Influence of Adenosine Phosphates and Magnesium on Photosynthesis in Chloroplasts from Peas, *Sedum*, and Spinach

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ABSTRACT

14CO2 photoassimilation in the presence of MgATP, MgADP, and MgAMP was investigated using intact chloroplasts from *Sedum prostratum*, a Crassulacean acid metabolism plant, and two C3 plants: spinach and peas. Inasmuch as free ATP, ADP, AMP, and uncomplexed Mg2+ were present in the assays, their influence upon CO2 assimilation was also examined. Free Mg2+ was inhibitory with all chloroplasts, as were ADP and AMP in chloroplasts from *Sedum* and peas. With *Sedum* chloroplasts in the presence of ADP, the time course of assimilation was linear. However, with pea chloroplasts, ADP inhibition became progressively more severe, resulting in a curved time course. ATP stimulated assimilation only in pea chloroplasts. MgATP and MgADP stimulated assimilation in all chloroplasts. ADP inhibition of CO2 assimilation was maximal at optimum orthophosphate concentrations in *Sedum* chloroplasts, while MgATP stimulation was maximal at optimum or below optimum concentrations of orthophosphate. MgATP stimulation in peas and *Sedum* and ADP inhibition in *Sedum* were not sensitive to the addition of glycerate 3-phosphate (PGA).

PGA-supported O2 evolution by pea chloroplasts was not inhibited immediately by ADP; the rate of O2 evolution slowed as time passed, corresponding to the effect of ADP on CO2 assimilation, and indicating that glycerate 3-phosphate kinase was a site of inhibition. Likewise, upon the addition of AMP, inhibition of PGA-dependent O2 evolution became more severe with time. This did not mirror CO2 assimilation, which was inhibited immediately by AMP. In *Sedum* chloroplasts, PGA-dependent O2 evolution was not inhibited by ADP and AMP. In chloroplasts from peas and *Sedum*, the magnitude of MgADP and MgATP stimulation of PGA-dependent O2 evolution was not much larger than that given by ATP, and it was much smaller than MgATP stimulation of CO2 assimilation. Analysis of stromal metabolite levels by anion exchange chromatography indicated that ribulose 1,5-bisphosphate carboxylase was inhibited by ADP and stimulated by MgADP in *Sedum* chloroplasts.

The appearance of label in the medium was measured when [U-14C] ADP-loaded *Sedum* chloroplasts were challenged with ATP, ADP, or AMP and their Mg2+ complexes. The rate of back exchange was stimulated by the presence of Mg2+. This suggests that ATP, ADP, and AMP penetrate the chloroplast slower than their Mg2+ complexes. A portion of the CO2 assimilation and O2 evolution data could be explained by differential penetration rates, and other proposals were made to explain the remainder of the observations.

There have been a number of studies on the influence of adenosine-P (ATP, ADP, AMP) on photosynthesis in intact, isolated chloroplasts. With spinach chloroplasts effects are small or nil (22), although penetration through the chloroplast membrane has been shown to occur via an adenosine-P translocator (14). In contrast, pea (25, 31) and wheat (9) chloroplasts from very young plants are sensitive to adenosine-P. In all of these studies, scant attention was paid to the effect of Mg2+, even though MgATP is required for the kinases of the reductive pentose-P cycle (26), and MgADP is used by coupling factor (4). In addition, transport across the chloroplast membrane of externally added adenosine-P (without Mg2+) could result in chloroplast Mg2+, affecting the activity of the Mg2+-requiring enzymes, fructose and sedoheptulose bisphosphatase (6, 24) and RuBP carboxylase (2). In this paper, the dependence of CO2 assimilation upon the addition of adenosine-P with and without Mg2+ has been investigated in isolated chloroplasts from two C3 plants, peas and spinach, and *Sedum*, a CAM species. An attempt was made to pinpoint reaction steps of the reductive pentose-P cycle or other factors which are responsive to adenosine-P and Mg2+, using chloroplasts from peas and *Sedum*.

In studying the interaction of Mg2+ with adenosine-P, it is essential that the real concentration of the Mg2+ complex (as opposed to free adenosine-P) be taken into consideration. Such an analysis begins by calculation of the apparent metal-ligand stability constant at the medium pH. For any metal binding ligand L which has two different relevant states of ionization, both of which can bind a metal ion M, the following equilibria apply, where $K_a$ is the acid dissociation constant of the ligand, and $K_1$ and $K_2$ are the metal stability constants for the more protonated and less protonated forms of the ligand, respectively.

\[
\begin{align*}
\text{LH}_n^a & \iff K_a \text{LH}_{n-1}^a + H^+ \\
\text{LH}_n^a & \iff K_1 \text{LH}_{n-1}^{a+1}
\end{align*}
\]

The charge of the protonated ligand is $-a$, and the metal ion is assumed to be divalent. The apparent metal binding capacity of the ligand at any pH is given by 30

\[
K_{\text{app}} = \frac{K_1 + K_2 (K_a/H^+)}{1 + K_a/H^+}
\]

In this paper, $K_{\text{app}}$ values (with the subscript dropped) will be used.

1 Abbreviations: RuBP, ribulose 1,5-bisphosphate; FBP, fructose 1,6-bisphosphate; FCCP, carbonylcyanide-4-trifluoromethoxy phenylhydrazone; G3P, glyceraldehyde 3-phosphate; HMP, hexose monophosphates; PGA, glyceral 3-phosphate; Ru5P, ribulose 5-phosphate; SBP, sedoheptulose 1,7-bisphosphate; TP, triose phosphate.

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as necessary with reference given for the true acid dissociation and stability constants. This treatment is an oversimplification, since it fails to account for competition between Na⁺ or K⁺ and the divalent metal (21). In practice, this results in only a minor error which is partially compensated because $K_1$ and $K_2$ values are determined in the presence of some-salt. Of somewhat greater importance may be differences in the mode of action of Mg²⁺-adenosine-P complexes with their state of protonation. Such a differentiation is beyond the scope of this paper, and only the sum of all forms of free or complexed adenosine-P will be considered.

It should be noted, however, that the predominant form of adenosine-P at the pH values used here has its phosphate ester fully ionized.

The standard Mg²⁺ chloroplast medium contains 5 mM EDTA, as well as Mn²⁺. At 25°C and pH 7.6, log $K_{	ext{Mg}}^{\text{EDTA}} = 6.3$, and log $K_{	ext{Mn}}^{\text{EDTA}} = 11.2$ (28); EDTA strongly prefers to bind Mn²⁺. In the presence of 6 mM Mg²⁺ and 1 mM Mn²⁺, the concentration of MnEDTA is approximately 1 mM, and the concentration of MgEDTA is approximately 4 mM, with only a trace of free Mn²⁺ and approximately 2 mM free Mg²⁺. Log $K_{	ext{Mg}}^{\text{TP}}$, log $K_{	ext{Mg}}^{\text{ADP}}$, and log $K_{	ext{Mg}}^{\text{AMP}}$ are 4.0, 3.1, and 1.9, respectively, at 25°C and pH 7.6 (29). The stability constant for the strongest Mg²⁺ chelator, ATP, is about 2 orders of magnitude lower than that of EDTA. If Mn²⁺ is considered, this difference is much larger. Hence, the binding of Mg²⁺ or Mn²⁺ to EDTA is essentially unaffected by the addition of any adenosine-P. When 5 mM ATP, ADP, or AMP is added to the medium containing 2 mM free Mg²⁺, the concentration of MgATP, MgADP, or MgAMP is 1.9, 1.7, or 0.6 mM, respectively. The concentration of free Mg²⁺ is, therefore, 0.1, 0.3, or 1.4 mM with added ATP, ADP, or AMP. This difference is important, since free Mg²⁺ can be a good inhibitor of CO₂ assimilation in intact chloroplasts (8). In all cases, there is a considerable amount of uncomplexed adenosine-P present in the medium. The experimental conditions used here keep free Mg²⁺ to a minimum but suffer the disadvantage of variable component composition.

### MATERIALS AND METHODS

**Chemicals and Supplies.** [¹⁴C]Sodium bicarbonate was supplied by Amersham. MgCl₂·6H₂O was from Fisher Scientific. The sodium salts of ATP (equine muscle), ADP (Grade III), and AMP (Type II) were supplied by Sigma, as was PGA. Aluminum plantchets were purchased from Coy Laboratory Products, Ann Arbor, MI. All other chemicals were reagent grade. Water was distilled from an all glass apparatus.

**Plants.** *Sedum proeaeulm* and peas (*Pisum sativum* var. Progress No. 9) were cultivated in a greenhouse. Spinach (*Spinacia oleracea*) was cultivated under controlled conditions (12 h light, 22°C/12 h dark, 18°C). Spinach and *Sedum* were grown in a vermiculite-soil mixture (1:1), and peas were grown in vermiculite, after overnight soaking in aerated water. Pea plant age was measured from the day of planting. All plants were fertilized weekly with one-quarter strength Hoagland solution.

**Chloroplast Isolation.** *Sedum* chloroplasts were isolated from protoplasts, as previously described (23). Spinach and pea chloroplasts were isolated by mechanical homogenization of leaves and were purified on a Percoll cushion (20). Chloroplasts were 80 to 90% intact based on ferricyanide assay (17).

**¹⁴CO₂ Assimilation.** For those experiments with Mg²⁺-asimilation with *Sedum* and pea chloroplasts, was conducted in a 1 ml reaction medium containing 0.3% sorbitol, 50 mM Hepes-NaOH (pH 7.6), 5 mM NaH₁⁴CO₃, 1000 units of catalase, 0.25 mM KH₂PO₄, 5 mM EDTA, 5 mM MgCl₂, 1 mM MnCl₂, 5 mM adenosine-P, and 20 μM tricine (pH 8.1) was used. The reaction medium was the same except that 0.05 μM tricine-NaOH (pH 8.1) was used. The experimental protocol was as follows. A stock solution was prepared in dim light containing chloroplasts and all of the components listed above except that the adenosine-P was deleted and the MgCl₂ concentration was only 1 mM. A solution of adenosine-P plus MgCl₂ was added into 55 × 17 mm culture tubes held in a water bath. This solution was previously titrated to the appropriate pH with NaOH. After mixing the stock solution, aliquots were placed in each culture tube. CO₂ assimilation was initiated by banks of 150-w flood lamps providing 620 w/m². The temperature was 30°C with *Sedum* and 25°C for peas and spinach. For those experiments lacking Mg²⁺, CO₂ assimilation was conducted as described above, except the EDTA concentration was 2 mM.

Assimilation rates were determined by spotting 100-μl aliquots at 3- to 4-min intervals on aluminum plantchets containing lens paper and 0.1 ml 0.5 N HCl. Upon drying, the sample radioactivity was determined using a Nuclear Chicago gas-flow counter with an efficiency of about 14%. In most cases after an initial lag period of 5 to 5 min, the rates of assimilation were linear for the next 20 min. Unless otherwise specified, assimilation rates were calculated from data taken between 6 and 20 min.

**PGA-Dependent O₂ Evolution.** O₂ evolution was measured polarographically in a Clark-type O₂ electrode at 25°C with peas and 30°C with *Sedum*. The reaction chamber was illuminated with a 500-w projector lamp. The incident beam passed through 5 cm water and provided 430 w/m². The assay volume was 1 ml, and the reaction medium was that used for CO₂ assimilation, except that 5 mM PGA was substituted for bicarbonate. Solutions of adenosine-P were prepared in concentrated form such that addition of 20 μl gave a final concentration of 5 mM. The influence of adenosine-P upon O₂ evolution was determined as follows. Chloroplasts were assayed in the light with PGA until a linear trace was obtained (about 4 min). The adenosine-P was added, and the light was switched off for 2 to 3 min. The light was then switched on, and O₂ evolution was assayed until a linear trace was obtained (about 4 min). The dark incubation period was required for maximum adenosine-P effect. Control experiments showed that the intervening dark period had no effect on the basilar rate of O₂ evolution.

**Transport of Adenosine-P across the Chloroplasts Membrane.** *Sedum* chloroplasts were incubated in 0.1 mM [U-¹⁴C]ADP (49.6 μCi/μmol) on ice for 1.5 h in a 1 ml medium containing 0.33 mM sorbitol, 50 mM Hepes-NaOH (pH 7.6), 2 mM EDTA, 2 mM MgCl₂, 0.5 mM MnCl₂, and 0.4% (v/v) BSA. After separation of the intact and broken chloroplasts on a Percoll gradient (23), the chloroplasts were washed once with Mg⁺⁺-free reaction medium and challenged with exogenously added adenosine-P (5 mM) in a medium (1.15 ml) containing 0.33 mM sorbitol, 50 mM Hepes-NaOH (pH 7.6), and 6 mM MgCl₂, 5 mM EDTA, or 2 mM EDTA when no Mg²⁺ was added. After addition of the adenosine-P at 4°C, the chloroplasts were separated from the medium (0.15-ml aliquots) using silicone oil centrifugation at 15- to 20-s intervals for the first min, and at 1-min intervals after that. Exchange was about 50% complete in 4 min with MgATP.
14CO₂ incorporation was measured at 4-min intervals. The rates of assimilation were calculated from data taken after the lag period except for pea chloroplasts in ADP where rates were calculated from data taken between 11 and 23 min. All data were obtained from a single batch of chloroplasts, except that with Sedum the data were taken from a number of experiments. The control rates of assimilation in μmol CO₂/mg Chl-h were 3 to 7 for Sedum, 15 for spinach, 14 for 12-d peas, and 2.5 for 21-d peas. Normal assimilation mediums were used.

Table 1. Effect of Adenosine-P on the Rate of CO₂ Photoassimilation by Isolated Chloroplasts

<table>
<thead>
<tr>
<th>Condition</th>
<th>Sedum</th>
<th>Spinach</th>
<th>Pea</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>12-d</td>
<td>21-d</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>ATP</td>
<td>100</td>
<td>100</td>
<td>266</td>
</tr>
<tr>
<td>MgATP</td>
<td>383</td>
<td>138</td>
<td>894</td>
</tr>
<tr>
<td>ADP</td>
<td>43</td>
<td>100</td>
<td>21</td>
</tr>
<tr>
<td>MgADP</td>
<td>430</td>
<td>134</td>
<td>132</td>
</tr>
<tr>
<td>AMP</td>
<td>23</td>
<td>95</td>
<td>2</td>
</tr>
<tr>
<td>MgAMP</td>
<td>41</td>
<td>41</td>
<td>16</td>
</tr>
</tbody>
</table>

Table II. Effect of MgCl₂ on CO₂ Assimilation in Sedum, Spinach, and Peas

The data for spinach were taken from the work of Demmig and Gimmmer (8). Assimilation was conducted as usual except that EDTA and Mg²⁺ were deleted from the reaction mixture. The control rates of assimilation were 2, 82, 5, and 15 μmol CO₂/mg Chl-h for Sedum, spinach, pea 1, and pea 2, respectively. The pea chloroplasts were prepared from plants which were 11 d old.

<table>
<thead>
<tr>
<th>MgCl₂</th>
<th>Sedum</th>
<th>Spinach</th>
<th>Pea 1</th>
<th>MgCl₂</th>
<th>Pea 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>mM</td>
<td>%</td>
<td>mM</td>
<td>%</td>
<td>mM</td>
<td>%</td>
</tr>
<tr>
<td>0</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>0</td>
<td>100</td>
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<tr>
<td>0.05</td>
<td>114</td>
<td>100</td>
<td>0.01</td>
<td>86</td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>88</td>
<td>42</td>
<td>96</td>
<td>0.05</td>
<td>48</td>
</tr>
<tr>
<td>1</td>
<td>72</td>
<td>38</td>
<td>101</td>
<td>0.1</td>
<td>43</td>
</tr>
<tr>
<td>5</td>
<td>69</td>
<td>18</td>
<td>73</td>
<td>5</td>
<td>55</td>
</tr>
</tbody>
</table>

MgAMP is stimulatory, but its influence is masked by free AMP and Mg²⁺. It is also likely that free Mg²⁺ (0.3 mM) diminished the observed MgADP stimulation in pea chloroplasts.

Since Sedum and pea chloroplasts showed the greatest sensitivity to adenosine-P, their response to these compounds was investigated in some detail. Figure 1 shows the time course of CO₂ assimilation in the presence of adenosine-P by pea chloroplasts from 11-d-old plants. Two controls were run, one with 2 mM MgAMP is stimulatory, but its influence is masked by free AMP and Mg²⁺. It is also likely that free Mg²⁺ (0.3 mM) diminished the observed MgADP stimulation in pea chloroplasts.

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EdTA and 1 mM Mn²⁺, another with 5 mM EdTA, 1 mM Mn²⁺, and 1 mM Mg²⁺. The rates of assimilation were identical in either case, showing that only free Mg²⁺ influences assimilation, and that EdTA does not affect the chloroplasts directly. Except with ADP, the rate of assimilation was linear to about 25 min. Reproducibly, the initial rate of steady state assimilation at 3 to 7 min with ADP was the same or somewhat greater than the control rate, but after 11 to 15 min, assimilation nearly ceased. In contrast, with Sedum chloroplasts, ADP inhibition was fully evident after the lag period (data not shown).

ATP without Mg²⁺ did not enhance the rate of assimilation in Sedum (Table I), but the lag period was visibly shortened (Fig. 2). Determination of the exact length of a lag period is difficult experimentally. However, if the amount of CO₂ assimilated in a short time period after the start of illumination is determined, the apparent rate of incorporation reflects the length of the lag period, i.e. a low rate is indicative of a long lag. The apparent rate of assimilation 0 to 8 min was measured and designated the 'lag rate.' With no ATP added, the lag rate was about one-sixth of the linear rate (Fig. 2). At concentrations of ATP above 10 μM, the lag rate increased to equal the linear rate, indicating the complete absence of a lag period. Hence, these data show that ATP can penetrate the Sedum chloroplast membrane, and ATP is a limiting factor during the lag period, but not during steady state CO₂ assimilation.

The contention that ATP is not a limiting factor during CO₂ assimilation by Sedum chloroplasts is supported by the data shown in Figure 3. Here the effects of MgATP and ATP were observed in the presence of FCCP, an inhibitor of photophosphorylation. At 0.1 μM FCCP, ATP does provide stimulation, although neither ATP nor MgATP could restore assimilation rates to control values, possibly due to inhibition of the adenosine-P translocator by FCCP. Chlorocarbonylcyanide phenylhydrazone has been reported to inhibit the translocator of spinach chloroplasts (14). Transport of Adenosine-P across the Chloroplast Membrane. Back exchange from Sedum chloroplasts loaded with [U-¹⁴C]ADP was measured (Table III). Even when no adenosine-P was added to the loaded chloroplasts, some label appeared in the medium; all rates were corrected for this. The data show that the rate of exchange decreased in the order ATP > ADP > AMP. The addition of Mg²⁺ had a pronounced stimulatory effect on the rate. The Mg²⁺-complexed nucleotides may be preferentially exchanged, but it is also possible that the activity of the translocator is Mg²⁺ dependent.

Because of this finding, it seemed possible that ATP did not stimulate assimilation in pea chloroplasts as well as MgATP, only because ATP had a lower penetration rate, resulting in lower chloroplastic levels of adenosine-P. To check this idea, pea chloroplasts were incubated for various lengths of time in dim light.

Table III. Back Exchange from Sedum Chloroplasts Loaded with [U-¹⁴C]ADP

<table>
<thead>
<tr>
<th>Addition</th>
<th>Exchange Rate relative</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgATP</td>
<td>100</td>
</tr>
<tr>
<td>MgADP</td>
<td>72</td>
</tr>
<tr>
<td>MgAMP</td>
<td>42</td>
</tr>
<tr>
<td>ATP</td>
<td>84</td>
</tr>
<tr>
<td>ADP</td>
<td>18</td>
</tr>
<tr>
<td>AMP</td>
<td>17</td>
</tr>
</tbody>
</table>

Table IV. Influence of the Time of Incubation in the Dark upon the Stimulation of CO₂ Assimilation in Pea Chloroplasts by MgATP and ATP

The reaction media were prepared as for CO₂ assimilation and contained ATP or MgATP. The medium was placed into a 2.4 × 11 cm glass centrifuge tube covered with aluminum foil and kept at room temperature. At zero time, the chloroplasts (13-d-old peas) were added. At the indicated time, a 1.0-ml aliquot was removed to an illuminated reaction tube at 25°C. ¹⁴CO₂ incorporation was measured at 4-min intervals.

* Per cent incorporation is shown in parentheses.

Table V. Effect of Adenosine-P on the Rate of PGA-Supported O₂ Evolution

The data for Sedum were obtained from a single batch of chloroplasts, and the assays were conducted at 30°C with 51 μg Chl/ml. Pea chloroplasts were obtained from 11-d-old plants. PGA-dependent O₂ evolution (16.5 μg Chl/ml) and CO₂ assimilation in the absence of PGA (22.0 μg Chl/ml) were conducted with a single batch of chloroplasts at 25°C.

* Per cent incorporation is shown in parentheses.
with either MgATP or ATP, and then the rate of CO₂ assimilation in the light was determined. In Table IV are shown the results of two such experiments with 13-d-old peas. In the first experiment, stimulation with both ATP and MgATP increased somewhat with increasing incubation time, but by less than 2-fold; this increase is small compared to the 9-fold stimulation given by MgATP compared to control rates (Table I). In the second experiment, increasing incubation time did not influence the rate with ATP, but did slightly with MgATP. Hence, these data demonstrate that the role of Mg²⁺ in the stimulation of CO₂ assimilation is not due to its ability to enhance chloroplastic levels of adenosine-P.

PGA-Supported O₂ Evolution. In the reductive pentose-P cycle, MgATP is required for the conversion of PGA to G3P and for the synthesis of RuBP from Ru5P. The rate of MgATP utilization in the production of G3P can be monitored by measuring the rate of PGA-supported O₂ evolution (5 mM PGA is substituted for 5 mM bicarbonate in the reaction medium). The results of such studies on Sedum and pea chloroplasts are shown in Table V. In addition, for peas, CO₂ assimilation rates are shown which were obtained with the same batch of chloroplasts and at the same time as the O₂ evolution data. In order to mimic the conditions of CO₂ assimilation, the chloroplasts were subjected to a dark incubation period (2 min for peas, 3 min for Sedum) with adenosine-P, before O₂ evolution in the light was recorded. Unlike CO₂ assimilation, lag period in O₂ evolution was less than 0.5 min.

The control rates of PGA-dependent O₂ evolution were much higher than rates of CO₂ assimilation for peas and somewhat higher for Sedum. However, during steady state CO₂ assimilation only two-thirds of the available MgATP is utilized for G3P formation. Multiplying the control and MgATP O₂ evolution rates (Table V) for pea chloroplasts yields 45.2 and 74 μmol/mg Chl·h. For MgATP, the corrected rate of G3P formation is equal to the rate of CO₂ assimilation. Hence, the rate of G3P formation is a limiting factor for CO₂ assimilation in the presence of MgATP, possibly because the supply of NADPH is limiting. This conclusion assumes, however, that the penetration rate of PGA into the chloroplast during the O₂ evolution assay is not a limiting factor; this seems likely, given the high rates of PGA transport measured with spinach chloroplasts (10). For the control, the ‘corrected’ rate of G3P formation was substantially higher than the rate of CO₂ assimilation. This suggests that some step(s) of the reductive pentose-P cycle after G3P formation is rate determining. Addition of MgATP may accelerate G3P formation, but its main influence is expected in other parts of the reductive pentose-P cycle. There is a possible pitfall to this argument, however, and this concerns the levels of reductive pentose-P cycle intermediates, and whether these are saturating to the same degree in the presence of 5 mM PGA as with HCO₃⁻. It is possible that the acceleration in CO₂ assimilation with MgATP results from increased levels of cycle intermediates as well as ATP. To check this possibility, the stimulation in CO₂ assimilation by MgATP was measured in the presence of a range of PGA concentrations. With pea chloroplasts, MgATP had the ability to accelerate assimilation even in the presence of PGA, and the per cent stimulation by MgATP was only slightly lower with PGA present than without PGA (Fig. 4). Both MgATP and MgADP also provided substantial stimulation in CO₂ assimilation in the presence of 2 mM PGA or FBP plus aldolase with Sedum chloroplasts (data not shown). With Sedum chloroplasts, ATP was less effective in the stimulation of PGA-dependent O₂ evolution than MgATP (Table V), although any stimulation is unexpected, because ATP had no effect on CO₂ assimilation (Table I). This is explicable if the rate of G3P formation is fast relative to other reaction steps and not rate determining for CO₂ assimilation, even before ATP is added. As with pea chloroplasts, this suggests that MgATP (or MgADP after photophosphorylation to ATP) enhances CO₂ assimilation by accelerating a reaction step(s) of the reductive pentose-P cycle.

![Figure 4. Influence of PGA on MgATP stimulation of CO₂ assimilation in chloroplasts from 15-d-old pea plants.](https://plantphysiol.org)
Metabolite Levels in Sedum. As discussed above, PGA-supported O₂ evolution was not inhibited by the addition of ADP to Sedum chloroplasts. To gain an understanding of the site of ADP inhibition, ¹⁴CO₂ assimilation was conducted for 20 min and the labeled products were separated by anion exchange chromatography (23). For comparison, experiments were also performed with MgADP and FCCP. In Table VI, the levels of a number of reductive pentose-P intermediates are shown. These intermediates contained the bulk of labeled carbon; the remainder was found in starch and neutral saccharide fraction. Most of the label in TP was found in dihydroxyacetone phosphate, indicating the activity of triose-P isomerase was large enough to establish near thermodynamic equilibrium. Fructose 6-P was the main component of HMP, with the remaining being glucose 6-P.

On the right side of Table VI are shown the metabolite levels with 0.1 μM FCCP, which inhibited CO₂ assimilation about 50%. The percentage change in PGA was much smaller than that of TP, this being most evident in the chloroplast medium where the bulk of these two compounds was found. As expected, these data indicate a block in TP formation which results from inhibition of PGA kinase, as a consequence of a reduced chloroplastic ATP/ADP ratio.

The metabolite levels with 5 mM ADP or MgADP are shown on the left side of Table VI. ADP inhibited the rate of CO₂ assimilation by 30%. Examination of the stromal levels of PGA and TP revealed a relatively large decrease in TP. In this case, however, the medium levels did not reflect this change, and the TP/PGA ratio of the medium increased by about 18% over the control value. Consideration of the amount of PGA exported to the medium and the assimilation rate shows that the flux from PGA to TP in the stroma is greatly reduced in the presence of ADP. Yet the stromal level of PGA is not much lower than the control value. Thus, an inhibition by ADP of enzymatic activity is indicated. This results in a paradox, since PGA-supported O₂ evolution was not inhibited in the presence of ADP. It was estimated that the concentration of binding sites of RuBP carboxylase lies between 1.5 and 4 μM in spinach chloroplasts (3), but in Sedum in the presence of ADP, the sum of the concentrations of PGA and RuBP is only 0.2 μM (assuming a stromal volume of 25 μl/mg Chl). Thus, a considerable amount of PGA may be bound to carboxylase, and that available for conversion to TP is much lower than the value displayed in Table VI. In earlier ¹⁴CO₂ labeling studies of the reductive pentose-P cycle (3), it was found that PGA was initially labeled to a higher degree than expected. We concluded that only one molecule of newly formed PGA was in equilibrium with the PGA pool, and that the other three carbons of RuBP were either bound or were directly converted to some other molecule.

The percentage increase in stromal RuBP was over 200% in the presence of ADP, indicating that the primary site of inhibition is RuBP carboxylase. Inasmuch as inhibition by ADP was observed in the presence of exogenously added PGA which serves to increase the levels of reductive pentose-P intermediates, it is likely that the catalytic capacity (Ｖ₅₉₉) of carboxylase is being affected by ADP.

In the presence of MgADP, the stromal level of RuBP was slightly lower than the control value, in spite of an over 2-fold increase in the rate of CO₂ assimilation; the activation of carboxylase is thus indicated. The net flux of TP to the medium in the presence of MgADP is larger than the control by about a factor of 2. Since the stroma concentration was not increased, the activity of the Pi translocator toward TP must be enhanced. The stromal level of HMP increased by over 2-fold and the medium level over 3-fold, but increases in stromal levels of FBP and SBP were considerably smaller. This suggests that a reaction step after HMP formation is rate determining.

Effects of Pi. Figure 5 shows that 6 mM Pi depressed stimulation of CO₂ assimilation by MgATP in Sedum chloroplasts. This suggests that the rate-determining step of the reductive pentose-P cycle in the presence of high Pi is not affected by MgATP. An alternative explanation is that Pi inhibits the penetration of MgATP into the chloroplast through competitive inhibition of the adenine-P translocator. To be effective at a concentration of only 6 mM (the MgATP concentration is 1.9 mM) indicates a tight binding constant which seems unlikely, given the structural disparity involved. Since at pH 7.6 log K⁺₅₉₉ is only 1.6 (29) versus 4.0 for ATP, it is also unlikely that Pi exerts its effect by sequestering Mg²⁺.

The inhibition of ADP was apparent at optimum Pi concentrations. Perhaps at very low Pi, photophosphorylation becomes rate determining. At very high Pi concentrations, ADP inhibition was also minimal, which supports the contention that the rate-deter-
mining step of the reductive pentose-P cycle has changed and is not affected by ADP. The possibility that ADP and Pi compete for a common inhibitory binding site on carboxylase can be ruled out, because product analysis with a high concentration of Pi shows no inhibition of carboxylase (23).

DISCUSSION

Stimulation of PGA-Supported 02 Evolution. A one-to-one exchange of adenosine-P via a translocator of the chloroplast membrane will not result in a net increase in chloroplastic adenosine-P. It has been shown, however, that PPI can exchange for adenosine-P (25), and there may be other compounds in the chloroplast capable of this. It is likely that stimulation of PGA-supported 02 evolution by MgADP, ADP, and MgAMP in Sedum, and MgATP and ATP in Sedum and peas (Table V) results from increases in the chloroplastic levels of adenosine-P.

Stimulation of CO2 Assimilation. It was shown that Mg2+ enhances the penetration rate of adenosine-P (Table III). An increased penetration rate can explain why MgATP and MgADP enhanced CO2 assimilation in spinach, while ATP and ADP had no effect. If penetration rate is the only factor involved, it is difficult to understand why free ADP is a potent inhibitor of CO2 assimilation in both Sedum and pea chloroplasts (Table I, Fig. 1).

The experiments in which pea chloroplasts were incubated in MgATP or ATP before assimilation (Table IV) negate the possibility that differences in chloroplastic levels of adenosine-P are responsible for the increased stimulation by MgATP relative to ATP alone. It is possible, however, that MgATP or ATP are providing substrate amounts of ATP during assimilation, and the increased penetration rate of MgATP, compared to ATP, explains the superiority of MgATP in CO2 assimilation by pea chloroplasts (Table I). With Sedum chloroplasts, this explanation is not viable, since ATP was shown to penetrate the chloroplast by the back exchange experiment (Table III), and by its enhancement of PGA-supported 02 evolution (Table V). ATP was also shown to have an effect upon the lag period of CO2 assimilation (Fig. 2); yet ATP provided no stimulation in Sedum chloroplasts, while MgATP did (Table I). Furthermore, an enhancement of assimilation in Sedum by ATP could only be observed when photophosphorylation was deliberately crippled by FCCP (Fig. 3).

That MgATP and MgADP have special effects upon assimilation other than just the contribution of adenosine-P is supported by the metabolite analysis with Sedum chloroplasts (Table VI) which demonstrated that RuBP carboxylase is stimulated by MgADP. This observation is completely consistent with the CO2 assimilation (Table I) and PGA-supported 02 evolution data (Table V) from peas and Sedum which showed that some reaction step of the reductive pentose-P cycle after TP formation is stimulated by MgADP or MgATP. One possible explanation for the data is that the free Mg2+ concentration in Sedum and pea chloroplasts is suboptimal, and that Mg2+ is carried into the chloroplast along with either ATP or ADP.

Various measurements of stromal Mg2+ have revealed that its concentration is rather high and can increase by several millimolar upon illumination (15). This increase is partially responsible for light activation of CO2 assimilation. All of these studies have been performed with spinach chloroplasts, the plastids least sensitive to MgATP. Studies using the ionophore A23187 and EDTA suggest that about one-half of the Mg2+ and all Ca2+ are bound in the spinach chloroplast stroma (19). It is possible that Sedum or young pea chloroplasts are deficient in free Mg2+, but the total concentration of Mg2+ is still high.

32P incorporation into intact Ehrlich ascites tumor cells indicated that adenosine-P are not saturated with Mg2+ (12). Measurement of adenosine-P levels has indicated an energy charge which is strongly inhibitory toward nitrogenase in vivo (7). To explain the high activity of this enzyme, it has been suggested that low Mg2+ limits the concentration of MgADP (the inhibitory form of ADP). In liver cells, less than 10% of total Mg2+ is free (33). Thus, there is some evidence in other biological systems that Mg2+ concentration is not always excessive.

It cannot be ascertained whether the proposed deficiency of Mg2+ is an artifact of chloroplast preparation. However, the pea chloroplasts were prepared quite rapidly by mechanical grinding, and the whole procedure required only about 15 min. The lack of any significant stimulation by free Mg2+ suggests that Mg2+ by itself does not penetrate the chloroplast membrane and, therefore, would not leak out during preparation. The membrane of spinach chloroplasts has been shown to be nearly impermeable to free Mg2+ (11).

Recently, it has been reported that MgPPI can stop the deactivation of RuBP carboxylase in older (about 20 min) spinach chloroplasts (32). Perhaps as the chloroplasts age, Mg2+ leaks out or is bound to the thylakoids, and MgPPI penetrates the chloroplast to replenish free Mg2+. Thus, Mg2+ may be transported into the chloroplast by a variety of carriers.

Inhibition by ADP and AMP. ADP inhibited CO2 assimilation in Sedum and pea chloroplasts (Table I). In pea chloroplasts, inhibition by ADP of PGA kinase (Table V) was partially or perhaps completely responsible for inhibition of CO2 assimilation. The kinetics are strange, however, and initially ADP often stimulated PGA-supported 02 evolution and CO2 assimilation. It is possible that ADP initially increased chloroplastic levels of adenosine-P but as time proceeded depleted MgATP from the chloroplast through counterexchange. If this were occurring, it is difficult to understand why ATP does not have a similar effect, unless it is hypothesized that the adenosine-P translocator is selective in its release of Mg2+.

It is possible that ADP serves two roles. One is to penetrate the chloroplast membrane, increasing internal levels of adenosine-P. A second role is to bind to the external (solubil-impermeable) membrane and thereby affect internal processes. A pertinent example is the binding of ADP (without Mg2+) to a surface receptor of human blood platelets which in turn inhibits adenylate cyclase (18). If this scheme is operative, Mg2+ must regulate the binding or action of adenosine-P on the outer membrane. In this context, the role of Mg2+ is indirect. A differentiation between direct penetration of Mg2+ and an indirect effect must await experiments with radiolabeled Mg2+.

AMP is a more effective inhibitor than ADP in both Sedum and pea chloroplasts (Table I). In pea chloroplasts, AMP inhibition was immediate upon illumination (Fig. 1), whereas ADP inhibition was not. One site of AMP inhibition must be after TP formation in chloroplasts from either plant. In a reconstituted spinach chloroplast system, AMP was shown to inhibit the reductive pentose-P cycle after FBP formation (16). However, chloroplastic fructose bisphosphatase of spinach (5) as well as Ru5P kinase of peas (1) have been reported to be insensitive to AMP.

The relevance of these studies is questionable, inasmuch as they were performed with activated enzyme. In the studies discussed here, AMP was added to the chloroplasts in the dark when the enzymes were in the inactive, nonreduced state, and the state of activation may influence the sensitivity to inhibitors. Alternatively, AMP may be inhibiting at some other as yet undetected site.

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