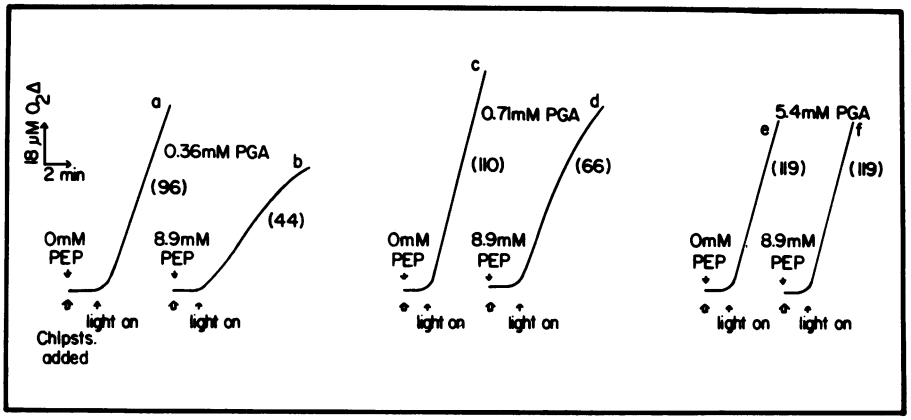

FIG. 1. Recorder traces of light and PGA-dependent O_2 evolution from mesophyll chloroplasts of crabgrass. The chloroplasts (12 to 16 μg) were added at the open arrow, 2 min before illumination, with varying amounts of PGA and with or without PEP. Values in parentheses represent the μmol O_2 evolved mg^{-1} Chl h^{-1}.

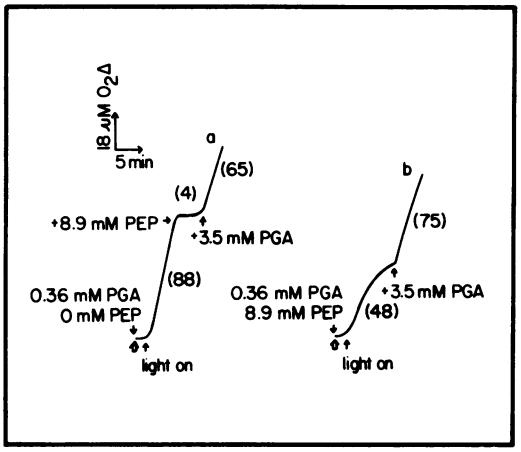

FIG. 2. PGA-dependent O_2 evolution by crabgrass chloroplasts. PEP and/or PGA were added in the dark or following illumination at the concentrations indicated. Numbers in parentheses are μmol of O_2 evolved mg^{-1} Chl h^{-1}.

tained for several minutes with only PGA (no PEP or Pi) in the medium (Fig. 1).

PEP Inhibition. PEP inhibited PGA-dependent O_2 evolution of crabgrass chloroplasts whether it was included during preincubation in the dark (Fig. 1, B and D; Fig. 2B) or added after several minutes of illumination of the chloroplasts in the presence of PGA (Fig. 2A). When added in the dark, PEP inhibited O_2 evolution by decreasing the initial slope and, particularly at very low PGA concentrations, resulted in a more rapid decline from the maximum linear rate (Figs. 1 and 2). Addition of PEP to illuminated chloroplasts produced an immediate inhibition of O_2 evolution, which was linear and without a lag period (Fig. 2A). The inhibition by PEP observed in Figure 2, A and B, could be reversed by the subsequent addition of PGA. Although not shown, addition of Pi (0.05–10 mM) did not overcome the inhibition by PEP.

The inhibition of PGA-dependent O_2 evolution by PEP was only found to occur when the PGA concentrations were low and a high ratio of PEP to PGA was maintained (Figs. 1–3). In some experiments PEP was kept constant (9 mM) and the PGA concentration varied to give different ratios of PEP/PGA. In this case, when the PEP/PGA ratio was 25 the per cent inhibition was 54 to 70% (Figs. 1, A and B; 2A; 3A). When the ratio was reduced to 5 by increasing PGA, the inhibition was reduced to about 20% (Figs. 2A and 3A), and usually no inhibition was seen at ratios of PEP to PGA <2 (Figs. 1, E and F; 2A; 3A).

PEP was found to inhibit O_2 evolution in both crude (Fig. 4) and Percoll-purified (Fig. 3) chloroplasts regardless of the difference in activity of PEP carboxylase between the two preparations. Using 9 mM PEP, the maximum inhibition recorded at very low PGA concentrations (0.14 mM) was about 65 to 75%. At such

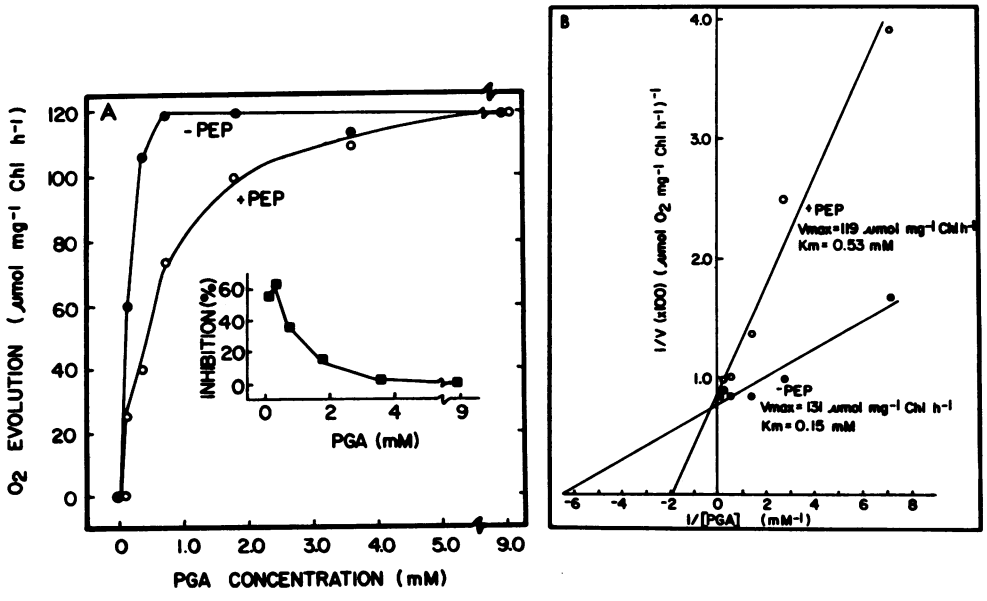

FIG. 3. A, Influence of PGA concentration on the rate of O_2 evolution in the presence or absence of 9 mM PEP by Percoll-purified chloroplasts of crabgrass. Inset shows the percentage inhibition of O_2 evolution by the addition of PEP. B, Double reciprocal plot of O_2 evolution *versus* PGA concentration and with or without PEP from the data in A.

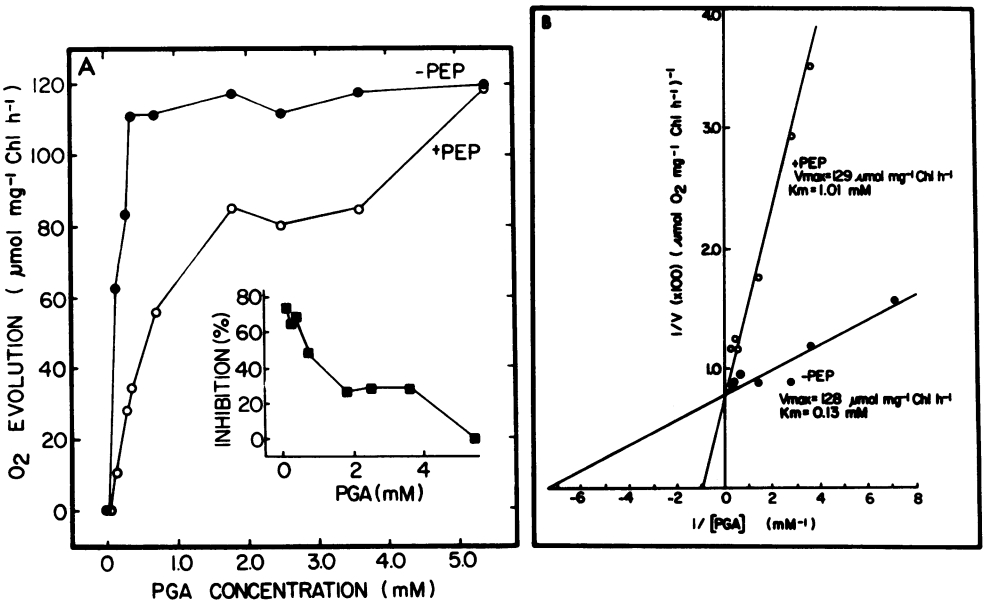

FIG. 4. A, Influence of PGA concentration on the rate of O_2 evolution in the presence or absence of 9 mM PEP by crude mesophyll chloroplast preparations of crabgrass. Inset shows inhibition of O_2 evolution by the addition of PEP. B, Double reciprocal plot of O_2 evolution *versus* PGA concentration in the presence or absence of PEP from the data in A.

low PGA concentrations, it became difficult to measure the exact rates due to nonlinearity of O_2 evolution in the presence of PEP. As the PGA was increased, there was a continual decline in the per cent inhibition by 9 mM PEP in Percoll-purified chloroplasts, with inhibition becoming insignificant at PGA concentrations greater than about 3.0 mM (Fig. 3A, inset). In the crude preparation, inhibition by PEP remained at about 25 to 30% until PGA was increased to at least 4.0 mM. At 5.4 mM PGA, no inhibition by PEP was observed (Fig. 4A, inset).

Double reciprocal plots of the rate of O_2 evolution *versus* PGA concentration indicated that PEP was a competitive inhibitor of PGA-dependent O_2 evolution in both crude (Fig. 4B) and purified (Fig. 3B) crabgrass mesophyll chloroplasts. The apparent K_m for PGA (without PEP) was 0.13 and 0.15 mM increasing to 0.53 and 1.01 mM, with the addition of 9 mM PEP, in purified and crude chloroplast preparations, respectively. The theoretical V_{max}s were similar to those observed experimentally in each case and did not differ significantly in crude *versus* purified chloroplasts.

Interactions between Pi, PEP, and PGA. The effect of Pi addition on PGA-dependent O_2 evolution by crabgrass chloroplasts was investigated using saturating (Fig. 5) and low, nonsaturating (Fig. 6) amounts of PGA, in the presence and absence of PEP. In Figure 5, both PGA and PEP (where added) equalled 9 mM. From the previous data (Figs. 1 and 3A) and as shown here (Fig. 5), at a PEP to PGA ratio of 1, and in the absence of Pi, no inhibition of O_2 evolution by PEP was observed. Pi (200 μM) stimulated O_2 evolution 44% and 52% in the presence and absence of PEP, respectively. When the Pi concentration was near optimum, there was some inhibition by PEP (up to 22%; Fig. 5, inset). With increasing Pi, the per cent inhibition by PEP decreased, approaching zero at 5 mM Pi.

In Figure 6, PEP was again 9 mM (where added), but the PGA concentration was reduced to 0.7 mM and hence there was significant inhibition of O_2 evolution by PEP without Pi present. As in Figure 5, Pi stimulated O_2 evolution in both the presence and absence of PEP, but at much lower concentrations of Pi and to a lesser extent (Fig. 6). Without PEP, the Pi optimum was 20 μM and shifted to 100 μM with the addition of PEP. With increasing Pi, the inhibition of O_2 evolution by PEP decreased from a maximum of 50% to 20% (Fig. 6, inset). This was mainly due to Pi having little effect on O_2 evolution in the presence of PEP. Without PEP, the rate of O_2 evolution decreased from a

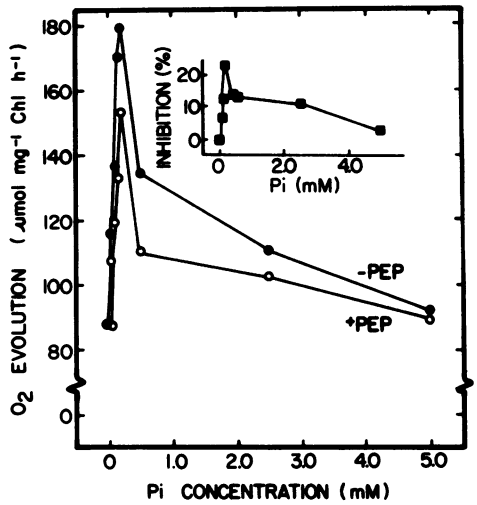

FIG. 5. Influence of Pi concentration on the rate of PGA-dependent O_2 evolution with 9 mM PGA and ±9 mM PEP. Inset shows percentage inhibition by PEP compared to the control without PEP.

maximum of 105 to 59 μmol mg^{-1} Chl h^{-1} at 1.0 mM Pi, compared to a change of only 64 to 47 μmol mg^{-1} Chl h^{-1} in the presence of PEP (Fig. 6).

ATP and PEP Inhibition. Since the adenine nucleotide translocase has been proposed to exchange ATP for PEP in maize mesophyll chloroplasts (26), we examined, in two ways, the possibility that PEP was inhibiting O_2 evolution in crabgrass mesophyll chloroplasts by depleting the chloroplasts of ATP. First, chloroplasts were preincubated with PGA and with or without PEP for 0 to 10 min and then the light and PGA-dependent O_2 evolution rates were recorded (Table I). There was essentially no effect of preincubation in the dark on the percentage inhibition of PGA-dependent O_2 evolution by PEP. A slight decrease in O_2 evolution was observed in both the control and control with PEP treatments with increasing dark incubation time past 2 min.

Second, various concentrations of ATP, Mg^{2+}, and PEP were added in the dark or after a 2-min light period to chloroplasts with PGA. Whether ATP (1.0–4.5 mM) was added in the dark or light, it did not prevent PEP inhibition of O_2 evolution (Table

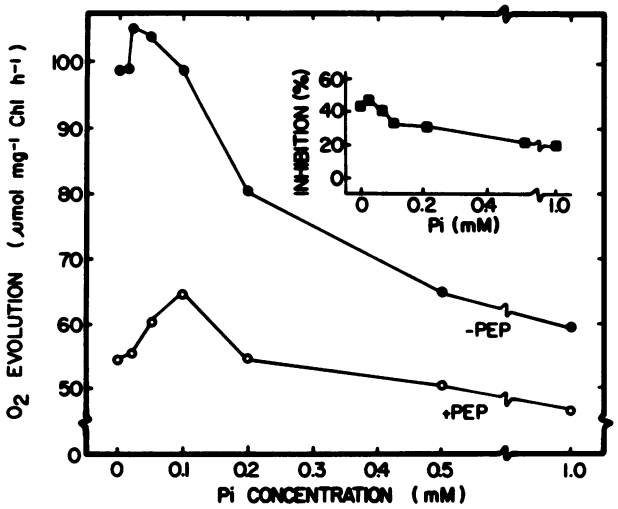

FIG. 6. Influence of Pi concentration on the rate of PGA-dependent O_2 evolution with 0.71 mM PGA and ±9 mM PEP. Inset shows percentage inhibition by PEP.

Table I. *Effect of Dark Incubation Time on PGA-Dependent O_2 Evolution With and Without PEP*

Crabgrass chloroplasts were incubated in the dark with 0.71 mM PGA and with or without 9 mM PEP for the times indicated, and then the rate of O_2 evolution recorded.

Dark Incubation Time	O_2 Evolution		Inhibition by PEP
	−PEP	+PEP	
min	*μmol mg⁻¹ Chl h⁻¹*		%
0	100.6	63.6	36.8
2	117.4	73.6	37.3
5	110.7	69.0	37.7
10	107.8	63.3	41.3

Table II. *Effect of ATP on PEP Inhibition of PGA-Dependent O_2 Evolution*

Crabgrass chloroplasts were incubated for 2 min in the dark with 0.71 mM PGA and with or without the compounds indicated before illumination and measurement of O_2 evolution (first rate). After 2 min illumination, PEP or ATP was added (where indicated) and the second rate recorded.

Compounds Added in Dark			First Rate	Compounds Added after 2 Min Light		Second Rate
PEP	ATP	Mg^{2+}		PEP	ATP	
mM			*μmol mg⁻¹ Chl h⁻¹*	*mM*		*μmol mg⁻¹ Chl h⁻¹*
0	0	0	115.0	9	0	57.5
9	0	0	73.6	0	1.0	55.2
9	1.8	0	57.5			
9	1.8	1.0	57.5			
9	4.5	1.0	64.4			
0	1.8	1.0	115.0	9	0	46.0
0	4.5	1.0	115.0	9	0	46.0

II). Likewise, addition of Mg^{2+} with ATP had no protective effect. Even if Mg-ATP was present with PGA in the dark and PEP was not added until 2 min after illumination, the same degree of inhibition occurred.

K_i (PEP) in Spinach *versus* Crabgrass. PGA-dependent O_2 evolution was also measured in mechanically isolated C_3 chloroplasts of spinach, with and without PEP. The maximum rates achieved varied from 103 to 120 μmol O_2 mg^{-1} Chl h^{-1} (Fig. 7). PEP inhibited O_2 evolution, but to a much lesser extent than in crabgrass mesophyll chloroplasts. Inhibition by PEP (9 mM) was maximum at very low PGA (0.14 mM) and decreased rapidly to about 25 to 20% and remained in this range until PGA levels were greater than 4.0 mM (Fig. 7, inset). As before, there may be significant variation at the lowest PGA concentration due to difficulty in obtaining a measurable linear rate.

Inhibition constants (K_i) for PEP can be calculated by plotting the O_2 evolution rate at two different substrate (PGA) levels *versus* inhibitor (PEP) concentration, *i.e.* a Dixon plot (22). This was done for both spinach (Fig. 8A) and crabgrass (Fig. 8B) chloroplasts. Using the relation, $[I] = -K_i$ where the two lines intersect, the apparent K_i (PEP) for spinach was calculated to be 20.9 mM (18.6 mM in a second experiment) and 3.0 mM for crabgrass.

DISCUSSION

Transport of PEP across the C_4 mesophyll chloroplast envelope is essential for carboxylation by PEP carboxylase located in the cytoplasm of C_4 plants (7). Previous reports have suggested that PEP is transported on a phosphate translocator (2, 9, 15), and more recently, that the adenine nucleotide translocator may catalyze the exchange of PEP and ATP (26) (see Fig. 9). Evidence for these translocators arose out of studies on transport using the silicone oil centrifugation technique. It is reasonable to expect that PEP could inhibit PGA-dependent O_2 evolution of C_4 mesophyll chloroplasts through its effect on carrier mediated transport (via competing for PGA uptake, or exchanging adenylates or Pi out of the chloroplast). However, conflicting reports have appeared in the literature (see "Introduction") and there is the further question of whether PEP may regulate PGA reduction *in vivo*.

Chloroplast Purity and PEP Inhibition. Hallberg and Larsson (9) were recently able to show inhibition of PGA-dependent O_2 evolution by PEP using *Digitaria sanguinalis* (crabgrass) chloroplasts. While this was the first evidence that PEP and PGA can interact in transport during metabolism of the C_4 chloroplast, there are limitations in interpreting the data. In their effort to eliminate PEP carboxylase and achieve very pure chloroplasts, a long (1.5 h after protoplast breakage) preparation period was

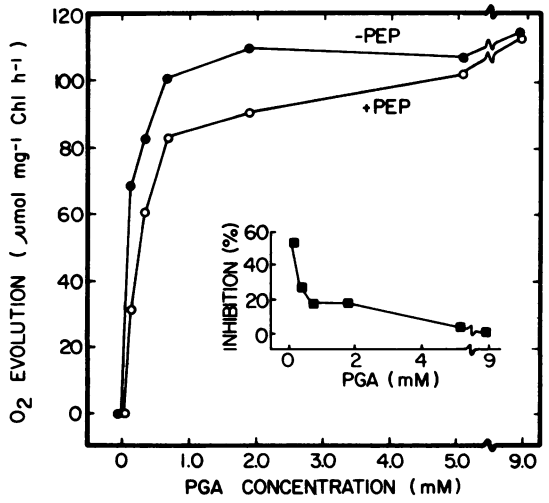

FIG. 7. Influence of PGA concentration on the rate of O_2 evolution with or without 9 mM PEP in Percoll-purified spinach chloroplasts. Inset shows the percentage inhibition by PEP. The assay included 3.6 mM glycolaldehyde (see "Materials and Methods").

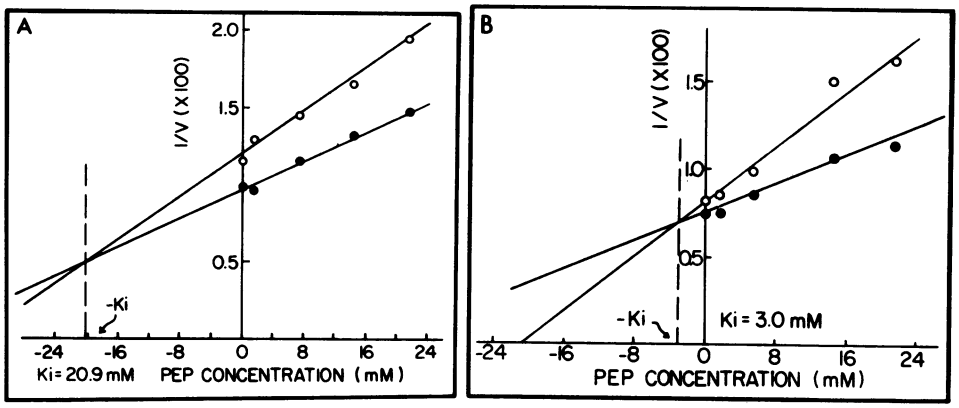

FIG. 8. A, Dixon plot of the rate of O_2 evolution *versus* PEP concentration at 0.71 mM (O) and 1.8 mM (●) PGA from spinach chloroplasts. The *r*-values (correlation coefficients) were 0.993 (●) and 0.996 (O), respectively. B, Dixon plot of the rate of O_2 evolution *versus* PEP concentration at 0.71 mM (O) and 1.8 mM (●) PGA in mesophyll chloroplasts of crabgrass. The *r*-value in each case equalled 0.983.

used. This resulted in low activities of PGA-dependent O_2 evolution. As they noted, essential metabolites and nucleotides may have been lost from the chloroplasts during preparation, which would affect the rates. In evaluating PEP inhibition of O_2 evolution, the control rates were only about 32 μmol O_2 mg^{-1} Chl h^{-1} and decreased 30% in 25 min even without PEP. Thus, the assay of PEP inhibition of O_2 evolution by consecutively adding PEP in the light over a 25-min period (9) is questionable in its accuracy (requires a correction of 30% on a low rate and no O_2 evolution traces were presented), and would also be unreliable for determining the nature of the inhibition by PEP. A further complication is that Pi was included in the assays when PEP was evaluated as an inhibitor (9). As shown in the present study, low amounts of Pi stimulated PGA-dependent O_2 evolution in crabgrass mesophyll chloroplasts (Fig. 5) and resulted in better linearity with time (not shown). However, when looking at PEP or Pi inhibition, analyses would be complicated by addition of a basal level of Pi (Figs. 5 and 6).

We have shown here that *Digitaria* mesophyll chloroplasts can be isolated with minimum preparation time by breaking protoplasts with a Yeda press and either pelleting the chloroplasts directly or further purifying with Percoll centrifugation. Although the PEP carboxylase was somewhat higher in the crude than in the Percoll-purified chloroplasts, both preparations exhibited PEP inhibition of PGA-dependent O_2 evolution to about the same extent. The PEP carboxylase in the Percoll purified chloroplasts was similar to that of Hallberg and Larsson (9). While they suggested previous failure to observe PEP inhibition may be due to the presence of PEP carboxylase, they only made comparisons between protoplast extracts (which would have very high PEP carboxylase activity) and the Percoll-purified chloroplasts. In most of our experiments, PEP was preincubated with the chloroplast suspension plus PGA (no Pi) in the dark for 2 min before recording O_2 evolution, although inhibition by PEP could be observed by its addition in the light. When varying the concentration of a metabolite, a separate assay was used for each concentration rather than consecutive additions to one assay, due to the problem already noted in making corrections for nonlinear rates. Reliable rates of O_2 evolution could be achieved until the PGA added was reduced to about 0.14 mM. Furthermore, high concentrations of PEP had no direct effect on the activities of PGA-kinase or NADP-glyceraldehyde phosphate dehydrogenase (enzymes required for PGA reduction) in the chloroplast extract (data not shown).

The reported lack of inhibition of PGA-dependent O_2 evolution by PEP in maize (3) and *Panicum miliaceum* (5) mesophyll chloroplasts is still uncertain, but may be due in part to the following reasons. In the maize studies, a relatively high concentration of PGA (2.5 mM) was added initially and then Pi was added before addition of 5 mM PEP. The Pi stimulation of O_2 evolution could mask any PEP inhibition. In addition, with a PEP to PGA ratio of 2, very little if any inhibition would be expected according to our data with crabgrass. With *P. miliaceum*, a PEP/PGA ratio of 5 or 10 resulted in no inhibition of O_2 evolution even with only 0.3 mM PGA (5). The capacity of the phosphate translocator in these chloroplasts may well exceed the capacity for reduction of PGA in the chloroplast such that the uptake of PGA is not limited even in the presence of high PEP. Alternatively, these chloroplasts may be less sensitive to PEP (higher K_i value), or PEP and PGA may be transported on separate carriers. More experiments with both of these species will be required to determine why inhibition by PEP was not observed.

Effect of Pi on PGA-Dependent O_2 Evolution. The stimulation of PGA-dependent O_2 evolution by Pi with the crabgrass chloroplasts was particularly apparent at high concentrations of PGA (Fig. 5). Day *et al.* (3) also observed stimulation of PGA-dependent O_2 evolution by Pi in maize mesophyll chloroplasts. At high concentrations of PGA, the chloroplast may be depleted of Pi which could limit photophosphorylation. Thus, addition of a low concentration of Pi may prevent excessive exchange of Pi from the chloroplast. On the other hand, when the PGA concentration was low (Fig. 6), there was little stimulation of PGA-dependent O_2 evolution by addition of Pi. Rather, under a low concentration of PGA, Pi readily inhibited PGA-dependent O_2 evolution apparently by preventing uptake of PGA into the chloroplast on the phosphate translocator. Pi appears to be a much better inhibitor of PGA-dependent O_2 evolution than PEP. Pi, at 1 mM, inhibited O_2 evolution about 40% at 0.7 mM PGA and without PEP (Fig. 6). At the same PGA concentration, about 9 mM PEP was required to inhibit O_2 evolution 40% (Fig. 3A).

PEP Translocators. Three possible means of transporting PEP across the mesophyll chloroplast envelope to the cytoplasm in *Digitaria* are depicted in Figure 9. We have demonstrated competitive inhibition of PGA-dependent O_2 evolution by PEP in *Digitaria* suggesting PEP is translocated on the phosphate

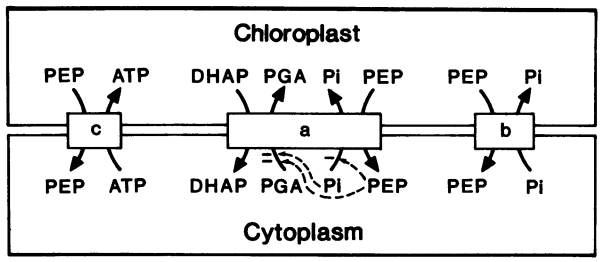

FIG. 9. Proposed translocators for PEP transfer from the mesophyll chloroplast to the cytosol in C_4 plants. A, Phosphate translocator (modified from Day and Hatch [2]); B, a specific PEP/phosphate translocator; C, adenine nucleotide translocator. Dashed arrows with (−) indicate inhibition of transport at that site by the indicated compound.

translocator with PGA (Fig. 9A) which is consistent with the conclusions from transport studies on maize using silicone oil centrifugation techniques (2). From direct studies on transport, spinach (C_3) and crabgrass chloroplasts have similar K_m values for PGA uptake, while the spinach chloroplasts transport PEP poorly (2, 8). The K_i values for PEP against Pi uptake are approximately 10 times greater in spinach than in the C_4 chloroplast (8, 15). Our K_i (PEP) values relative to PGA-dependent O_2 evolution of 3 and 21 mM for crabgrass and spinach, respectively, reflect this difference in PEP recognition by the phosphate translocator. The K_i value (PEP) for PGA uptake based on silicone oil centrifugation studies in maize was 1.5 mM (2), or about one-half of what we determined from O_2 evolution studies with crabgrass.

In particular, then, this study does not support the existence of a distinct PEP/Pi translocator in *Digitaria* mesophyll chloroplasts (Fig. 9B) as the means of transporting PEP. In such a case, PEP and PGA would not be expected to show competitive interactions. Furthermore, if PEP/Pi exchange and PGA/triose-P exchange occurred on separate carriers, PEP might inhibit by exchanging out Pi, in which case inhibition should be reversed by exogenous Pi. However, addition of Pi could not overcome the inhibition of PGA-dependent O_2 evolution by PEP (Fig. 6). This suggests that it is not the depletion of internal Pi by PEP, but rather a limitation on PGA uptake which results in the inhibition of the O_2 evolution observed.

Huber and Edwards (13) have shown that *Digitaria* chloroplasts can transport ATP on the adenine nucleotide translocator at minimum rates of 30 to 40 μmol mg^{-1} Chl h^{-1}. Woldegiorgis *et al.* (26) have shown that PEP can exchange with ATP involving the adenine nucleotide translocator (Fig. 9C) in pea and maize. If this could occur at significant rates in crabgrass, then PEP might inhibit PGA-dependent O_2 evolution via exchanging adenylates from the chloroplasts. However, there are several reasons why this would not appear to be the means of PEP inhibition in the present study. PEP was a competitive inhibitor with respect to PGA, suggesting that PEP and PGA are competing for uptake on the phosphate translocator, and PGA can overcome the PEP inhibition. Preincubation of the chloroplasts in the dark with PEP for up to 10 min did not result in a significant increase in PEP inhibition of PGA-dependent O_2 evolution, suggesting PEP is not exchanging adenylates out of the chloroplasts (Table I). Also, addition of ATP in the light or dark with PEP did not prevent inhibition of O_2 evolution which might be expected if PEP was capable of exchanging out adenylates (Table II). An exchange of cytoplasmic PEP with chloroplast adenylates would not appear to be of physiological importance in C_4 photosynthesis.

Studies on the interaction of metabolites on PGA-dependent O_2 evolution in C_4 mesophyll chloroplasts provides some qualitative information relative to transport mechanisms. There is reason to suggest from the present study that PEP and PGA exchange on a common phosphate translocator (Fig. 9A), and that PEP exchange with Pi on a distinctive translocator (Fig. 9B) and PEP exchange with adenylates (Fig. 9C) are not significant means of PEP transport. However, it is clear that experiments with a metabolic system do not provide quantitative evidence on rates of transport of metabolites or full interpretation relative to kinetic constants for carrier mediated transport of metabolites. For example, if the capacity of transport exceeds by far the capacity for PGA-dependent O_2 evolution, then a partial inhibition of transport would not influence the rate of PGA reduction. Transport rates in the light or dark will depend not only on the kinetic constants for substrate binding to the carrier, but the relative concentrations of substrates which are recognized by the carrier (*e.g.* Pi, PGA, triose-P) in both the chloroplast and medium. Thus, complete interpretation of transport in a quantitative way will only be possible by measuring transport in both directions directly (*e.g.* PEP import *versus* PEP export in a silicone oil centrifugation system) and by evaluating metabolite concentrations.

PEP Inhibition *In Vivo*. Metabolite transport is essential to the continued operation of the C_4 cycle. We have examined indirectly whether PEP may interfere with PGA transport in C_4 mesophyll chloroplasts by following PGA-dependent O_2 evolution. One point that should be addressed is the likelihood or possible role of competitive interactions between PEP and PGA for uptake *in vivo*. We have shown that high concentrations of PEP are required to inhibit PGA-dependent O_2 evolution. If the ratio of PEP/PGA should become unusually high in the mesophyll cell, further synthesis of PEP might become limited. A high PEP/PGA ratio would limit PGA reduction in the mesophyll cells and the return of triose-P to the bundle sheath cells for regeneration of RuBP. Then, the decarboxylation of malate through malic enzyme, which is linked to PGA reduction (7), would be limited which would cut off pyruvate synthesis which is the precursor for the formation of PEP. As PEP is metabolized, the ratio of PEP/PGA would then decrease and become more favorable for PGA reduction. The *in vivo* concentration of PGA in mesophyll cells of illuminated maize leaves has been estimated to be 8 mM and the PEP/PGA ratio about 0.5 (16). From our data, at least 40 mM PEP would be required to inhibit O_2 evolution about 20% if the PGA concentration was 8 mM. Less than 16 mM PEP would result in no inhibition. With such a low ratio of PEP/PGA in the mesophyll cell it seems unlikely that PEP may ever reach high enough levels *in vivo* to be inhibitory.

If the PGA concentration available to the mesophyll cells is relatively high (*i.e.* 5 or 10 mM), the main limitation on PGA utilization may be on the reductive phase in the chloroplast (enzyme capacity and provision of energy) such that even high PEP levels would be without effect. This could be the reason why no inhibition is seen by PEP when both it and PGA concentrations are relatively high (*i.e.* 9 mM as in Fig. 5). It is known from previous work (12) that PGA is at a branch point in metabolism in the cytoplasm of the mesophyll cell, in that it can be converted to PEP through phosphoglyceromutase and enolase, or taken up by the chloroplast and reduced to triose-P. This flexibility in the metabolism of PGA likely allows a balance between the function of the C_4 cycle and the reductive pentose phosphate pathway. Thus, it appears that circumstances may exist in C_4 photosynthesis such that PEP and PGA can be transported on the phosphate translocator in the C_4 mesophyll chloroplast without PEP interfering with PGA reduction.

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