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Influence of Organic-Phosphates on 3-Phosphoglycerate Dependent O_2 Evolution in C_3 and C_4 Mesophyll Chloroplasts

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In C_4 plants phosphoenolpyruvate (PEP) of the C_4 cycle may be transported on a chloroplast transporter which also transports 3-phosphoglycerate (3-PGA) and triose-phosphates. In C_3 plants PEP is not considered to be effectively transported on the chloroplast phosphate translocator. The influences of certain organic phosphates, having a similar structure to either PEP or triose-phosphates, on 3-PGA dependent O_2 evolution by C_4 (*Digitaria sanquinalis* L. Scop.) and C_3 (*Hordeum vulgare* L.) mesophyll chloroplasts were investigated. In the C_4 mesophyll chloroplasts phosphoglycolate was a competitive inhibitor (K_i=2.1 mM) of 3-PGA dependent O_2 evolution, and was as effective as previously reported for PEP. 2-Phosphoglycerate was also a competitive inhibitor (K_i=8.6 mM) of O_2 evolution in the C_4 mesophyll chloroplasts with 3-PGA as substrate, while phospholactate was a weak inhibitor and glyphosate had no effect. Neither PEP, phosphoglycolate nor 2-phosphoglycerate were effective inhibitors of 3-PGA dependent O_2 evolution in the C_3 chloroplasts. Phosphohydroxypyruvate was a competitive inhibitor of 3-PGA dependent O_2 evolution in both chloroplast types. The selectivity in inhibition of O_2 evolution with 3-PGA as substrate suggests that the C_4 mesophyll chloroplasts can recognize certain organic phosphates with the phosphate in the C-2 or C-3 position but that the C_3 mesophyll chloroplasts can only effectively recognize certain organic phosphates with the phosphate in the C-3 position. The results also support the view that 3-PGA and PEP are transported on the same phosphate translocator in C_4 mesophyll chloroplasts.

Key words: Chloroplast (phosphate translocator, O_2 evolution) — C_4 plant — *Digitaria sanquinalis* L. — *Hordeum vulgare* L. — 3-Phosphoglycerate.

During photosynthesis in C_4 plants, exchanges between PEP/P_i and 3-PGA/triose-P occur in the mesophyll chloroplasts. The PEP/P_i exchange is associated with the C_4 cycle. PEP is formed in the chloroplast through the action of pyruvate,P_i dikinase, and exported in exchange for P_i (Hatch and Osmond 1976). In the cytosol PEP is then utilized in CO_2 fixation through PEP carboxylase, with oxaloacetate and P_i as products. The mesophyll chloroplasts also contribute to the reduction of 3-PGA to triose-P. Part of the 3-PGA formed in bundle sheath cells is

Abbreviations: Chl, chlorophyll; K_i, inhibition constant; PEP, phosphoenolpyruvate; 3-PGA, 3-phosphoglycerate; 2-PGA, 2-phosphoglycerate; P-glycolate, phosphoglycolate; P-hydroxypyruvate, phosphohydroxypyruvate; P-lactate, phospholactate; triose-P, triose phosphates (dihydroxyacetonephosphate+glyceraldehyde 3-phosphate); DIDS, 4,4′-diisothiocyano-2,2′-disulfonic acid stilbene.

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transported to mesophyll chloroplasts and reduced to triose-P, resulting in 3-PGA import and triose-P export (Edwards and Walker 1983).

Although the phosphate translocator(s) of C_4 mesophyll chloroplasts has not been isolated, there are results which suggest that the exchange of PEP/P_i and 3-PGA/triose-P occur on a single common translocator of the chloroplast envelope (Day and Hatch 1981, Hallberg and Larsson 1983, Rumpho and Edwards 1984, 1985). Evidence for a PEP translocator which exchanges PEP/P_i in C_4 mesophyll chloroplasts was provided by Huber and Edwards (1977), while C_3 chloroplasts have been shown to transport PEP poorly (Fliege et al. 1978, Day and Hatch 1981). Direct studies on metabolite transport with C_4 mesophyll chloroplasts have shown a competitive inhibition of 3-PGA uptake by PEP and P_i (Day and Hatch 1981). PEP was shown to be a competitive inhibitor of 3-PGA dependent O_2 evolution in the C_4 mesophyll chloroplasts of *Digitaria sanguinalis* with a K_i of 3 mM (Rumpho and Edwards 1984). More direct evidence was provided by use of DIDS which, in micromolar levels, irreversibly inhibited 3-PGA dependent O_2 evolution in C_4 mesophyll chloroplasts. This inhibition could be prevented by preincubation with PEP, 3-PGA or P_i before addition of DIDS (Rumpho and Edwards 1985). Furthermore, DIDS inhibited 3-PGA dependent O_2 evolution in barley chloroplasts. This inhibition could be prevented by preincubation with 3-PGA but not by preincubation with PEP.

Since these data suggest 3-PGA and PEP are transported on a common carrier in C_4, but not in C_3, mesophyll chloroplasts, it was of interest to evaluate whether certain naturally occuring or synthetic organic phosphates which have some similarity in structure to PEP may selectively inhibit 3-PGA dependent O_2 evolution in the C_4 mesophyll chloroplasts. In the present study we examined the effect of several such compounds on the 3-PGA dependent O_2 evolution by mesophyll chloroplasts of *Digitaria sanguinalis* (C_4) and *Hordeum vulgare* (C_3).

Materials and Methods

Plant material—Digitaria sanguinalis L. Scop. (crabgrass) and *Hordeum vulgare* L. var. Marex (barley) were grown in growth chambers as previously described (Rumpho and Edwards 1985). Crabgrass Leaves (4–6 cm in length) were used for protoplast isolation from seedlings 14 to 16 days old. For barley protoplast isolation, the leaves were 6 to 8 cm long and taken from seedlings 8 to 10 days old.

Isolation of chloroplasts from protoplasts—Chloroplasts were isolated from enzymatically prepared mesophyll protoplasts of either crabgrass or barley as previously detailed (Rumpho and Edwards 1984, 1985). The chloroplasts were purified by centrifugation through a 20% (v/v) Percoll solution (Mills and Joy 1980). The resuspension medium consisted of 330 mM sorbitol, 10 mM EDTA, 0.2% BSA and 50 mM HEPES (pH 7.8). The chloroplast preparations were stored on ice throughout.

Oxygen evolution—3-PGA-dependent O_2 evolution was measured polarographically at 30°C with Rank Brothers (Cambridge, U.K.) O_2 electrodes. The photosynthetic photon flux density was 800 to 1,000 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at the chamber surface. Chl, ranging from 17–19 μg for barley and 16–24 μg for crabgrass, was added to the reaction medium containing 330 mM sorbitol, 10 mM EDTA, 50 mM HEPES (pH 7.8), and 200 units/ml catalase, with a final volume of 1.4 ml. In addition, 3.6 mM glycolaldehyde was added to the reaction medium for barley chloroplasts to prevent any CO_2 dependent O_2 evolution (Sicher 1984). The time of addition of chloroplasts, substrates/inhibitors (all at pH 7.8), was 1.5 min prior to illumination. All rates of O_2 evolution refer to the initial maximum linear rate. Chl was determined by the method of Wintermans and De Mots (1965).

Reagents—The sodium salts of 3-PGA and 2-PGA, and the potassium salt of PEP were purchased from Sigma, St. Louis, Missouri, U.S.A. The dimethylketal, cyclohexylammonium

salt of P-hydroxypyruvate was converted to the potassium salt by treating with Dowex 50(H+) resin, neutralizing with KOH, and standing at room temperature for 24 h; the cyclohexylammonium salt of P-glycolate was converted to the potassium salt by treatment with dowex 50(H+) resin and neutralizing with KOH as detailed by Sigma. Glyphosate was a gift from Dr. John Franz of Monsanto Agricultural Products Co., St. Louis, Missouri, and P-lactate and P-glycolate were generously supplied by Dr. Marion O'Leary, University of Wisconsin, Madison, and Dr. C. E. Ballou, University of California, Berkeley.

Results

Without addition of other potential effectors, 3-PGA dependent O_2 evolution by crabgrass (C_4) mesophyll chloroplasts had a maximum rate of approximately 120–170 μmol·(mg Chl)$^{-1}$·h^{-1} (Fig. 1 and 2). The rates with barley (C_3) chloroplasts, with saturating levels of 3-PGA in the absence of other organic phosphates, were about 50 to 100 μmol·(mg Chl)$^{-1}$·h^{-1} among separate

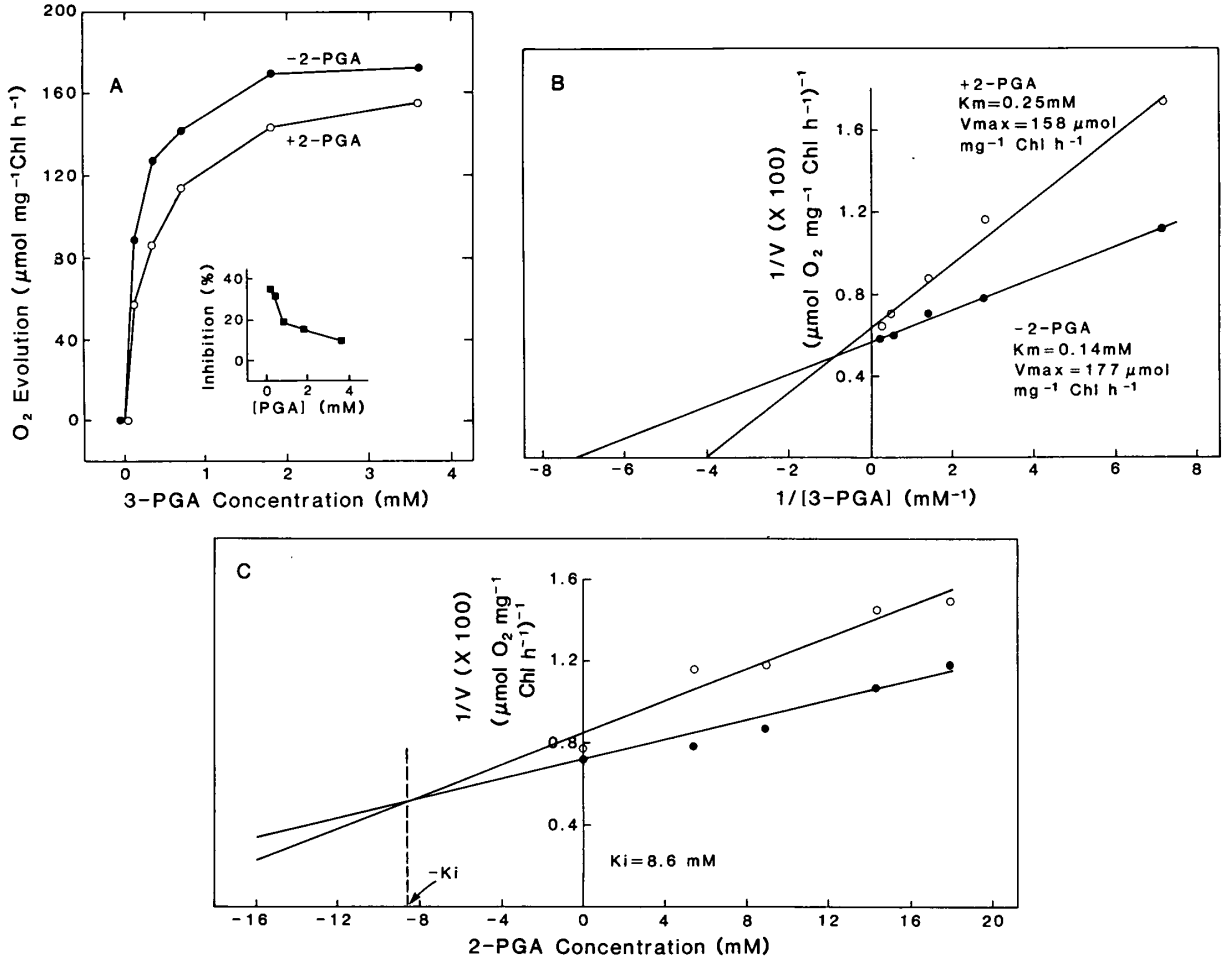


Fig. 1 A. The effect of 2-PGA (8.9 mM) on the rate of photosynthetic O_2 evolution by mesophyll chloroplasts of crabgrass with varying 3-PGA concentrations as substrate. Each point represents a separate assay. Inset shows the percentage inhibition of O_2 evolution by the addition of 2-PGA. B. Double reciprocal plot of O_2 evolution *versus* 3-PGA concentration and with or without 2-PGA from the data in panel A. C. Dixon plot of the rate of O_2 evolution *versus* 2-PGA concentration at 0.36 mM (○) and 0.71 mM (●) 3-PGA. The *r*-values (correlation coefficients) were 0.968 (○) and 0.972 (●), respectively.

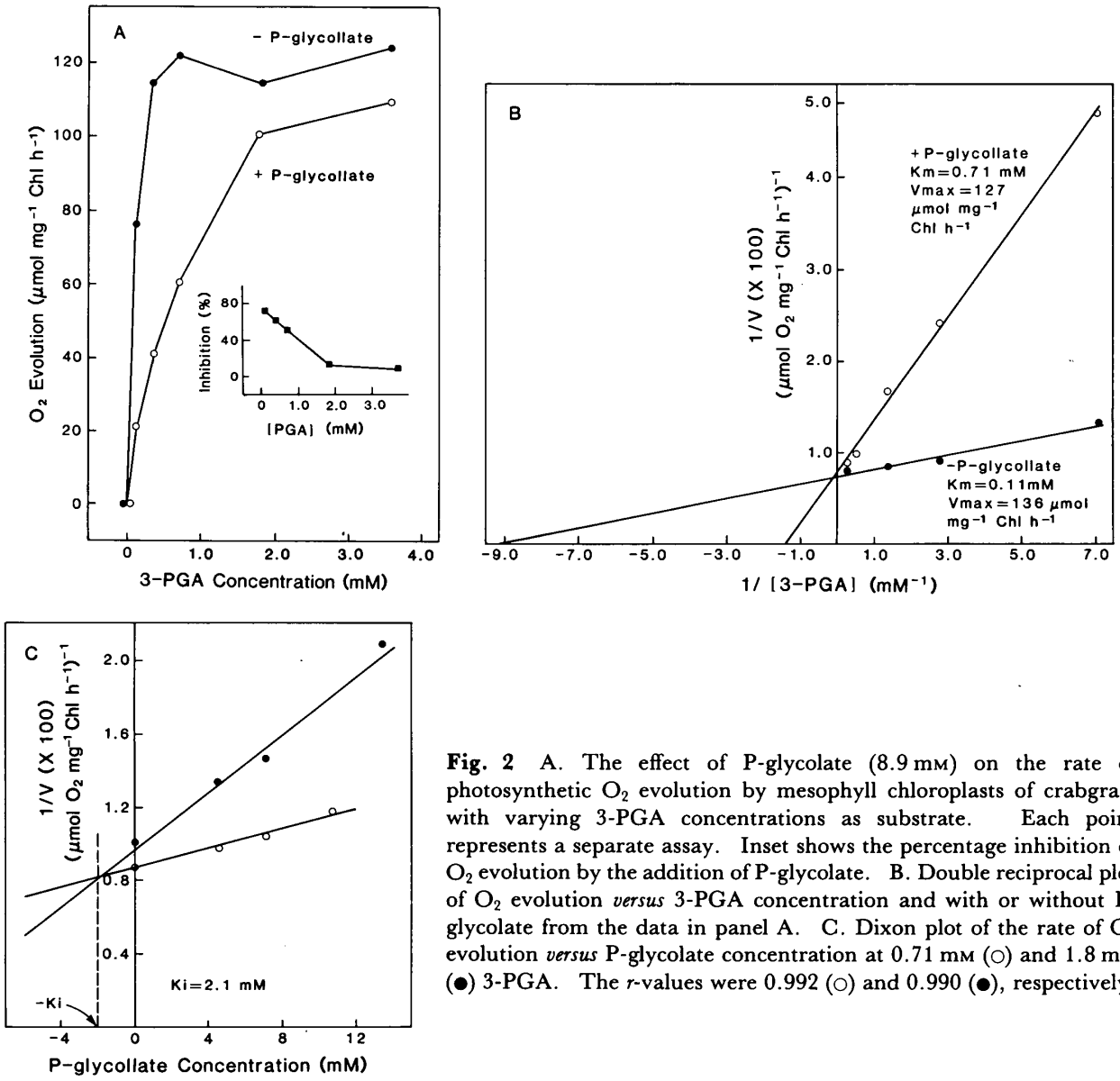


Fig. 2 A. The effect of P-glycolate (8.9 mM) on the rate of photosynthetic O_2 evolution by mesophyll chloroplasts of crabgrass with varying 3-PGA concentrations as substrate. Each point represents a separate assay. Inset shows the percentage inhibition of O_2 evolution by the addition of P-glycolate. B. Double reciprocal plot of O_2 evolution *versus* 3-PGA concentration and with or without P-glycolate from the data in panel A. C. Dixon plot of the rate of O_2 evolution *versus* P-glycolate concentration at 0.71 mM (○) and 1.8 mM (●) 3-PGA. The *r*-values were 0.992 (○) and 0.990 (●), respectively.

preparations (Table 2).

The effect of 8.9 mM 2-PGA on 3-PGA dependent O_2 evolution by the C_4 mesophyll chloroplasts is shown in Fig. 1. There was no O_2 evolution in the presence of 8.9 mM 2-PGA alone, which indicates that the C_4 chloroplast can not take up 2-PGA and metabolize it to 3-PGA. The lack of O_2 evolution in the presence of 2-PGA could either be due to limited uptake or lack of chloroplastic 3-phosphoglyceromutase for the enzymatic conversion from 2-PGA to 3-PGA. With 3-PGA alone the double reciprocal plot of the rate of O_2 evolution versus substrate concentration was linear, giving an apparent K_m for 3-PGA of 0.14 mM and a theoretical V_{max} of 177 $\mu mol \cdot (mg\ Chl)^{-1} \cdot h^{-1}$ (Fig. 1B). In the presence of 8.9 mM 2-PGA there was inhibition of O_2 evolution, which was strongest at low levels of 3-PGA (Fig. 1A). The double reciprocal plots suggested 2-PGA was a competitive inhibitor, and the Dixon plot gave a K_i value of 8.6 mM (Fig. 1C). In contrast, there was little or no inhibitory effect of 2-PGA (8.9 mM) on O_2 evolution of barley chloroplasts at a ratio of 2-PGA/3-PGA greater than 10 (Table 2), while there was

Table 1 The effect of several organic phosphates on 3-PGA dependent oxygen evolution by mesophyll chloroplasts of crabgrass

Compound tested	[Compound] (mM)	[3-PGA] (mM)	Control rate (μmol·(mg Chl)$^{-1}$·h^{-1})	Rate with compound (μmol·(mg Chl)$^{-1}$·h^{-1})	Inhibition (%)
Glyphosate	3.8	0.36	74	71	4
	3.8	0.71	96	95	0
P-lactate	7.0	0.71	121	106	12
	14.2	0.71	121	101	17
	21.2	0.71	121	91	25
PEP [1]	8.9	0.14	61	25	58
	8.9	0.36	108	40	62
	8.9	0.71	120	75	39

The chloroplasts were incubated with 3-PGA and in the presence or absence of various concentrations of other organic phosphates for 1.5 min in the dark, and then the rate of light- and 3-PGA dependent oxygen evolution recorded. The percentage inhibition was calculated by taking the [(control rate minus the rate with addition of organic phosphate)/control rate] × 100.

[1] Data from Rumpho and Edwards (1984).

Table 2 The effect of several organic phosphates on 3-PGA dependent oxygen evolution by mesophyll chloroplasts of barley

Compound tested	[Compound] (mM)	[3-PGA] (mM)	Control rate (μmol·(mg Chl)$^{-1}$·h^{-1})	Rate with compound (μmol·(mg Chl)$^{-1}$·h^{-1})	Inhibition (%)
Experiment I					
2-PGA	8.9	0.71	36	32	11
	8.9	3.5	47	61	+23 [a]
P-glycolate	8.9	0.71	36	29	19
	8.9	3.5	47	79	+41
Experiment II					
PEP	8.9	0.14	31	24	23
	8.9	0.36	44	37	16
	8.9	0.71	52	45	13
	8.9	1.8	79	67	15
Experiment III					
P-hydroxypyruvate	8.9	0.71	61	20	67
	8.9	3.5	99	90	9
PEP	8.9	0.71	61	63	+3
	8.9	3.5	99	125	+21
Inorganic phosphate	8.9	0.71	61	21	66
	8.9	3.5	99	73	26

See Table 1 for conditions.

[a] A "+" sign indicates percent stimulation.

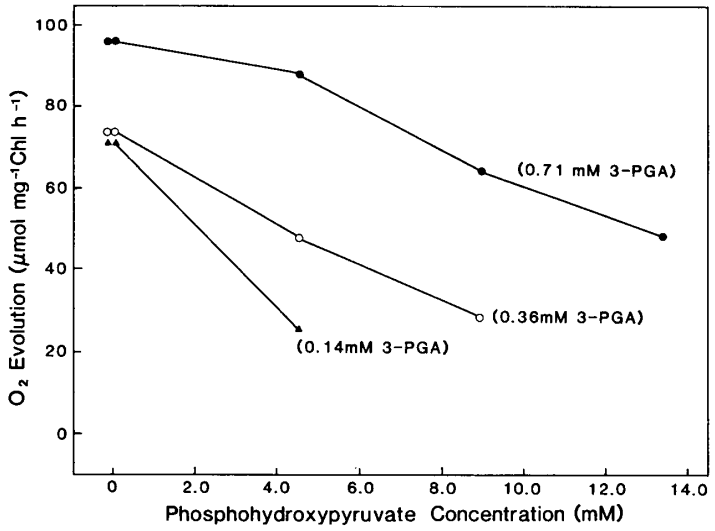


Fig. 3 The effect of varying P-hydroxypyruvate concentrations on photosynthetic O_2 evolution by mesophyll chloroplasts of crabgrass with three different concentrations of 3-PGA as substrate. Each point represents a separate assay.

some stimulation by 2-PGA at the higher concentration of 3-PGA.

The results with P-glycolate were similar to those with 2-PGA, except with the crabgrass chloroplasts P-glycolate was a stronger competitive inhibitor than 2-PGA (Fig. 2). With the crabgrass chloroplasts, 8.9 mM P-glycolate severely reduced the rate of O_2 evolution at low levels of 3-PGA, while it caused little inhibition at high levels of 3-PGA. Double reciprocal plots (Fig. 2B) showed competitive type inhibition, and from a Dixon plot the K_i value for P-glycolate was 2.1 mM (Fig. 2C). In contrast, with barley chloroplasts addition of P-glycolate caused a slight inhibition at low levels of 3-PGA (P-glycolate/3-PGA ratio greater than 10) and stimulation at a higher level of 3-PGA (P-glycolate/3-PGA ratio of 2.5). The addition of 8.9 mM PEP to barley chloroplasts had little effect at low levels of 3-PGA, but in one experiment caused some stimulation at high (3.5 mM) 3-PGA (Table 2). On the other hand, PEP inhibited 3-PGA dependent O_2 evolution in crabgrass chloroplasts (Table 1) and the inhibition was competitive with respect to 3-PGA (K_i=3 mM, Rumpho and Edwards 1984).

Two other organic phosphates, P-lactate and glyphosate (*N*-[phosphonomethyl]glycine), were examined for their effect on 3-PGA dependent O_2 evolution by the C_4 mesophyll chloroplasts. P-lactate, at concentrations from 7 to 21 mM, was a much weaker inhibitor than P-glycolate, and the nature of this inhibition was not further characterized (Table 1). Glyphosate, at a concentration of 3.8 mM, caused essentially no inhibition of 3-PGA dependent O_2 evolution by crabgrass chloroplasts, even with a ratio of glyphosate/3-PGA as high as 10 (Table 1).

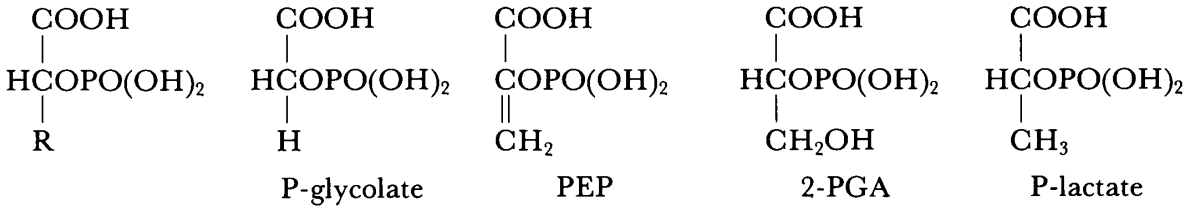
P-hydroxypyruvate was found to effectively inhibit 3-PGA dependent O_2 evolution by both crabgrass and barley chloroplasts at low concentrations of 3-PGA (Fig. 3 and Table 2). High concentrations of 3-PGA tended to prevent inhibition by P-hydroxypyruvate, which suggests the substrate and inhibitor were competing for the same binding site on the carrier.

High levels of P_i (8.9 mM) also inhibited photosynthetic O_2 evolution in barley chloroplasts with 3-PGA as substrate, with the greatest inhibition occurring at a low level of 3-PGA (Table 2). Similar results were previously obtained with P_i in crabgrass chloroplasts (Rumpho and Edwards 1984). Therefore, P-hydroxypyruvate and P_i are compounds which inhibit 3-PGA dependent O_2 evolution in both chloroplast types.

Discussion

The results of the present study are consistent with the view that 3-PGA and PEP are

transported on a common phosphate translocator in C_4 mesophyll chloroplasts, and that PEP is poorly transported by the phosphate translocator in C_3 chloroplasts. Two compounds P-glycolate and 2-PGA, as shown below, have similarities in structure to PEP. They are competitive inhibitors of 3-PGA dependent O_2 evolution in crabgrass chloroplasts, but cause little or no inhibition of 3-PGA dependent O_2 evolution in barley chloroplasts.

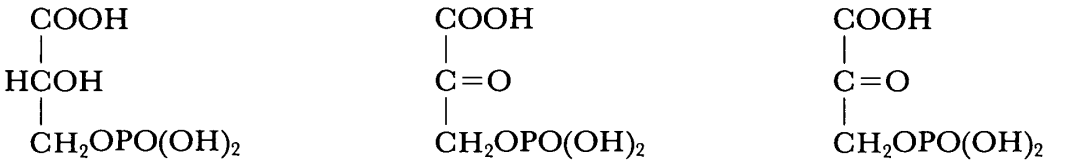


When R is an H or CH_2 group, there is similar inhibition, when R is a CH_2OH group it is less effective, and when R is a CH_3 group there is very little inhibition. Thus, when the phosphate group is at the second carbon there appears to be some specificity for recognition based on the structure at C-3.

P-glycolate is an inhibitor of PEP carboxylase (O'Leary 1982), and the implication of the present study is that it is recognized by the phosphate translocator of crabgrass, which transports PEP as well as P_i, 3-PGA, and DHAP. 2-PGA is also recognized by the phosphate translocator of crabgrass although it is not a strong inhibitor. This is consistent with the fact that 2-PGA has some ability to prevent inhibition of 3-PGA dependent O_2 evolution by DIDS in crabgrass chloroplasts (Rumpho and Edwards 1985). Previous studies showed that the phosphate translocator of C_3 chloroplasts had a much lower affinity for 2-PGA than for 3-C compounds having the phosphate on C-3 (Fliege et al. 1978).

The results with barley are consistent with the hypothesis that the phosphate translocator in C_3 chloroplasts can effectively recognize organic phosphates when the phosphate group is on C-3, but not when the phosphate group is on C-2. Thus PEP, 2-PGA and P-glycolate had little inhibitory effect on 3-PGA dependent O_2 evolution even with ratios of the compound/3-PGA greater than 10 fold. At higher concentrations of 3-PGA (3.5 mM) there was some evidence for stimulation of the rate of O_2 evolution by addition of 8.9 mM of PEP, 2-PGA, or P-glycolate. The stimulation of O_2 evolution may be a direct effect or the result of low levels of inorganic phosphate either being carried with the substrate or being generated in the assay medium due to phosphatase activity. It was previously shown (Rumpho and Edwards 1985) that very low concentrations of P_i (e.g. 50 to 100 μM) stimulated O_2 evolution by crabgrass chloroplasts at high concentrations of 3-PGA (9 mM 3-PGA). This is perhaps more likely with P-glycolate since C_3 chloroplasts have P-glycolate phosphatase and some activity could appear in the assay medium if a small percentage of the chloroplasts were broken during assay. In the presence of rather high concentrations of 3-PGA, and in the absence of any external inorganic phosphate, there may be exchange of P_i out of the chloroplasts, thereby limiting ATP synthesis. Low concentrations of P_i in the external medium may be sufficient to prevent this loss of P_i from the chloroplasts in the presence of high levels of 3-PGA.

In contrast to the above results with phosphate in the C-2 position, P-hydroxypyruvate was an effective inhibitor of photosynthetic O_2 evolution by barley chloroplasts at low levels of 3-PGA but not at high levels of 3-PGA. Similarly, in crabgrass P-hydroxypyruvate inhibited O_2 evolution, and this inhibition could be reduced by increasing concentrations of 3-PGA. These results support the view that the phosphate translocator of both chloroplast types can recognize certain organic phosphates which have phosphate in the C-3 position. As shown below, P-hydroxypyruvate is very similar in structure to 3-PGA and dihydroxyacetone phosphate.



3-PGA dihydroxyacetonephosphate P-hydroxypyruvate

While glyphosate is a potent inhibitor of 5-enolpyruvylshikimic acid-3-phosphate synthase (Steinrucken and Amrhein 1980), in which PEP and 3-phosphoshikimate are substrates, this herbicide did not inhibit 3-PGA dependent O_2 evolution by crabgrass chloroplasts. This suggests that the phosphonomethyl group attached to the *N* of glycine is not recognized by the phosphate translocator.

The use of labelled PEP and 3-PGA for direct studies on the effect of various C-2 and C-3 phosphates on their uptake by the two chloroplasts types will be required for full characterization of the differences between the phosphate translocators in their recognition of organic phosphates. As noted previously (Rumpho and Edwards 1984), if the capacity of transport exceeds, by far, the capacity for PGA-dependent O_2 evolution, then a partial inhibition of transport would not be reflected in the rate of PGA reduction. In addition, whether or not compounds which act as inhibitors can actually be transported needs to be examined, for example by measuring their effect on the back exchange of labelled 3-PGA or PEP which has been preloaded into the chloroplasts.

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