Plant Physiol. (1991) 97, 580–587
0032-0889/91/97/0580/08/$01.00/0

Received for publication March 4, 1991
Accepted May 17, 1991

Surface Charge-Mediated Effects of Mg^{2+} on K^+ Flux across the Chloroplast Envelope Are Associated with Regulation of Stromal pH and Photosynthesis[1]

Weihua Wu, Jeanne Peters, and Gerald A. Berkowitz*

Horticulture Department, Cook College, Rutgers—The State University of New Jersey, New Brunswick, New Jersey 08903

ABSTRACT

Studies of *Spinacia oleracea* L. were undertaken to characterize further how Mg^{2+} external to the isolated intact chloroplast interacts with stromal K^+, pH, and photosynthetic capacity. Data presented in this report were consistent with the previously developed hypothesis that millimolar levels of external, unchelated Mg^{2+} result in lower stromal K^+, which somehow is linked to stromal acidification. Stromal acidification directly results in photosynthetic inhibition. These effects were attributed to Mg^{2+} interaction (binding) to negative surface charges on the chloroplast envelope. Chloroplast envelope-bound Mg^{2+} was found to decrease the envelope membrane potential (inside negative) of the illuminated chloroplast by 10 millivolts. It was concluded that Mg^{2+} effects on photosynthesis were likely not mediated by this effect on membrane potential. Further experiments indicated that envelope-bound Mg^{2+} caused lower stromal K^+ by restricting the rate of K^+ influx; Mg^{2+} did not affect K^+ efflux from the stroma. Mg^{2+} restriction of K^+ influx appeared consistent with the typical effects imposed on monovalent cation channels by polyvalent cations that bind to negatively charged sites on a membrane surface near the outer pore of the channel. It was hypothesized that this interaction of Mg^{2+} with the chloroplast envelope likely mediated external Mg^{2+} effects on chloroplast metabolism.

Mg^{2+} external to the chloroplast has been shown to have regulatory effects on the photosynthetic activity of isolated chloroplasts. Several aspects of this interaction have been abundantly clear for quite some time. Photosynthetic inhibition in isolated, intact chloroplasts incubated in solutions containing millimolar unchelated Mg^{2+} is associated with impaired stromal alkalization and altered stromal K^+ levels (2, 3, 11, 12, 14). High stromal pH (*i.e.* light-induced alkalization) is a critical regulator of several enzymes of the photosynthetic carbon reduction cycle and, hence, is required for maximal photosynthetic activity (9). It has been asserted, therefore, that the low stromal pH induced by external Mg^{2+} is the direct cause of photosynthetic inhibition in the presence of external Mg^{2+} (2, 11, 14). These studies have also shown that Mg^{2+} impairment of stromal alkalization is likely the result of altered H^+ fluxes across the chloroplast envelope; altered H^+ pumping across the thylakoid membrane is thought not to be involved.

Mg^{2+} effects on H^+ movement across the chloroplast envelope are known to be linked to K^+ counterflux (2, 11, 14). However, the specific molecular mechanisms that facilitate fluxes of those monovalent cations and allow for Mg^{2+} regulation of the system are not well characterized. Work from this laboratory (21) has established that it is not Mg^{2+} external to the chloroplast but rather Mg^{2+} bound or associated with the chloroplast envelope that exerts the regulatory effect. It is also clear from several different lines of evidence (19, 21) that millimolar levels of free external Mg^{2+} can greatly affect chloroplast metabolism even though Mg^{2+} movement across the envelope into the stroma does not occur under these conditions.

Two hypotheses have been presented in the literature regarding the specific mechanisms that allow for Mg^{2+} regulation of H^+/K^+ counterfluxes. The results of work by Huber and Maury (11) and Maury *et al.* (14) suggest that H^+ and K^+ movement across the chloroplast envelope both occur via a specific antiport enzyme. They postulated that this transport protein, when activated, can facilitate either K^+ influx/H^+ efflux or K^+ efflux directly linked to H^+ influx. These researchers speculated that Mg^{2+} external to chloroplast activates this antiport enzyme and impairs stromal alkalization in the presence of low external K^+ due to net K^+ efflux from the stroma (coupled directly to H^+ influx).

Demmig and Gimmler (2, 3) speculated that other mechanisms facilitate K^+ and H^+ fluxes across the envelope and that Mg^{2+} may influence this system in a different manner. They proposed that a Donnan system (fixed negative charges) develops in the stroma of the illuminated chloroplast, increasing the E_m[2] (inside negative) between the stroma and extrachloroplast medium. They concluded that this Donnan system-generated E_m drives monovalent cation influx. At high external K^+, influx of K^+ occurs. At low external K^+, impaired stromal alkalization was thought to occur due to H^+ influx. Mg^{2+} was thought to influence this system by altering external solution ionic strength; the E_m was proposed to be dependent on ionic strength of the medium. This model, however, does

[1] New Jersey Agricultural Experiment Station, Publication No. 12149-3-91. This material is based upon work supported by the U. S. Department of Agriculture Competitive Research Grants Office under Award No. 90-37262-5586. W. W. received partial support from the National Science Foundation of China.

[2] Abbreviations: E_m, membrane potential; dimethonium, ethane-1,2-bis-trimethylammonium; TPP, tetraphenylphosphonium.

not explain the H^+ efflux that was observed to occur in the presence of high external K^+.

Studies presented in this report focus on further characterizing the mechanisms mediating Mg^{2+} regulation of K^+ and H^+ fluxes across the chloroplast envelope. This research attempted to elucidate the specific biophysical nature of Mg^{2+} interaction with the chloroplast envelope.

MATERIALS AND METHODS

Plant Material

Spinach (*Spinacia oleracea* var 'Melody') seeds were planted in pots containing 2000 cm^3 of potting mix (1:1 peat:vermiculite, v/v) and thinned to three to five plants/pot after 2 weeks. Pots were watered twice/week with tap water and once/week with complete (Peter's Geranium Special plus micronutrients) fertilizer. Plants were grown in a growth chamber with a 10-h light (480 μmol/m^2/s PAR) period at 21°C (day)/16°C (night). Fully expanded, nonsenescing leaves taken from 6- to 8-week-old plants were used for chloroplast isolation.

Chloroplast Isolation

Intact (routinely >90%) chloroplasts were isolated from spinach using procedures described previously (21). Briefly, leaves were homogenized in 50 mL grind medium containing 0.33 M sorbitol, 50 mM Hepes-NaOH (pH 6.8), 2 mM Na_2EDTA, 1 mM $MgCl_2$, and 1 mM $MnCl_2$. After the solution was centrifuged (750*g* for 50 s), pelleted chloroplasts were resuspended in 5 mL grind medium and layered on top of an 8-mL cushion (40% [v/v] Percoll and 0.5% [w/v] BSA in grind medium) in 15-mL tubes. These step gradients were then centrifuged (2000*g* for 1 min) in a swinging bucket rotor. Intact chloroplasts were recovered in the pellet and resuspended in a small volume (0.5–1.0 mL) of grind medium. All steps in the isolation procedure were carried out at 0 to 2°C.

Photosynthesis

CO_2-supported O_2 evolution of intact chloroplasts was measured with Hansatech (Decagon Institute, Seattle, WA) O_2 electrodes at 25°C, and 1500 μmol/m^2/s PAR were supplied by the 500 W bulb of a slide projector focused through a water heat filter. Typically, 50 μL of grind medium containing 20 to 30 μg Chl was added to 1 mL final volume reaction medium (0.33 M sorbitol, 50 mM Hepes-NaOH [pH 7.6], 2 mM Na_2EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 0.25 mM NaH_2PO_4, 5 mM $NaHCO_3$, and 1000 units/mL catalase) with additions as noted in the text.

Stromal K^+

Chloroplasts (50 μg Chl/mL) in 180 μL photosynthesis reaction medium were incubated in 400-μL microfuge tubes at 25°C and 2200 μmol/m^2/s PAR for 5 min. These tubes had 20 μL 14% (v/v) $HClO_4$ and 100 μL silicone oil below the reaction medium. Chloroplasts were separated from the medium and pelleted into the $HClO_4$ layer of the tubes by centrifugation in a Beckman B microcentrifuge. K^+ in the $HClO_4$ layer was measured using a Perkin-Elmer 2280 atomic absorption spectrophotometer (Norwalk, CT). All samples and standards were made up to contain 0.2% (w/v) $LaCl_3$; absorbance was measured at a wavelength of 766.5 nm.

In some experiments K^+ movement into or out of the chloroplast was measured (in contrast to total stromal K^+). For these studies, $^{86}Rb^+$ was used as a radiolabeled analog of K^+, and chloroplasts were incubated in silicone oil microcentrifugation tubes under the general conditions described above, with additions to the reaction medium as noted in the text. For measurement of K^+ uptake, chloroplasts were incubated in photosynthetic reaction medium with both K^+ and $^{86}Rb^+$ added as the chloride salts. Both [K^+] and $^{86}Rb^+$ specific activities are noted for each experiment in table headings and figure legends. Results are expressed as nanomoles K^+ taken up into the stroma per milligram Chl; the radiolabel associated with the chloroplasts in the $HClO_4$ fraction of the silicone oil microcentrifugation tubes was converted to nanomoles of K^+ by calculating the specific activity (Ci $^{86}Rb^+$/mol K^+) external to the chloroplast in each experiment. $^{86}Rb^+$ in the $HClO_4$ fraction was measured using liquid scintillation spectrophotometry assuming 100% counting efficiency.

K^+ efflux from chloroplasts was measured by preloading chloroplasts with $^{86}Rb^+$ and then incubating them in photosynthetic reaction medium before silicone oil microcentrifugation. $^{86}Rb^+$ efflux was ascertained by measuring the $^{86}Rb^+$ retained in the chloroplast (*i.e.* in the $HClO_4$ fraction after microcentrifugation) after varying durations of time. Chloroplasts were preloaded with $^{86}Rb^+$ during the isolation procedure. After the first centrifugation, the pelleted chloroplasts were resuspended in 5 mL grind medium which had 10 mM KCl and 20 μCi $^{86}Rb^+$/mL added. The chloroplasts were incubated in this solution for 30 min at 0 to 2°C and then removed from this medium by centrifugation through the 8-mL 40% Percoll step gradient.

E_m

E_m across the chloroplast envelope was ascertained by measuring equilibration of the lipophilic cation TPP across the chloroplast envelope as described by Demmig and Gimmler (3) and Prased and Hoefer (20). Chloroplasts were incubated under the same conditions used for the stromal pH determinations in photosynthetic reaction medium with 2 μM [^{3}H] TPP (300 Ci/mol) and additions as noted in the text. After 5 min in the light, incubations were terminated by centrifugation of the microfuge tubes. Demmig and Gimmler (3) have established that TPP equilibration across the chloroplast envelope is relatively fast as compared to these incubation times and should not affect the results. Calculated values for [TPP] in the stroma and external to the chloroplast were used in the Nernst equation to estimate E_m across the envelope.

Chloroplast Volume

The dual label technique developed by Heldt (8) as modified by Sen Gupta and Berkowitz (21) was used to ascertain stromal volume and sorbitol-permeable space associated with chloroplasts. Chloroplasts were incubated in photosynthetic

reaction medium that contained 12.5 μCi/mL [^{14}C]sorbitol and 5 μCi/mL [^{3}H]H_2O. After 5 min, chloroplasts were separated from the medium above the silicone oil layer in the 400-μL tubes by microcentrifugation. ^{3}H and ^{14}C in the supernatant and $HClO_4$ fraction of the tubes were ascertained by liquid scintillation spectrophotometry using dual label dpm programs with external standards ratio quench correction. The water volume associated with the chloroplasts represented the sum of the stromal and sorbitol-permeable space. The [^{14}C]sorbitol space represented the sorbitol-permeable (*i.e.* extrastromal) volume of reaction medium associated with the chloroplasts in the $HClO_4$ fraction. The difference between the two was taken to be a measure of stromal volume.

For all pertinent studies (*e.g.* stromal K^+, E_m), the stromal volume and sorbitol-permeable space were measured on aliquots of the chloroplast preparation subjected to the various treatments. For all experiments that involved the measurement of the stromal level of a compound, the amount calculated to be in the (extrachloroplastic) sorbitol-permeable space of the chloroplast pellet was subtracted from the total amount in the $HClO_4$ fraction of the microcentrifuge tubes.

Mg^{2+} Treatments

Chloroplasts were subjected to two different types of high Mg^{2+} treatments in the experiments described in this report. In some cases, 5 mM Mg^{2+} (as a chloride salt) was added to the standard reaction medium. It should be noted that the standard reaction medium already contained 1 mM Mg^{2+}, 1 mM Mn^{2+}, and 2 mM EDTA. As discussed previously (21), the levels of free (*i.e.* unchelated) Mg^{2+} and free EDTA in this solution were estimated to be 44.7 nM; free Mn^{2+} is essentially zero. Therefore, incubation in this medium subjects chloroplasts to a very low level of free Mg^{2+} (and total divalent cations). Adding an additional 5 mM Mg^{2+} to this medium, then, exposes chloroplasts to a relatively high level of free Mg^{2+}, which chlorotetracycline fluorescence studies (21) have indicated causes a marked increase in chloroplast envelope-bound Mg^{2+}.

A second method of high Mg^{2+} treatment involved a transient incubation of chloroplasts in medium with high Mg^{2+} and removal from this medium before the use of the chloroplasts in subsequent studies. In this case, during the isolation procedure, chloroplasts were resuspended in 5 mL grind medium to which 5 mM Mg^{2+} had been added and were incubated at 0 to 2°C for 5 min. Chloroplasts were then centrifuged (750*g* for 50 s) again, and the pelleted plastids were resuspended in 8 mL standard grind medium and centrifuged (2000*g* for 1 min) through the 40% Percoll cushion as described previously. This procedure allowed for an increase in chloroplast envelope-bound Mg^{2+} (as assayed using both $^{28}Mg^{2+}$ and chlorotetracycline fluorescence; 21) and yet also facilitated the quantitative removal of the chloroplasts from solutions containing high levels of free Mg^{2+}. For experiments involving this "Mg^{2+} wash" treatment, control chloroplasts were "washed" by incubation in standard grind medium at 0 to 2°C for 5 min during isolation.

Reagents

[^{14}C]Sorbitol and [^{3}H]H_2O were obtained from ICN (Irvine, CA) and [^{3}H]TPP and ^{86}RbCl from Amersham (Chicago, IL). Silicone oils were from William F. Nye Co. (New Bedford, MA) and Hepes from Research Organics (Cleveland, OH). A23187 was from Boehringer Mannheim (Indianapolis, IN). Dimethonium was obtained as a gift from R. D. Nathan (Texas Tech University School of Medicine, Lubbock, TX) and was originally synthesized by A. McLaughlin. All other reagents were from Sigma (St. Louis, MO).

RESULTS AND DISCUSSION

E_m and Photosynthesis

Demmig and Gimmler (3) speculated that the E_m across the envelope of the illuminated chloroplast is an important regulatory factor that affects photosynthetic capacity. To test this assertion, treatment effects on E_m and E_m effects on photosynthesis were studied. E_m values of chloroplasts incubated at 25°C for 5 min in the dark and in the light were -114 ± 0.9 and -123 ± 1.3 mV, respectively, demonstrating the light-induced increase in E_m previously reported by Demmig and Gimmler (3). Chloroplasts exposed to a 5 mM Mg^{2+} wash treatment were found to have E_m values of -109 ± 2.6 and -111 ± 1.0 mV in the dark and light, respectively. The Mg^{2+} wash treatment, which was found previously to increase envelope-bound Mg^{2+} (21), was found to decrease the E_m across the envelope of the illuminated chloroplast by 12 mV in this experiment. Further studies were undertaken to investigate whether decreases in E_m across the chloroplast envelope by 12 mV can directly affect photosynthetic capacity. The E_m across the envelope was broken down by exposing isolated chloroplasts to increasing concentrations of TPP, a lipophilic cation. Both E_m and photosynthesis at varying TPP concentrations were monitored. Results are shown in Figure 1. Increasing external TPP decreased E_m; a substantial decrease of E_m by 80 mV occurred at the highest [TPP] tested (Fig. 1A). Photosynthesis was also found to be inhibited with increasing [TPP], although not much inhibition was noted (Fig. 1A). The relationship between the decrease in E_m and concomitant photosynthetic inhibition is shown in Figure 1B. A 20-mV decrease in E_m corresponds to only a 10% inhibition in photosynthesis. The 12-mV breakdown in E_m associated with high chloroplast envelope-bound Mg^{2+} should, in and of itself, have directly caused only negligible inhibition of photosynthesis. However, exposure of chloroplasts to either a 5 mM Mg^{2+} wash treatment or 5 mM free Mg^{2+} in the photosynthetic reaction medium typically inhibits photosynthesis to a greater extent. In a series of experiments, exposure of chloroplasts to 5 mM Mg^{2+} in the reaction medium inhibited photosynthesis (mean control rate of 183 μmol O_2/mg Chl/h) by an average of 33% ($n = 9$). The Mg^{2+} wash treatment was found to inhibit photosynthesis by 40% ($n = 3$). Mg^{2+} inhibition of photosynthesis cannot be explained, therefore, by direct effects of the changes in E_m associated with this treatment.

These photosynthesis data also address the speculation of Demmig and Gimmler (3) that Mg^{2+} affects metabolism of the isolated chloroplast by changing the ionic strength of the

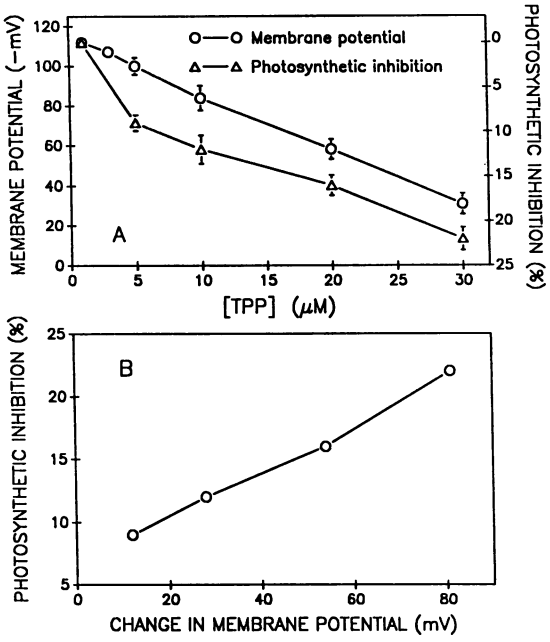

Figure 1. Effect of E_m breakdown on chloroplast photosynthesis. Illuminated chloroplasts were incubated in photosynthesis reaction medium with TPP (1–30 μM) added. A, E_m and photosynthesis were measured on the same chloroplast preparation. Maximal photosynthetic rate in this experiment was 87 μmol O_2/mg Chl/h. Points, means; bars, SEM. B, Resultant photosynthetic inhibition is plotted as a function of the extent of E_m breakdown. All assays contained 0.6 μCi/mL [^{3}H]TPP. It should be noted that, because 2 μM TPP is used in the E_m assay, E_m at O TPP could not be ascertained.

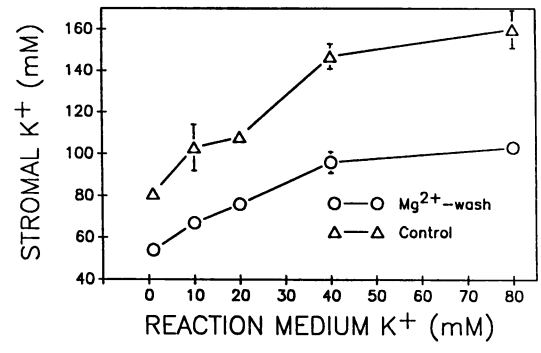

Figure 2. Effect of a Mg^{2+} wash treatment on stromal [K^+] of illuminated chloroplasts incubated in photosynthetic reaction medium with varying external K^+. Chloroplasts were illuminated for 5 min before separation from the reaction medium by silicone oil microcentrifugation. Total K^+ associated with the chloroplast was measured using atomic absorption spectrophotometry. Stromal K^+ concentration was calculated by measuring stromal volume. Points, means; bars, SEM.

Table I. *Reversal of Mg^{2+} Inhibition of Photosynthesis by NH_4^+*

Chloroplast photosynthesis was measured in the presence and absence of 5 mM Mg^{2+}, and 0.75 mM NH_4^+ was added to the reaction medium. Data are presented from two experiments (with one replication/treatment in each experiment). Data in parentheses are results from a second experiment.

Treatment	Photosynthesis	% Change[a]
	μmol O_2/mg Chl·h	
Control	136 (150)	
Mg^{2+}	86 (94)	−37 (−37)
Mg^{2+}, NH_4^+	131 (156)	−4 (+4)
NH_4^+	139 (148)	+2 (−1)

[a] In each experiment, treatment effects on photosynthesis are compared to the control rate.

incubation medium. We noted a substantial inhibition of photosynthesis (40%) due to a Mg^{2+} wash treatment, which was at least as great as when chloroplasts are exposed to high Mg^{2+} during photosynthesis. The Mg^{2+} wash treatment allows for an increase in envelope-bound Mg^{2+} (21) without subjecting chloroplasts to a photosynthetic reaction medium that contains free Mg^{2+} and, therefore, an increased ionic strength. These data, then, suggest that Mg^{2+} external to the chloroplast is likely not influencing chloroplast metabolism by affecting the external solution ionic strength, but rather by an increase in the extent of envelope-bound Mg^{2+}.

In contrast to the effect of Mg^{2+} on E_m across the envelope, Mg^{2+} inhibition of photosynthesis is more likely directly linked to an impairment of light-induced stromal alkalization. In agreement with previous studies (3, 11), we found (using the assay developed by Heldt [8] as modified by Peters and Berkowitz [18]) stromal pH of the illuminated chloroplast to be reduced in the presence of Mg^{2+}. In two separate experiments, a stromal pH of 8.01 ± 0.005 and 7.89 ± 0.017 was decreased to 7.81 ± 0.012 and 7.8 ± 008 ($n = 5$), respectively, after the addition of 5 mM Mg^{2+} to the photosynthetic reaction medium. In summary, then, the experiments involving study of Mg^{2+} and Mg^{2+} wash effects on photosynthesis, E_m, and stromal pH are consistent with a model involving Mg^{2+} interaction with the chloroplast envelope which results in impaired stromal alkalization and concomitant photosynthetic inhibition.

Testing the Antiporter Model: Mg^{2+} Effects on K^+ Fluxes

An important aspect of the Mg^{2+}-activated K^+/H^+ antiport scheme developed by Maury *et al.* (14) and Huber and Maury (11) is the hypothesis that, under appropriate assay conditions, the presence of high levels of chloroplast envelope-bound Mg^{2+} should stimulate K^+ fluxes in either direction across the chloroplast envelope. As discussed previously, Mg^{2+}-induced decreases in stromal [K^+] should be associated with impaired stromal alkalization and concomitant photosynthetic inhibition. Data consistent with this model are presented in Figure 2. High envelope Mg^{2+} did reduce stromal K^+ concentration (Fig. 2). As noted previously, photosynthetic inhibition and lower stromal pH result from exposure of chloroplasts to high Mg^{2+}.

Table II. *Effect of High Reaction Medium Mg^{2+} on K^+ Efflux from the Stroma*

Chloroplasts were preloaded with $^{86}Rb^+$ during isolation and then removed from the $^{86}Rb^+$ incubation medium before the experiment. For experiment 1, chloroplasts were incubated in 4 mL grind medium with 10 mM KCl and 15 μCi/mL $^{86}Rb^+$ added. The chloroplasts were left at 0 to 2°C for 10 min and then brought to 20°C for an additional 15 min. For experiment 2, chloroplasts were incubated in a medium containing 20 mM KCl and 15 μCi/mL $^{86}Rb^+$ for 10 min at 0 to 2°C and then 20°C for 20 min. K^+ (*i.e.* $^{86}Rb^+$) efflux was ascertained by incubation of illuminated chloroplasts in photosynthetic reaction (with and without 5 mM Mg^{2+} added) for various time periods before silicone oil microcentrifugation.

Experiment and Incubation Time	Stromal $^{86}Rb^+$	
	Control	$+Mg^{2+}$
min	*μCi/g Chl*	
Exp. 1		
0	104.1 ± 0.7[a]	109.8 ± 0.4
7	34.3 ± 0.4	38.1 ± 0.4
Net efflux	69.8	71.7
% Efflux[b]	67.1	65.3
Exp. 2		
0	165.8 ± 1.3	152.7 ± 3.2
5	85.6 ± 0.3	82.3 ± 0.7
Net efflux	80.2	70.4
% Efflux[b]	48.4	46.1

[a] Mean ± SEM. [b] % Efflux = net efflux/stromal $^{86}RB^+$ at time zero.

Demmig and Gimmler (2) indicated that direct effects of K^+ loss from the stroma could not be definitively ruled out as a factor contributing to high Mg^{2+}-induced photosynthetic inhibition. Data presented in Table I suggest that the reduction (by approximately 35%, see Fig. 2) of stromal K^+ caused by high chloroplast envelope-bound Mg^{2+} is not directly inhibitory to chloroplast metabolism. Low (<1 mM) concentrations of NH_4^+ have been demonstrated to increase stromal pH of isolated chloroplasts without causing substantial uncoupling of photophosphorylation (7). The presence of NH_4^+ in the reaction medium was found to completely reverse Mg^{2+} inhibition of photosynthesis (Table I). The presence of monovalent cations in the reaction medium was found previously to have no effect on the interaction of Mg^{2+} with the chloroplast envelope (21). Additionally, the Mg^{2+} effect of reducing stromal K^+ was still evident when measured in the presence of NH_4^+ (data not shown). The effect of NH_4^+, therefore, should be to only increase stromal pH without reversing Mg^{2+} effects on stromal [K^+]. These data, then, support the contention made by Huber and Maury (11) and Maury *et al.* (14) that it is Mg^{2+} effects on stromal pH, and not concomitant effects on stromal [K^+], that directly result in photosynthetic inhibition.

Table III. *Effect of High External Mg^{2+} on K^+ Uptake by Chloroplasts*

Illuminated chloroplasts were incubated in photosynthetic reaction medium which had 10 mM K^+ and 20 μCi/mL $^{86}Rb^+$ added. The reaction medium either had no extra Mg^{2+} (control) or 5 mM Mg^{2+} added. Data from one experiment are shown as means ± SE (n = 5). Results presented as K^+ uptake refer to the $^{86}Rb^+$ associated with the chloroplast pellet after silicone oil microcentrifugation. These data do not reflect total stromal K^+.

Incubation Time	K^+ Uptake		% Reduction in Uptake
	Control	$+Mg^{2+}$	
min	*nmol/mg Chl*		
0	41 ± 1.5	47 ± 1.3	
7	838 ± 5.3	691 ± 3.7	
Net uptake	796	644	19.2

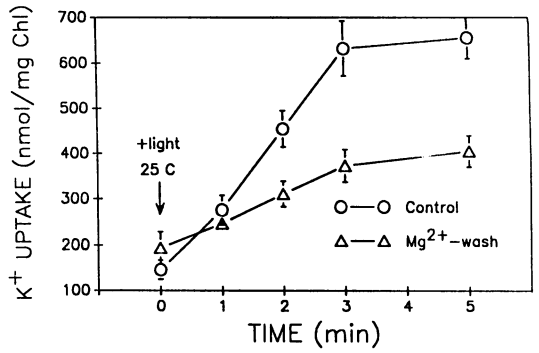

Figure 3. Effect of a Mg^{2+} wash treatment on the kinetics of K^+ uptake into illuminated chloroplasts. Chloroplasts were incubated in photosynthesis reaction medium that had 10 mM K^+ and 25 μCi/ml $^{86}Rb^+$ added. After varying periods of illumination, chloroplasts were separated from the medium by silicone oil microcentrifugation. Points, means; bars, SEM.

Although high external Mg^{2+} was found to reduce stromal [K^+] in our studies (Fig. 2), the results of further work examining Mg^{2+} effects on K^+ fluxes across the chloroplast envelope were not consistent with the model developed by Huber and Maury (11) and Maury *et al.* (14). In no case was high envelope-bound Mg^{2+} or high external free Mg^{2+} found to stimulate a K^+/H^+ antiporter; *i.e.* increase the rate of K^+ flux across the plastid envelope. The net effect of a lower stromal [K^+] due to external Mg^{2+} as shown in Figure 2 could be caused by either a Mg^{2+}-induced increase in the rate of K^+ efflux or an inhibition in the rate of K^+ uptake.

Treatment effects on K^+ efflux were studied by preloading intact chloroplasts with $^{86}Rb^+$ and then measuring $^{86}Rb^+$ loss from chloroplasts after transfer to Rb^+- (and K^+-) free medium (Table II). In two separate experiments, the presence of Mg^{2+} in the external medium had virtually no effect on the rate of $^{86}Rb^+$ efflux from chloroplasts. In contrast, high external Mg^{2+} did have an effect on $^{86}Rb^+$ (*i.e.* K^+) uptake into chloroplasts. In the experiment shown in Table III, K^+ ($^{86}Rb^+$) uptake was inhibited when chloroplasts were incubated in 5 mM free Mg^{2+}. Kinetic analysis also demonstrated a clear inhibition of K^+ influx in the presence of high envelope-bound Mg^{2+} (Fig. 3). It appears, then, that the specific molecular mechanisms allowing for Mg^{2+} regulation of K^+ transport are not consistent with the action of a Mg^{2+}-activated K^+/H^+ bidirectional antiporter. If the antiporter model was correct in its entirety, envelope-bound Mg^{2+} should have stimulated both K^+ uptake and efflux under the proper assay conditions.

A New Model: Surface Charge-Modulated, Inwardly Rectified Monovalent Cation Channels in the Chloroplast Envelope?

Work involving a broad spectrum of animal cell types has shown that Mg^{2+} interaction with the exterior surface of a cell membrane can restrict K^+ influx through ion channels (6, 16, 22). On a theoretical basis, divalent cation regulation of inward current through K^+ channels can be facilitated by two different membrane:cation interactions. As delineated in the Gouy-Chapman-Stern theory, Mg^{2+} could act by binding to fixed negative charges on the membrane and altering its surface potential or by exerting a screening effect on the negative surface charge of the membrane (15).

Binding involves direct chemical absorption of a cation to the negatively charged sites on the membrane, and screening is the result of electrostatic attraction of the cation to an area near the sites (17). The fixed negative charges near the outer pore of a K^+ channel could theoretically interact with a divalent cation in either manner, thus restricting K^+ flux into the interior of the biomembrane (17). Either interaction would affect K^+ currents. Overall flux through the channel is a function of both the electrical driving force across the channel (Mg^{2+} binding would reduced the surface potential and thus decrease this component of the driving force for K^+ influx) and the local concentration of permeant ion (*i.e.* chemical gradient) at the channel pore (17). Mg^{2+} screening at the channel pore would reduce the latter component. Mg^{2+} binding to the negatively charged moieties on the membrane surface would reduce both components.

We speculated that, in the case of the chloroplast envelope, Mg^{2+} could restrict K^+ flux into the stroma by either or both of the afore-mentioned mechanisms. This hypothesis seemed plausible in light of our recent work in which we postulated the presence of K^+ channels in chloroplast envelopes (18).

It should be noted that, in addition to the negatively charged phosphate "head" groups of phospholipids, acidic carbohydrate moieties of glycolipids can contribute significantly to the total fixed negative charges on membrane surfaces that exert gating properties and alter localized [K^+] at the pore of K^+ channels (17). Interestingly, the chloroplast inner envelope is composed primarily of polar lipids with a particularly high (*i.e.* nearly 50%) fraction of the total lipid made up of the glycolipids mono- and digalactosyldiacylglycerol (4). Also consistent with the hypothesis regarding the nature of Mg^{2+} interaction with the chloroplast envelope and resultant effects on K^+ currents through channels is the work done previously in this laboratory in which we examined the $^{28}Mg^{2+}$ association with the chloroplast envelope (21). The extent of Mg^{2+} association with the chloroplast envelope in the presence of millimolar levels of unchelated external Mg^{2+} was quantified in this previous work. External Mg^{2+} of 5 mM caused 3.5 μmol Mg^{2+}/mg Chl to be associated with the chloroplast envelopes (no distinction was made between inner and outer envelope binding). This level of Mg^{2+}, associated with the envelope(s), is apparently required for photosynthesis, stromal K^+, and stromal pH to be affected by the external Mg^{2+} (11, 14, 21). This level of divalent cation association with the envelope is far in excess of that required if one Mg^{2+} molecule (per ion channel) was binding to an individual receptor site. This level of Mg^{2+} association with the chloroplast envelope is more on the level of the "uniformly smeared surface charge" which is postulated in the Guoy-Chapman-Stern equation to be responsible for divalent cation effects on membranes (17).

Several experiments were undertaken to test the hypothesis that Mg^{2+} restriction of inward K^+ currents across the chloroplast envelope was mediated by the afore-mentioned binding and/or screening effects. Mg^{2+} effects on K^+ influx were compared with the effects of the divalent organic cation dimethonium and the trivalent cation La^{3+}. Dimethonium has been shown to exert only a screening effect on the surface potential (15); it has been demonstrated that this cation has an exceedingly small binding constant with lipid membranes (15, 17). If Mg^{2+} restriction of K^+ influx was due only to screening at a K^+ channel pore, dimethonium should mimic the effect of Mg^{2+}. Experimental evidence has supported this assertion; dimethonium has been found to mimic the effects of divalent cations and alter currents through certain types of K^+ channels when the action of the divalent cation was attributed to negative surface charge screening (5). With other membrane systems, dimethonium was found not to duplicate the effects of divalent cations, which were predicted to bind to negative charges on the membrane surface (1).

In the Gouy-Chapman-Stern equation (13, 15) describing cation effects on the surface potential of biomembranes, the equilibrium constant for cation binding is affected by cation valence in an exponential manner. This relationship predicts that monovalent cations will have negligible effects as compared to divalent cations, and the presence of divalent cations on the membrane surface at the pore of an ion channel should severely restrict monovalent cation influx. This prediction has

Table IV. *Effect of Polyvalent Cations in the Reaction Medium on K^+ Uptake into Illuminated Chloroplasts*

K^+ ($^{86}Rb^+$) uptake in standard photosynthetic reaction medium is compared to uptake in the presence of Mg^{2+} (5 mM), La^{3+} (2 mM), and dimethonium (5 mM) added to the reaction medium. All data are presented as means ± SE (n = 4). In all cases, 10 mM KCl and 20 μCi/mL $^{86}Rb^+$ were added. Results presented as K^+ uptake refer to the $^{86}Rb^+$ associated with the chloroplast pellet after silicone oil microcentrifugation.

Experiment and Incubation Time	K^+ Uptake			
	Control	Mg^{2+}	La^{3+}	Dimethonium
min	*nmol/mg Chl*			
Exp. 1				
0	50 ± 5.6	47 ± 7.4	46 ± 3.8	31 ± 3.5
7	994 ± 14.5	714 ± 10.1	658 ± 14.9	949 ± 11.7
Net uptake	944	667	612	968
% Change[a]	0	−29	−35	+3
Exp. 2				
0	75 ± 4.7	70 ± 7.7	69 ± 3.4	79 ± 2.5
7	856 ± 11.6	615 ± 9.4	563 ± 14.1	857 ± 10.4
Net uptake	781	545	494	778
% Change[a]	0	−30	−37	0

[a] The net uptake under the various treatments is compared with uptake in the control treatment.

Table V. *Effect of Polyvalent Cation Addition to the Reaction Medium on Photosynthesis*

For the control treatment, chloroplasts were incubated in standard photosynthetic reaction medium. For other treatments, polyvalent cations were added to the treatment as noted. Presented data are from single measurements in each experiment. Results from three experiments are presented.

Treatment	Concentration	Photosynthesis			% Inhibition[a]		
		Exp. No.			Exp. No.		
		1	2	3	1	2	3
	mM	μmol O_2/mg Chl·h					
Control		165	209	191			
Mg^{2+}	5	97	157	90	41	25	53
La^{3+}	1	85	105	74	48	50	61
La^{3+}	2	43			76		
La^{3+}	5	37			78		
Dimethonium	5	159			4		
Dimethonium	10	156	196		6	6	

[a] Photosynthesis under each treatment is compared with the control rate for each experiment.

been found to be in agreement with results in a wide variety of experimental systems (6, 10, 13, 15–17, 22). The equation also predicts that trivalent cations should have substantially greater effects on membrane surface potential, and, thus, restrict flux through an ion channel, to a greater extent than equimolar concentrations of divalent cations.

The results of several experiments involving the comparison of Mg^{2+} effects on K^+ uptake into the stroma with dimethonium and La^{3+} effects are shown in Table IV. In both experiments, the presence of external Mg^{2+} was again found to reduce K^+ ($^{86}Rb^+$) influx into chloroplasts. Dimethonium was found to have no effect at all on K^+ uptake. In these experiments, chloroplasts were exposed to lower concentrations of La^{3+} (2 mM) than Mg^{2+} (5 mM). However, in both experiments K^+ uptake was reduced from control levels to a greater extent with La^{3+} than with Mg^{2+} (Table IV).

Polyvalent cation effects on photosynthesis were also studied (Table V). The Mg^{2+} inhibition of K^+ influx (Table IV) was associated with a substantial inhibition of photosynthesis (Table V). However, in three separate experiments, photosynthesis was more sensitive to La^{3+} than Mg^{2+} (Table V). As expected, dimethonium had virtually no adverse effect on photosynthesis in two separate experiments. The results presented in Tables IV and V are consistent with the hypothesis that external Mg^{2+} influences chloroplast metabolism by altering the envelope exterior membrane surface charge, effectively reducing localized [K^+] near the outer pore of a K^+ channel and the localized surface potential. Divalent cations which do not bind to the envelope surface have no effect on either K^+ currents or photosynthesis. Trivalent cations which have higher binding coefficients to negatively charged surfaces of biomembranes have a greater effect than Mg^{2+} on both K^+ currents and photosynthesis.

CONCLUSION

One of the most critical issues that needs to be resolved with regard to Mg^{2+} effects on chloroplast physiology is the understanding of exactly how Mg^{2+} external to the chloroplast impairs stromal alkalization (*i.e.* net H^+ influx). The work presented in this report, unfortunately, has not resolved that research problem. However, two current theories regarding this effect were critically evaluated; neither was fully consistent with the results reported here.

The results reported in this study do offer a new perspective with which external Mg^{2+} effects on chloroplast physiology can be examined. It has been shown that photosynthetic inhibition in the presence of external Mg^{2+} is likely due to the interaction of the metal ion with the exterior surface of the membrane. One clear effect of Mg^{2+} binding to the envelope is an inhibition of K^+ uptake. However, the data reported here support the results of previous studies (2, 11, 14, 18) in that Mg^{2+} inhibition of photosynthesis was demonstrated to be caused directly by stromal acidification. The data suggest that Mg^{2+}-induced stromal acidification is not due to the action of a Mg^{2+}-activated K^+/H^+ antiporter or to changes in the ionic strength of the bulk solution external to the chloroplast but possibly due to the metal ion's effects on the current through monovalent cation channels in the envelope.

It should be noted that Mg^{2+} effects on both stromal pH and photosynthesis are modulated equally well by K^+ or Na^+ (14). The reaction media used in the experiments reported here contained approximately 25 mM Na^+ (due to HCO_3, EDTA, and PO_4 addition as Na^+ salts and pH adjustment with NaOH). Mg^{2+} effects on stromal pH and photosynthesis are likely mediated by Mg^{2+} modulation of either Na^+ or K^+ uptake. This hypothesis will be addressed in further studies.

LITERATURE CITED

1. **Bers DM, Philipson KD, Peskoff A** (1985) Calcium at the surface of cardiac plasma membranes—cation binding, surface charge screening and Na-Ca exchange (abstract). Biophys J **47:** 491a
2. **Demmig B, Gimmler H** (1979) Effect of divalent cations on cation fluxes across the chloroplast envelope and on photosynthesis of intact chloroplasts. Z Naturforsch **24C:** 233–241
3. **Demmig B, Gimmler H** (1983) Properties of isolated intact chloroplasts at cytoplasmic K^+ concentrations. I. Light-induced cation uptake into intact chloroplasts is driven by an electrical potential difference. Plant Physiol **73:** 169–174
4. **Douce R, Block MA, Dorne AJ, Joyard J** (1983) The plastid envelope membranes: their structure, composition, and role in chloroplast biogenesis. Subcell Biochem **10:** 1–84
5. **Fermini B, Nathan RD** (1990) Sialic acid and the surface charge associated with hyperpolarization-activated, inward rectifying channels. J Membr Biol **114:** 61–69
6. **Gilbert DL, Ehrenstein G** (1969) Effect of divalent cations on potassium conductance of squid axons: determination of surface change. Biophys J **9:** 447–463
7. **Heath RL, Leech RM** (1978) The stimulation of CO_2-supported O_2 evolution in intact spinach chloroplasts by ammonium ion. Arch Biochem Biophys **190:** 221–226
8. **Heldt HW** (1980) Measurement of metabolite movement across the envelope and of the pH in the stroma and the thylakoid space in intact chloroplasts. Methods Enzymol **69:** 604–613
9. **Heldt HW, Werdan K, Milovancev M, Geller G** (1973) Alkalization of the chloroplast stroma caused by light dependent proton flux into the thylakoid space. Biochim Biophys Acta **314:** 224–241
10. **Hille B, Woodhull M, Shapiro BI** (1975) Negative surface charge near sodium channels of nerve: divalent ions, monovalent ions, and pH. Phil Trans R Soc Lond B **270:** 301–318
11. **Huber SC, Maury W** (1980) Effects of magnesium on intact chloroplasts. I. Evidence for activation of (sodium) potassium/

proton exchange across the chloroplast envelope. Plant Physiol **65:** 350–354

12. **Kaiser WM, Urbach W, Gimmler H** (1980) The role of monovalent cations for photosynthesis of isolated intact chloroplasts. Planta **149:** 170–175
13. **Kostzuk PG, Mironov SL, Doroshenko PA, Ponomarev VN** (1982) Surface charge on the outer side of mollusc neuron membrane. J Membr Biol **70:** 171–179
14. **Maury WJ, Huber SC, Moreland DE** (1981) Effects of magnesium on intact chloroplasts. II. Cation specificity and involvement of the envelope ATPase in (sodium) potassium/proton exchange across the envelope. Plant physiol **68:** 1257–1263
15. **McLaughlin A, Eng WK, Vaio G, Wilson T, McLaughlin S** (1983) Dimethonium, a divalent cation that exerts only a screening effect on the electrostatic potential adjacent to negatively charged phospholipid bilayer membranes. J Membr Biol **76:** 183–193
16. **Mozhazeva GN, Naumov AP** (1970) Effect of surface charge on the steady-state potassium conductance of nodal membrane. Nature **228:** 164–165
17. **Nathan RD** (1986) Negative surface charge: its identification and regulation of cardiac electrogenesis. *In* RD Nathan, ed, Cardiac Muscle: the Regulation of Excitation and Contraction. Academic Press, Orlando, FL, pp 55–86
18. **Peters JS, Berkowitz GA** (1991) Studies on the system regulating proton movement across the chloroplast envelope. Effects of ATPase inhibitors, Mg^{2+}, and an amine anesthetic on stromal pH and photosynthesis. Plant Physiol **95:** 1229–1236
19. **Portis AR, Heldt HW** (1976) Light-dependent changes of the Mg^{2+} concentration in the stroma in relation to the Mg^{2+} dependency of CO_2 fixation in intact chloroplasts. Biochim Biophys Acta **449:** 434–446
20. **Prased R, Hoefer M** (1986) Tetraphenylphosphonium is an indicator of negative membrane potential in *Candida albicans*. Biochim Biophys Acta **861:** 377–380
21. **Sen Gupta A, Berkowitz GA** (1989) Development and use of chlorotetracycline fluorescence as a measurement assay of chloroplast envelope-bound Mg^{2+}. Plant Physiol **89:** 753–761
22. **Stanfield PR** (1988) Intracellular Mg^{2+} may act as a co-factor in ion channel function. Trends Neurosci **11:** 475–477