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INORGANIC PYROPHOSPHATASE AND PHOTOSYNTHESIS BY ISOLATED CHLOROPLASTS

II. THE CONTROLLING INFLUENCE OF ORTHOPHOSPHATE

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SUMMARY

1. It has been proposed that Mg^{2+}, inorganic pyrophosphate and a protein fraction which exhibits fructose-1,6-diphosphatase activity may interact to regulate photosynthesis by isolated chloroplasts.

2. Evidence is presented which confirms the interaction and regulation but shows that these effects are indirectly attributable to pyrophosphatase activity rather than fructose-1,6-diphosphatase

3. When provided with Mg^{2+} and PP_i the pyrophosphatase simply alters the proportions of orthophosphate and PP_i in the reaction mixture. As the P_i concentration is increased, it first stimulates and then inhibits, the degree of inhibition being enhanced by additional Mg^{2+}. PP_i ameliorates the inhibition, possibly by chelation of Mg^{2+}.

4 It is concluded that the proposed regulation is ultimately governed by the P_i concentration and the known relationship between P_i uptake and triose phosphate export across the chloroplast envelope.

INTRODUCTION

The inhibitory effect of orthophosphate on photosynthesis by isolated chloroplasts was first recognised by Arnon *et al.*[1] and has been extensively studied in this laboratory[2–4] The proposal[5] that "a direct obligatory exchange between orthophosphate (outside) and sugar phosphate (inside) could account for the inhibition of photosynthesis by orthophosphate and its reversal by sugar phosphates" has been supported by the work of Heldt *et al.*[6–9], and clearly bears on the probability that the principal traffic of metabolites across the chloroplast envelope in C3 plants

Abbreviations HEPES, *N*-2-hydroxyethylpiperazine-*N'*-ethanesulphonic acid, MES, 2-(*N*-morpholino)-ethanesulphonic acid

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involves import of P_i and CO_2 and corresponding export of phosphoglycerate and dihydroxyacetone phosphate[10-12]. Accordingly, the suggestion that photosynthesis may be regulated by a mechanism involving Mg^{2+}, inorganic pyrophosphate and a protein fraction isolated from leaves or chloroplasts[13-15] should be examined to see if it might bring about its effects by altering the P_i–sugar phosphate balance. This paper explores the possibility that PP_i exerts its effects primarily by determining the rate of formation of P_i in the external medium[16] and that this, in turn, governs the rate of synthesis and export of photosynthetic intermediates.

METHODS

Chloroplasts were prepared from spinach and assayed according to the methods of Bassham *et al*[13-15] under conditions otherwise identical to those previously described[16].

Each of the three solutions used in the isolation and assay of chloroplasts therefore contained: 0.33 M sorbitol, 2 mM $NaNO_3$, 2 mM EDTA, 2 mM sodium isoascorbate, 1 mM $MnCl_2$, 1 mM $MgCl_2$ and 0.5 mM K_2HPO_4. In addition, Soln A contained 0.02 M NaCl, 0.05 M 2-(*N*-morpholino)-ethanesulphonic acid (MES) (pH 6.1); Soln B contained 0.02 M NaCl, 0.05 M *N*-2-hydroxyethylpiperazine-*N'*-ethanesulphonic acid (HEPES) (pH 6.7); Soln C contained 5 mM $Na_4P_2O_7$, 0.05 M HEPES (pH 7.6).

Inorganic pyrophosphatase was prepared from spinach chloroplasts and assayed by P_i formation as described previously[16].

RESULTS

Effect of P_i on the kinetics of photosynthesis by isolated chloroplasts

Fig. 1 illustrates some effects of P_i on photosynthesis. In Fig. 1, Curve 1, P_i was deficient so that although the lag was minimal and the rate quickly approached the maximum rate observed in Fig 1, Curve 2, it soon fell away. 3-Phosphoglycerate then had little effect[2] but the rate was restored by the addition of P_i. In Fig. 1, Curve 2, P_i was near optimal. The lag was slightly longer and 3-phosphoglycerate produced only a small stimulation. In Fig. 1, Curve 3, P_i was inhibitory so that the lag was increased and the maximal rate depressed until the inhibition was reversed by addition of 3-phosphoglycerate[3,12]. In Fig. 2 the chloroplasts and assay medium were unchanged but the addition of bicarbonate was delayed for 3 min. This latter approach, essentially the same as that employed by Bassham *et al.*[13-15] for measurements of CO_2 fixation, avoids (or, according to the investigator's point of view, fails to disclose) the relationship between P_i concentration and induction made apparent by the procedure of continuous recording[2,3,12].

The comparison between these methods is included here to emphasise the fact that in any study of the effects of P_i on photosynthesis it is more than usually important to consider the way in which the experiment was conducted. Accordingly, to permit sensible comparison with data of Bassham *et al.*[13-15], we have used their procedure and measured photosynthesis as total change in O_2 during the 5 min following the addition of bicarbonate, for the purposes of this paper. However, it is evident from Figs 1 and 2 that continuous recording of photosynthesis yields much more information and for all other purposes should be preferred.

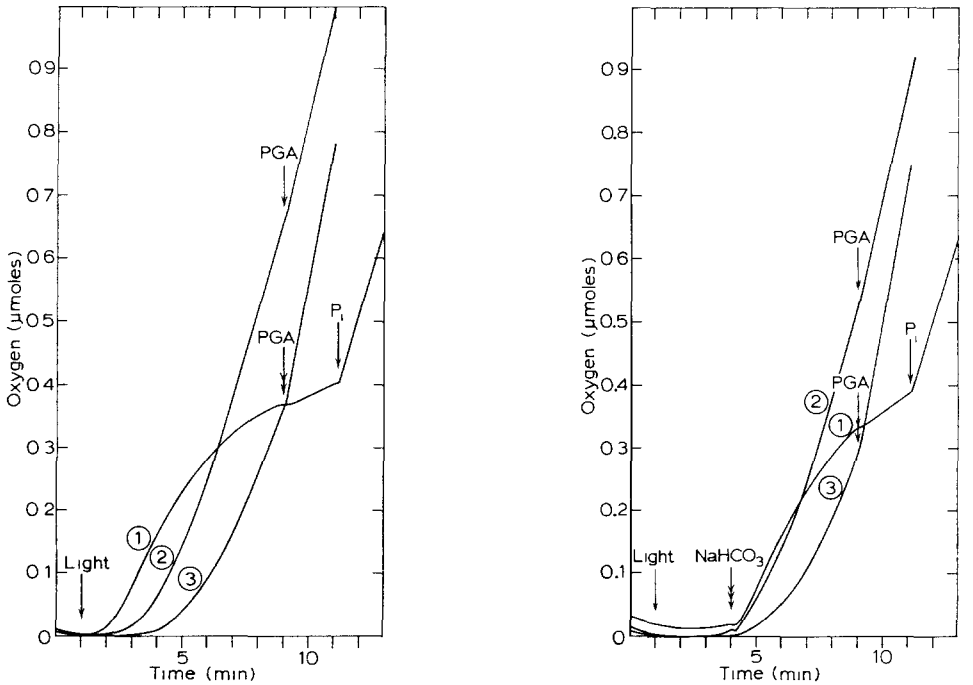

Fig. 1. Effects of orthophosphate on photosynthesis Oxygen evolution was measured as reported previously[16] The reaction mixtures contained 10 mM $NaHCO_3$ and chloroplasts equivalent to 200 μg chlorophyll in a final volume of 2 0 ml Soln C with PP_i omitted Initial P_i concentrations were as follows Trace 1, P_i omitted, Trace 2, 0 5 mM P_i, Trace 3, 2 mM P_i. The complete reaction mixtures were illuminated after 1 min in the dark 3-Phosphoglycerate (2 μmoles), and P_i (1 μmole) were added as indicated.

Fig 2. Effects of orthophosphate on photosynthesis. Conditions as for Fig. 1 except that the $NaHCO_3$ (20 μmoles) was added to all reaction mixtures after 3 min preillumination Subsequent additions as for Fig. 1.

Effects of P_i and PP_i on photosynthetic O_2 evolution following pre-illumination in the absence of added CO_2

The effects of P_i and PP_i on photosynthesis are illustrated in Fig. 3. As the P_i concentration was increased, photosynthesis exhibited a sharp optimum between 0 and 0.5 mM. Beyond this optimum the rate of O_2 evolution declined and inhibition became almost total at about 10 mM P_i (in other experiments the concentration required for complete inhibition varied according to the pre-treatment of the leaf, being lowest in chloroplasts prepared from dark-stored tissue[17]). When PP_i was increased at constant (0.5 mM) P_i there was an increase in the rate of oxygen evolution, but as previously reported[2], there was no significant inhibition at the highest concentration used. At 0.5 mM, P_i was almost certainly in excess of the optimal concentration, and the acceleration in rate brought about by PP_i is attributable to its ameliorating effect on P_i inhibition When the P_i concentration was increased in the presence of 5 mM PP_i, as in the work of Bassham *et al.*[13-15], the stimulation was again observed, and the rates were higher over a wider range of P_i concentration, due to the ameliorating effect of PP_i.

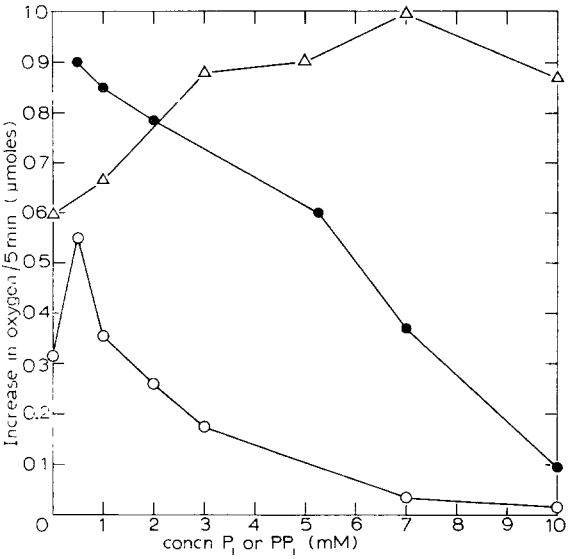

Fig 3 Effects of P_i and PP_i on photosynthesis. Method as for Fig. 2. Initial concentrations of P_i and PP_i adjusted to the values indicated. ○–○, effect of increasing P_i concentration, no PP_i present; ●–●, effect of increasing P_i concentration with constant (5 mM) PP_i present; △–△, effect of increasing PP_i concentration with constant (0.5 mM) P_i present.

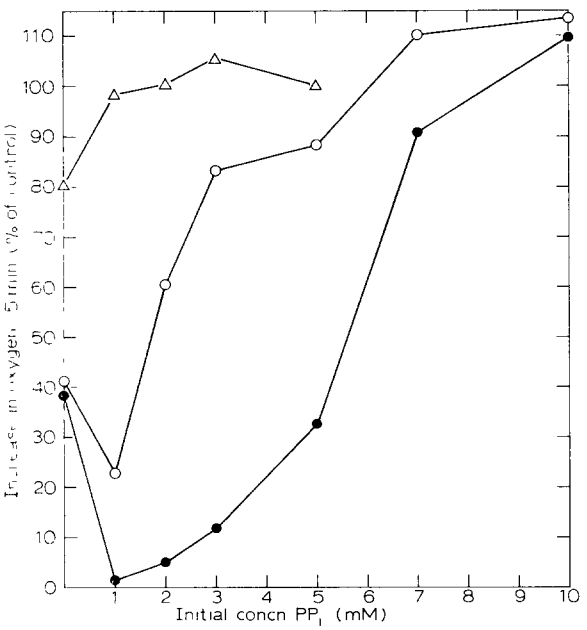

Fig. 4 Effects on photosynthesis of increasing initial PP_i concentration in the presence of added $MgCl_2$ and pyrophosphatase. Method as for Fig. 2. Reaction medium Soln C with appropriate adjustments to PP_i and Mg^{2+} concentrations. △–△, 1 mM Mg^{2+} and no added pyrophosphatase; ○–○, 3 mM $MgCl_2$ and added pyrophosphatase containing 26 μg protein; ●–●, 6 mM $MgCl_2$ and added pyrophosphatase containing 70 μg protein Oxygen evolution expressed as percent of rate achieved by a control containing 5 mM PP_i, 1 mM $MgCl_2$ and no added pyrophosphatase. Control rate. 1.0 μmole O_2 evolved in 5 min.

Stimulation and inhibition of photosynthesis by PP_i and pyrophosphatase

Striking figures have been published[13,15] showing that in the presence of protein fractions from spinach leaves containing fructose-1,6-diphosphatase activity, PP_i is inhibitory at low concentrations and stimulatory at higher concentrations Fig. 4 shows essentially the same behaviour, but was obtained with an inorganic pyrophosphatase fraction isolated from spinach chloroplasts and shown to contain only slight fructose-1,6-diphosphatase activity (see ref. 16, Table I) In the absence of the added enzyme fraction, PP_i brought about a stimulation, whereas in its presence the response was more complex so that PP_i was at first increasingly inhibitory and then producing a stimulation as the concentration was increased As in the previous work[13-15] the concentration of PP_i required to produce a stimulation increased as the Mg^{2+} and enzyme protein concentration was increased. The final concentrations of P_i and PP_i in Fig. 4 are recorded in Table I. Several features may be noted: (a) the P_i concentration in the absence of added pyrophosphatase remained more or less constant at low PP_i concentrations, implying only slow hydrolysis by endogenous pyrophosphatase under these conditions, (b) in the presence of added Mg^{2+} and pyrophosphatase, the P_i concentration increased as the amount of PP_i added was

TABLE I

RATE OF PHOTOSYNTHESIS AS A FUNCTION OF INITIAL AND FINAL CONCENTRATIONS OF P_i, PP_i, PYROPHOSPHATASE AND $MgCl_2$

Following the measurement of oxygen evolution (Fig. 4) the P_i concentration was determined at the end of each experiment (see Methods). The final PP_i concentrations was calulated from the extra P_i formed during the reaction, less that esterified during photosynthesis The latter was estimated from the amount of oxygen evolved. Oxygen evolution as % control (5 mM PP_i, 1 mM $MgCl_2$, no added pyrophosphatase), data from Fig 4.

	$MgCl_2$ added (mM)	*Pyrophosphatase added (μg protein)*	*PP_i added (mM)*	*Final PP_i (mM)*	*Final P_i (mM)*	*Oxygen evolution (% control)*
(a)	1	0	0	0	0.37	80
			1	0 95	0.42	98
			2	1 99	0 34	100
			3	2 96	0 39	106
			5	4.78	0.76	100
(b)	3	26	0	0	0 32	41
			1	0 13	2 20	23
			2	1 29	1 81	60
			3	2 15	2 05	83
			5	4 77	0 80	88
			7	6 68	0 95	110
			10	9 83	0 65	114
(c)	6	70	0	0	0 32	38
			1	0.12	2 25	2
			2	0.35	3.78	5
			3	1.59	3.30	12
			5	3 69	3.45	33
			7	6 16	2.03	91
			10	9 46	1 40	110

increased, but maxima were reached in the range of 1–3 mM PP_i for 3 mM $MgCl_2$ and 2–5 mM for 6 mM $MgCl_2$. This limited accumulation of P_i is consistent with enzymic hydrolysis of $MgPP_i^{2-}$ which becomes inhibited by P_i and PP_i as their concentrations are increased[1,6], (c) the rate of photosynthesis was highest when optimal P_i was combined with relatively high PP_i, (d) in the absence of added PP_i, P_i was more inhibitory at high than at low Mg^{2+} concentrations.

For the experiment of Fig. 5, concentrations of P_i and PP_i were selected to give values close to those actually recorded at the end of the appropriate assays

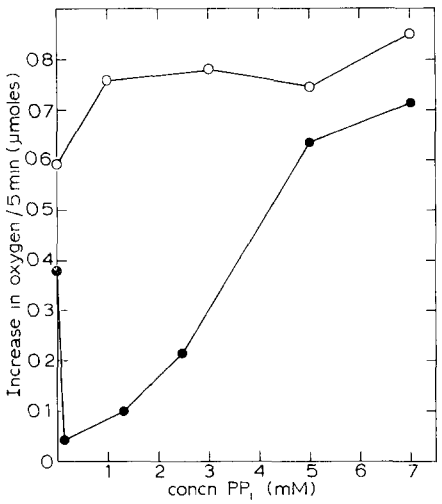

Fig 5. Effects of selected concentrations of P_i and PP_i on photosynthesis in the absence of added pyrophosphatase. Method as for Fig. 2. Reaction medium: Soln C with appropriate adjustments to P_i, PP_i and Mg^{2+} concentrations ○–○, 1 mM Mg^{2+} 0·5 mM P_i, ●–●, 3 mM Mg^{2+}, the P_i concentrations for each point are given in Table II

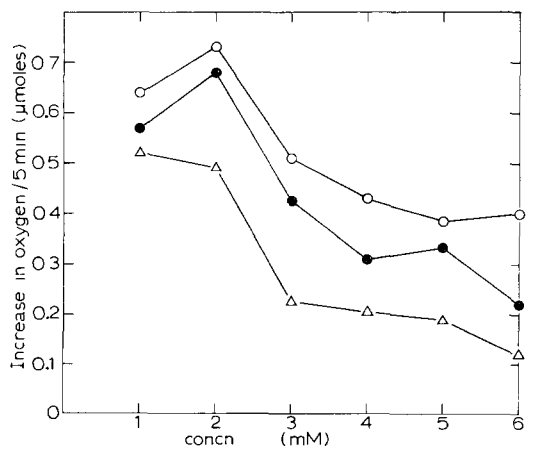

Fig. 6. Effects of increasing Mg^{2+} concentration on photosynthesis Method as for Fig 2. Reaction medium Soln C with appropriate adjustments to Mg^{2+} and P_i concentrations but with PP_i omitted. ○–○, 0.5 mM P_i, ●–●, 1 mM P_i; △–△, 2 mM P_i.

TABLE II

CONCENTRATIONS OF ORTHOPHOSPHATE AND PYROPHOSPHATE USED TO SIMULATE "REGULATION OF PHOTOSYNTHESIS"

Photosynthetic O_2 evolution determined as in Fig 2, with appropriate alterations to P_i and PP_i concentrations

Actual final concentrations (mM) of P_i and PP_i in experiment of Fig 4 which shows "regulation of photosynthesis" (Values from Table Ib)		*Initial concentrations (mM) of P_i and PP_i used to simulate "regulation of photosynthesis"*		
PP_i	P_i	PP_i	P_i	*μmoles O_2 evolved in 5 min*
0	0 32	0	0 5	0 378
0 13	2 20	0 13	2 0	0 044
1 29	1 81	1 30	2 0	0.100
2.15	2 05	2 15	2 0	0 246
4 77	0 80	5 0	1 0	0 635
6.88	0 95	7 0	1 0	0 715

(Table I) in the experiment of Fig. 4. The rates of photosynthesis measured with these concentrations of P_i and PP_i, but in the absence of added pyrophosphatase, are given in Table II and plotted in Fig. 5 This figure shows quite clearly that the characteristic responses to increasing PP_i seen in the presence and absence of additional Mg^{2+} and protein fractions can be achieved simply by omitting the protein and adding the appropriate quantities of P_i which would be derived by hydrolysis in its presence.

Inhibition by increasing Mg^{2+} at constant P_i concentration

An apparent inhibitory effect of Mg^{2+}, seen in Figs 4 and 5 and in previously published work[15], is confirmed in Fig. 6. In the presence of P_i an increase in Mg^{2+} concentration from 1 to 3 mM was sufficient to depress photosynthesis by about 50 %. Sequestration of Mg^{2+} to this extent might therefore be expected to stimulate the low rates observed in the presence of high Mg^{2+} and high P_i. However, although the substitution of EDTA for PP_i brought about some slight amelioration, it was not nearly so effective as PP_i at comparable concentrations. The low solubility of magnesium pyrophosphate suggested that precipitation of this compound might be a factor in the apparent opposing effects of Mg^{2+} and PP_i on the inhibitory effects of high P_i concentrations. However, analyses of Mg^{2+} remaining in solution in centrifuged resuspending medium showed that precipitation is not significant at concentrations of up to 10 mM PP_i and 6 mM Mg^{2+}.

DISCUSSION

We believe that the results presented in this paper necessitate a re-evaluation of the conclusions drawn by Bassham *et al.*[13–15] with respect to a proposed regulatory mechanism involving PP_i and fructose-1,6-diphosphatase. Whether or not the fruc-

tose-1,6-diphosphatase used in their experiments also contained pyrophosphatase activity is not stated, but a similar preparative procedure yielded extracts containing both enzymic activities[16]. At least some of their results can be reproduced by a pyrophosphatase with only traces of fructose-1,6-diphosphatase activity (Fig. 4), and even by certain proportions of P_i and PP_i in the absence of added protein fractions (Fig. 5). Mixtures of PP_i, pyrophosphatase and Mg^{2+} will inevitably yield varying proportions of PP_i and P_i after incubation (Table I) and any interpretation of the observed effects of these mixtures on photosynthesis must then take into account the known control exerted by P_i[2-4] and the way this may be modified by Mg^{2+} (Fig. 6), and by PP_i which does not readily penetrate the chloroplast envelope[16]. The inhibition of photosynthesis by P_i is immediately and completely reversed by triose phosphates and 3-phospholycerate which are known to move freely across the chloroplast envelope[3,4,10-12,17]. These observations, together with the direct measurements of Heldt *et al*[6-9], favour the concept that high external P_i produces its effect by exchanging with intermediates from the photosynthetic cycle. Seen in this light the inhibition by high external P_i would be an artificial disturbance of the normal translocation mechanism which imports P_i and CO_2 and exports stoichiometric quantities of triose phosphate and/or phosphoglycerate[12].

These results show that appropriate mixtures of Mg^{2+}, PP_i and pyrophosphatase can maintain external P_i at near optimal concentrations during photosynthesis by isolated chloroplasts. Hydrolysis yields P_i to maintain the photosynthetic requirement, excess hydrolysis is inhibited by free PP_i and any inhibitory effect of excess P_i would be offset by the ameliorating effect of PP_i. However, the basic reasons for the amelioration by PP_i, and the accentuation by Mg^{2+} of the P_i inhibition of photosynthesis are not known.

Mg^{2+} may influence the rate of exchange across the chloroplast envelope of external P_i for triose phosphates and phosphoglycerate, or may produce its effects indirectly by influencing the rate of formation of carbon cycle intermediates such as hexose monophosphates[18] that are not rapidly exchanged with external P_i. Whatever the mechanism of magnesium action it seems probable that much of the PP_i amelioration of P_i inhibition could be brought about by PP_i chelation of magnesium.

Bassham *et al.*[15] concluded "that the primary effect of added fructose-1,6-diphosphatase on photosynthesis by isolated chloroplasts is not produced by means of its function in converting Fru-1,6-P_2 to Fru-6-P". In view of the present results it is difficult to avoid the further conclusion that the fructose-1,6-diphosphatase activity was entirely coincidental and that the effects produced relate to the release of P_i from PP_i by pyrophosphatase activity. Present evidence certainly suggests that all of the observed effects including increased transport of metabolites to the medium could be reproduced with appropriate mixtures of P_i and PP_i.

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