

Photosynthesis by Isolated Pea Chloroplasts

SOME EFFECTS OF ADENYLATES AND INORGANIC PYROPHOSPHATE¹

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ABSTRACT

When added singly to chloroplasts isolated from young pea (*Pisum sativum*) leaves, both inorganic pyrophosphate (PPi) and small quantities (0.2 mM) of ADP inhibit photosynthesis, but when added together they cause a marked stimulation. ATP (at 0.2 mM) is less inhibitory (or not inhibitory) when added alone, but like ADP, stimulates when added in the presence of PPi. This behavior is in marked contrast to that of spinach (*Spinacia oleracea*) chloroplasts which are normally stimulated rather than inhibited by PPi and which are largely unresponsive to exogenous adenylates. The inhibitory behavior of PPi with pea chloroplasts was observed under conditions where external hydrolysis to Pi is negligible. It is proposed that the exchange of organic and PPi across the chloroplast envelope may be more rapid in chloroplasts from young pea leaves than in chloroplasts from spinach and that interaction between these two processes could account for the principal observations.

The question of adenylate transport across the chloroplast envelope in C₃ plants has been debated for some years. Once it had been established that photophosphorylation could occur at high rates (2), it seemed unlikely that this process would not contribute directly to the energy metabolism of the cell. Nevertheless, it became evident at an early stage (27) that photophosphorylation of exogenous ADP was much more rapid if the limiting envelopes were removed by osmotic shock and that the relatively low rates achieved by "intact" chloroplasts could be ascribed to the inevitable presence of a small percentage of damaged chloroplasts in the same preparation (22). Similarly, the work by Heldt and his colleagues on the adenylate translocator appeared to put the fastest rate of transport via this mechanism at about 5 $\mu\text{mol} \cdot \text{mg}^{-1} \text{Chl} \cdot \text{hr}^{-1}$ and continuous exchange at 1/10 of this value or less (7). Indeed, Heldt *et al.* (9) have concluded that the adenine nucleotide translocator "does not participate in the photophosphorylation of cytoplasmic ADP." Nevertheless, there have been reports from time to time which put a contrary view (see 3, 11, 14, 19, 26). The earliest of these prompted Stokes and Walker (20) to devise what they believed might be a definitive experiment. In it they showed that ATP could not restore PGA-dependent O₂ evolution in uncoupled chloroplasts with intact envelopes but could bring about such a restoration in a reconstituted chloroplast system in which any barrier to ATP entry had been removed. It was accepted, of course, that this approach would fail to detect ATP import dependent on coupled electron transport but it seemed to pro-

vide good evidence that there was no *rapid* direct transfer of ATP (entry via the translocator being considered too slow to be effective in this regard). It is now clear that the DHAP/PGA shuttle may bring about a rapid exchange of "reducing power" between the chloroplast and the cytoplasm so that the absence of direct rapid transport of adenylate is no longer as puzzling as it once appeared (6).

The demonstration by Robinson and Wiskich (15) that ATP and ADP can stimulate CO₂ fixation in isolated pea chloroplasts has now, however, reopened the entire question of adenylate movement. In this paper, we present evidence which confirms their findings and provide additional results which point to an involvement of PPi.

MATERIALS AND METHODS

Spinach. (*Spinacia oleracea*, United States Hybrid 424, Ferry-Morse Seed Co., Mountain View, Calif.) was grown in water culture according to Lilley and Walker (13).

Peas. (*Pisum sativum* var. Feltham First, Suttons Seeds Ltd., Earley, Reading, Berkshire) seeds were germinated in a mixture of vermiculite and for a period of 11 to 14 days under field conditions. This treatment yielded shoots of about 4 to 5 cm with unexpanded leaves. Material of a greater physiological age gave less active chloroplasts.

Chloroplasts and Photosynthetic Assays. Chloroplasts were isolated from spinach leaves in sorbitol-pyrophosphate using conventional techniques (13). Pea chloroplasts were prepared by homogenizing 80 g of freshly harvested leaf + shoot in 250 ml of semifrozen grinding medium for 2 to 4 sec in a Polytron blender and squeezing the homogenate through two layers of muslin. The brei was then filtered through eight layers of muslin + cotton wool and spun in a swing-out Christ 17 centrifuge from rest to 6,000 rpm to rest in 90 sec. The pellet was rinsed in 1/25 dilution of resuspending medium containing 0.33 M sorbitol. The grinding medium also contained 0.33 M sorbitol + 0.1% (w/v) NaCl, 0.1% BSA, 0.2% sodium D-isoascorbate, and 0.1 M MES at pH 6.5. In addition to 0.33 M sorbitol, the resuspending medium contained 1 mM EDTA, 10 mM KCl, and 50 mM HEPES at pH 7.6.

Chloroplast extract was prepared by osmotic shock of intact chloroplasts as before (25).

O₂ evolution was followed polarographically in twin Clark-type electrode vessels purchased from Hansatech Ltd., Hardwick Industrial Estate, Kings Lynn, Norfolk.

CO₂ Fixation. This was measured as acid-stable radioactivity incorporated from NaH¹⁴CO₃ (18).

Reaction mixtures were illuminated by light from 150-w quartz-iodine slide projectors which was passed through filters (18) to give light mostly in the range of 590 to 750 nm at an irradiance of 300 w · m⁻².

The assays were made at 20 C with sorbitol-HEPES resuspending media as previously described for intact spinach chloro-

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plasts (13, 18) or for the reconstituted chloroplast system (which also contained ferredoxin, chloroplast extract etc.—for details see 13, 18, 25) or as described above for peas. Whole chloroplast mixtures were adjusted to pH 7.6 and the reconstituted system to pH 7.9. Mixtures contained chloroplasts equivalent to 100 μg Chl and 10 mM NaHCO_3 in a final volume of 2 ml unless otherwise stated.

Orthophosphate Assay. Pi was measured by a modification of Allen's method (1) in 200- μl samples taken from reaction mixtures containing intact chloroplasts in resuspending medium \pm 1 mM MgCl_2 . Pyrophosphatase activity in shocked chloroplasts was measured according to Schwenn *et al.* (16).

RESULTS AND DISCUSSION

Experiments with Spinach Chloroplasts. In previous experiments (20), it was shown that PGA-dependent O_2 evolution was inhibited by uncouplers (and by phloridzin) and not then restored by ATP except in the absence of the limiting envelopes. This observation can be extended, using spinach chloroplasts, to CO_2 -dependent O_2 evolution (thus ruling out the possibility that the uncouplers etc. which inhibited PGA-dependent O_2 evolution in the earlier experiments, might have acted by stopping PGA entry—it being clearly difficult to argue that there would not be enough CO_2 -bicarbonate within the chloroplast to allow any restoration of O_2 upon the subsequent addition of ATP). Conversely, in the reconstituted system, CO_2 -dependent O_2 evolution is readily inhibited by an uncoupler and as readily restored by the addition of ATP (20) or an ATP-generating system (25). Similarly, O_2 evolution is readily interrupted in the reconstituted system by the addition of ADP (because of its effect on the conversion of PGA to GBP [13, 17, 18]) whereas ADP appears to be without effect on rapid steady-state photosynthesis by intact spinach chloroplasts, and has only a marginal effect when present from the outset (Fig. 1).

These results would seem to reinforce the earlier contention that there is no direct transport of ATP (or ADP) into the intact spinach chloroplast other than at the slow rate permitted by the adenylate translocator. It should be noted, however, that the then unsuspected inhibition of O_2 evolution by ADP (13, 17, 18) could exaggerate the negative nature of this response (*i.e.* if ATP enters at a much slower rate than it is consumed internally in the phosphorylation of ribulose 5-P, its entry would not lead to a significant improvement in the ATP/ADP ratio and phosphoglycerate reduction would not be resumed).

Photosynthesis by spinach chloroplasts is facilitated by exogenous PPI (10, 12, 16). Unlike Pi, PPI does not penetrate the chloroplast at a rapid rate, but in the presence of Mg, there is usually sufficient PPIase activity in the external medium to produce Pi at a rate sufficient to meet the photosynthetic requirement (12). At low constant [Mg], this rate of supply does not increase with increasing [PPI] because Mg-PPI is the substrate for hydrolysis and anionic PPI is inhibitory. In addition, PPI alleviates Pi inhibition (5, 12) presumably by interfering with the action (4, 23) of the Pi translocator (8).

In most of the previously published work, the responses of spinach chloroplasts to Pi and PPI were examined in mixtures containing added Mg but Figure 1 (*cf.* 12) shows that the relationship was much the same when Mg was omitted. In these circumstances, PPIase activity is very low (16) and therefore the action of PPI will be relatively uncomplicated by external hydrolysis to Pi. The response of spinach chloroplasts to PPI (Fig. 1) contrasts strongly with the response of pea chloroplasts in similar reaction mixtures (see Fig. 3, lower curves). With spinach, PPI had no deleterious effect in the range of 0 to 5 mM when used in the presence of 0.25 mM Pi. There was a small but real stimulation by ADP under these conditions (*i.e.* in the absence of exogenous Mg and with ADP present from the outset) but,

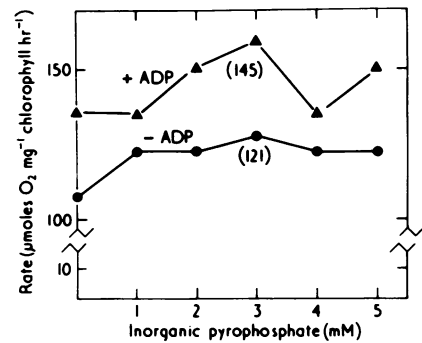


FIG. 1. CO_2 -dependent O_2 evolution by spinach chloroplasts as a function of [PPI] in the presence or absence of 0.2 mM ADP. Reaction mixtures as for Figure 3 with 0.25 mM Pi and no MgCl_2 . Mean rates in parentheses.

because of the lack of inhibition by PPI, the stimulation (on average about 10% greater than experimental error) was much less marked in spinach than in peas (see Fig. 3). The stimulation by PPI in these experiments is less marked than in some earlier reports (12) but this simply reflects a fortuitous choice of Pi concentration as the [Pi] optimum is not only sharp but variable (12).

Experiments with Pea Chloroplasts. To simplify interpretation, most of the experiments in this section were carried out with pea chloroplasts prepared in media containing no added Pi or Mg, but essentially similar results were obtained with chloroplasts isolated in more conventional mixtures (see 13) and the original medium used for the isolation of pea chloroplasts (21) continued to give the most active preparations.

We had been aware for some years that pea chloroplasts (or possibly chloroplasts from immature tissues) behaved somewhat differently from chloroplasts isolated from mature spinach leaves and had more recently concluded that pea chloroplasts (unlike spinach chloroplasts) were inhibited by PPI. We had not observed stimulation by exogenous adenylates, and when Robinson and Wiskich reported this effect (15), we were immediately struck by the fact that they had included PPI in their reaction mixtures. Accordingly, we carried out experiments such as those illustrated in Figures 2 and 3 from which it emerges that both PPI and ADP inhibit when added singly but stimulate when added together. Figure 2 shows simultaneous measurements of CO_2 fixation and O_2 evolution by pea chloroplasts and makes the point that ADP (0.2 mM) inhibits in the absence of 5 mM PPI but stimulates in its presence. (The imbalance between CO_2 fixation and O_2 evolution in Fig. 2B in the presence of ADP is significant and may reflect ADP inhibition of PGA reduction (13, 17, 18). Clearly, such imbalance could not persist at high rates of fixation because the CO_2 acceptor would soon become exhausted but at low rates over short periods selective inhibition of O_2 evolution by this mechanism seems feasible.) Figure 3 shows O_2 evolution by two preparations of pea chloroplasts plotted as a function of [PPI] in the presence and absence of ADP (0.2 mM) and contrasts sharply with the behavior of spinach chloroplasts in a similar experiment (Fig. 1). It will be seen that the inhibition by ADP at zero PPI concentration was variable but that in each case the single addition of ADP or PPI brought about inhibition whereas the combined addition brought about a stimulation.

Table I again shows the characteristic inhibition by PPI and ADP when added singly and the contrasting stimulation brought about by a combination of both additives. It also shows that this pattern of response was less sharp but still clearly detectable in the presence of intermediates of the reductive pentose phosphate pathway when added in either substrate or catalytic quantities. Secondly, ATP was either not inhibitory or much less inhibitory than ADP, whereas ADP usually brought about a

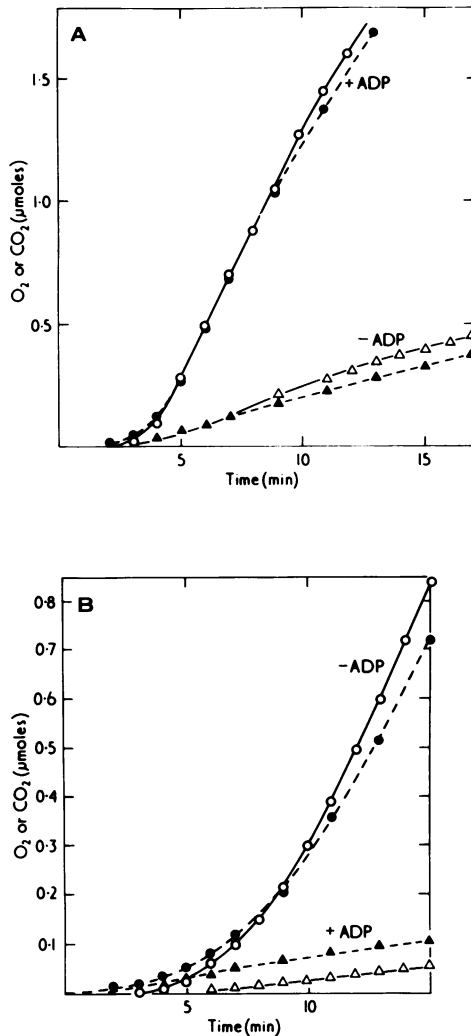


FIG. 2. A (top): photosynthesis by pea chloroplasts. (○, △: O₂ evolution; ●, ▲: CO₂ fixation.) Stimulation by ADP in the presence of PPI. Reaction mixtures (2 ml) containing intact chloroplasts ($\approx 200 \mu\text{g Chl}$) NaH¹⁴CO₃ (10 mM, 120 μCi), catalase (400 units), Pi (0.25 mM), PPI (5 mM), and ADP (+/- 0.2 mM, as indicated) in addition to resuspending medium. B (bottom): inhibition by ADP in the absence of PPI. As for A, but without PPI.

moderate to strong inhibition (see the range of response in Figs. 2 and 3 and Table I). Conversely, ATP was as effective as ADP when added in the presence of PPI, *i.e.* like ADP, small concentrations (about 0.2 mM) of ATP converted the PPI inhibition into a stimulation.

Figure 4, which again confirms the observations of Robinson and Wiskich (15), shows the kinetics of the response when ADP was added during illumination to a reaction mixture containing 5 mM PPI. As in the previous report (15), some minutes elapsed before a maximum was reached. Comparable, slowly attained, reversals followed the addition of PPI to mixtures illuminated in the presence of ADP.

Alleviation of Pi Inhibition by PPI. Inhibition by Pi of photosynthesis by spinach chloroplasts is less severe in the presence of PPI (5, 12). This was originally attributed to interference with Pi entry (5) and this notion has since been elaborated in terms of the effect of PPI on the phosphate translocator (4, 23). In these terms, Pi inhibition is seen as enforced transport of metabolites (23, 24) of the reductive pentose phosphate pathway and PPI alleviation as a sparing effect on the Pi-metabolite exchange. Convincing evidence of diminished export in the presence of PPI has been reported by Bamberger *et al.* (4).

Figure 5 shows that in the presence of ADP, PPI not only stimulates photosynthesis by pea chloroplasts but also alleviates Pi inhibition. In this, as in the other effects reported, pea chloroplasts respond to PPI + ADP in much the same way as spinach chloroplasts respond to PPI alone (12).

Inorganic Pyrophosphatase Activity. Although the above experiments with peas were carried out in the absence of exogenous Mg and although it is known that intact spinach chloroplasts will not hydrolyze PPI under these conditions (16), the possibility that external conversion of PPI to Pi might contribute to the above results could not be ignored. Accordingly, the Pi content of reaction mixtures was measured in light and dark in the presence and absence of exogenous Mg. When Mg was omitted, no increase in Pi could be detected, whereas in the presence of (1 mM) Mg, the Pi concentration of a 1-ml reaction mixture containing 100 $\mu\text{g Chl}$ increased by a maximum of 0.65 μmol in 10 min. When osmotically shocked and assayed for PPIase activity according to Schwenn *et al.* (16), Pi increased at a rate of approximately 400 $\mu\text{mol} \cdot \text{mg}^{-1} \text{Chl} \cdot \text{hr}^{-1}$. It is therefore clear that although the pea chloroplast contains an active PPIase,

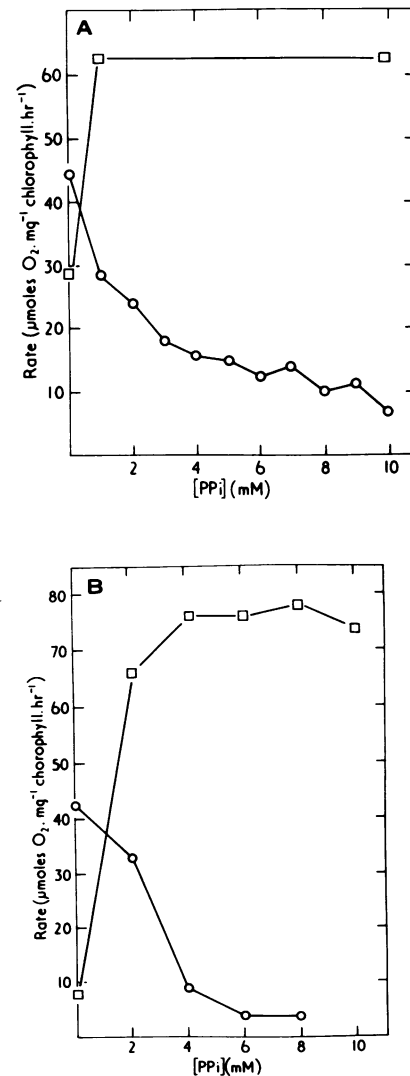


FIG. 3. CO₂-dependent O₂ evolution by pea chloroplasts as a function of [PPI] in the presence (□) or absence (○) of 0.2 mM ADP. Note striking inhibition by PPI in the absence of ADP contrasting sharply with behavior of spinach chloroplasts. Chloroplasts (100 $\mu\text{g Chl}$) in resuspending medium for peas + 0.25 mM Pi, 10 mM NaHCO₃, and 200 units catalase. A (top) and B (bottom) illustrate results obtained with two separate preparations.

there is no external hydrolysis of PPi in the absence of exogenous Mg nor, if PPi enters the intact chloroplast, is there any detectable increase in external orthophosphate.

If external PPi exchanges with internal adenylate, it would not need to do so at a high rate in order to affect photosynthesis (*cf.*

Table 1
The effect of ADP, ATP and PPi on CO₂-dependent O₂ evolution by pea chloroplasts

Additives						Rate
R5P	PGA	Pi	PPi	ADP	ATP	
-	-	0.25	-	-	-	55
-	-	"	-	0.2	-	32
-	-	"	-	-	0.2	54
-	-	"	5.0	0.2	-	89
-	-	"	-	-	-	48
-	-	"	-	0.2	-	23
-	-	"	5.0	-	-	19
-	-	"	5.0	0.2	-	81
2.0	-	"	-	-	-	55
"	-	"	-	0.2	-	49
"	-	"	5.0	-	-	32
"	-	"	5.0	0.2	-	72
-	0.2	"	-	-	-	53
-	"	"	-	0.2	-	43
-	"	"	5.0	-	-	40
-	"	"	5.0	0.2	-	72
-	-	"	-	-	-	45
-	-	"	-	0.2	-	13
-	-	"	-	-	0.2	31
-	-	"	-	-	-	31
-	-	"	-	0.2	-	15
-	-	"	-	-	0.2	27

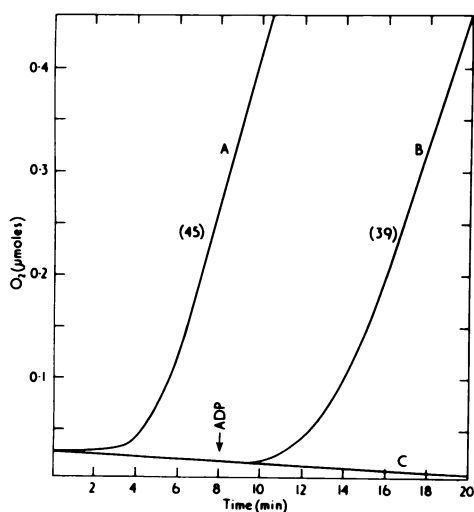


Fig. 4. Stimulation by ADP, of CO₂-dependent O₂ evolution by pea chloroplasts in the presence of PPi. Curve B: kinetics of response to ADP (0.2 mM) following addition (as indicated) during illumination. Controls (A and C) contained ADP from outset (A) or no ADP (C). Each reaction mixture as for Figure 3 but with PPi at 5 mM. Rates in $\mu\text{mol}\cdot\text{mg}^{-1}\text{Chl}\cdot\text{hr}^{-1}$ given in parentheses. (Note: the fastest rate recorded during this work with conditions as in A was 114.)

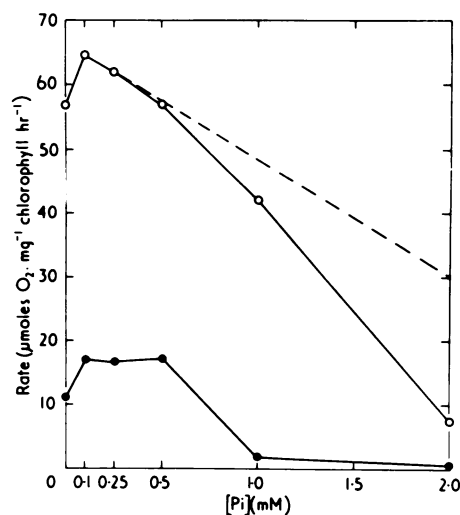


Fig. 5. Alleviation of Pi inhibition (●) by PPi + ADP (○) in pea chloroplasts. With spinach chloroplasts, Pi inhibition is relieved by PPi alone (5, 12). This figure shows that pea chloroplasts behave like spinach chloroplasts if reaction mixtures also contain ADP (0.2 mM). Broken line indicates the extent to which the activity of the pea chloroplasts declined during the 3-hr period during which measurements were taken. Each pair of values (+ and - ADP at a given [Pi]) were, however, recorded simultaneously using twin O₂ electrodes.

15). The Pi assay used in this work would detect changes of about $3\ \mu\text{mol}\cdot\text{mg}^{-1}\text{Chl}\cdot\text{hr}^{-1}$ over a 10-min period. The fact that no increase in Pi could be detected, either in the light or in the dark, under conditions of PPi inhibition suggests that the rate of PPi exchange, if it occurs, cannot exceed $1\ \mu\text{mol}\cdot\text{mg}^{-1}\text{Chl}\cdot\text{hr}^{-1}$ if it is hydrolyzed internally as rapidly as it penetrates. Even so, depletion of internal adenylate at this rate would account for the observed inhibition (given an original internal concentration of approximately $34\ \text{nmol}\cdot\text{mg}^{-1}\text{Chl}$, the adenylate content would be exhausted in about 2 min if enforced exchange with PPi occurred at $1\ \mu\text{mol}\cdot\text{mg}^{-1}\text{Chl}\cdot\text{hr}^{-1}$).

CONCLUSIONS

Although our present results may pose several new problems, there seems no doubt that the behavior of chloroplasts isolated from young pea leaves is markedly different from that of chloroplasts isolated from mature spinach leaves. This, in itself, could resolve some apparent discrepancies (for a discussion see ref. 15) which may now be seen to follow from an unwarranted assumption that chloroplasts from different C₃ species were likely to have essentially similar characteristics. On the other hand, the present results do not in themselves call for a revision of the now widely accepted thesis that in C₃ species direct movement of adenylates across the inner envelope is a very slow process mediated by a specific translocator. A consistent feature of results obtained with some 50 preparations (in which chloroplasts were isolated either as described or in other commonly employed media such as sorbitol-pyrophosphate) was that assay mixtures containing ADP + PPi were markedly superior to those containing PPi alone, thus confirming the recent report by Robinson and Wiskich (15). We can now add the additional fact that when ADP and PPi are added together they stimulate, whereas when added alone they inhibit to a greater or smaller extent. In short, ADP does not stimulate unless PPi is also present. In order to act as it does, ADP must enter the pea chloroplast more readily than it enters the spinach chloroplast. (The difference may be a question of the relative maturity of the leaves rather than a difference in species [15], but this remains to be resolved.) The fact that (in the absence of PPi) ADP inhibits whereas ATP does not (or is less inhibitory) could be explained

if exogenous ADP exchanged with endogenous ATP at a rate sufficiently large to diminish the internal ATP/ADP ratio. The inhibition by P_i is more difficult to understand. As in spinach (16), hydrolysis to P_i in the absence of exogenous Mg is too slow to be significant. Moreover, P_i stimulates when used with spinach chloroplasts (16) or with pea chloroplast supplemented with ADP. With spinach chloroplasts, there is good evidence that P_i stimulates by interfering with the action of the P_i-translocator (4, 23) thereby diminishing the export of metabolites which can otherwise facilitate autocatalytic acceleration of photosynthesis. We are therefore driven to the conclusion that P_i may slowly enter the pea chloroplast via the adenylate translocator (7). If the maximum rate of exchange were of the same order as that for ADP in spinach (about 0.2 μmol, mg⁻¹ Chl · hr⁻¹), the drain on the total adenylate would probably be too small to slow photosynthesis appreciably in short term experiments. If, however, the adenylate translocator acts more rapidly in young pea leaves (say, by a factor of 5), exchange of internal adenylate with external P_i or ADP could become inhibitory in a matter of minutes. If, in addition, P_i interferes with the P_i-translocator in peas (as in spinach), its presence would become beneficial and stimulation would ensue, once the ADP loss had been made good or halted.

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