Plant Physiol. (1977) 59, 422-427

Pyrophosphate Inhibition of Carbon Dioxide Fixation in Isolated Pea Chloroplasts by Uptake in Exchange for Endogenous Adenine Nucleotides[1]

Received for publication September 8, 1976 and in revised form October 18, 1976

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ABSTRACT

Carbon dioxide-dependent O_2 evolution by isolated pea (*Pisum sativum*) chloroplasts was inhibited by inorganic pyrophosphate (PPi). Oxygen evolution was also inhibited by high concentrations of orthophosphate (Pi) and the inhibition was relieved by 3-phosphoglycerate. In contrast, the inhibition by PPi was not relieved by 3-phosphoglycerate, indicating that hydrolysis of PPi and accumulation of inhibitory concentrations of Pi were not occurring. In agreement with this suggestion, the percentage of ^{14}C-labeled products diffusing out of the chloroplasts was increased by Pi but not by PPi. The inhibition of O_2 evolution by PPi was reversed by ATP. The concentration of PPi required for 50% inhibition was 1.2 to 1.4 mM and the subsequent stimulation by ATP was half-maximal at 16 to 25 μM. Carbon dioxide-dependent O_2 evolution by spinach chloroplasts, or chloroplasts isolated from older pea plants, was not significantly inhibited by PPi.

Chloroplasts were preloaded with ^{14}C-ATP and release of the labeled nucleotides was measured to assess the activity of adenine nucleotide transport across the inner chloroplast envelope membrane. A rapid exchange was promoted by the addition of exogenous ATP. Addition of PPi also resulted in a release of endogenous nucleotides. We suggest that PPi inhibits CO_2 fixation by entering the chloroplast in exchange for endogenous adenine nucleotides via the transporter on the inner envelope membrane. The subsequent depletion of the internal adenine nucleotide pool would result in decreased CO_2 fixation due to insufficient ATP. Addition of ATP to PPi-inhibited chloroplasts apparently results in uptake of catalytic amounts of ATP and restoration of the internal adenine nucleotide pool thus relieving the inhibition of CO_2 fixation.

Assimilation of CO_2 by chloroplasts requires NADPH and ATP. A high percentage of chloroplasts isolated by current methods retain their outer membranes and their stromal matrix containing the endogenous pyridine and adenine nucleotides. Although 50% of the endogenous adenine nucleotides may be lost from chloroplasts during the isolation procedure, the chloroplasts are still capable of high rates of CO_2 fixation (8). There is evidence for transfer of adenine nucleotides between chloroplast and cytosol (8, 16) but the evidence for direct transfer of adenine nucleotides into isolated chloroplasts is contradictory (3, 11, 17, 19). It appears that substrate quantities do not rapidly penetrate the chloroplast envelope membranes and the transfer observed *in vivo* may be indirect *i.e.* via shuttles of photosynthetic intermediates (6, 21). Heldt (9) reported the presence of an adenine nucleotide transporter on the chloroplast inner envelope membrane which apparently moved ATP into the chloroplast, although the rates of transfer were low. A second transporter on the chloroplast envelope membranes exchanges Pi for sugar phosphates (6, 10, 21). Inhibition of CO_2 fixation by high concentrations of Pi is considered to be the result of obligatory transfer of sugar phosphate intermediates out of the chloroplast in exchange for the added Pi, thus depleting the pools of photosynthetic intermediates and inhibiting CO_2 fixation (5, 21).

Pyrophosphate has often been included in the reaction medium for measurement of CO_2 fixation in isolated chloroplasts (3, 11, 17, 19). The PPi supplies Pi for photosynthesis via hydrolysis by an inorganic pyrophosphatase present in chloroplast preparations. Pyrophosphate also appears to decrease inhibition of CO_2 fixation by Pi (5, 13). Schwenn *et al.* (18) reported an inhibition of CO_2 fixation by PPi in the presence of added inorganic pyrophosphatase but the inhibition was reversed by PGA[2] and was, therefore, considered to be due to the release of inhibitory concentrations of Pi by the added enzyme. Pi inhibition of CO_2 fixation is known to be reversed by PGA (5, 21). Direct uptake of PPi by chloroplasts appears to be slow (18, 21). Release of labeled Pi or PGA from chloroplasts by the addition of PPi was only 2 to 13% of that promoted by Pi or PGA (10). In addition, the data of Heldt and Rapley (10) show that the uptake of Pi or PGA is only inhibited 10 to 20% by a 10-fold excess of PPi. Both of these experiments suggest that the Pi translocator has a low affinity for PPi. However, the results of Bamberger *et al.* (2) suggest that PPi may inhibit triose-P efflux from chloroplasts.

A recent report (15), showed that catalytic amounts of adenine nucleotides stimulated CO_2 fixation in isolated pea chloroplasts. We suggested that the adenine nucleotide pool was effectively depleted in these chloroplasts and that exogenous nucleotides could enter via the adenine nucleotide transporter to increase the internal pool size and thus increase the rate of CO_2 fixation. We now find that the stimulation by adenine nucleotides is greatly reduced if PPi is omitted from the reaction medium and propose that the decrease in adenine nucleotide pool in the chloroplasts is caused by pyrophosphate uptake in exchange for internal adenine nucleotides. Evidence for this proposal is presented in this report.

MATERIALS AND METHODS

Pea seedlings (*Pisum sativum* var. Massey Gem) were grown in vermiculite for 2 to 3 weeks in a glasshouse. Spinach plants (*Spinacia oleracea* var. True Hybrid 102) were grown in soil for 4 to 6 weeks. BSA was obtained from the Commonwealth Serum Laboratories (Melbourne, Australia), enzymes were ob-

[1] Financial support was provided by a Commonwealth Postgraduate Research Award to S. P. R. and by the Australian Research Grants Committee.

[2] Abbreviations: OAA: oxaloacetate; PGA: 3-phosphoglycerate; R-5-P: ribose-5-phosphate.

tained from Boehringer and Soehne (Mannheim, Germany), and other biochemicals from the Sigma Chemical Co.

Isolation of Chloroplasts. Pea shoots or deribbed spinach leaves (60–80 g) were ground in a Polytron blender for 2 to 3 sec in 200 ml of ice-cold medium containing 400 mM sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM $NaNO_3$, 20 mM NaCl, 0.5 mM Pi, 2 mM isoascorbate, 50 mM MES, and 0.4% BSA adjusted to pH 6.2. The brei was squeezed through a double layer of Miracloth containing a layer of cotton wool and the filtrate was centrifuged at 2,000g for 30 sec in an M.S.E. Super Minor centrifuge. The chloroplast pellet was rinsed once with a medium containing 400 mM sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM $NaNO_3$, 20 mM NaCl, 0.5 mM Pi, 50 mM HEPES, and 0.4% BSA adjusted to pH 6.7. The pellet was resuspended in 1 ml of the same medium using a glass rod wrapped with cotton wool. All procedures were carried out at 2 C using chilled solutions and apparatus.

Assay Procedures. Oxygen evolution was measured in a Rank O_2 electrode connected to Rikadenki B-261 recorder. The vessel was illuminated with a Rank-Aldis 150-w projector giving a light intensity of 2×10^5 ergs·cm^{-2}·sec^{-1} at the center of the vessel. The vessel was maintained at 20 C. Chlorophyll was determined from 80% acetone extracts using the method of Arnon (1).

Diffusion of ^{14}C-labeled Intermediates. Distribution of ^{14}C-labeled photosynthetic products between chloroplasts and reaction medium was determined by rapidly centrifuging the chloroplast suspension and determining the percentage of ^{14}C-labeled products in the supernatant. Chloroplasts (133 μg Chl) were illuminated in 2 ml of standard reaction mixture (400 mM sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, and 50 mM HEPES, pH 7.6) containing 5 mM $NaHCO_3$. When a linear rate of O_2 evolution was achieved, ^{14}C-labeled $NaHCO_3$ (400 nmol; 50 μCi/μmol) was added. After 5 min, two samples (0.2 ml) were removed and one was immediately centrifuged in a Beckman model 152 Microfuge for 1 min. Samples (0.1 ml) of the supernatant and of the chloroplast suspension were acidified, dried, and counted in a Nuclear-Chicago gas flow counter. From these results, total CO_2 fixation (counts in chloroplast suspension) and percentage of labeled intermediates which had leaked out of the chloroplasts (counts in supernatant divided by counts in chloroplast suspension) were calculated. The percentage of label in the pellet was obtained by subtraction.

Adenine Nucleotide Back Exchange. Back exchange of adenine nucleotides was measured according to the method of Heldt (9). The adenine nucleotide pool of the chloroplasts was labeled by incubation of the chloroplasts (1.5 mg Chl/ml) for 60 min at 0 C in a medium containing 330 mM sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 0.4% BSA, 20 mM Tricine (pH 8.4), and 0.2 mM ^{14}C-ATP (20 μCi/μmol). The chloroplasts were washed twice and resuspended in the above medium minus ATP. For assay of back exchange, chloroplasts (80 μg Chl) were added to 2 ml of a medium containing 330 mM sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, and 50 mM HEPES (pH 7.6). The suspension was maintained at 20 C and illuminated as for O_2 evolution measurements. Two samples (0.2 ml) were withdrawn at various time intervals and one was immediately centrifuged in a Beckman model 152 Microfuge for 1 min. Aliquots (0.1 ml) of this supernatant and of the untreated chloroplast suspension were added to 10 ml of scintillation fluid (Toluene/Brydet, 2:1, plus 0.4% PPO plus 0.02% POPOP) and counted in a Packard liquid scintillation counter. Release of the labeled nucleotides was calculated from the appearance of ^{14}C label in the supernatant.

RESULTS

Effect of PPi on O_2 Evolution. Previously, we have described adenine nucleotide stimulation of CO_2-dependent O_2 evolution by isolated pea chloroplasts (15). In those experiments, the reaction medium contained PPi to provide a source of Pi for continued CO_2 fixation. Figure 1, a and b, shows the stimulatory effect of ATP in the presence of PPi. Addition of catalytic amounts of ATP (or ADP) increased the rate of O_2 evolution two to 20 times. If PPi was omitted from the reaction medium, the rate of O_2 evolution was increased and was stimulated by ATP to a much lesser extent (Fig. 1, c and d). In the presence of PPi, stimulation of O_2 evolution by ATP was catalytic and therefore not the result of ATP acting as a source of Pi (15). In the absence of PPi and with no exogenous Pi source present, the stimulation by ATP may have been the result of ATP acting as a source of Pi. Cockburn *et al.* (5) have shown that ATP can act as a Pi source in spinach chloroplasts. The ATP-consuming reaction in pea chloroplast preparations (15) which would release Pi was fast enough to account for the stimulation of O_2 evolution by ATP in the absence of PPi. Low concentrations of Pi were also stimulatory and gave rates of O_2 evolution similar to those with PPi + ATP (Fig. 1e). Oxygen evolution with Pi was not significantly stimulated by ATP. Thus, ATP, Pi, and PPi + ATP all stimulated O_2 evolution by providing a source of Pi for continued CO_2 fixation. The effects of PPi were confused by its apparent inhibition of O_2 evolution in the absence of ATP.

Addition of PPi to chloroplasts evolving O_2 at a steady rate resulted in a gradual inhibition of O_2 evolution (Fig. 2). In some preparations, PPi initially stimulated O_2 evolution before inhibition occurred. The time taken for maximum inhibition by PPi varied for different preparations but was never less than 2 to 3 min. The PPi inhibition was relieved by ATP (Fig. 2) with the maximum rate being achieved after 1 to 1.2 min. The effect of varying PPi concentration on O_2 evolution is shown in Figure 3. Half-maximal inhibition occurred at 1.2 to 1.4 mM PPi with maximum inhibition requiring 4 to 6 mM PPi. In the presence of ATP, O_2 evolution was stimulated by PPi (Fig. 3). Although ATP stimulated O_2 evolution in the absence of PPi (presumably by acting as a Pi source), the rate of O_2 evolution was further increased by PPi, suggesting that ATP was a limited source of Pi. This is in agreement with the results of Cockburn *et al.* (5).

The effect of PPi, Pi, and ATP on O_2 evolution with different substrates is shown in Table I. Reduction of PGA, which requires but does not consume Pi, was less dependent on the addition of Pi. O_2 evolution with PGA was also inhibited by PPi and stimulated by Pi or PPi + ATP. O_2 evolution with R-5-

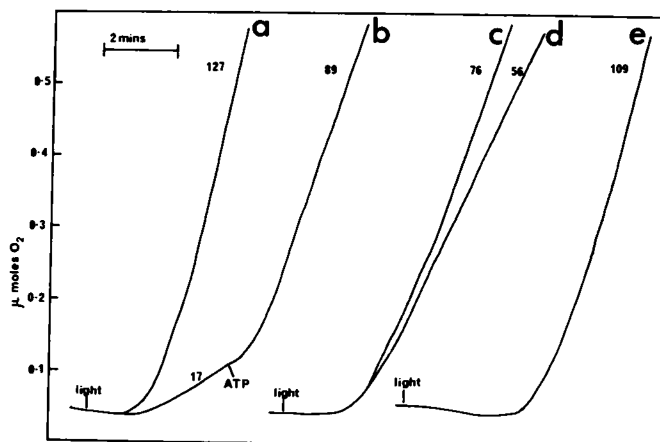

FIG. 1. O_2 evolution by isolated pea chloroplasts showing the effect of PPi and Pi. Oxygen evolution was measured in a medium containing 400 mM sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 50 mM HEPES (pH 7.6), 4 mM $NaHCO_3$, and chloroplasts equivalent to 120 μg Chl in a total volume of 2.2 ml. The following additions were made: (a) 5 mM PPi, 0.8 mM ATP; (b) 5 mM PPi. ATP added as indicated to 0.8 mM; (c) 0.8 mM ATP; (d) no addition; (e) 0.2 mM Pi. Numbers along the traces indicate rates of O_2 evolution expressed as μmol O_2 mg Chl^{-1}·hr^{-1}.

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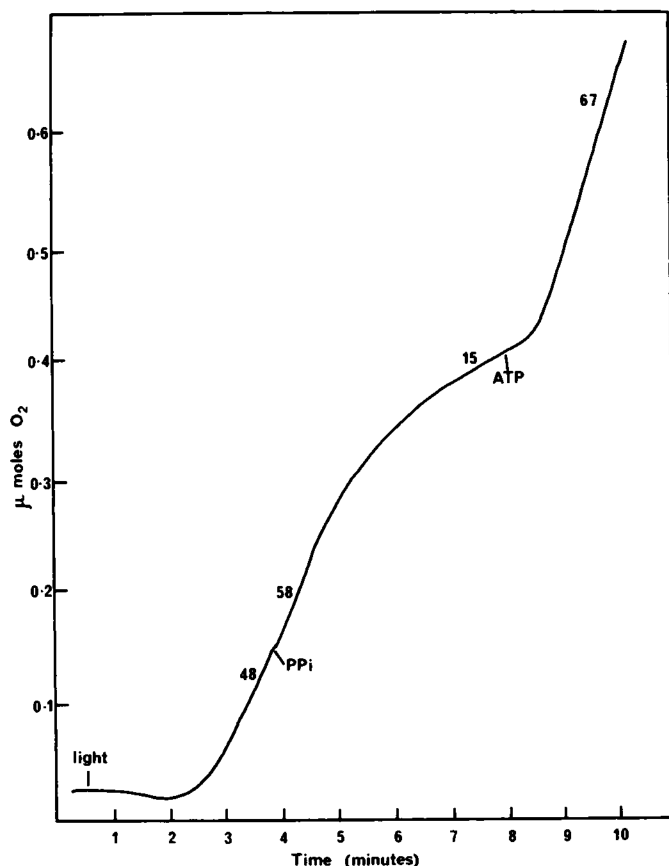

FIG. 2. O_2 electrode trace showing inhibition of O_2 evolution by PPi and subsequent relief by ATP. O_2 evolution was measured as described in Figure 1 but with chloroplasts equivalent to 148 μg Chl. PPi and ATP were added as indicated to final concentrations of 5 mM and 0.9 mM, respectively. Chloroplasts were isolated from young pea shoots.

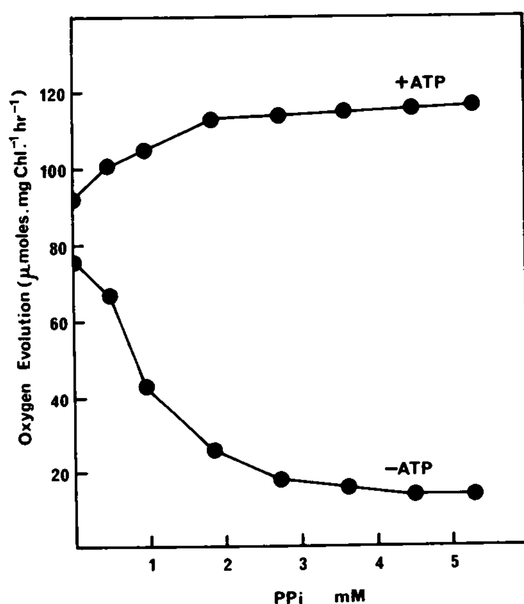

FIG. 3. Effect of PPi concentration on CO_2-dependent O_2 evolution by pea chloroplasts. O_2 evolution was measured as described in Figure 1 except that chloroplasts equivalent to 104 μg Chl were added. PPi was added to the reaction mixture initially and ATP was added to a final concentration of 0.8 mM after a steady rate of O_2 evolution was achieved.

P+CO_2 ceased after 2 to 5 min in the absence of an exogenous Pi source. This was probably the result of Pi depletion caused by increased phosphoribulokinase activity (5). O_2 evolution with R-5-P+CO_2 was also inhibited by PPi and stimulated by Pi or PPi + ATP. Oxaloacetate-dependent O_2 evolution by chloroplasts is stimulated by uncouplers and does not directly involve any adenine nucleotide-dependent reactions (7, 14). Oxaloacetate-dependent O_2 evolution by pea chloroplasts was inhibited by PPi but to a much lesser extent than for other substrates (Table I). In the presence of an uncoupler, PPi inhibition of OAA-dependent O_2 evolution was reduced to 10 to 20%.

Effect of Pi on O_2 Evolution. Low concentrations of Pi stimulated O_2 evolution and this rate was not significantly affected by ATP (Fig. 1 and Table I). The optimum Pi concenration was 0.2 to 0.5 mM and higher concentrations of Pi were inhibitory (Fig. 4). Although the optimum concentration of Pi was sharply defined for any single chloroplast preparation, the optimum varied with different preparations. For this reason, it was difficult to

TABLE I.

Effect of Pi, PPi and ATP on Rates of Oxygen Evolution with different substrates.

Oxygen evolution was measured as described in Fig. 1. Substrates were 2 mM except for $NaHCO_3$ which was 4 mM. With OAA, 10 mM D,L-glyceraldehyde was included to prevent CO_2-dependent O_2 evolution. NH_4Cl was 10 mM. Final concentrations of additions were: Pi : 0.2 mM, PPi : 5 mM, ATP : 0.8 mM. Percent inhibition by PPi was calculated relative to the rate in the presence of Pi. In the absence of added Pi, O_2 evolution with R-5-P + $NaHCO_3$ ceased after 2 to 5 min. Chloroplasts were isolated from young pea shoots.

ADDITION	SUBSTRATE				
	$NaHCO_3$	PGA	R-5-P +$NaHCO_3$	OAA	OAA +NH_4Cl
	μmol mg Chl^{-1}hr^{-1}				
None	68	86	44,0	75	111
Pi	114	110	119	73	117
PPi	8	23	14	49	97
PPi + ATP	161	114	128	54	120
Pi + ATP	111	...	...	..	...
% Inhibition	93	79	88	33	17

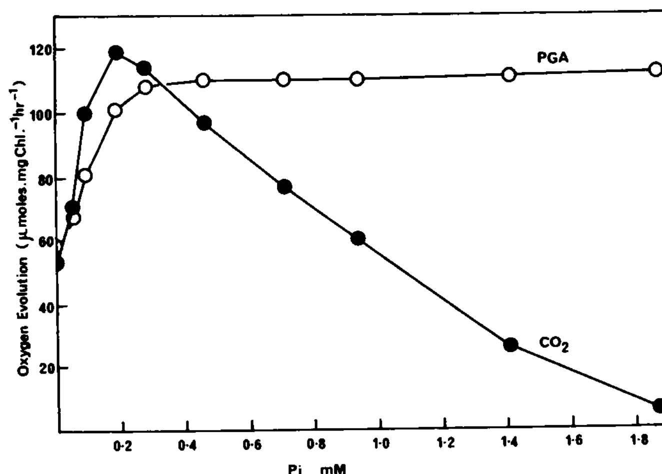

FIG. 4. Effect of Pi concentration on CO_2-dependent and PGA-dependent O_2 evolution. O_2 evolution was measured as described in Figure 1 but with varying concentrations of Pi. Bicarbonate was replaced by 2 mM PGA for measurement of PGA-dependent O_2 evolution.

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obtain the maximum rate of O_2 evolution with Pi for every preparation. O_2 evolution with PGA was also stimulated by Pi but was not inhibited at higher Pi concentrations (Fig. 4). Low concentrations of Pi also stimulated O_2 evolution with R-5-P+CO_2 but had no effect on OAA-dependent O_2 evolution (Table I). Inhibition of O_2 evolution by higher concentrations of Pi occurred more rapidly than did inhibition by PPi. While PPi inhibition was relieved by ATP but not by PGA, inhibition by higher concentrations of Pi was relieved by PGA but not by ATP (Fig. 5). Thus, the PPi inhibition could not be explained as rapid hydrolysis of PPi resulting in the formation of inhibitory concentrations of Pi.

Efflux of Labeled Products. The induction period (or lag) before maximum rates of O_2 evolution are observed is interpreted as the time taken for accumulation of sufficient quantities of photosynthetic intermediates to allow the Calvin cycle enzymes to operate at optimum rates (20). The induction period increases with increasing phosphate concentration (5), apparently due to the removal of photosynthetic intermediates from the chloroplasts in obligatory exchange for Pi uptake (21). The induction period was increased in the presence of Pi but was unaffected by PPi or PPi+ATP (Fig. 1). To determine the effect of Pi and PPi on the efflux of intermediates from the chloroplasts, the percentage of ^{14}C-labeled intermediates was determined in the supernatant after rapid centrifugation of chloroplasts photosynthesizing at a steady rate. The effects of PPi, Pi, and ATP on CO_2 fixation were similar to those observed for O_2 evolution (Table II). In the absence of Pi and PPi, 56% of the label was found in the supernatant after 5 min of photosynthesis and this value was not significantly altered in the presence of ATP (Table II). Addition of 0.5 mM Pi only inhibited CO_2 fixation to a minor extent but increased the percentage of label in the supernatant to 75%. This increase was not reversed by ATP (Table II). In contrast, PPi severely inhibited CO_2 fixation, but the percentage of label in the supernatant was lower than for the control experiment. In the presence of PPi, ATP stimulated CO_2 fixation, and the percentage of label in the supernatant was unaffected (Table II). These results are in agreement with those of Heldt and Rapley (10) that ATP and PPi do not enter chloroplasts via the Pi/sugar phosphate transporter. The increased efflux of labeled intermediates in the presence of Pi is in agreement with the results of Kaiser and Urbach (12). Although

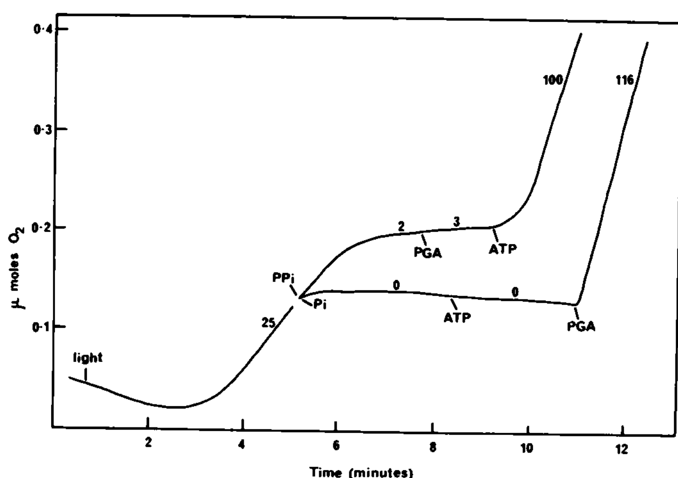

FIG. 5. O_2 electrode traces showing inhibition of O_2 evolution by PPi and Pi and subsequent effect of PGA and ATP. O_2 evolution was measured as described in Figure 1 except that chloroplasts equivalent to 148 μg Chl were added. PPi and Pi were added to a final concentration of 5 mM as indicated. PGA and ATP were added to final concentrations of 2 mM and 0.8 mM, respectively. Chloroplasts were isolated from young pea shoots.

TABLE II.

Effect of Pi, PPi and ATP on rates of CO_2 fixation and distribution of label with isolated pea chloroplasts.

ADDITIONS	CO_2 FIXATION μmol mg $Chl^{-1}.hr^{-1}$	% ^{14}C FIXED Supernatant	Pellet
None	33	56	44
1 mM ATP	33	57	43
0.5 mM Pi	29	75	25
Pi + ATP	20	83	17
5 mM PPi	9	45	55
PPi + ATP	55	45	55

TABLE III

Effect of PPi, Pi and ATP on rates of CO_2-dependent oxygen evolution with chloroplasts prepared from young or old pea shoots or from spinach leaves.

ADDITION	O_2 EVOLUTION		
	YOUNG PEAS 11 days old	OLD PEAS 19 days old	SPINACH
	μmol mg $Chl^{-1}hr^{-1}$		
None	95	40	52
5 mM PPi	6	35	65
5 mM PPi + 1 mM ATP	142	120	65
0.2 mM Pi	127	65	76
0.5 mM Pi	103	113	66

the percentage of ^{14}C label in the supernatant appears high, it can be calculated that if the concentration of intermediates in the chloroplasts was at equilibrium with that of the reaction medium, less than 0.3% of the label would be recovered in the chloroplast pellet for the experiment in Table III.

Adenine Nucleotide Exchange. As PPi inhibition was relieved by ATP but not by PGA, we considered the possibility that PPi inhibition was due to the obligatory efflux of adenine nucleotides from the chloroplasts in exchange for external PPi. Because the adenine nucleotide transporter is apparently an exchange-diffusion carrier, it is possible to study the uptake of various metabolites by the transporter by measuring the concurrent efflux of labeled adenine nucleotides from the chloroplast (9). The results of such an experiment with pea chloroplasts are shown in Figure 6. In the absence of added nucleotides, there was a slow leakage of labeled nucleotides out of the chloroplasts. The addition of inhibitory concentrations of Pi did not promote nucleotide efflux, suggesting that Pi cannot enter chloroplasts via the adenine nucleotide transporter. Addition of ATP promoted a rapid efflux of labeled nucleotides. When PPi replaced ATP, the efflux of nucleotides was slower, but 80% exchange was achieved after 3 min. In our experiments, the first sample was removed 15 sec after addition of ATP by which time 80% of the nucleotides were exchanged. The calculated values for 50% exchange of nucleotides ($t_{0.5}$) were 9 sec for ATP and 54 sec for PPi. Because of the rapid response to addition of ATP, the calculated $t_{0.5}$ for ATP was probably an overestimate. The $t_{0.5}$ for PPi-mediated exchange was probably an accurate estimate as the exchange was considerably slower than for ATP. The $t_{0.5}$ for ATP is similar to that reported by Heldt (9) for spinach chloroplasts, even though the pH of the medium used in our experiments was probably not optimal (9). The absolute rate of transport depends on $t_{0.5}$ and on the size of the active adenine nucleotide pool (9). Because of the low yield of chloroplasts from young pea plants, we were unable to estimate the size of the active pool and calculate absolute rates of transport.

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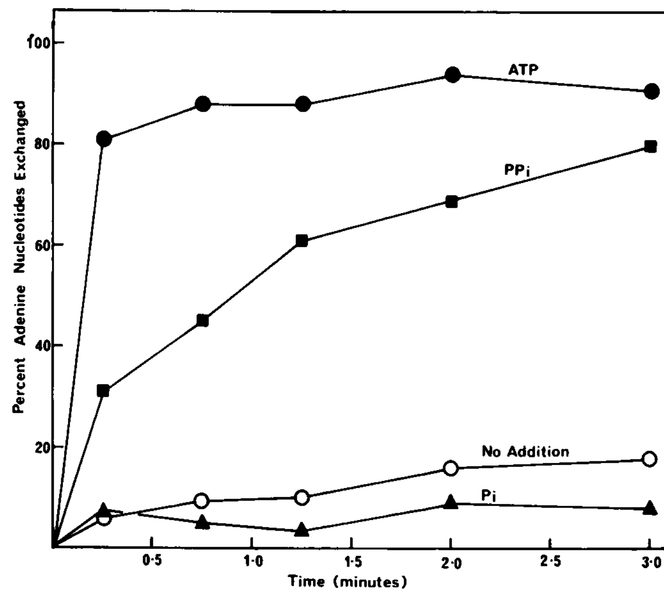

FIG. 6. Back exchange of labeled adenine nucleotides initiated by ATP and PPi. The per cent exchange was measured as described under "Materials and Methods." Additions (at zero time) were 0.9 mM ATP, 5 mM PPi, or 1 mM Pi as indicated. Chloroplasts were isolated from young pea shoots. Back exchange was corrected for nonspecific leakage of adenine nucleotides from the chloroplasts (and rupture of chloroplasts during sampling) by subtraction of values obtained for samples prior to addition of metabolites (see ref. 9).

Differences in Plant Material. In the presence of PPi, the stimulation of O_2 evolution by ATP was dependent on the age of the pea plants used for isolation of chloroplasts (15). Chloroplasts isolated from young pea shoots showed high stimulation of O_2 evolution by ATP, but chloroplasts isolated from older pea plants or from spinach leaves showed little or no stimulation by ATP (15). Table III shows the effect of PPi, Pi, and ATP on O_2 evolution by chloroplasts isolated from the three different tissues. O_2 evolution by chloroplasts isolated from young pea plants was strongly inhibited by PPi. Chloroplasts from older pea plants were inhibited only slightly by PPi, and O_2 evolution in spinach chloroplasts was slightly stimulated by PPi. Chloroplasts from young peas were less dependent on added Pi than chloroplasts from older peas and were inhibited by lower Pi concentrations (Table III).

DISCUSSION

The inhibition of CO_2-dependent O_2 evolution by PPi was not the result of PPi hydrolysis and formation of inhibitory concentrations of Pi. This was supported by the failure of PPi to lengthen the induction period (Fig. 1) or to increase the efflux of photosynthetic intermediates from the chloroplasts (Table II), and by the failure of PGA to relieve PPi inhibition (Figs. 4 and 5). The interaction between PPi and ATP suggests that specific uptake of PPi into chloroplasts is likely to be via the adenine nucleotide transporter. The concentration of PPi required for 50% inhibition (1.2 to 1.4 mM) was much higher than the ATP concentration required for half-maximal stimulation of PPi-inhibited chloroplasts (16-25 μM) (15). This suggests that the affinity of the adenine nucleotide transporter for PPi is much lower than for ATP.

Addition of high concentrations of PPi to a pea chloroplast suspension would result in uptake of PPi in exchange for internal adenine nucleotides. As the internal adenine nucleotide concentration decreased, CO_2 fixation would decrease due to insufficient ATP. The gradual decrease in rate of O_2 evolution after addition of PPi (Fig. 2) was probably a reflection of this effect. The time taken for inhibition by PPi (2-3 min) was in agreement with the time taken for maximum adenine nucleotide exchange (3-4 min). Once outside the chloroplasts, endogenous adenine nucleotides would be diluted by the reaction medium. For a reaction medium of 2 ml containing chloroplasts equivalent to 120 μg Chl, removal of all of the endogenous nucleotides into the medium would result in the concentration being diluted from approximately 1 mM inside the chloroplasts to less than 2 μM in the reaction medium. At this concentration, re-entry of the adenine nucleotides would be too slow to counter PPi-mediated adenine nucleotide efflux. Because the rate of transport of ATP was much higher than for PPi when both were at optimal concentrations (Fig. 6), addition of ATP to PPi-inhibited chloroplasts would result in uptake of sufficient ATP (in exchange for internal PPi) to restore the internal adenine nucleotide pool and thus allow CO_2 fixation. The time taken to achieve maximum rates of photosynthesis after addition of ATP (1-1.2 min) is much longer than the time for maximum adenine nucleotide exchange (15-30 sec), and probably reflects the time taken for ATP to enter the chloroplasts and restore the pools of photosynthetic intermediates to levels sufficient to maintain a higher rate of CO_2 fixation. The lag after addition of ATP may thus be considered as a second induction period (20). When PPi and ATP were both present, the exchange would favor ATP entry rather than PPi entry, and thus would not reduce the levels of internal adenine nucleotides. The PPi would be hydrolyzed outside of the chloroplasts to yield Pi for CO_2 fixation (18). As the Pi translocator is apparently much faster than the adenine nucleotide transporter (21), inhibition by Pi would be expected to occur more rapidly than PPi inhibition (Fig. 5).

The mechanism for PPi inhibition proposed above is analogous to the mechanism suggested for Pi inhibition of O_2 evolution (21). In both cases, the inhibitor is required as a Pi source for maximum rates of CO_2 fixation. With Pi, the concentration can be kept low enough to supply Pi without significantly inhibiting CO_2 fixation (Fig. 4). Concentrations of PPi sufficient to provide optimum amounts of Pi for CO_2 fixation were inhibitory (Fig. 3). It should be stressed that reversal of PPi inhibition required only catalytic amounts of adenine nucleotides to enter the chloroplast and restore the internal pool (15).

The low inhibition of O_2 evolution by PPi with chloroplasts isolated from older pea plants or from spinach leaves (Table III) suggests that the rate of adenine nucleotide transport may be lower in chloroplasts from these tissues. A lower rate of PPi/adenine nucleotide exchange should increase only the time taken for PPi to inhibit O_2 evolution. However, the relatively short time period of measurement of O_2 evolution (5-10 min) may not be sufficient to observe significant PPi inhibition if the rate of exchange is low.

The reason for the apparent decrease in adenine nucleotide transport with increasing age of the pea plants is not clear. The permeability of the chloroplast envelope membranes to amino acids is known to change with plant age (4). Possibly these permeability changes reflect changes in the demand for synthetic precursors with plant development. In developing plants, ATP may be transported into the chloroplasts to support continued protein synthesis in the dark. This would not require high rates of adenine nucleotide transport. It is possible that the ATP-consuming reaction measured with intact pea chloroplasts, which also decreases with increasing plant age (15), is a reflection of the rate of adenine nucleotide transport.

Previously, we attributed the stimulation of CO_2 fixation by adenine nucleotides in chloroplasts from young pea shoots to the presence of an ATP-consuming reaction with a high affinity for ATP (15). In the presence of Pi, ATP did not significantly stimulate O_2 evolution (Table I), suggesting that the ATP-consuming reaction did not decrease the rate of CO_2 fixation. As chloroplasts are capable of producing ATP/NADPH in the ratio 2:1 (14) *i.e.* in excess of that required for CO_2 fixation, it

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appears that chloroplasts are able to produce sufficient ATP to allow high rates of CO_2 fixation even in the presence of an additional ATP-consuming reaction. It is possible that the ATP-consuming reaction is suppressed during CO_2 fixation. Nevertheless, the presence of ATP transport, perhaps in exchange for PPi, may be an important aspect of chloroplast function, especially during development.

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