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Stimulation of Carbon Dioxide Fixation in Isolated Pea Chloroplasts by Catalytic Amounts of Adenine Nucleotides[1]

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ABSTRACT

Carbon dioxide-dependent O_2 evolution by isolated pea (*Pisum sativum* var. Massey Gem) chloroplasts was increased two to 12 times by the addition of ATP. O_2 evolution was also stimulated by ADP and to a lesser extent by AMP. The ATP effects were not due to broken chloroplasts present in the preparations nor was ATP acting as a phosphate source. We concluded that the adenine nucleotides were acting catalytically. The concentration of ATP required for half-maximum rate of O_2 evolution was 16 to 25 μM. The degree to which ATP stimulated O_2 evolution depended on the age of pea plants from which the chloroplasts were isolated. Spinach (*Spinacia oleracea* var. True Hybrid 102) chloroplasts did not show a consistent stimulation of O_2 evolution by adenine nucleotides.

The adenine nucleotide content of pea chloroplasts was not lower than that of spinach chloroplasts, but pea chloroplasts which showed a large stimulation of O_2 evolution by ATP contained an ATP-hydrolyzing reaction with rates of 10 to 50 μmol ATP hydrolyzed mg chlorophyll^{-1} hour^{-1}. The rate of the ATP-consuming reaction was much lower in spinach chloroplasts and in chloroplasts from older pea plants which did not show large stimulation of O_2 evolution by ATP. We propose that the ATP-consuming reaction, with a high affinity for ATP, decreased the effective size of the ATP pool available for CO_2 fixation. Added adenine nucleotides could be transported into the chloroplasts increasing the concentration of internal nucleotides. Calculations showed that the adenine nucleotide transporter on the outer chloroplast membranes could operate at a sufficient rate to produce such an effect.

Photosynthetic assimilation of CO_2 requires NADPH and ATP. Trebst *et al.* (23) demonstrated dark fixation of CO_2 by chloroplast extracts which were supplied with exogenous NADPH plus ATP and showed that these two cofactors could be produced by the light reactions of chloroplasts. Experiments with nonaqueous fractionation of leaf tissue suggested that the level of ATP increased upon illumination not only in the chloroplast but also in the nonchloroplast part of cells, indicating a transfer of adenine nucleotides between the chloroplast and cytosol (8, 16, 17). Evidence for direct transfer of adenine nucleotides into intact chloroplasts has been confused by a number of contradictory reports. Walker (25) showed that rupture of the outer membrane of intact chloroplasts increased the rate of cyclic phosphorylation, suggesting that penetration of ADP was slow and rate-limiting. Robinson and Stocking (15) showed that ADP increased the rate of O_2 evolution with phosphoglycerate, while Stocking *et al.* (21) showed that ATP decreased the inhibition of O_2 evolution by uncoupling agents. These reports suggest that adenine nucleotides penetrate intact chloroplasts to some extent. Jensen and Bassham (11) showed that ATP stimulated CO_2 fixation to a small extent in intact chloroplasts, and that in the dark, ATP increased the level of ribulose-1,5-diphosphate. Studies of diffusion of compounds from isolated chloroplasts suggested that ATP and ADP diffuse out of chloroplasts but can re-enter and be used metabolically (2). Heldt (9) reported the presence of an adenine nucleotide transporter on the chloroplast outer membrane which apparently operates primarily to move ATP into the chloroplast, although the rates of transfer were low (5 μmol mg Chl^{-1} hr^{-1}). The experiments of Heber and Santarius (8) similarly suggested low rates of adenine nucleotide transport into chloroplasts. Stokes and Walker (22) suggested that the failure of ATP to reverse uncoupler inhibition of P-glycerate-dependent O_2 evolution indicated impermeability of the chloroplast outer membrane to adenine nucleotides. It should be noted, however, that the adenine nucleotide transporter is inhibited by uncouplers (9), and that reversal of uncoupler inhibition requires the accumulation of substrate quantities of ATP.

Stimulation of CO_2-dependent O_2 evolution by ATP does not necessarily imply entry of ATP into chloroplasts. The ATP could be stimulating CO_2 fixation by envelope-free chloroplasts present in the preparation, but this is unlikely, as other cofactors should also be required (27). Alternatively, ATP could be acting as a source of Pi (5). Restoration by ATP of O_2 evolution in phosphate-depleted media may be due to external hydrolysis of ATP and entry of Pi rather than any direct uptake of ATP. Thus, the stimulatory effect of ATP and of PPi reported by Vose and Spencer (24) was probably the result of phosphate deficiency in the chloroplasts. There have been two recent reports of ATP stimulation of CO_2 fixation by chloroplasts in media containing Pi or PPi. Schürmann *et al.* (19) suggested that additional ATP from cyclic phosphorylation was required for CO_2 fixation and that exogenous ATP could meet this need in the absence of cyclic phosphorylation. However, the rates of CO_2 fixation were low and the stimulation by ATP could be explained on the basis of the slow rate of ATP uptake via the adenine nucleotide transporter (9). Peavey and Gibbs (14) reported ATP stimulation of CO_2 fixation by spinach chloroplasts, although the effect was not repeatably observed.

If movement of adenine nucleotides between the chloroplast and cytosol is not by direct transfer (via the adenine nucleotide transporter) but by indirect transfer (via shuttles of intermediates) (8), the role of the adenine nucleotide transporter becomes obscure. It is important to distinguish between transport of substrate amounts of adenine nucleotides (*e.g.* providing ATP for CO_2 fixation) and transport of catalytic amounts of adenine nucleotides which could serve to increase the pool size within the chloroplast. We have found that catalytic amounts of adenine nucleotides can stimulate CO_2 fixation in isolated chloroplasts under certain conditions where the effective pool size of adenine nucleotides in the chloroplasts is diminished.

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MATERIALS AND METHODS

Pea seedlings (*Pisum sativum* var. Massey Gem) were grown in vermiculite for 1 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 obtained from Boehringer and Soehne (Mannheim, Germany), and other biochemicals from Sigma Chemical Co.

Isolation of Chloroplasts. Pea shoots or deribbed spinach leaves (60–80 g) were ground in a Polytron blendor 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 2000*g* 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. O_2 evolution was measured in a Rank O_2 electrode connected to a 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. Chl was determined from 80% acetone extracts using the method of Arnon (1).

The percentage of intact chloroplasts in a preparation was determined by measuring O_2 evolution with 1.3 mM ferricyanide and 5 mM NH_4Cl in a medium containing 400 mM (intact chloroplasts) or 100 mM (shocked chloroplasts) sucrose, 5 mM $MgCl_2$, 10 mM Pi, and 50 mM HEPES (pH 7.6) (8). The rate of ADP production was measured enzymically with pyruvate kinase and lactic dehydrogenase. The chloroplasts were added to a 1-cm cuvette containing the above medium, and in addition, 0.5 mM P-enolpyruvate, 60 mM KCl, 18 mM $MgSO_4$, 0.3 mM NADH, 0.05 mg lactic dehydrogenase, and 0.05 mg pyruvate kinase in a total volume of 2.2 ml. The reaction was started by the addition of ATP to a final concentration of 2.5 mM and the reaction rate was determined from the decrease in absorbance at 340 nm. The enzyme solutions were freed of ammonium sulfate by dialysis.

Estimation of Adenine Nucleotides. Chloroplast pellets (20–40 mg Chl) were resuspended in 50 mM HEPES (pH 7.6) and extracted at 2 C with 0.5 N perchloric acid for 10 min. The extract was centrifuged at 5000*g* for 10 min and the supernatant neutralized to pH 7.5 with KOH. The $KClO_4$ precipitate was removed by centrifugation at 3000*g* for 5 min. Aliquots of the supernatant were assayed for adenine nucleotides. ADP and AMP were determined with pyruvate kinase and lactic dehydrogenase. The reaction mixture contained 150 mM triethanolamine hydrochloride (pH 7.5), 0.3 mM NADH, 60 mM KCl, 18 mM $MgSO_4$, 0.5 mM P-enolpyruvate, and 0.05 mg lactic dehydrogenase. Further additions were made in the following order: 0.05 mg pyruvate kinase, 0.1 mM ATP, 0.02 mg adenylate kinase. The decrease in absorbance at 340 nm was measured after each addition, and the levels of ADP and AMP calculated. ATP was determined with hexokinase and glucose-6-P dehydrogenase, in a reaction mixture containing 150 mM triethanolamine hydrochloride (pH 7.5), 1 mM NADP, 1 mM glucose, 2.5 mM $MgCl_2$, and 0.25 mg hexokinase. The reaction was started with the addition of 0.02 mg glucose-6-P dehydrogenase, and the levels of ATP calculated from the increase in absorbance at 340 nm. All spectrophotometric measurements were made with a Beckman Acta C III recording spectrophotometer.

RESULTS AND DISCUSSION

The isolated pea chloroplasts were 60 to 90% intact as determined by ferricyanide penetration. Rates of CO_2-depenedent O_2 evolution in the absence of any added Calvin cycle intermediates were 4 to 40 μmol mg Chl^{-1} hr^{-1}. The addition of ATP to the reaction mixture increased rates of O_2 evolution to 70 to 130 μmol mg Chl^{-1} hr^{-1}. Figure 1 shows traces of O_2 evolution by pea chloroplasts with ATP included in the reaction mixture initially or added after a steady rate of O_2 evolution had been achieved. The addition of ATP after O_2 evolution had reached a steady rate resulted in a 2- to 15-fold stimulation of the rate of O_2 evolution. The time taken to reach a new steady rate of O_2 evolution after addition of ATP was relatively constant in all preparations and was 1 to 1.2 min. Addition of ATP to the reaction mixture initially always gave higher rates of O_2 evolution than addition after O_2 evolution had commenced (Fig. 1). The lag period (26) was not consistently shortened by the addition of ATP in contrast to the results of Schürmann *et al.* (19). In many cases, the induction period with ATP present was slightly longer than that in the absence of ATP even though ATP increased the rate of O_2 evolution more than 5-fold. The lag period after addition of ATP to chloroplasts evolving O_2 at a steady rate presumably reflects the time taken to increase the intermediates of the Calvin cycle to levels sufficient to maintain a new, higher rate of CO_2 fixation (26). It may also reflect the time taken for ATP to be transported into the chloroplast.

Experiments with $^{14}CO_2$ showed that CO_2 fixation was also stimulated by ATP. In one experiment, rates of O_2 evolution were 14 and 40 μmol mg Chl^{-1} hr^{-1} and rates of $^{14}CO_2$ fixation were 14 and 51 μmol mg Chl^{-1} hr^{-1} in the absence and presence of 0.9 mM ATP, respectively. It is possible that the stimulation by ATP was a result of its effect on the broken chloroplasts present in the preparations. If this were so, decreasing the sorbitol concentration in the reaction medium should have less effect on O_2 evolution in the presence of exogenous ATP than in its absence. Figure 2 shows that O_2 evolution by pea or spinach chloroplasts or by pea chloroplasts with ATP all decreased at sorbitol concentrations below 300 mM in a uniform fashion. This suggests that ATP alone cannot promote CO_2 fixation by ruptured chloroplasts. In addition, the percentage of broken chloroplasts in the preparation was usually 20 to 30%, which should only result in a 20 to 30% increase in rates of CO_2-dependent O_2 evolution if all of the broken chloroplasts were stimulated to fix CO_2 by ATP.

Table I shows the effect of other nucleotides on CO_2-dependent O_2 evolution. ATP gave the greatest stimulation although ADP and AMP were also stimulatory. Other nucleotides tested did not significantly affect O_2 evolution (Table I). The time taken to reach a new steady rate of O_2 evolution was lowest for ATP and highest for AMP. This may reflect the specificity of the adenine nucleotide transporter on the chloroplast envelope (9) or the increased time taken to convert AMP and ADP to ATP for use by the Calvin cycle enzymes.

The stimulation by AMP suggests that the effect was not upon broken chloroplasts present in the preparation. The chloroplasts were assayed in a medium containing 5 mM PPi which should provide sufficient Pi for photosynthesis from the inorganic pyrophosphatase present in pea chloroplast preparations (20). Pi (0.3–0.8 mM) did not substitute for ATP. The stimulation by AMP also suggests that ATP was not acting as a source of Pi.

The effect of ATP on O_2 evolution with various substrates is shown in Table II. CO_2-dependent O_2 evolution showed the greatest stimulation, although O_2 evolution with PGA,[2] R-5-P,

[2] Abbreviations: PGA: 3-phosphoglycerate; R-5-P: ribose-5-phosphate; OAA: oxaloacetate; CCCP: carbonyl cyanide *m*-chlorophenyl.

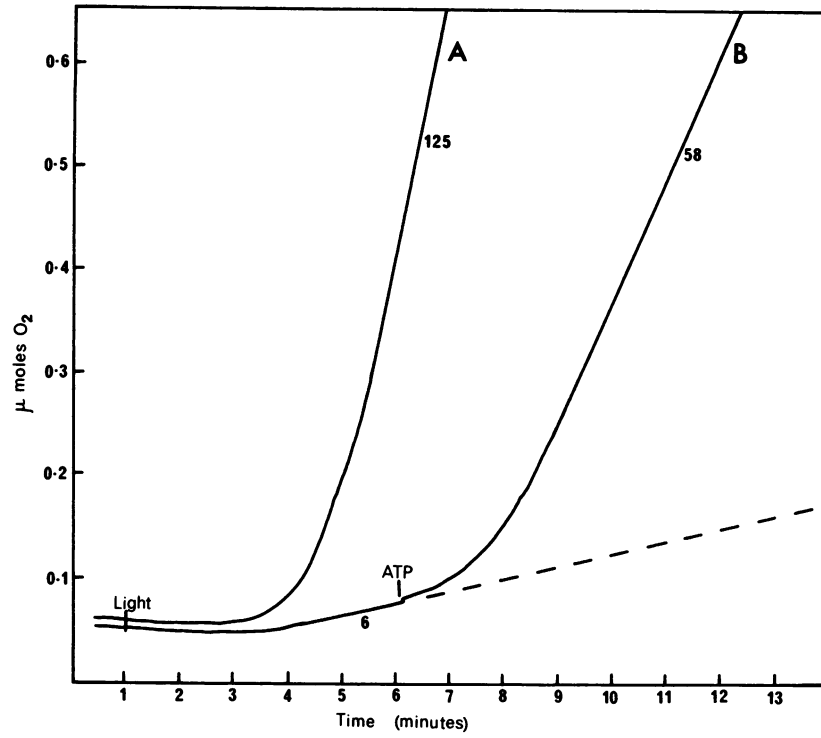

FIG. 1. Effect of ATP on CO_2-dependent O_2 evolution by pea chloroplasts. Assay medium contained 400 mM sorbitol, 2 mM EDTA, 1 mM $MnCl_2$, 1 mM $MgCl_2$, 10 mM $NaHCO_3$, 5 mM PPi, 50 mM HEPES (pH 7.6), and chloroplasts equivalent to 132 μgm Chl in a total volume of 2.4 ml. ATP was added initially to the reaction mixture in trace A and added as indicated in trace B to a final concentration of 0.9 mM. The chloroplasts were isolated from young (11-day-old) pea shoots. Numbers along the traces indicate the rate of O_2 evolution expressed as μmol O_2 mg Chl^{-1} hr^{-1}.

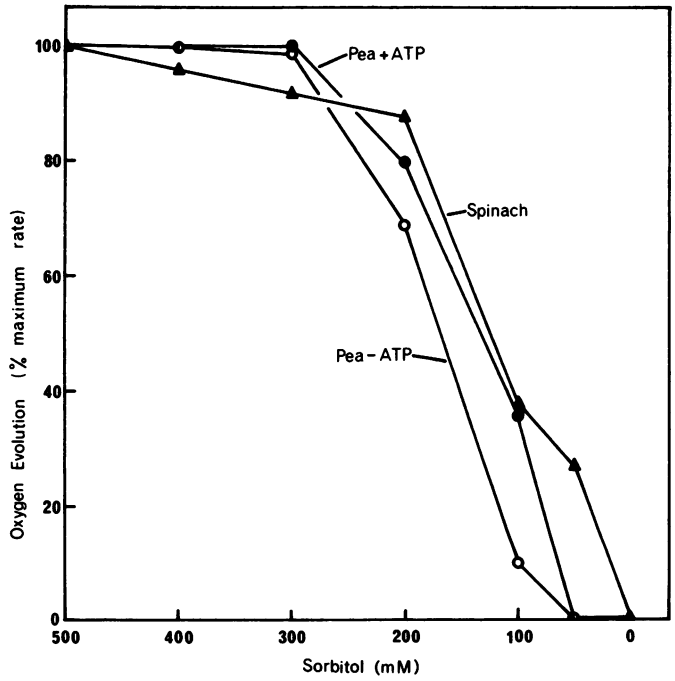

FIG. 2. Effect of decreasing sorbitol concentration on CO_2-dependent O_2 evolution with spinach chloroplasts and pea chloroplasts ± ATP. O_2 evolution was measured as described in Figure 1. ATP was added initially to give a final concentration of 0.9 mM where indicated. Maximum rates of O_2 evolution were 65 μmol mg Chl^{-1} hr^{-1} (spinach), 84 μmol mg Chl^{-1} hr^{-1} (pea + ATP), and 11 μmol mg Chl^{-1} hr^{-1} (pea − ATP).

and R-5-P plus CO_2 were also stimulated. Oxaloacetate-dependent O_2 evolution was not stimulated by ATP. Reduction of PGA requires 1 ATP/NADPH provided glyceraldehyde-3-P accumulates and ribulose-5-P is not simultaneously phosphorylated to ribulose-1,5-DiP. Rates of O_2 evolution with PGA in the absence of exogenous ATP were higher than with CO_2 but showed less stimulation by ATP (Table II). A deficiency of ATP would have less effect on PGA reduction than on CO_2 reduction because of the lower ATP requirement. Ribose-5-P reduction would be similar to CO_2 reduction in that 1.5 ATP/NADPH are required. Ribose-5-P reduction would result in CO_2 fixation and the addition of R-5-P would merely decrease the induction period by increasing the levels of photosynthetic intermediates. The rates of O_2 evolution with R-5-P or R-5-P + CO_2 were similar to those with CO_2 alone and were stimulated by ATP to a similar extent (Table II). The time taken to reach a new steady rate of O_2 evolution after addition of ATP was similar for CO_2, R-5-P, and R-5-P + CO_2 but was less for PGA (Table II). Because the full Calvin cycle is probably not operating during PGA reduction, higher levels of intermediates would not be required to maintain a higher rate of O_2 evolution. Thus, the

Table I. *Nucleotide Specificity for Stimulation of CO_2-dependent O_2 Evolution by Pea Chloroplasts*

O_2 evolution was measured as described in Figure 1 except that the final volume was 1.2 ml and the Chl concentration was 82 μg Chl/ml. After a steady rate of O_2 evolution was achieved, the nucleotides were added to give a final concentration of 1 mM. Lag refers to the time (min) taken to reach a new steady rate of O_2 evolution after addition of the nucleotide.

Nucleotide	Oxygen Evolution		Stimulation	Lag
	Absent	Present		
	μmoles mg $Chl^{-1}hr^{-1}$		%	Min
ATP	9	72	700	1.2
ADP	9	63	600	2.2
AMP	9	35	289	3.3
UTP	9	12	33	...
ITP	8	12	50	...
CTP	9	7	-29	...
GTP	7	7	0	...

Table II. *Effect of ATP on O_2 Evolution with Different Substrates*

O_2 evolution was measured in a medium containing 400 mM sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 5 mM PPi, 50 mM HEPES (pH 7.6), and chloroplasts equivalent to 72 μg Chl in a final volume of 1.2 ml. Substrates were 2 mM except for $NaHCO_3$, which was 10 mM. D,L-Glyceraldehyde (10 mM) was included with OAA to prevent CO_2-dependent O_2 evolution. NH_4Cl was added to a final concentration of 8 mM where indicated. ATP was added to a final concentration of 0.8 mM. Lag = time (min) taken to attain a new steady rate of O_2 evolution after addition of ATP to chloroplasts which had reached a steady rate of O_2 evolution.

Substrate	Oxygen Evolution			Stimulation	Lag
	- ATP	+ ATP	+ ATP initially		
	μmoles mg Chl^{-1} hr^{-1}			%	min
HCO_3^-	7	44	78	529	1.2
PGA	36	42	63	17	0.5
R-5-P	8	21	64	163	1.6
R-5-P + CO_2	7	31	69	343	1.3
OAA	21	21	23	0	...
OAA + NH_4Cl	49	49	50	0	...

Table III. *Effect of Uncouplers and Inhibitors on ATP-stimulated O_2 Evolution*

CO_2-dependent O_2 evolution was measured as described in Figure 1. ATP was added initially to the reaction mixture, and the various compounds were added after a steady rate of O_2 evolution was achieved. The chloroplasts were isolated from young (11- to 13-day-old) pea plants. The rate of CO_2-dependent O_2 evolution in the absence of added ATP was 11 to 16 μmol O_2 mg Chl^{-1} hr^{-1}.

Compound	Concentration	Oxygen Evolution		Inhibition
		- Compound	+ Compound	
	mM	μmoles O_2 mg Chl^{-1} hr^{-1}		%
Nigericin	0.05	50	0	100
CCCP	0.017	57	0	100
Arsenate	3	57	0	100
NH_4Cl	8	60	26	57
Valinomycin	0.006	72	36	50
Valinomycin + NH_4Cl	as above	72	0	100
Phlorizin	2.5	66	0	100
Atractylate	0.05	34	32	6
Antimycin A	0.006	45	51	-13
Glyceraldehyde	10	36	0	100
Azide	2	41	15	63
Pi	10	51	5	90

time taken for stimulation of PGA reduction by ATP (0.5 min) probably reflects more accurately the time taken for ATP to be transported into the chloroplasts. The reduction of OAA to malate by chloroplasts does not appear to involve any adenine nucleotide-dependent reactions and is stimulated by uncouplers (7). Thus, OAA-dependent O_2 evolution should not be affected by ATP (Table II).

The ATP-stimulated O_2 evolution was sensitive to uncoupling agents as shown in Table III. It is interesting to note that NH_4Cl and valinomycin showed synergistic uncoupling of CO_2-dependent O_2 evolution. The uncoupler inhibition suggests that ATP was not being utilized in substrate amounts and that photophosphorylation was still required for CO_2 fixation. This is in agreement with the results of Stokes and Walker (22). The O_2 evolution in the presence of ATP was also inhibited by phlorizin and by inhibitors of the Calvin cycle enzymes (azide, D,L-glyceraldehyde, and Pi). Atractylate did not prevent ATP stimulation of O_2 evolution, but is known to be ineffective in inhibiting the chloroplast adenine nucleotide transporter (8, 9). Schürmann *et al.* (19) suggested that antimycin A inhibited cyclic phosphorylation and decreased the rates of CO_2 fixation. They further suggested that ATP could overcome antimycin inhibition. In our chloroplast preparations, antimycin did not significantly inhibit CO_2-dependent O_2 evolution in the absence or presence of exogenous ATP. A slight stimulation of O_2 evolution by antimycin was frequently observed (Table III). Such a stimulation has been reported previously although PPi decreases the effect (3, 13).

Figure 3 shows that the stimulatory effect of ATP on O_2 evolution by pea chloroplasts was saturated at 0.2 to 0.3 mM. Double reciprocal plots showed that half-maximal stimulation was obtained at 16 to 25 μM ATP. In one experiment, 36 nmol ATP were added to the reaction medium and the rate of O_2 evolution was increased from 3 to 31 μmol mg Chl^{-1} hr^{-1}. The rate was maintained until 382 nmol O_2 had been evolved. If used directly, the 36 nmol ATP would only result in the evolution of 12 nmol O_2. If ATP were acting as a Pi source, 216 nmol O_2 would be evolved if all of the ATP was hydrolyzed to AMP and Pi. Thus, ATP does not act as a Pi source but appears to act catalytically in stimulating O_2 evolution.

Pea chloroplasts prepared using sorbitol-PPi media (6) also showed stimulation of O_2 evolution by ATP. The stimulation of oxygen evolution was dependent on the age of the pea plants used for chloroplast isolation (Fig. 4). With chloroplasts isolated from young (10- to 12-day-old) pea shoots, the rate of O_2 evolution was stimulated five to nine times by ATP. As the age of the pea plants increased the per cent stimulation decreased, and chloroplasts from 22- to 25-day-old pea plants showed little stimulation. The average rate of O_2 evolution (−ATP) increased with increasing plant age and was optimal for chloroplasts from 14- to 18-day-old plants. The over-all rate of O_2 evolution (+ATP) decreased with plant age.

Spinach chloroplasts, isolated by the same methods, showed little stimulation of oxygen evolution by ATP (Table IV). In some preparations of spinach chloroplasts, the rate of O_2 evolu-

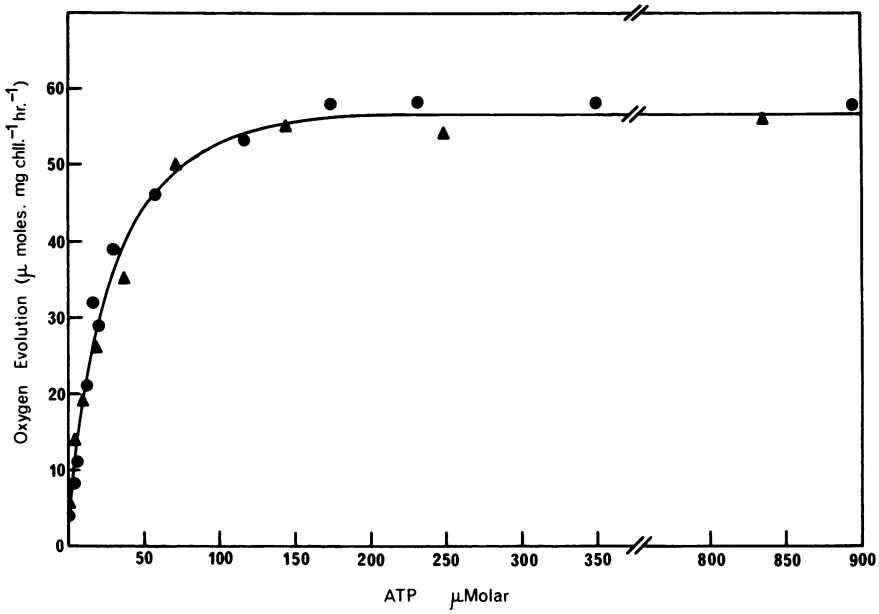

FIG. 3. Effect of ATP concentration on the rate of CO_2-dependent O_2 evolution by pea chloroplasts. Data for two chloroplast preparations are shown. O_2 evolution was measured as described in Figure 1 except that the final volume was 1.2 ml and the Chl concentrations were 82 and 91 μg Chl/ml. ATP was added initially to the reaction mixture. Chloroplasts were isolated from young (11-day-old) pea shoots.

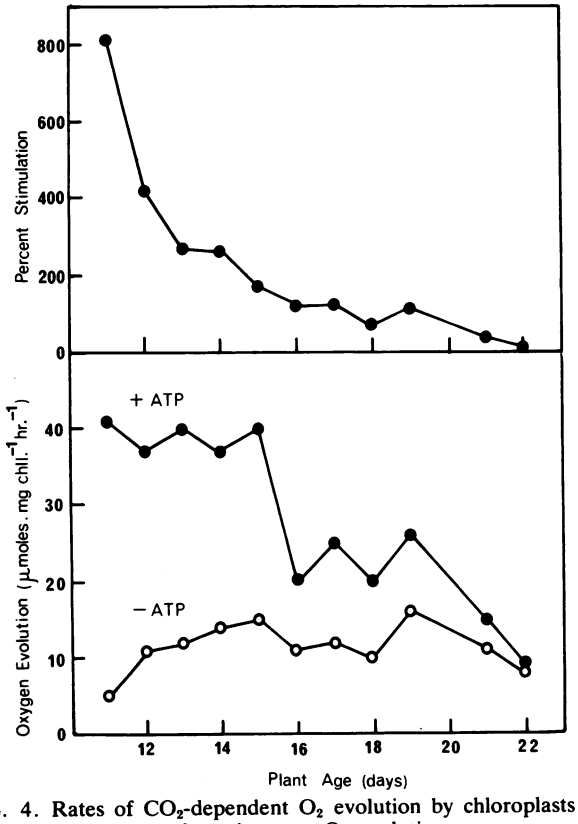

FIG. 4. Rates of CO_2-dependent O_2 evolution by chloroplasts isolated from pea plants of varying age. O_2 evolution was measured as described in Figure 1. ATP was added to a final concentration of 0.5 to 1.1 mM after a steady rate of O_2 evolution was achieved. Data are the averaged results of 50 chloroplast preparations.

tion was increased 1.2 to 1.8 times when ATP was present in the reaction medium initially. The rate was rarely stimulated by the addition of ATP after a steady rate of O_2 evolution had been achieved.

As ATP appeared to be acting catalytically, it may have been the result of small amounts of ATP entering the chloroplasts and increasing the internal pool of adenine nucleotides. During isolation, it is possible that smaller molecules such as adenine nucleotides are washed out of the chloroplasts without rupture of the outer envelope. Heber and Santarius (8) have shown that up to 50% of the chloroplast adenine nucleotides are lost during isolation in aqueous media. They noted, however, that the chloroplasts were still capable of high rates of CO_2 fixation, suggesting that the decreased adenine nucleotide content did not affect the capacity for CO_2 fixation (8). Chloroplasts from young pea plants (which showed large stimulation by ATP) and from older pea plants or spinach leaves (which did not show large stimulation by ATP) were analyzed for their adenine nucleotide contents. The results (Table V) showed that chloroplasts from young pea plants had almost double the adenine nucleotide content of chloroplasts isolated from older pea plants. Spinach chloroplasts had an adenine nucleotide content similar to chloroplasts from young pea plants, and the values (25–38 nmol mg Chl^{-1}) were similar to those reported by Heber and Santarius (8). While chloroplasts from young pea plants did not show lower adenine nucleotide contents than spinach or older pea chloroplasts, it should be realized that the data are calculated on a Chl basis, and the concentration of adenine nucleotides will depend on chloroplast internal volume.

Another possible reason for the lower ATP stimulation in chloroplasts isolated from older pea plants was a difference in the rate of transport of adenine nucleotides. The permeability of the outer membrane to amino acids is known to alter with the age of the chloroplast (4). While this possibility cannot be ignored, it is unlikely, as the time taken for a new steady rate of O_2 evolution to be achieved after the addition of ATP (which probably reflects the rate of ATP uptake) was relatively constant (1.1–1.2 min) and was independent of plant age.

The effective pool size of ATP would be decreased if other ATP-consuming reactions, not involved in CO_2 fixation, were operating. In pea chloroplast preparations, such a reaction occurred, and rates of hydrolysis of ATP ranged from 10 to 50 μmol mg Chl^{-1} hr^{-1}. Figure 5 shows rates of ATP hydrolysis by pea chloroplasts isolated from plants of differing age. The rate of ATP hydrolysis was approximately doubled when the chloroplasts were shocked to disrupt the outer membranes. Rates of ATP hydrolysis by intact or shocked chloroplasts decreased with

Table IV. *Effect of ATP on CO_2-dependent O_2 Evolution by Spinach Chloroplasts*

O_2 evolution was measured as described in Figure 1. ATP hydrolysis was measured for shocked chloroplasts as described under "Materials and Methods."

Preparation No	Oxygen Evolution				ATP hydrolysis
	- ATP	+ ATP	+ ATP initially	Stimulation	
	μmoles mg $Chl^{-1}hr^{-1}$			%	μmoles mg $Chl^{-1}hr^{-1}$
1	56	73	102	30	9.9
2	22	25	...	14	7.0
3	65	54	61	-17	6.4

Table V. *Adenine Nucleotide Content of Chloroplasts Isolated from Young Pea Plants, Older Pea Plants, and Spinach*

Total adenine nucleotides (AMP + ADP + ATP) were estimated from extracts as described under "Materials and Methods" and the values corrected for the percentage of broken chloroplasts present in the preparation. O_2 evolution was measured as described in Figure 1. ATP was added after a steady rate of O_2 evolution was achieved.

Plant Material	Total Adenine Nucleotide	Oxygen Evolution		Stimulation	Chloroplasts intact
		- ATP	+ ATP		
	nmoles mg Chl^{-1}	μmoles mg $Chl^{-1}hr^{-1}$		%	%
Young Peas	28.0	5	31	520	80
	34.4	8	41	413	78
	40.8	4	58	1350	78
Average	34.4	5.7	43	761	79
Old Peas	16.4	14	24	71	59
	18.0	13	21	62	60
	25.2	21	32	52	77
Average	19.9	16	26	62	65
Spinach	37.2	21	21	0	40

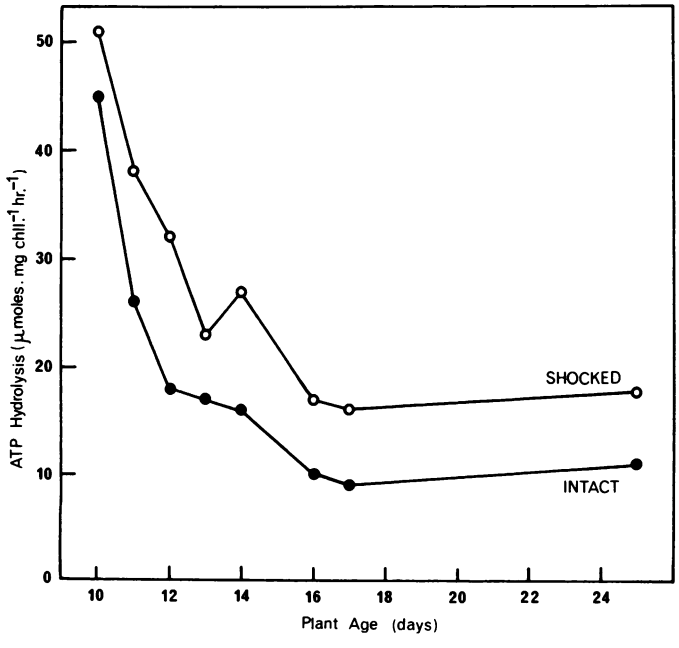

FIG. 5. Rates of dark ATP hydrolysis by intact and shocked pea chloroplasts isolated from plants of differing ages. ATP hydrolysis was determined by ADP analysis as described under "Materials and Methods." Data are the averaged results of 25 chloroplast preparations.

increasing plant age in a parallel fashion to the decrease in stimulation by ATP (*cf.* Figs. 4 and 5). The rate of ATP hydrolysis by tobacco chloroplasts also decreases with increasing leaf age (18). Chloroplasts isolated from older pea plants showed rates of ATP hydrolysis of 4 to 11 μmol mg Chl^{-1} hr^{-1} and 7 to 18 μmol mg Chl^{-1} hr^{-1} for intact and shocked chloroplasts, respectively. The ATP-consuming reaction was present in spinach chloroplasts, although rates were always less than 10 μmol mg Chl^{-1} hr^{-1} (Table IV). The low rate of ATP hydrolysis by spinach chloroplasts was consistent with the lack of stimulation of O_2 evolution by ATP.

For the ATP-consuming reaction to be capable of decreasing the effective pool size of ATP, the reaction must have a higher affinity for ATP than the enzymes involved in CO_2 fixation. The two adenine nucleotide-dependent reactions involved in CO_2 fixation are mediated by P-glycerate kinase which has a *Km* (ATP) of 2 mM and ribulose-5-P kinase which has a *Km* (ATP) of 0.42 mM (12). Preliminary experiments with pea chloroplasts have shown that the ATP-consuming reaction has an apparent *Km* (ATP) of 20 to 40 μM. Although the nature of the reaction is unknown, it does not appear to be related to the normal CO_2 fixation cycle.

CONCLUSIONS

The reason for the difference in rates of O_2 evolution between ATP added initially or added after O_2 evolution had reached a steady rate is not clear. Transport of ATP into the chloroplast may be slower in the light than in the dark, or it may be affected

by the levels of photosynthetic intermediates in the chloroplast. The decrease in rates of O_2 evolution with decreasing sorbitol concentrations (Fig. 2), stimulation by AMP (Table I), and failure of Pi to replace ATP stimulation all suggest that the stimulation by ATP was not the result of CO_2 fixation by broken chloroplasts nor of ATP acting as a Pi source. That the adenine nucleotides were acting catalytically was confirmed by the sensitivity to uncouplers (Table III), by the concentration of ATP required to stimulate O_2 evolution (Fig. 3), and by direct measurement of the amount of O_2 evolved with known amounts of added ATP.

One possibility which can not be eliminated is that ATP was affecting the transporters on the outer membrane, resulting in a decreased efflux of intermediates from the chloroplasts.

If the adenine nucleotides were being transported into the chloroplasts to increase the internal pool size, the rates of transport reported by Heldt (9) are more than sufficient. Thus, while the rate of ATP uptake of 5 μmol mg Chl^{-1} hr^{-1} does not provide substrate amounts of ATP for continued CO_2 fixation at high rates, the increase in concentration of internal adenine nucleotides would be large. For example, using the above rate of transport and assuming a chloroplast volume of 25 μl mg Chl^{-1} (10), the internal concentration of ATP would increase by 3.3 mM/min. In addition, if the adenine nucleotide content is 25 nmol mg Chl^{-1}, the internal concentration would be 1 mM. Thus, in 1 min, the internal concentration of adenine nucleotides would more than treble.

Under normal conditions, isolated chloroplasts apparently retain sufficient adenine nucleotides to maintain high rates of CO_2 fixation (8). However, if ATP-utilizing side reactions have a higher affinity for ATP than the Calvin cycle enzymes, the effective pool of ATP available for CO_2 fixation would be decreased. In chloroplasts from young pea plants, the ATP-consuming reaction could hydrolyze most of the ATP formed and a concentration of ATP sufficient to maintain high rates of turnover of the Calvin cycle enzymes would only accumulate after the ATP-consuming reaction had been saturated. The fact that pea chloroplasts can maintain high rates of CO_2 fixation in the presence of an ATP-consuming reaction suggests that the chloroplasts can produce more than 1.5 ATP/NADPH. In chloroplasts from older pea plants or from spinach leaves, the rate of ATP hydrolysis was lower (Fig. 5, Table IV) and would create less of a drain on the ATP levels in the chloroplasts. The rate of CO_2 fixation in these chloroplasts was limited by some factor other than effective adenine nucleotide pool size.

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