Effects of Magnesium on Intact Chloroplasts

I. EVIDENCE FOR ACTIVATION OF (SODIUM) POTASSIUM/PROTON EXCHANGE ACROSS THE CHLOROPLAST ENVELOPE

STEVEN C. HUBER and WENDY MAURY
United States Department of Agriculture, Science and Education Administration, Agricultural Research Division, Crop Science, and Botany, North Carolina State University, Raleigh, North Carolina 27650

ABSTRACT
Exogenous Mg²⁺ (2 millimolar) altered the stromal pH of intact spinach chloroplasts. Without added KCI in the medium, Mg²⁺ decreased the stromal pH in the dark by approximately 0.3 pH unit. External KCI (25 millimolar) largely prevented the acidification caused by Mg²⁺. Effects on the stromal pH were not caused by changes in H⁺ pumping across the thylakoid membrane because Mg²⁺ had no effect on the light-induced quenching of stromal fluorescence by intact chloroplasts. However, Mg²⁺ affected H⁺ fluxes across the envelope. Addition of Mg²⁺ to intact chloroplasts in the dark caused a significant acidification of the medium that was dependent on the presence of K⁺.

External K⁺ or Na⁺ also prevented the inhibition of CO₂-dependent O₂ evolution by Mg²⁺, whereas chloride was less effective. The combination of Mg²⁺ and K⁺ stimulated O₂ evolution at suboptimal pH, inhibited O₂ evolution at optimal and superoptimal pH, and prevented the inhibition of photosynthesis caused by acetate. In the absence of added K⁺, Mg²⁺ was most inhibitory to O₂ evolution at suboptimal pH.

The results suggested that Mg²⁺ activated a reversible (Na⁺)K⁺/H⁺ exchange across the chloroplast envelope. It is postulated that changes in the stromal pH may explain the inhibition of photosynthesis caused by the presence of exogenous Mg²⁺.

Millimolar concentrations of Mg²⁺ have been shown to inhibit CO₂-dependent O₂ evolution by isolated chloroplasts of spinach (8, 9, 13, 17), barley (8, 9, 11), and lettuce (2). Results obtained with spinach and barley chloroplasts suggested that Mg²⁺ inhibits photosynthesis by preventing the light activation of NADP-glyceraldehyde-3-P dehydrogenase, phosphoribulokinase, and fructose-1,6-bisphosphatase (9). It was later postulated that Mg²⁺ inhibits O₂ evolution and the light activation of photosynthetic enzymes by stimulating Pi exchange across the chloroplast envelope (8). The postulate was supported by several lines of evidence. First, Mg²⁺ reduced the optimal Pi concentration required for O₂ evolution (8) and inhibition by Mg²⁺ of both O₂ evolution and the light activation of photosynthetic enzymes was prevented by metabolites which compete with Pi for uptake on the phosphate translocator (11). Second, the activation of photosynthetic enzymes by light in a reconstituted system (stromal proteins plus thylakoid membranes) was inhibited by Pi but not by Mg²⁺ (10).


Because the chloroplast envelope is impermeable to divalent cations (3), the above observations suggested that Mg²⁺ stimulated Pi exchange indirectly, perhaps by interaction with some component of the chloroplast envelope.

Recent results from this laboratory have suggested that the Pi dependence of chloroplast photosynthesis is sensitive to pH (8). Specifically, reduction of the stromal pH apparently stimulated Pi exchange (8). The objectives of the present study were to determine whether Mg²⁺ affected the stromal pH, and if so, the mechanism involved.

MATERIALS AND METHODS
Chloroplast Isolation. The spinach (Spinacia oleracea L.) plants were grown in soil in a growth chamber with a 12-h photoperiod and 22_C/17_C temperature regime. Intact chloroplasts were isolated by the method of Lilley and Walker (14). The blending medium contained 0.33 mM sorbitol, 10 mM Na₃P₂O₇, 5 mM MgCl₂, and 2 mM isocitrate, adjusted to pH 7.6. Following centrifugation (200g, 90 s), the pellet was washed once and resuspended in 0.33 mM sorbitol, 1 mM MgCl₂, 1 mM MnCl₂, 2 mM EDTA, and 50 mM Hepes-NaOH (pH 7.6). The final preparation contained 50-70% intact chloroplasts, based on the ferricyanide reduction assay.

O₂ Evolution. O₂ evolution was followed polarographically with Clark-type electrodes in 1.8-ml water-jacketed vessels maintained at 25 C. The basic reaction mixture contained 0.33 mM sorbitol, 50 mM Hepes-NaOH (pH 7.6), 1 mM MgCl₂, 1 mM MnCl₂, 2 mM EDTA, 0.5 mM Na₃P₂O₇, 6 mM NaHCO₃, and 600 units/ml of catalase. The concentration of Chl was 20–50 μg/ml. Illumination was provided by a 75-w floodlamp to give a quantum flux density of 80 nE/cm²·s between 400 and 700 nm at the face of the cuvette.

Measurement of Stromal pH. The 200-μl chloroplast incubation medium contained 0.33 mM sorbitol, 50 mM Hepes-NaOH (pH 7.6), 1 mM MgCl₂, 1 mM MnCl₂, 2 mM EDTA, 0.5 mM Na₃P₂O₇, 600 units/ml of catalase and 5 mM NaH¹⁴CO₃ (0.75 μCi/μmol). Reactions were run at 25 C and were typically initiated by the addition of chloroplasts (15–30 μg Chl) and terminated after 1 min of illumination by centrifugation through a layer (70 μl) of silicone oil (Wacker AR 200) into a bottom layer of 200 μl of 2.5 mM NaOH as previously described (19). Illumination was provided by an overhead 75-w floodlamp that produced 60 nE/cm²·s (400–700 nm) at the side of the polyethylene centrifuge tube. After centrifugation, a 50-μl aliquot of the top layer was counted in scintillation fluid to determine total dpm in the incubation mixture and the entire bottom layer was excised and placed in scintillation fluid to count the dpm.
determine dpm in the chloroplast pellet. Quench correction was by external standard. The amount of label in the chloroplast pellet was corrected for nonosmotic uptake and absolute volumes were determined by uptake of $^3$H$_2$O and $^{14}$C$\text{Sucrose}$ as previously described (18). The pH of the stroma was determined in accordance with the relationship (18)

$$\Delta pH = pH_{\text{int}} - pH_{\text{ext}} = \log \left( \frac{[\text{H}^+\text{CO}_3]_{\text{ext}}}{[\text{H}^+\text{CO}_3]_{\text{int}}} \right)$$

**pH Electrode Measurements.** Changes in the pH of the medium were measured with a combination pH electrode at 25 C in the dark. The 2-ml reaction mixture contained 0.33 M sorbitol, 0.5 mM HEPES-NaOH (pH 7.0), 0.5 mM Pi, and chloroplasts (50–75 $\mu$g Chl/ml). The buffering capacity of the mixture was determined at the end of each experiment by addition of 0.1 $\mu$mol NaOH. The initial pH was adjusted to pH 7.0 to minimize the spontaneous acidification of the medium observed when the initial pH was greater than 7.0.

All experiments were repeated at least three times using different chloroplast isolations.

**RESULTS**

**Effects of Mg$^{2+}$ on Stomatal pH.** The stomatal pH of intact spinach chloroplasts after 1 min of illumination was about pH 8.1 (Table I), which concurs with previous findings (6, 18, 19). MgCl$_2$ decreased the stomatal pH in the light by approximately 0.3 pH units (Table I). The stomatal pH was not affected by exogenous KCl; however, KCl largely prevented the acidification caused by Mg$^{2+}$ (Table I). Occasionally, the combination of Mg$^{2+}$ + K$^+$ caused an increase in absolute stomatal pH relative to the control pH. The chloroplast stomatal volume was also affected by Mg$^{2+}$ (Table I). Mg$^{2+}$ decreased the stomatal volume by 36%. External KCl (25 mM) caused only a slight decrease in volume, a result which may be attributed to the increase in medium osmolarity (50 mM total). The decrease in stomatal volume caused by Mg$^{2+}$ was prevented by K$^+$ (Table I).

The acidification of the stomatal pH caused by Mg$^{2+}$ (Table I) could be explained by reduced pumping of H$^+$ across the thylakoid membrane or by increased permeability of the chloroplast envelope to H$^+$. In experiments not reported here, exogenous Mg$^{2+}$ (3 mM) had no significant effect on the light-dependent quenching of atebin fluorescence (12), which reflects acidification of the intrathylakoid space. The results indicated that exogenous Mg$^{2+}$ probably did not affect the stomatal pH by affecting H$^+$ translocation through the thylakoid membrane.

If changes in the stomatal pH (Table I) were caused by movement of protons across the chloroplast envelope, changes in the pH of the medium should be observed. Typical results are presented in Figure 1. Addition of 4 mM MgCl$_2$ in the dark to chloroplasts suspended in a medium of low buffering capacity (at pH 7.0) caused a significant acidification of the medium that was dependent on external K$^+$. Without added K$^+$, addition of Mg$^{2+}$ usually caused an alkalinization of the medium (Fig. 1) and occasionally, a slight acidification (data not shown). No pH changes were observed when chloroplasts were omitted from the reaction mixture. Some of the acidification caused by Mg$^{2+}$ may be attributed to displacement of protons bound to lipid groups in the membrane. Hence, quantitative evaluation of the data may not be justified. However, the increased acidification of the medium by Mg$^{2+}$ in the presence of 40 mM K$^+$ (Fig. 1) suggested that some of the released protons were obtained from the stromal space (Table I) and that Mg$^{2+}$ affected the envelope rather than the thylakoid membrane.

**Reversal of Mg$^{2+}$ Inhibition of O$_2$ Evolution by Salts.** Studies were conducted to determine whether exogenous K$^+$ would prevent Mg$^{2+}$ inhibition of CO$_2$-dependent O$_2$ evolution. Mg$^{2+}$ (4 mM) produced nearly complete inhibition of O$_2$ evolution, whereas KCl (30 mM), in the absence of Mg$^{2+}$, had no effect (Fig. 2). Similar effects of Mg$^{2+}$ and K$^+$ were reported previously (10). Inhibition of O$_2$ evolution by Mg$^{2+}$ was completely prevented by K$^+$ when both were added before illumination and addition of K$^+$ in the light to Mg$^{2+}$-inhibited chloroplasts caused a rapid rise in O$_2$ evolution (Fig. 2).

Other monovalent salts were tested for effects on O$_2$ evolution in the presence and absence of Mg$^{2+}$. The chloride salts of K$^+$, Na$^+$, and choline did not produce significant inhibition of O$_2$ evolution but prevented Mg$^{2+}$ inhibition of O$_2$ evolution to varying degrees (Table II). At a concentration of 50 mM, Na$^+$ and K$^+$ almost completely reversed the inhibition by Mg$^{2+}$, whereas choline did not (Table II). LiCl produced significant inhibition of O$_2$ evolution in the presence or absence of Mg$^{2+}$, a result which must be ascribed to inhibition by Li$^+$. The sulfate salts of the monovalent cations were inhibitory, which is consistent with previous observations (1, 5).

The concentration dependence for K$^+$ and choline reversal of Mg$^{2+}$ inhibition of O$_2$ evolution is presented in Figure 3. Up to concentrations of 30 mM, K$^+$ and choline had no effect on the rate of O$_2$ evolution in the absence of Mg$^{2+}$ (Fig. 3). Without added salt, O$_2$ evolution was inhibited greater than 90% by 2 mM Mg$^{2+}$, and 97% by 4 mM MgCl$_2$. Relatively low concentrations of K$^+$ were considerably more effective than equimolar amounts of choline in preventing Mg$^{2+}$ inhibition of O$_2$ evolution. The action of K$^+$ was not affected by increasing the concentration of Mg$^{2+}$ from 2 to 4 mM (Fig. 3). The results suggested that prevention of Mg$^{2+}$ inhibition by K$^+$ was not caused simply by an ionic strength effect, because in that case, choline would be expected to be as effective as K$^+$ and prevention by the monovalent salt should decrease as the concentration of Mg$^{2+}$ was increased.

### Table I. Effects of Mg$^{2+}$ and K$^+$ on Stomatal pH and Volume of Spinach Chloroplasts after 1 min of Illumination at 25 C

<table>
<thead>
<tr>
<th>Additions</th>
<th>Stomatal pH</th>
<th>Stomatal Volume</th>
<th>µl/mg Chl</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>8.10</td>
<td></td>
<td>27.5</td>
</tr>
<tr>
<td>4 mM MgCl$_2$</td>
<td>7.78</td>
<td></td>
<td>17.5</td>
</tr>
<tr>
<td>25 mM KCl</td>
<td>8.14</td>
<td></td>
<td>25.0</td>
</tr>
<tr>
<td>4 mM MgCl$_2$+ 25 mM KCl</td>
<td>8.02</td>
<td></td>
<td>25.0</td>
</tr>
</tbody>
</table>

**FIG. 1.** Changes in pH of external medium by addition of 4 mM MgCl$_2$ in presence and absence of 40 mM KCl. (---): Change in pH without addition of Mg$^{2+}$.---: Change in pH with addition of Mg$^{2+}$.---: Change in pH without addition of Mg$^{2+}$.---: Change in pH with addition of Mg$^{2+}$.---: Change in pH without addition of Mg$^{2+}$.---: Change in pH with addition of Mg$^{2+}$.
of Mg$^{2+}$ was largely reversed by exogenous K$^+$. As shown by the results presented in Figure 4, Mg$^{2+}$ + K$^+$ increased O$_2$ evolution even above control values at suboptimal pH (pH 7.0-7.5) and inhibited O$_2$ evolution at higher pH (Fig. 4). The results suggested that Mg$^{2+}$ caused acidification of the stroma in the absence of K$^+$ and alkalization in the presence of exogenous K$^+$.

The alkaline-shifted pH optimum for photosynthesis in the presence of Mg$^{2+}$ (Fig. 4) may reflect both acidification of the stroma (Table I) and the increasing concentration of Na$^+$ (used to neutralize the buffer) with increased pH. Because Na$^+$ was equivalent to K$^+$ in reversing Mg$^{2+}$ inhibition of photosynthesis, a complete evaluation of the effects of Mg$^{2+}$ and monovalent cations will have to be done using a buffer system neutralized with a nonpermeating base. It is apparent that at least at low pH (<8.0), the Na$^+$ contributed by the buffer was not sufficient to prevent Mg$^{2+}$ inhibition (Fig. 4). At pH 8.5, the rate of photosynthesis in the control was similar to the rate in the presence of Mg$^{2+}$ and Mg$^{2+}$ + K$^+$ (Fig. 4), which may indicate that the postulated Mg$^{2+}$-dependent changes in stromal pH do not occur at high external pH.

The postulate predicted that Mg$^{2+}$ + K$^+$ should reverse inhibition of O$_2$ evolution caused by weak acids that act by causing acidification of the stroma. Heldt et al. (6) have shown that Ac$^-$ causes acidification of the stroma which was suggested to occur by diffusion of HAc across the envelope followed by internal dissociation to produce H$^+$ + Ac$^-$. The interpretation was supported by the demonstration that mM concentrations of NaAc inhibited spinach chloroplast O$_2$ evolution at suboptimal pH and stimulated O$_2$ evolution at superoptimal pH (6, 8).

Typical results showing inhibition of CO$_2$-dependent O$_2$ evolution by NaAc are presented in Figure 5. Ac inhibited the rate of O$_2$ evolution about 35% (Fig. 5, trace D). The presence of 30 mM KCl alone caused only a slight inhibition of rate (Fig. 5, trace B), whereas 2 mM MgCl$_2$ produced greater than 95% inhibition (Fig. 5, trace F). In the absence of exogenous K$^+$, Mg$^{2+}$ accentuated inhibition of O$_2$ evolution by Ac (Fig. 5, trace G). Inhibition of O$_2$ evolution by NaAc was not affected by KCl; however, inhibition was completely reversed by Mg$^{2+}$ + K$^+$ (Fig. 5, trace C).

**DISCUSSION**

The purpose of this study was to determine the basis for inhibition of chloroplast photosynthesis by Mg$^{2+}$ (2, 9, 13). The results presented herein suggested that exogenous Mg$^{2+}$ altered the pH of the stroma by affecting H$^+$ movements across the envelope. The direction of the pH change was apparently dependent on the concentration of K$^+$ in the medium. Admittedly, the effects of Mg$^{2+}$ are complex and not entirely understood. However, as a working model we postulate that Mg$^{2+}$ activated a reversible (Na$^+$)K$^+$/H$^+$ exchange across the chloroplast envelope (Fig. 6).

Several lines of evidence indicated that when the concentration of (Na$^+$)K$^+$ in the medium was low, Mg$^{2+}$ caused acidification of the stroma (Fig. 6A). First, the stromal pH in the light was significantly reduced by Mg$^{2+}$ (Table I), which was probably caused by an influx of H$^+$ from the medium (Fig. 1). Second, Mg$^{2+}$ was most inhibitory to O$_2$ evolution at suboptimal pH (Fig. 4 and ref. 8). Previously, Ac (19) and nitrite (16) have been shown to reduce the stromal pH and inhibit O$_2$ evolution preferentially at suboptimal pH. Third, it was demonstrated previously with barley chloroplasts that Mg$^{2+}$ inhibition was prevented by NH$_4$Cl (8) which presumably caused alkalization of the stroma. The stromal content of K$^+$ has been estimated to be approximately 20-30 mM (3); however, the concentration of free K$^+$ in the stroma may be considerably less. The mechanism schematically presented (Fig. 6A) suggests that influx of H$^+$ may be coupled to the efflux of stromal K$^+$ down its concentration gradient. The resultant

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**Table II. Effect of Various Salts on CO$_2$-dependent O$_2$ Evolution by Spinach Chloroplasts in the Presence and Absence of MgCl$_2$**

<table>
<thead>
<tr>
<th>Added Salt (50 mM)</th>
<th>O$_2$ Evolution</th>
<th>µmol O$_2$/mg Chl-h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-MgCl$_2$</td>
<td>+ 2 mM MgCl$_2$</td>
</tr>
<tr>
<td>None*</td>
<td>70</td>
<td>5</td>
</tr>
<tr>
<td>LiCl</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>KCl</td>
<td>60</td>
<td>65</td>
</tr>
<tr>
<td>NaCl</td>
<td>65</td>
<td>59</td>
</tr>
<tr>
<td>Choline-Cl</td>
<td>74</td>
<td>35</td>
</tr>
</tbody>
</table>

* Reaction mixtures contained about 30 mM Na$^+$ used to neutralize the Hepes buffer.

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**Fig. 2. Typical results showing prevention and reversal of Mg$^{2+}$ inhibition of spinach chloroplast O$_2$ evolution by KCl.** At the arrow, 30 mM KCl was added to a reaction mixture that contained 4 mM MgCl$_2$. All other additions were made in the dark. Maximum rates of O$_2$ evolution, expressed as µmol O$_2$/mg Chl-h, are shown parenthetically.
stromal acidification may inhibit photosynthesis by increasing Pi exchange (ref. 8; see Fig. 6A) and decreasing the activation of certain photosynthetic enzymes by light (9). Another potential factor limiting photosynthesis is that the photosynthetic enzymes would have to function at suboptimal pH. The extent to which these factors are related remains an open question, although it is clear that stromal pH does not affect apparent Pi exchange by decreasing Calvin cycle activity (Huber, manuscript in preparation). The magnitude of the pH decrease caused by MgCl₂ (0.32 pH units, Table I) may be sufficient to account for the inhibition of photosynthesis. Heldt et al. (6) have shown that a 1 pH unit decrease of the medium pH results in a decreased stromal pH of approximately 0.5 pH unit. Hence, a decrease in stromal pH of 0.3 unit may be analogous to decreasing the pH medium by 0.6 pH units. Such a change in medium pH (i.e. from pH 7.6 to 7.0) gave complete inhibition of O₂ evolution with 50 mM KCI. An ionic strength of 0.3 unit, pH 7.0 (Table I); (b) prevention of stromal acidification caused by MgCl₂ alone and occasionally an increase in the stromal pH above the control values (Table I); (b) stimulation of CO₂-dependent O₂ evolution in stromal pH at (A) low and (B) high concentration of external K⁺ and suggested effects of stromal pH on phosphate translocator. It is postulated that the K⁺/H⁺ exchange occurs only in the presence of exogenous MgCl₂. (+): activation; (-): inhibition.

at suboptimal pH (Fig. 4); (c) prevention of Ac inhibition of O₂ evolution (Fig. 5); and (d) acidification of the medium (Fig. 1). The results may be explained on the basis that influx of K⁺, driven by the existing concentration gradient, was coupled to an efflux of H⁺, thereby causing alkalization of the stroma and reduced phosphate exchange (Fig. 6B).

Because the chloroplast envelope is impermeable to divalent cations (3), it may be adduced that MgCl₂ represents some "site" on the inner membrane of the envelope. It was important to determine whether exogenous K⁺ simply reversed the effect of MgCl₂ by causing anionic strength displacement of MgCl₂ from the envelope. An ionic strength effect seemed unlikely because choline was less effective than K⁺ or Na⁺ in reversing MgCl₂ inhibition of O₂ evolution (Table II and Fig. 3). Further, K⁺ did not simply reverse

![Figure 3](image-url)  Effect of K⁺ and choline chloride on spinach chloroplast O₂ evolution in the presence and absence of MgCl₂.

![Figure 4](image-url)  Effect of pH on O₂ evolution by spinach chloroplasts in the presence and absence of 2 mM MgCl₂ and 30 mM KCl. Reaction mixtures were buffered with 50 mM HEPES, adjusted to the indicated pH with NaOH.

![Figure 5](image-url)  Prevention of Ac inhibition of spinach chloroplast O₂ evolution by MgCl₂ + K⁺. The indicated salts were added before illumination. Maximum rates of O₂ evolution, expressed as μmol O₂/mg Chl·h are shown parenthetically.

![Figure 6](image-url)  Schematic diagram of effect of MgCl₂ induced K⁺/H⁺ exchange on stromal pH at (A) low and (B) high concentration of external K⁺ and suggested effects of stromal pH on phosphate translocator. It is postulated that the K⁺/H⁺ exchange occurs only in the presence of exogenous MgCl₂. (+): activation; (-): inhibition.
the effect of Mg$^{2+}$, but actually caused additional effects. For example, at suboptimal pH, the combination of Mg$^{2+}$ + K$^+$ stimulated O$_2$ evolution well above the control rates (Fig. 4) and prevented inhibition of O$_2$ evolution by acetate (Fig. 5) which causes acidification of the stroma.

The (Na$^+$)K$^+$/H$^+$ antiporter postulated to be presented in the chloroplast envelope (Fig. 6) may be mechanistically similar to the Na$^+$/H$^+$ antiporter in the inner membrane of rat liver mitochondria (15) and the (Na$^+$)K$^+$/H$^+$ antiporter of plant mitochondria (7). The mechanism postulated in the present study seemed to account for many of the experimental observations concerning the effects of Mg$^{2+}$ on isolated chloroplasts. Experiments are underway to correlate H$^+$ and K$^+$ fluxes across the chloroplast envelope and to determine whether the envelope ATPase is involved.

Because many cytoplasmic enzymes require Mg$^{2+}$ for activity, it is likely that the chloroplast in situ must function in an environment containing this cation. From the results presented herein, it appears that whether Mg$^{2+}$ is inhibitory to chloroplast photosynthesis is directly dependent on pH and the concentration of K$^+$ and indirectly on factors affecting the phosphate translocator (11). The potential may exist for the control of both chloroplastic and extrachloroplastic processes, such as sucrose formation, by the concentration of cytoplasmic Mg$^{2+}$.

Note. During review of this manuscript, we became aware of a paper by B. Demming and H. Gimmel (Z Naturforsch 34c: 233–241) in which they have independently demonstrated acidification of the stroma and decrease in stromal K$^+$ caused by Mg$^{2+}$. These authors also reported prevention of Mg$^{2+}$ inhibition of O$_2$ evolution by K$^+$ and derived conclusions similar to our own.

LITERATURE CITED