Stromal Phosphate Concentration Is Low during Feedback Limited Photosynthesis

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ABSTRACT

It has been hypothesized that photosynthesis can be feedback limited when the phosphate concentration cannot be both low enough to allow starch and sucrose synthesis at the required rate and high enough for ATP synthesis at the required rate. We have measured the concentration of phosphate in the stroma and cytosol of leaves held under feedback conditions. We used nonaqueous fractionation techniques with freeze-clamped leaves of Phaseolus vulgaris plants grown on reduced phosphate nutrition. Feedback was induced by holding leaves in low O₂ or high CO₂ partial pressure. We found 7 millimolar phosphate in the stroma of leaves in normal oxygen but just 2.7 millimolar phosphate in leaves held in low oxygen. Because 1 to 2 millimolar phosphate in the stroma may be metabolically inactive, we estimate that in low oxygen, the metabolically active pool of phosphate is between negligible and 1.7 millimolar. We conclude that halfway between these extremes, 0.85 millimolar is a good estimate of the phosphate concentration in the stroma of feedback-limited leaves and that the true concentration could be even lower. The stromal phosphate concentration was also low when leaves were held in high CO₂, which also induces feedback-limited photosynthesis, indicating that the effect is related to feedback limitation, not to low oxygen per se. We conclude that the concentration of phosphate in the stroma is usually in excess and that it is sequestered to regulate photosynthesis, especially starch synthesis. The capacity for this regulation is limited by the coupling factor requirement for phosphate.

During photosynthesis, phosphate is required by the coupling factor for the production of ATP from ADP. At the same time, phosphate inhibits starch (15) and sucrose (32) synthesis, as well as many of the reactions of the carbon reduction cycle (19, 24). Sharkey (25) suggested that the conflicting requirements of ATP synthesis for phosphate and starch and sucrose synthesis for low phosphate can limit the overall rate of photosynthesis at high rates of photosynthesis. This condition is called the feedback limitation of photosynthesis (26). Because the affinity of the coupling factor for phosphate is high (1, 16, 23) and the sensitivity of starch and sucrose synthesis to low phosphate is also high, it was predicted that the concentration of phosphate in the stroma would fall to 1 mM, perhaps even less, during feedback-limited photosynthesis (25). Furbank et al. (6) suggested that RuBP carboxylase reduces the affinity of the coupling factor for phosphate and so the phosphate level need not fall to such low levels to limit photosynthesis.

The concentration of phosphate in the stroma has been measured in the past (3, 21, 36), but it has never been measured in feedback-limited leaves, that is leaves which exhibit O₂-insensitive photosynthesis. We decided to measure the concentration of phosphate in the stroma and cytosol of leaves exhibiting feedback-limited photosynthesis. This condition can be induced by feeding the phosphate sequestering agent mannose (9) and can also occur under natural conditions (13, 20).

The measurement of stromal phosphate concentration is difficult because plants grown on luxuriant levels of phosphate, as is common practice in research, usually have a large amount of metabolically inactive phosphate in the vacuole (4, 39). This problem can be overcome by growing plants with more realistic phosphate nutrition. When the phosphate supply is restricted, the phosphate concentration in the vacuole can be substantially reduced with little or no effect on photosynthesis (5, 22). Rebielle et al. (17) found that sycamore cells starved for phosphate lose primarily vacuolar phosphate, preserving the concentration of phosphate in the cytoplasm. This observation justifies the study of stromal phosphate concentration in plants grown on restricted phosphate nutrition, especially if it can be shown that such plants have normal stromal phosphate concentrations under some conditions. We used plants fertilized with Hoagland solution modified to contain 15% of the usual amount of phosphate.

Phosphate is extremely soluble in water. Therefore, fractionation of plant material for compartmental analysis of phosphate must be done nonaqueously. We chose the method developed by Gerhardt and Heldt (8).

We report here measurements which confirm the prediction that the concentration of metabolically active phosphate in the stroma can fall to 1 mM or less. We measured the concentration of phosphate in the stroma and cytosol of feedback limited leaves.

MATERIALS AND METHODS

Plant Culture

Plants of Phaseolus vulgaris L. cv Linden were grown in a growth chamber in 4 L pots containing a soil:peat:perlite:rice hull (3:3:3:2) mix. Plants were grown under a 12 h photoperiod with 24/17°C day/night temperature, 60% RH with a photon flux density of 500 μmol m⁻² s⁻¹. The plants were fertilized 5 time per week with Hoagland’s solution B (10)

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modified to contain just 15% of the normal concentration of phosphate (0.15 mM P).

Gas Exchange and Leaf Sampling

Gas exchange measurements were carried out in an open gas exchange system as described in (34). Air was mixed from N₂, O₂, and CO₂ so that the partial pressures of O₂ and CO₂ could be controlled. Once the gas exchange characteristics of each leaf were determined, the leaf was quickly frozen in a freeze-clamp apparatus. We estimate that fewer than 250 ms elapsed between the time the light illuminating the chamber was interrupted and the time the temperature of the leaf material was less than 0°C. Six leaf samples were combined to make one sample for nonaqueous fractionation. Samples for different treatments within an experiment were taken from opposite leaflets of trifoliolates for uniformity. Samples were stored at -80°C for up to 1 week.

Nonaqueous Fractionation

Nonaqueous fractionation of the leaf material was carried out using methods similar to those of Gerhardt and Heldt (8). The frozen leaf samples were ground in liquid N₂ in a mortar. The leaf powder was lyophilized in a Virtils 10-324 until the pressure was 5 mmorr. This usually required about 3 h. We made comparisons between this method which allows the sample to warm up once the water is lost and the slower method of holding the sample at -40°C during the freeze-drying. The latter method requires three days for complete drying, and we found the results to be indistinguishable. The dry powder was transferred to 20 mL heptane and sonicated with a Branson Sonifier 250 on power setting 8 and 50% duty cycle. The tube containing the leaf powder suspended in heptane was held in a beaker containing heptane kept partially frozen by the addition of liquid N₂. The suspension was concentrated by centrifugation then put on a 7 mL discontinuous density gradient of heptane and tetrachloroethylene. The gradient was made by putting one ml of the following densities: 1.60, 1.55, 1.50, 1.475, 1.45, 1.40, and 1.35. The gradient was centrifuged at 25,000 g in an HB-4 swinging bucket rotor in an RC-5B refrigerated centrifuge for 2.5 h. After centrifugation, the gradient was divided into six 1 to 2 mL fractions. Each fraction was divided with one third of the fraction for enzyme assays and two-thirds for metabolite assays. Heptane was added to each sample, then each sample was centrifuged in a microcentrifuge for 4 min. The pellet was allowed to dry overnight under reduced pressure in a desiccator containing paraffin and silica gel.

Marker Enzyme Assays

For enzyme assays, the samples were resuspended in 500 μL 100 mM Bicine (pH 7.8), 5 mM MgCl₂, and 1 mM EDTA. The samples were sonicated for 30 s. After 5 min, the samples were centrifuged for 5 min in a microcentrifuge. The supernatant was used for enzyme assays and the pellet was used for Chl determinations. All manipulations were carried out on ice.

Chl was used for the chloroplast marker. In many experiments we also measured NADP-dependent glyceraldehyde 3-phosphate dehydrogenase and found no significant differences in distribution of these two chloroplast markers in the density gradient. Chl was determined by adding 1 mL 95% ethanol to the pellet, sonicating for 30 s, then centrifuging for 2 min in a microcentrifuge. The A at 654 nm was read and converted to Chl amount using the equations in Wintemans and DeMots (37).

PEPCase was used as the cytosol marker. PEPCase was assayed at 22°C by adding 10 to 50 μL of extract to 250 μL 100 mM Bicine (pH 7.8), 5 mM MgCl₂, 1 mM EDTA, 550 μM NADH, 15 units malate dehydrogenase, 4 mM NaHCO₃, with 2.5 Ci mol⁻¹ NaH¹⁴CO₃. The reaction was started by adding PEP to give a final concentration of 2 mM. After 5 min, the reaction was stopped by adding 500 μL 2 N HCl. The assays were dried overnight. The next morning, 100 μL of water and 3 mL of Bio-Safe II scintillation cocktail was added to each sample. After vortexing, the samples were counted in a scintillation counter.

For the vacuolar marker we used α-mannosidase. This enzyme was assayed by adding 10 to 50 μL extract to 400 μL 50 mM citrate (pH 4.5) and 400 μL 5 mM p-nitrophenyl α-D-mannoside. After 30 min at 37°C, the assay was stopped by adding 400 μL 0.8 M borate (pH 9.8). The A at 405 nm was read.

Inorganic phosphate was measured after extraction of the metabolite fraction in 600 μL 3.5% perchloric acid. The extracts were neutralized to pH 6 to 7 by adding a solution of 2 N KOH, 150 mM Hepes (to help stabilize the pH), and 10 mM KCl (to help the precipitation of KClO₄). The phosphate assay was the malachite green enhanced-molybdate assay taken from Itaya and Ui (11) and Penny (14). An assay solution of 2 g L⁻¹ malachite green (Sigma M9636) and 10 mM ammonium molybdate in 0.8 M HCl was made up at least two days prior to assay. This solution was filtered through Whatman No. 1 filter paper. Plant extract (10–50 μL) was added to 800 μL of molybdate reagent. After 1 min, 100 μL 1 M trisodium citrate was added to the assay. After 1 further min, 100 μL of 1% Extrn 1000 detergent was added to the assay. The optical density at 650 nm was read after 30 min and compared with standards made with dried KH₂PO₄.

Data Analysis

In principle it is possible to set up simultaneous equations to solve for the phosphate concentration in each of the three compartments, stroma, cytosol, and vacuole (8). In practice, we found that a two-compartment analysis as described by Gerhardt and Heldt (8) and as we describe below gave more consistent and believable results. The two-compartment analysis is as follows (8). The amount of phosphate in the stroma, Pₛₘ, is related to the amount of stromal marker. We used Chl as the stromal marker. The amount of phosphate in

2 Abbreviations: PEPCase, phosphoenolpyruvate carboxylase; PEP, phosphoenolpyruvate; RuBP, ribulose bisphosphate; PGA, 3-phosphoglyceric acid.
the cytosol is related to the amount of the cytosolic marker PEPCase.

\[ a = \frac{P_{\text{str}}}{\text{Chl}} \]  

\[ b = \frac{P_{\text{cyt}}}{\text{PEPCase}} \]

In each fraction, denoted by a subscript i, we assume that the total phosphate present comes only from the stroma and cytosol.

\[ P_{i}^{\text{total}} = P_{i}^{\text{str}} + P_{i}^{\text{cyt}} \]

Substitute Equations 1 and 2 into Equation 3

\[ P_{i}^{\text{total}} = a \cdot \text{Chl}_i + b \cdot \text{PEPCase}_i \]

Divide by PEPCase

\[ \frac{P_{i}^{\text{total}}}{\text{PEPCase}_i} = \frac{a}{\text{PEPCase}} \cdot \text{Chl}_i + b \cdot \frac{1}{\text{PEPCase}} \]

Given Equation 5, if we plot \( P_i / \text{PEPCase} \) versus \( \text{Chl}_i / \text{PEPCase} \), the slope is a, the ratio of stromal phosphate to Chl and the y intercept is the ratio of cytosolic phosphate to PEP carboxylase. By multiplying the y intercept by the ratio of PEP carboxylase to Chl, the ratio of cytosolic phosphate to Chl is obtained.

Because this analysis assumes that phosphate in each fraction is derived only from the stroma or the cytosol, it is necessary to estimate the contribution of the vacuole to determine the error of this analysis. The distribution of markers for one of the gradients is shown in Figure 1. Over 70% of the vacuolar marker was in the two heaviest two fractions. The top four fractions contained less than 10% each of the vacuolar marker. We used only the top three or four fractions in the analyses reported in the paper so that vacuolar contamination was kept to a minimum. To estimate the error introduced by this contamination, we assumed that all of the phosphate in the heaviest fraction was vacuolar (an overestimation). The ratio of phosphate to vacuolar marker calculated this way was then applied to the top fractions. This analysis indicated that less than 10% of the phosphate in the top fractions came from vacuolar contamination. If the ratio of vacuolar content to cytosolic content remained constant over the top four fractions then the cytosolic phosphate content may be overestimated by 10%. Because we were looking for low phosphate levels, and ignoring the vacuolar phosphate was likely to cause an overestimation of the phosphate concentration in the stroma and the cytosol, we believe that it is a conservative and justifiable approach to ignore the vacuolar phosphate.

Chloroplasts from leaves which had been freeze-clamped in low \( \text{p(O}_2\text{)} \) had a greater density, so we often could only use the top three fractions of the gradient in the analysis instead of the top four as for normal \( \text{p(O}_2\text{)} \) samples. Although the concentration of phosphate in the stroma and cytosol can be determined from just two points, using three or four points serves as a check on the internal consistency of the data. Therefore, we report the number of points used and the correlation coefficient of the linear regression (r) for each measurement reported.

RESULTS

Leaves freeze-clamped in low \( \text{p(O}_2\text{)} \) had much less stromal phosphate than leaves clamped in normal \( \text{p(O}_2\text{)} \) (Fig. 2). While only two data points are required for the determination of the slope, using up to four data points averages out experimental error to give a more reliable estimate of the stromal phosphate concentration. The fact that all four points approximate a straight line is an indication of the internal consistency of the measurement. Chloroplast material from leaves freeze-clamped in low \( \text{p(O}_2\text{)} \) was heavier than that from leaves freeze-clamped in normal \( \text{p(O}_2\text{)} \), and so the greatest ratio of Chl to

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**Figure 1.** Distribution of markers in the fractions of a density gradient of freeze-dried plant material. Each marker is given as a proportion of the total amount of that marker in the entire gradient.

**Figure 2.** Plot of \( P_i / \text{PEP carboxylase} \ versus \text{Chl}_i / \text{PEP carboxylase} \ as described in the "Materials and Methods." The normal oxygen level is 200 mbar and the low level is 20 mbar. The open triangles are from fractions 5 and 6 of the low oxygen sample and are not included in the calculations. The filled triangles are for fractions 5 and 6 of the normal oxygen sample. The slope of the normal oxygen line (excluding fractions 5 and 6) is 0.248, and so there is 248 nmol phosphate/mg Chl in the stroma, whereas the slope of the low oxygen line (excluding fractions 5 and 6) is 0.053, indicating 53 nmol phosphate/mg Chl in the stroma of the leaves frozen in low oxygen. The correlation coefficients were 0.97 for the normal oxygen treatment and 0.96 for the low oxygen treatment.
PEP carboxylase was less in the low compared to that in the normal \( p(O_2) \) sample. We do not know why chloroplasts from feedback limited leaves were denser but speculate that it was related to the differences in metabolite contents.

The measurement of phosphate was repeated three other times over a period of 6 months. The degree of phosphate starvation of the plants varied from one experiment to the next and so the rate of photosynthesis was different in the three experiments (Table I). The variation in the degree of phosphate starvation was caused by variations in age of the plants and in phosphate binding capacity of the soil used (and probably other factors as well). Nevertheless, within each experiment, no stimulation of photosynthesis was found upon switching to low \( p(O_2) \). The rate of photosynthesis varied from well below normal (experiment 1) to essentially normal (experiment 3).

Although the plants used for each measurement were physiologically different, the results were similar enough to justify calculation of an average for all of the measurements that we made (values in Table I and Fig. 2). These averages are reported in Figure 3. It is readily apparent that leaves held in low \( p(O_2) \), under conditions where low \( p(O_2) \) does not stimulate photosynthesis, had a lower stromal concentration of phosphate but similar cytosolic concentration of phosphate compared to leaves in normal \( p(O_2) \).

It is believed that the effect of low oxygen on photosynthesis results primarily from feedback limitations in photosynthesis (25, 26). Therefore, many of the effects caused by low oxygen should also be observed in high \( p(CO_2) \). We measured the phosphate concentrations in leaves held in normal or high \( p(CO_2) \) (Table II). High \( CO_2 \) caused the phosphate concentration in the stroma fall, just as \( O_2 \) had.

We tested the effect of feeding mannose on the concentration of phosphate in the stroma and cytosol. After feeding mannose, the concentration of phosphate in the stroma fell (Table II). Mannose-fed leaves had less cytosolic phosphate (Table II).

The concentration of phosphate in the stroma and cytosol of phosphate-starved plants was measured over a range of photon flux densities to characterize the phosphate-starved plants (Fig. 4). The phosphate concentration in the stroma was relatively constant over a range of photon flux densities. In the cytosol, the phosphate concentration was very high in darkness and at 100 \( \mu \text{mol m}^{-2} \text{s}^{-1} \). At higher photon flux densities the cytosolic phosphate concentration fell.

### DISCUSSION

The concentration of phosphate in the stroma falls when leaves are put in low \( p(O_2) \). Assuming 25 \( \mu \text{L mg}^{-1} \text{Chl} \), the average phosphate concentration in the stroma of leaves held in low \( p(O_2) \) was 2.7 mm; in two experiments the concentration was just 2 mm and it was 2 mm in one of the measurements of leaves incubated in high \( CO_2 \). Some of this phosphate may not be readily available for metabolism (38). The metabolically unavailable phosphate in the stroma was estimated by Furbank et al. (7) by measuring the phosphate concentration inside chloroplasts unable to photosynthesize for lack of phosphate. They found between 1.5 and 2.5 mm phosphate inside such chloroplasts. Robinson and Giersch (18) found 1.1 to 1.8 mm phosphate in phosphate-starved chloroplasts when measured with a colorimetric assay, as employed in this study. However, when they assayed the amount of phosphate that readily exchanged with \(^3\text{P}\)-phosphate, they found only 0.2 mm phosphate. Therefore, between 1 and 2 mm stromal phosphate measured colorimetrically must be considered metabolically inactive. This amount could be bound to enzymes or inside membranes and released upon extraction. From the data reported here and the data of Robinson and Giersch (18) and Furbank et al. (7), we conclude that the stromal phosphate concentration in leaves in low \( p(O_2) \) may be anywhere from vanishingly small (the 2 mm measured in the experiment reported in Figure 2 and experiment 2 of Table I, minus 2 mm metabolically inactive phosphate) up to 1.7 mm (the average reported in Fig. 3 minus 1 mm metabolically inactive). In the absence of other compelling evidence our best guess is that the concentration of metabolically active phosphate in the stroma of feedback limited leaves is 0.85 mm and possibly lower.

In experiment 3, Table I, the phosphate concentration was low, even at normal \( p(O_2) \). We interpret this result to indicate that the leaves were feedback limited even in normal \( p(O_2) \) in this experiment. The concentration of phosphate in the stroma of leaves in normal \( p(O_2) \) was usually in the range of

<table>
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<tr>
<th>( p(O_2) )</th>
<th>Stroma</th>
<th>Cytosol</th>
<th>Correlation Coefficient</th>
<th>Assimilation</th>
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<tr>
<td>mbar</td>
<td>nmol P, mg(^{-1}) Chl</td>
<td>r (n)</td>
<td>( \mu \text{mol m}^{-2} \text{s}^{-1} )</td>
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<tr>
<td>Experiment 1</td>
<td></td>
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<tr>
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<td>148</td>
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<td>8.5 ± 1.2</td>
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<tr>
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<td>88</td>
<td>163</td>
<td>0.93 (3)</td>
<td>8.2 ± 1.2</td>
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<tr>
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<td>80</td>
<td>209</td>
<td>0.93 (3)</td>
<td>18.7 ± 0.7</td>
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</table>

**Figure 3.** Average and standard error for all measurements of stromal and cytosolic phosphate concentrations in leaves frozen in normal or low oxygen.
The decline in stromal phosphate concentration by 5 mM upon switching to low p(O2) under feedback conditions is easily accounted for by the buildup of RuBP (27) and PFK (29) that occurs under these conditions. The increase in PGA concentration is caused by a lowered ATP/ADP ratio (29) while the buildup of RuBP is caused by reduced carbamylation of RuBP carboxylase (2, 28). The decline in carbamylation is caused by the reduced ATP/ADP ratio (33).

Feeding mannose did not precisely mimic the naturally occurring feedback syndrome. Mannose sequesters phosphate in the cytosol (Table II) as expected (9, 31, 35), and reduced hexose monophosphate and phosphoglycerate concentration (data not shown). Leaves fed a similar phosphate sequestering agent, 2-deoxyglucose, have much less RuBP (28). Feedback limited leaves have increased RuBP and phosphoglycerate levels and constant or increased cytosolic phosphate concentration (this occurred in three of four low oxygen experiments and three of three high CO2 experiments).

During feedback limited photosynthesis, the stromal phosphate concentration is extremely low (Fig. 3), yet the concentration of RuBP is high (27). This condition makes no sense when the feedback limitation is viewed as a lack of phosphate or inadequate phosphate nutrition (12, 30, 31, 35). However, if the low concentration of phosphate in the stroma is viewed as an intermediary, reporting that the capacity for triose phosphate production exceeds the capacity for triose phosphate utilization, then the high concentration of RuBP is adaptive. Ordinarily, the concentration of phosphate inside the stroma is saturating for ATP synthesis. As the rate of photosynthesis increases, starch and sucrose synthesis must also increase so that they are matched to the capacity for triose phosphate production by the chloroplast. As light was increased up to 400 μmol m−2 s−1, the cytosolic phosphate level fell, but there was little decline in stromal phosphate concentration (Fig. 4). Presumably P-glycerate increased to stimulate starch synthesis. Upon switching to low O2 or high CO2 to upset the balance between triose phosphate production and consumption, the stromal phosphate concentration falls dramatically providing a strong signal for starch synthesis. The decline in stromal phosphate can be effected by decar-bamylation of RuBP carboxylase (2) leading to accumulation of RuBP at the expense of free phosphate.

However, there is a limit to how far the stromal phosphate concentration can fall before it will begin to limit ATP synthesis. Once this limit is reached, photosynthesis cannot increase further. A higher stromal phosphate concentration would inhibit starch synthesis while a lower concentration would inhibit ATP synthesis. Thus the high RuBP concentration is adaptive in lowering the stromal phosphate concentration to the point where starch synthesis is stimulated as much as possible; in extremis ATP synthesis is inhibited and photosynthesis becomes feedback limited.

The mechanism put forward above assumes that phosphate is ordinarily in excess supply, and that changes in RuBP carboxylase carbamylation sequesters more or less of the excess as RuBP to regulate photosynthesis. This view is different from the view put forward by Sivak and Walker (30, 31) and Walker and Sivak (35). Their view, that phosphate is in short supply and easily becomes limiting in its own right, fails to explain the high concentration of RuBP that is found in feedback limited leaves; our view explains that high concentration.

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**LITERATURE CITED**

protoplasts and chloroplasts from photosynthetic tissue using phosphorus-31 NMR. Biochem J 202: 429-434
30. Sivak MN, Walker DA (1986) Photosynthesis in vivo can be limited by phosphate supply. New Phytol 102, 499-512