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Evidence of a Low Stromal Mg^{2+} Concentration in Intact Chloroplasts in the Dark

I. STUDIES WITH THE IONOPHORE A23187

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ABSTRACT

The loss of Mg^{2+} upon the addition of the ionophore A23187 in the dark was prevented by less than 0.1 millimolar $MgCl_2$ with intact chloroplasts suspended in a sorbitol medium, but required 1 to 3 millimolar $MgCl_2$ if the chloroplasts were in a K^+-gluconate medium. Measurements of stromal pH in the dark indicated that, in the K^+-gluconate medium, the stromal pH is nearly the same as that of the medium, whereas in the sorbitol medium it is much more acidic as reported previously. These observations suggest that the free Mg^{2+} concentration in the stroma in the dark is between 1 and 3 millimolar. Other experiments on the inihibition by A23187 of CO_2 fixation in the light and in a system capable of catalyzing CO_2 fixation in the dark, and on the Mg^{2+} binding properties of thylakoid membranes, are consistent with this conclusion. The results provide further support for the hypothesis that light-induced Mg^{2+} concentration changes occur in the stroma that are important in the light-dark regulation of CO_2 fixation.

Changes in Mg^{2+} concentration in the stroma of intact chloroplasts have been postulated to have an important role in the light-dark regulation of CO_2 fixation (11) by modulation of several Calvin cycle enzymes including ribulose-1,5-bisphosphate carboxylase, fructose-1,6-bisphosphatase and sedoheptulose-1,7-bisphosphatase (12, 23). Direct evidence of light-dependent Mg^{2+} movements from the thylakoids (5, 9) that increase the Mg^{2+} concentration in the stroma (13, 20) has been obtained and Mg^{2+}-dependent changes in the CO_2 fixation rate have been observed in model systems (16, 19).

Although estimates of a stromal Mg^{2+} concentration as large as 10 mM have been made, recent evidence suggests that a light-induced change of 2 to 3 mM is likely (13, 20). Changes of Mg^{2+} concentration of this magnitude could be sufficient for a significant change in the activity of the three enzymes mentioned above only if the Mg^{2+} concentration in the stroma in the dark was relatively low, in the range of 1 to 3 mM. The high Mg^{2+} content of intact chloroplasts (400 to 1,000 nmol Mg^{2+}/mg Chl) (6, 20) in comparison to estimations of the extent of the light-induced efflux (20–100 nmol Mg^{2+}/mg Chl) (5, 9, 13, 20) emphasizes the necessity to obtain an accurate estimate of the actual free Mg^{2+} concentration in the stroma in the dark.

The dependence of the Mg^{2+} content of intact chloroplasts on the external Mg^{2+} concentration was measured in the presence of the divalent cation ionophore A23187 in an attempt to obtain an estimate of the dark stromal Mg^{2+} concentration. We also examined further the Mg^{2+} dependency of CO_2 fixation in the presence of the ionophore in the light and in a system capable of catalyzing CO_2 fixation in the dark (24). These results, and other experiments on the concentration dependence of Mg^{2+} binding to the thylakoids, provide evidence that the stromal Mg^{2+} concentration is about 2 mM in the dark.

MATERIALS AND METHODS

Chemicals. A23187 (lot 361-X17-284) was a gift from Eli Lilly[1] and Co. and was dissolved in ethanol. The isotopes used for pH determinations and $^{14}CO_2$ fixation were obtained from New England Nuclear. Silicone oils (AR 20 No. 2877, AR 200 No. 2628)[2] were obtained from SWS Silicones, Adrian, MI.

Chloroplast Isolation. Spinach (*Spinacia oleracea*—American Hybrid No. 424) was grown in water culture (17) under artificial light (PAR 400 $\mu E/m^2 \cdot s$) with a 10-h light period at 23 C, 60–70% RH and a 14-h dark period at 18 C. Intact chloroplasts were prepared from fully grown leaves according to the modified method of Heldt and Sauer (7), except that EDTA, $MnCl_2$, and $MgCl_2$ were omitted from Medium B. Thylakoid membranes were prepared by a 10-fold dilution of an intact chloroplast suspension with distilled H_2O. The medium was then made up to 50 mM sorbitol, 50 mM KCl, 50 mM Hepes-NaOH (pH 7.5), and washed twice by centrifugation (1,900 *g*, 3 min).

O_2 Evolution. Measurements of CO_2-dependent O_2 evolution were performed with a Clark-type O_2 electrode (Hansatech Ltd., Kings Lynn, Norfolk, U.K.) at 20 C, with light (PAR 1,000 $\mu E/m^2 \cdot s$) from a 500-w slide projector.

pH Determination. Stromal and thylakoid pH of intact chloroplasts were determined following the technique of Heldt *et al.* (8). The uptake of [^{14}C]DMO[3] and [^{14}C]methylamine was assayed in separate samples by rapid centrifugation of the chloroplasts through silicone oil (AR 20/AR 200, 1:4), using ^3H_2O as an indicator of trapping. Internal volumes were measured using [^{14}C]sorbitol and ^3H_2O.

Mg Determination. The amount of Mg^{2+} associated with intact chloroplasts and thylakoids was determined by centrifugation of the chloroplasts through a layer of silicone oil (AR 20/AR 200, 1:4 for intact chloroplasts; AR 20/AR 200, 3:1 for thylakoids) into a lower layer of 12% sorbitol (w/v) as described previously (20). The lower layer was resuspended in 500 μl of 1 mM EDTA (pH

[1] Mention of a trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the U. S. Department of Agriculture or University of Illinois and does not imply its approval to the exclusion of other products or vendors that may also be suitable.

[2] Variations in measured densities between different lots of these oils have been found (M. H. Spalding and A. R. Portis, unpublished).

[3] Abbreviation: DMO, 5,5 dimethyloxazolidine-2,4-dione.

8.5) and again centrifuged to remove the membranes and Chl. The Mg^{2+} content of the supernatants was assayed by atomic absorption spectroscopy and corrected for Mg^{2+} in the small amount of incubation medium carried along with the membranes, as determined by using [^{14}C]sorbitol in otherwise identical samples.

RESULTS AND DISCUSSION

The ionophore A23187, which has a high specificity for divalent cations, has been used extensively in recent studies of CO_2 fixation and the role of divalent cations in photosynthetic phenomena. Addition of the ionophore to intact chloroplasts markedly increases the permeability of the envelope to Mg^{2+}, allowing the external manipulation of the Mg^{2+} content and concentration. Since the ionophore catalyzes an electroneutral H^+/divalent cation exchange (18), at equilibrium the external and internal Mg^{2+} concentrations will be in equilibrium with the corresponding pH according to the relationship:

$$\log \frac{[Mg^{2+}]in}{[Mg^{2+}]out} = 2 \log \frac{[H^+]in}{[H^+]out} \quad (1)$$

This equation states that at equilibrium a pH difference between the stroma and medium of +0.1 will result in a 1.6-fold greater Mg^{2+} concentration in the stroma, and a pH difference of 0.2 will result in a 2.5-fold greater stromal Mg^{2+} concentration.

To determine the Mg^{2+} concentration in the stroma before addition of the ionophore, one can determine the external Mg^{2+} concentration that results in no net influx or efflux of Mg^{2+} on addition of the ionophore.

Inasmuch as there also should be no pH change on addition of the ionophore at this Mg^{2+} concentration, measurement of the pH difference between the stroma and the medium under these conditions allows the calculation of the stromal Mg^{2+} concentration according to equation (1).

In Figure 1, the results of a typical experiment on Mg^{2+} movement are shown. In this experiment, chloroplasts are suspended in the conventional sorbitol-Hepes medium at pH 8.0. Similar results were obtained at pH 7.0 and 7.5 (data not shown). In this medium, as the external Mg^{2+} concentration is increased, there is a large amount of Mg^{2+} binding (300 nmol Mg^{2+}/mg Chl at 2 mM) to the chloroplasts even before addition of the ionophore. Presumably, this represents electrostatic binding of Mg^{2+} to the envelope, since Mg^{2+} does not rapidly cross the envelope membrane (6). Addition of the ionophore causes an efflux of Mg^{2+}, as expected, but only a low external Mg^{2+} concentration is required before increased binding and net influx of Mg^{2+} is observed (≥150 nmol Mg^{2+}/mg

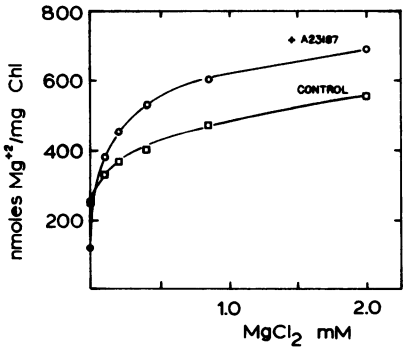

FIG. 1. Mg^{2+} binding to intact chloroplasts suspended in sorbitol in the presence (○) or absence (□) of 2.5 μM A23187. Reaction mixture contained 330 mM sorbitol, 50 mM Hepes-NaOH pH 8.0, 0.1 mM EDTA, 150 μg Chl/ml and the indicated concentration of $MgCl_2$. Five min after addition of the ionophore in the dark, 200-μl aliquots were centrifuged and Mg^{2+} content of the pellets assayed and corrected for trapping of the supernatant. Values shown are the average of 3 samples. Independent experiments on the time dependence of Mg^{2+} movements upon addition of A23187 indicated that 5 min was sufficient for equilibration.

Table I. *pH Dependence of Stromal and Thylakoid pH of Intact Chloroplasts*

Reaction mixtures contained 330 mM sorbitol or 160 mM K^+-gluconate, 50 mM Hepes-NaOH, 1 mM DMO, 30 μM methylamine, 100 μg Chl/ml. Samples were incubated for 5 min dark at 20 C for "dark," followed by 75 s in the light for "light." CO_2 fixation was 130 μmol/mg Chl·h (22C, sorbitol medium) and the chloroplasts were 72% intact. Medium pH was measured on reaction mixtures without isotopes but containing chloroplasts. In other experiments we found that 5 min dark incubation in K^+-gluconate was required to obtain routinely the equilibration of the stroma and medium pH.

Media	pH			ΔpH		
	Medium	Stroma		Dark Stroma-Med	L-D Stroma	Light Stroma-Thy
		Dark	Light			
Sorbitol	7.07	6.13	7.26	−0.94	1.13	2.63
	7.46	6.51	7.64	−0.95	1.13	2.76
	7.96	6.83	8.01	−1.13	1.18	2.93
K^+-gluconate	6.92	7.12	7.71	0.20	0.59	2.62
	7.47	7.33	7.85	−0.14	0.52	2.61
	7.86	7.80	8.02	−0.06	0.22	2.54

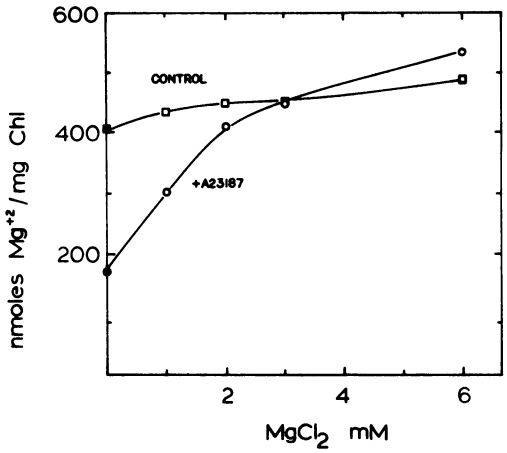

FIG. 2. Mg^{2+} binding to intact chloroplasts suspended in K^+-gluconate in the presence (○) or absence (□) of 2.5 μM A23187. Reaction mixtures contained 160 mM K^+-gluconate, 50 mM Hepes-NaOH (pH 8.0), 0.1 mM EDTA, 150 μg Chl/ml and the indicated concentrations of $MgCl_2$. Separate experiments indicated 5 min was sufficient for Mg^{2+} equilibration.

Table II. *Mg Dependence of the Effect of A23187 on Stroma pH*

Reaction mixtures contained 330 mM sorbitol or 160 mM K^+-gluconate, 50 mM Hepes-NaOH (pH 7.50), 1 mM DMO, ± 1 μM A23187 and 100 mg Chl/ml. Samples were incubated for 300 s or 120 s followed by 300 s in the presence of A23187 in the dark at 20 C. CO_2 fixation rate was 137 μmol CO_2/mg Chl·h Chl and the chloroplasts were 90% intact.

Media	MgCl	Stromal pH (±0.03)		Difference
		Control	+A23187	
	mM			
Sorbitol	0	7.00		
K^+-gluconate	0	7.49	7.18	−0.31
	1	7.41	7.36	−0.05
	3	7.40	7.45	+0.05
	6	7.34	7.44	+0.10

Chl at 1–2 mM).

Separate experiments indicated that the stromal pH was about 0.8 to 1.0 units more acidic than the medium in the presence or absence of the ionophore and Mg^{2+}. The stromal Mg^{2+} concentration could be more than 40-fold greater than that in the medium, accounting for the fact that the ionophore-induced loss of Mg^{2+} is prevented with such low external Mg^{2+} concentrations. Difficulties in accurately measuring the very acidic stromal pH and the Mg^{2+} concentration at which the ionophore-induced Mg^{2+} loss was prevented, did not permit very accurate estimations of the dark stromal Mg^{2+} concentration under these conditions. Therefore, we sought a means of increasing the pH of the stroma relative to the medium in the dark. After extensive testing, we found that K^+-gluconate was the most effective osmoticum in replacing sorbitol. The gluconate anion was previously determined to be impermeable to intact chloroplasts (6). K^+-gluconate nearly eliminated the pH difference between the stroma and the medium in the dark irrespective of the external pH (Table I). The light-induced alkalinization of the stroma was reduced by one half, but there was little effect on the ΔpH across the thylakoid membrane. The reasons for this effect on stromal pH are not clear at present. Since Na^+-gluconate was not as effective as K^+-gluconate (not shown), it appears that both a reduction of a Donnan potential (6) and K^+/H^+ exchange (4, 6, 10) across the envelope membranes may be involved.

Ionophore-induced Mg^{2+} movement with chloroplasts in the presence of K^+-gluconate at pH 8.0 is shown in Figure 2. In this medium, much higher concentrations of Mg^{2+} (always between 1 and 3 mM in various experiments) were required to prevent the ionophore-induced loss of Mg^{2+} from the stroma. The increase in the Mg^{2+} content of the chloroplasts upon increasing the external Mg^{2+} concentration from 3 to 6 mM in the presence of A23187 was only about 50 nmol/mg Chl. This is about what would be expected assuming an internal volume of 20 μl/mg Chl and suggests that the Mg^{2+} binding capacity of the stroma is saturated at the internal dark concentration equivalent to 3 mM external $MgCl_2$. Saturation of the Mg^{2+} binding capacity would be important for the light-induced Mg^{2+} efflux from the thylakoids to effectively increase the free Mg^{2+} concentration. Finally, there was much less binding of Mg^{2+} to the envelope as compared to the sorbitol medium, which is probably due to increased competition by the high K concentration. Results similar to those shown in Figure 2 were obtained at pH 7.0 and 7.5 (data not shown).

Measurements of the effect of ionophore addition on stromal pH are shown in Table II. As expected, ionophore-induced Mg^{2+} loss from the stroma in the medium containing no $MgCl_2$ led to acidification. Between 1 and 3 mM $MgCl_2$, very small changes in pH occurred, while at 6 mM the net influx of Mg^{2+} upon addition of the ionophore caused alkalinization. There was a slight acidification (<0.15 pH) of the stroma in the presence of $MgCl_2$ but it was much less than that reported for chloroplasts in sorbitol (4, 10). Therefore, in K^+-gluconate, Mg^{2+} loss upon addition of the ionophore A23187 is prevented by external $MgCl_2$ concentrations of 1 to 3 mM and little or no pH change is observed. The actual free Mg^{2+} concentration in these media is somewhat less than that added, as gluconate weakly binds Mg^{2+} (K_B = 0.7) (3). Our measurement of free Mg^{2+} in 160 mM K^+-gluconate medium and 1 to 3 mM $MgCl_2$ using 8-hydroxyquinoline (2) indicated that the free Mg concentration is only about 50% of that added (40% is calculated using K_B = 0.7). The stromal pH was not always exactly equal to that in the medium in various experiments, particularly after the addition of $MgCl_2$ (Table I). Allowing for a maximal difference of about 0.2 units, the reduced free Mg^{2+} concentration in K^+-gluconate would be exactly canceled out by the concentrating effect of the slightly more acidic pH of the stroma, suggesting a dark stromal Mg^{2+} concentration of less than 3 mM.

Mg^{2+}-dependence of CO_2 Fixation in the Presence of A23187. Inasmuch as much higher amounts of Mg^{2+} are required to prevent the ionophore-induced loss of Mg^{2+} from intact chloroplasts suspended in K^+-gluconate, the Mg^{2+} dependence of CO_2 fixation in the presence of A23187 in this medium was compared to previous results obtained with sorbitol (20). Much higher Mg^{2+} concentrations were required for maximal restoration of A23187 inhibited CO_2 fixation in K^+-gluconate than in sorbitol (Fig. 3). The 1/2 maximal Mg^{2+} concentration was shifted from 0.2 to 0.3 mM Mg^{2+} in sorbitol to about 1 mM Mg^{2+} in K^+-gluconate. Accounting for pH effects by independent measurements of stromal pH in the light under these conditions (0 to 0.2 units more acidic in various experiments) and Mg^{2+} binding in K^+-gluconate, a 1/2 maximal stromal Mg^{2+} concentration of about 1 to 2 mM is obtained for CO_2 fixation. This value compares well with the previous estimate in sorbitol (20) and suggests that the dark stromal Mg^{2+} concen-

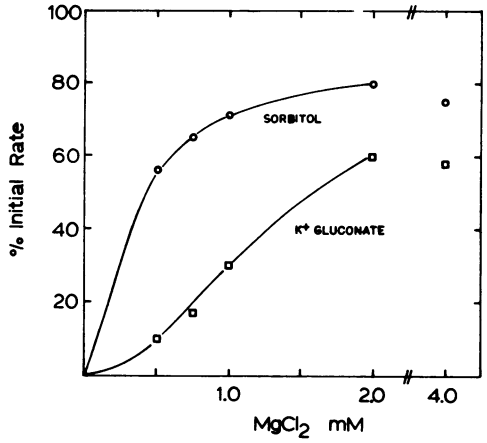

FIG. 3. Mg^{2+} dependence of CO_2 fixation, as measured by CO_2-dependent O_2 evolution after addition of the ionophore A23187, with chloroplasts suspended in sorbitol (○) and K^+-gluconate (□). O_2 evolution was measured with an O_2 electrode at 20 C in a 1-ml reaction mixture containing 37 μg Chl, 0.1 mM EDTA, 0.2 mM K_2HPO_4, 5 mM $NaHCO_3$, 50 mM Hepes-NaOH (pH 8.0) and either 330 mM sorbitol or 160 mM K^+-gluconate. After O_2 evolution reached a constant rate (2–3 min, 100% = 110–130 μmol O_2/mg Chl per h), 0.25 μM A23187 was added. Three min later, when total inhibition of O_2 evolution had occurred, the indicated concentration of $MgCl_2$ was added and illumination continued until a constant rate of O_2 evolution was attained.

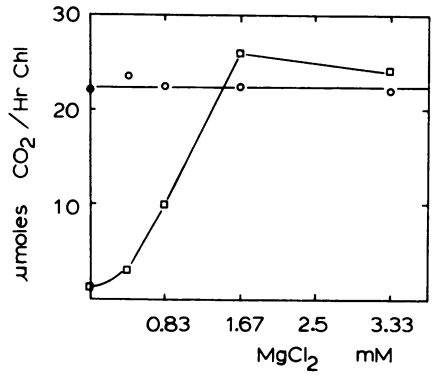

FIG. 4. Mg-dependence of dark CO_2 fixation in the presence of K^+-gluconate with (□) or without (○) 5 μM A23187. Intact chloroplasts (0.2 mg/ml) were preincubated for 10 min in 160 mM K^+-gluconate, 50 mM Hepes-NaOH (pH 8.0), 10 mM DTT, and 1.2 times the indicated $MgCl_2$ concentration. A23187 was added as indicated after 5 min preincubation. Dark CO_2 fixation was initiated by adding 1/6 the final volume of a solution of 60 mM $NaH^{14}CO_3$ (0.4 μCi/μmol), 30 mM dihydroxyacetone phosphate, 30 mM oxaloacetic acid, 96 mM K_2HPO_4. Aliquots were assayed at 6 and 12 min for the calculation of the rate.

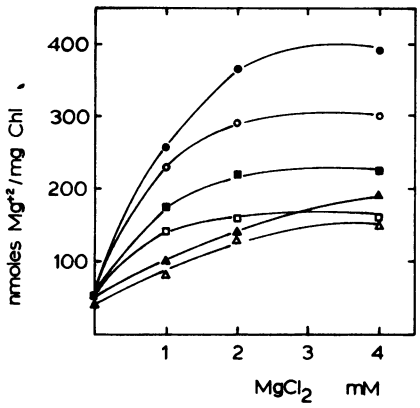

FIG. 5. Mg^{2+} binding to thylakoid membranes in light (○, □, △) and dark (●, ■, ▲) in various media: 100 mM sorbitol (○, ●); 50 mM KCl and 50 mM sorbitol (□, ■); 150 mM KCl (△, ▲). In addition to the above, the reaction mixtures contained 150 μg Chl/ml, 50 mM Hepes-NaOH (pH 7.5), and 2 mM K-ferricyanide. Mg^{2+} content of the pellets was determined and corrected for trapping of the supernatant. The concentrated thylakoid suspension used contained only 40 nmol Mg^{2+}/mg Chl of EDTA-releasable Mg^{2+}.

tration would not be sufficient for maximal rates of CO_2 fixation.

Mg-dependence of Dark CO_2 Fixation. Intact chloroplasts can fix CO_2 in the dark in the presence of dihydroxyacetone phosphate (24) or fructose bisphosphate and aldolase (22) with oxaloacetic acid and phosphate. However, the rates are only 5–20% of those obtained in the light even when the pH in the stroma is raised to pH 8.0 (sorbitol-Hepes medium pH 8.5–8.8) and DTT is added. It is possible that these low rates might be due to the low stromal Mg^{2+} concentration and therefore might be stimulated by adding A23187 and Mg^{2+}, but little stimulation was observed (Fig. 4). The experiment was conducted at pH 8.0, because the pH optimum for dark CO_2 fixation in K^+-gluconate is around pH 8.0 (not shown) due to effects of K^+-gluconate on stromal pH (Table I).

The lack of a large stimulation by A23187 and Mg^{2+} indicates that factors other than Mg^{2+} are limiting CO_2 fixation in this system. However, the previously reported inhibition of dark CO_2 fixation by A23187 (22) was prevented by the presence of less than 1.7 mM added Mg^{2+}. Measurements of free Mg^{2+} in K^+-gluconate containing 16 mM Pi with 8-hydroxyquinoline indicated a free Mg^{2+} concentration of about 0.7 mM Mg^{2+} at 1.7 mM of added $MgCl_2$. This would be a minimal estimate for the dark stromal Mg concentration with no pH gradient across the envelope and is consistent with the estimates made above from ionophore-induced changes in Mg^{2+} binding to intact chloroplasts.

Mg^{2+} Binding to Thylakoid Membranes. Very few reports (1, 20, 21) on the Mg^{2+} binding properties of thylakoid membranes have been published. Mg^{2+} binding to the thylakoids could account for a considerable amount of the Mg^{2+} content of intact chloroplasts. If extensive binding to the thylakoids occurs, a saturation of the binding might be expected at low Mg^{2+} concentrations. Otherwise Mg^{2+} concentration changes in the stroma due to Mg^{2+} efflux from the thylakoids would be greatly reduced due to rebinding to the outside of these membranes. Mg^{2+} binding is greatly dependent on light and K^+ concentration, as shown previously (9), varying from 125 to 400 nmol Mg^{2+}/mg Chl at saturation with the different conditions (Fig. 5). Assuming that these conditions are relevant to those occurring *in vivo*, it is possible that 20 to 50% of the Mg^{2+} present in intact chloroplasts is associated with the thylakoids. More importantly, saturation occurred at about 2 mM Mg^{2+} irrespective of K^+ concentration. This value agrees well with other studies of dark Mg^{2+}-proton exchange of thylakoids, indicating 1/2 maximal proton release at about 1 mM Mg^{2+} (1). These data suggest that the dark stromal Mg^{2+} concentration would have to be greater than 1 to 2 mM, otherwise light-induced Mg^{2+} efflux from the thylakoids (40–90 nmol) would be mostly rebound outside.

CONCLUSIONS

The data indicate that although the Mg^{2+} content of intact chloroplasts is high, the free Mg^{2+} concentration in the stroma is probably between 1 and 3 mM. This indicates that only 20 to 60 nmol Mg^{2+}/mg Chl are available of the total 400 to 1,000 nmol/mg Chl in chloroplasts. A large part of this excess is accounted for by the Mg^{2+} binding capacity of the thylakoids (Fig. 5). Our calculations indicate that the remainder can be accounted for by the large amounts of phosphorylated compounds (14) and dicarboxylic acids (15) in the chloroplasts, both of which can chelate Mg^{2+}. The low stromal concentrations of Mg^{2+} estimated in this report, coupled with the previous estimates of light-induced concentration increases of 1 to 3 mM, indicate that light-induced Mg^{2+} concentration changes are of sufficient magnitude to be involved in the light-dark regulation of CO_2 fixation.

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