Permeability of Chloroplast Envelopes to Mg²⁺[1]

EFFECTS ON PROTEIN SYNTHESIS

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ABSTRACT

When suspended in media lacking free Mg²⁺, chloroplasts from young pea plants (*Pisum sativum* CV Progress No. 9) lose 25 to 75% of their stromal Mg²⁺ content to the medium, without breakage. This effect amounts for the inhibition of protein synthesis in the dark by ATP in excess of the Mg²⁺ provided, since free ATP chelates Mg²⁺. The rate of loss is from 1 to 4.5 microgram-atoms Mg²⁺/milligram Chl/hour; and depleted chloroplasts take up Mg²⁺ from the medium at even faster rates, to a total amount not much more than that present originally (0.8 to 1.8 microgram-atoms/milligram Chl with an average of 1.33 ± 0.32 μg-atoms/mg Chl). Leakage is completely prevented by 0.25 to 0.40 millimolar external Mg²⁺. Addition of Mg²⁺ at a level sufficient to prevent leakage from intact chloroplasts results in approximately 20% stimulation in light-driven protein synthesis.

In previous work (7) intact chloroplasts from pea leaves, isolated from Percoll gradients, were shown to synthesize protein at high rates in darkness when provided with ATP. However the optimum ATP concentration was a function of the Mg²⁺ content of the medium. An excess of either Mg²⁺ (over the concentration of ATP present) or of ATP (over the Mg²⁺ concentration present) was inhibitory. In this paper we present evidence that the inhibition by excess ATP is due to its ability to chelate Mg²⁺. A low external concentration of free, unchelated Mg²⁺ is needed to prevent the loss of major amounts of stromal Mg²⁺. The permeability of chloroplast envelopes to Mg²⁺ appears greater in our experiments than was reported by Gimmler *et al.* (8).

MATERIALS AND METHODS

Methods for growth and treatment of pea plants (*Pisum savitum* cv Progress No. 9) for isolation of intact chloroplasts from Percoll gradients and for measurement of [³H]Leucine incorporation into protein were described earlier (6, 7). To study loss *versus* retention of stromal Mg²⁺, intact chloroplasts were resuspended in 0.45 to 0.60 ml of a medium containing 350 mM sorbitol, 33 mM Hepes-KOH (at pH 8.3, providing almost 30 mM K⁺), 0.90 mM DDT, chloroplasts at 100 μg Chl/ml, and other components as indicated in specific experiments. Zero-

time samples were kept undiluted on ice until the end of the experiment, then washed with resuspension buffer at the same time as the other samples. The usual incubation was for 30 min in the dark at 27°C. In early work the tubes were agitated by hand once every 5 min; in most of the work shown, the tubes were shaken continuously in a controlled temperature water bath. At the end of the incubation, 3 ml was added of an ice-cold solution containing the same components as above plus 0.10 mM MgCl₂ to decrease possible Mg²⁺ loss during washing. The diluted suspension was centrifuged for 7 min at top speed in a Sorvall clinical centrifuge model GLC-1, at 4°C. The chloroplast pellet was washed once in fresh resuspension medium. The final pellet, containing 40 to 80 μg of Chl was drained, then resuspended in 1.0 ml of a hypotonic solution containing 1 mM EDTA, 20 mM KCl (pH 6.9) to lyse the intact plastids. The suspension was incubated for 5 to 10 min at 25°C, with vigorous vortexing at the beginning and end of the incubation. All membranes were pelleted by centrifuging for 30 min at 15,600g in an Eppendorf micro-centrifuge. The supernatant, containing soluble stroma components, was removed, diluted with 2 to 4 ml of 1 mM EDTA, 20 mM KCl, and used for measurement of total Mg²⁺.

In some of the later experiments, a silicone oil centrifugal separation technique was used to remove intact plastids from the incubation medium. In this procedure micro-centrifuge tubes (400 μl) were prepared with 60 μl of a lysis solution containing 13.45% (w/v) Ficoll, 0.02% NaN₃, density 1.045 g/ml, overlaid with 80 μl of silicone oils General Electric Versilube F50 and Dow Corning 702 in a 16:3 ratio, w/w (density of 1.02 g/ml at 22°C). Aliquots of 200 μl of the incubation mixture were layered over the silicone oil, and the tubes centrifuged for 30 s in a Coleman microcentrifuge. Intact plastids moved through the silicone oil in this procedure, but broken chloroplasts (naked thylakoids) did not. The tubes were frozen, then cut with a razor blade and the bottom portion containing the Ficoll layer was placed upside down in an Eppendorf centrifuge tube. One min of centrifuging in the Eppendorf was adequate to remove the Ficoll layer from the original tube. These materials were diluted with 1 ml of 20 mM KCl, 1 mM EDTA, followed by vortexing, to ensure complete lysis of the chloroplasts, then centrifuged for 30 min in the Eppendorf to pellet all membranes. An aliquot of 0.90 ml was taken from the supernatant, diluted with 1.0 ml of KCl, EDTA, and used for Mg²⁺ determination. The pellet was redissolved in 1% SDS, and Chl measured at 654 nm by comparison with a standard curve using chloroplasts of known Chl concentration.

In these experiments the carry-through of the solute with the chloroplasts, or in the intramembrane space of the envelope, was estimated by adding to the remaining 250 μl suspension in the original tube, 10 μl containing 0.93 μCi of ³H₂O and 0.19 μCi of [¹⁴C]glucose. After mixing, 200 μl of the suspension was removed and centrifuged through silicone oil as before. Both the Ficoll

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layer underneath the silicone oil and the medium with components that did not pass through were collected in Eppendorf tubes and diluted. Following removal of thylakoid membranes by centrifugation, aliquots of the samples were counted in a Packard liquid scintillation spectrophotometer.

Total Mg^{2+} of the solubilized stroma was measured using a Perkin-Elmer model 290 Atomic Absorption Spectrophotometer equipped with a No. 322N Perkin-Elmer hollow cathode Ca/Mg lamp. The Mg^{2+} content of the samples was estimated by comparison with a standard curve for $MgCl_2$ between 2 and 30 μM. The concentration of our standards was checked against a commercial $Mg(NO_3)_2$ standard solution; the standard curves were always linear with $r^2 = 0.99$ (r = correlation coefficient). Chl was measured by the method of Arnon (1) and soluble chloroplast protein by the procedure of Markwell et al. (14) with BSA as a standard.

Pea seed was from the Agway Corp. Biochemicals were from Sigma, inorganic salts and organic solvents from Mallinckrodt. Percoll and Ficoll were obtained from Pharmacia Corp. Tritiated leucine (L-[4,5-³H]Leu) at 50 to 135 Ci/mmol was purchased from ICN. D-[U-¹⁴C]Glucose (329 mCi/mmol) and ³H₂O (0.18 mCi/mmol) were purchased from New England Nuclear Corp.

RESULTS

When chloroplasts were incubated as for protein synthesis in the dark (7) using 10 mM ATP but without Mg^{2+}, a very significant loss of the stroma Mg^{2+} content occurred (Fig. 1). This loss was almost entirely prevented by having 10 mM Mg^{2+} present with the ATP; a condition previously calculated (7) to result in having 0.5 mM free Mg^{2+} in the medium. Thus, retention of

stroma Mg^{2+} correlates with maximal rates of protein synthesis in the dark, and loss of Mg^{2+} with the much reduced time-course and smaller amount of leucine incorporation (7).

Similar leakage of Mg^{2+}, although not as extensive in these experiments, occurred if the chloroplasts were incubated with EDTA (at 0.05 or 0.22 mM) (data not shown) or even without any chelator at all present (Fig. 2). In various experiments the initial rate of leakage was estimated to vary between 1.0 and 4.5 μg-atoms Mg^{2+}/mg Chl·h. The total extent of loss was most extreme in the first curve of Figure 1; in other experiments the extent ranged from 25 to 75% of the Mg^{2+} present in zero-time samples, in 30 to 60 min.

In controlled experiments, the effect of chelators on loss of Mg^{2+} turned out to be not very significant (data not shown, except for the far left end of the curves shown in Fig. 3). The ones used—EDTA and ATP (at 10 mM)–caused only 10 to 20%

FIG. 2. Loss of Mg from chloroplasts suspended in incubation media without chelators. The normal incubation medium was used without any additions, or with 1.0 mM $MgCl_2$ as indicated. At the time shown aliquots were removed, brought to 0°C diluted, washed and then the stroma contents extracted. Separate experiments are labeled with triangles and circles.
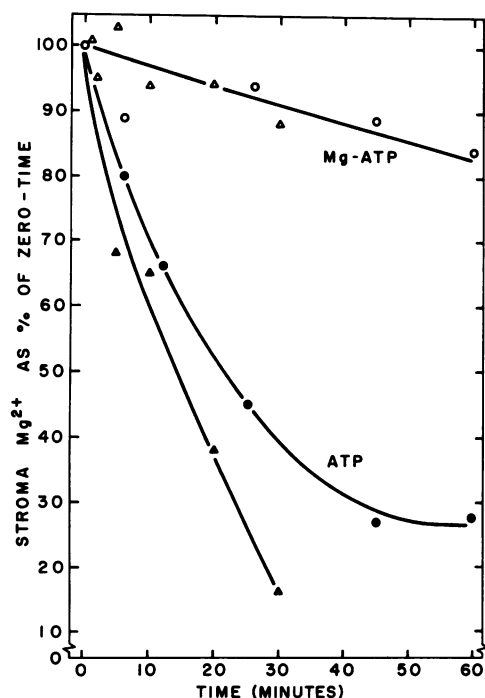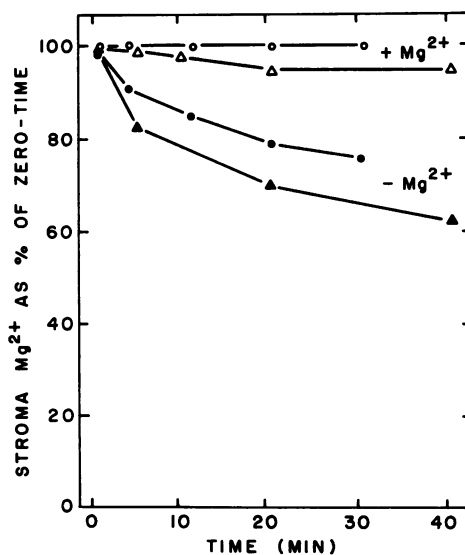
FIG. 1. Comparison of Mg^{2+} retention of chloroplasts incubated with either 10 mM ATP or 10 mM ATP + 10 mM $MgCl_2$. Intact chloroplasts were incubated as shown, with successive aliquots removed for measurement of stromal Mg^{2+} at different times. In the first experiment ($\triangle$, $\blacktriangle$), the chloroplasts were washed twice prior to extraction of stroma, and the zero-time level was 1.30 μg-atoms Mg^{2+}/mg Chl. In the second experiment (O, $\bullet$), aliquots were removed and chloroplasts sedimented through silicone oil to remove them from the medium. Corrections were made for carry-through of Mg^{2+} in the [¹⁴C]glucose space. The zero-time level was 1.88 μg-atoms Mg^{2+}/mg Chl.
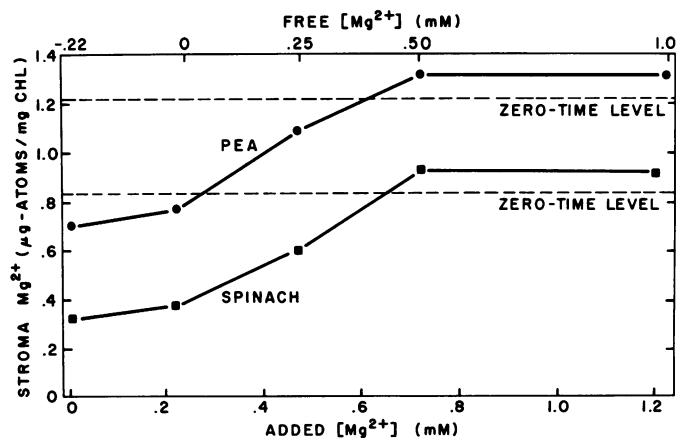
FIG. 3. Concentration of $MgCl_2$ needed to prevent Mg loss from chloroplasts. All incubation media contained 0.22 mM EDTA; with addition of $MgCl_2$ as shown on the bottom abscissa. Free Mg^{2+} concentrations (i.e. not chelated by the EDTA) are shown on the top abscissa. At the end of 25 min, aliquots were removed and chloroplasts separated from the medium by centrifuging through silicone oil layers.

further leakage above that in the controls. The effect of chelators saturated at or below 0.5 mM (data not shown).

Low external Mg^{2+} concentrations in the medium were able to prevent net loss of Mg^{2+} from these chloroplasts (Fig. 3; Table 1). With intact plastids from both very young pea plants and from mature spinach leaves, Mg^{2+} leakage was extensive but could be prevented by 0.23 to 0.40 mM $MgCl_2$. Chloroplasts from older (14 and 16 d) pea plants did not lose as much Mg^{2+}, however, only 10 and 15% of the initial stroma Mg^{2+} in two experiments. The curves for stroma Mg^{2+} as a function of external Mg^{2+} concentration indicate that leakage is directly related to the external free Mg^{2+} level. With no $MgCl_2$ added and 220 μM EDTA in the medium, the leakage was only slightly greater than when the EDTA and $MgCl_2$ were equimolar (Fig. 3). We were not able to detect a difference in the external Mg^{2+} concentration needed to prevent leakage from chloroplasts that were incubated in the light or in the dark; however, there was a necessary dark interval (at lowered temperature) between the end of the incubation and the centrifugal separation of chloroplasts from the medium, so the Mg^{2+} that moves out of the thylakoids in the light would have had a chance to return to the thylakoid space prior to lysis of the intact plastids. Using the more rapid and quantitative procedure for centrifuging plastids through a silicone oil layer to remove them from the medium resulted in slight higher values for stroma zero-time Mg^{2+} content—average of 1.56 ± 0.22 SD μg-atoms/mg Chl in seven experiments, rather than the average value of 1.15 ± 0.24 in nine experiments using the washing procedure. In three experiments with the silicone oil technique, the balancing external Mg^{2+} concentration was in the same range (0.23 to 0.43 mM) as that seen in the previous experiments. The only experimental drawback with the silicone oil method was the need to estimate solvent [^{14}C]glucose space with the sedimented chloroplasts.

Incubation of chloroplasts in Mg^{2+}-free media did not rupture the envelope membranes. Phase contrast microscope observation did not show any difference between chloroplasts incubated with or without Mg^{2+}. Second, no thylakoids from broken chloroplasts would sediment through the silicone oil layers when these were used; so the fraction of the Chl moving through could be used as a minimal criterion for intactness. On the average, 70% of the Chl did move through the silicone oil layer, independent of the presence or absence of Mg^{2+}, EDTA, or ATP in the medium. Third, no significant loss of soluble protein occurred from intact chloroplasts due to incubation in media lacking Mg^{2+} compared

Table I. *Concentration of External Mg^{2+} Needed to Prevent Any Leakage From Intact Pea Chloroplasts*

Conditions as in Figure 3, except that in experiments 5 and 6 the chloroplasts were separated from the medium by centrifuging through silicone oil. In all other experiments they were washed twice prior to extracting stromal contents and measuring Mg^{2+}. All incubations were for 30 min at 27°C, with 220 μM EDTA present.

Experiment	Treatment	Free Mg^{2+} Preventing Leakage	Initial Stroma Mg^{2+}
		mM	μg-atoms/mg Chl
1	Dark	0.26	1.39
2	Light	0.43	0.95
3	Dark	0.33	1.03
	Light	0.30	1.03
4	Dark	0.30	1.14
	Light	0.25	1.14
5	Dark	0.27	1.67
6	Dark	0.38	1.21
Average		0.315 ± 0.058 SD	1.23 ± 0.24

to media with Mg^{2+} (Table II). Although some loss of material at 259 nm (nucleotides, and possibly phenolic compounds) did occur during incubation, again the presence or absence of Mg^{2+} in the medium had no significant effect (Table III). Fourth, the ferricyanide reduction test for broken chloroplasts (9) when applied to these plastids before and after a 30-min incubation period, showed only negligible breakage had occurred whether the medium contained 0.5 mM Mg^{2+} to prevent leakage, or 0.05 to 0.20 mM EDTA to induce leakage of Mg^{2+} (Table IV). And finally, no other divalent cations tested (including Mn^{2+}, Ca^{2+}, Ba^{2+}, and Co^{2+}) were able to keep Mg^{2+} from leaking out (data not shown). Thus the loss of Mg^{2+} seems to reflect the intrinsic permeability of these chloroplast envelopes to Mg^{2+}.

It is not likely that any significant fraction of the Mg^{2+} we measure in stroma extracts represents cations bound to the outside of the envelopes by electrostatic association. In several control experiments, plastids were washed briefly at 4°C with 0.3 mM EDTA, and this did not affect the apparent zero-time Mg^{2+}

Table II. *ATP and EDTA Do Not Cause Loss of Soluble Protein from Intact Chloroplasts*

Chloroplasts were incubated for 30 min (experiment 1) or for the times shown (experiment 2) in incubation medium containing either 10 mM ATP and concentrations of $MgCl_2$ shown (experiment 1) or 100 μM EDTA (experiment 2). After incubation the chloroplasts were diluted washed and lysed as for Mg^{2+} measurement, and stroma protein content measured. Duplicate samples varied by no more than ± 0.2 mg/mg Chl.

Experiment	Incubation Conditions	Soluble Protein
		mg/mg Chl
1	Zero-time	10.1
	10 mM ATP, 0 mM $MgCl_2$	9.9
	10 mM ATP, 5.0 mM $MgCl_2$	9.5
	10 mM ATP, 7.5 mM $MgCl_2$	9.9
	10 mM ATP, 10 mM $MgCl_2$	9.3
2	Zero-time	9.8
	100 μM EDTA, 5 min	9.8
	100 μM EDTA, 15 min	9.7
	100 μM EDTA, 25 min	9.6

Table III. *Mg^{2+} and EDTA Do Not Affect Loss from Chloroplasts of Material Absorbing at 259 nm*

In experiment 1 standard conditions were used with the addition of EDTA and $MgCl_2$ as shown. After incubation for 30 min, the tubes were transferred to ice, then intact chloroplasts removed by 5 min centrifugation in the Eppendorf centrifuge. The supernatant was diluted with 0.6 ml of H_2O, the A measured at 259 nm and compared with a blank lacking chloroplasts. In experiment 2 similar conditions were used but the chloroplasts were separated from the medium by 7 min centrifugation in a clinical centrifuge at 4°C. The supernatant was further centrifuged for 15 min in the Eppendorf centrifuge and used for measuring A_{259}.

Experiment	EDTA	$MgCl_2$	Incubation	A_{259} of Supernatant
	mM	mM	min	
1	0.20		30	0.365, 0.369
	0.20	0.20	30	0.366, 0.396
	0.20	0.25	30	0.422, 0.391
	0.20	0.30	30	0.382, 0.352
2			0	0.235, 0.238
	0.10		5	0.305, 0.308
	0.10		10	0.318, 0.395
	0.10		15	0.389, 0.374
	0.10	0.15	5	0.274, 0.305
	0.10	0.15	10	0.315, 0.290
	0.10	0.15	15	0.323, 0.354

Table IV. *Ferricyanide Hill Reaction by Chloroplasts Incubated with or without Mg^{2+}*

Chloroplasts were taken from Percoll gradients, washed once, and incubated in a standard reaction mixture with either 0.5 mM $MgCl_2$ or 0.05 mM EDTA as shown, for 30 min at 27°C in the light (experiment 1) or in the dark (experiment 2), at a concentration of 0.10 mg Chl/ml. Either immediately after being put into this medium, or after 30 min of incubation, 0.3 ml aliquots were removed and put into a 2.0 ml volume reaction mixture containing 50 mM Tricine-NaOH (pH 8.0), 100 mM NaCl, 5 mM K_4 $(FeCN)_6$, 1 mM NH_4Cl, 0.5 mg/ml BSA, and either + or −350 mM sorbitol as shown. The reaction mixture was placed into an illuminated cell with a Clark type O_2 electrode, and O_2 evolution rates measured. Results are the average of duplicate determinations.

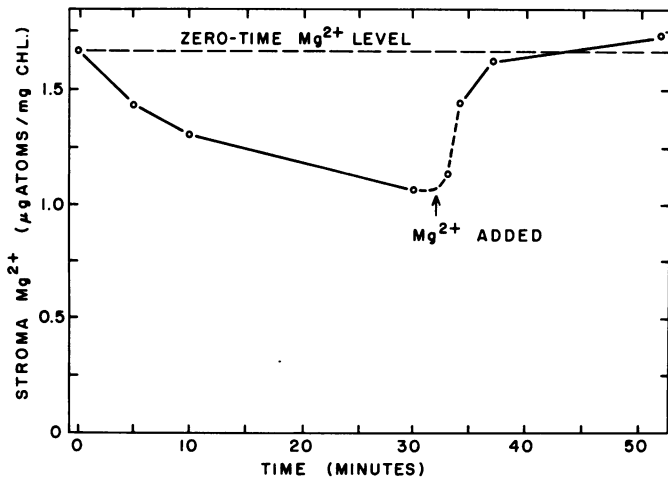| Experiment	Pre-incubation at 27°C			O_2 Evolution Rate		Estimated Intact Plastids
	Time	$MgCl_2$	EDTA	− sorbitol	+ sorbitol	
	min	*mM*	*mM*	*μ-atoms O_2/mg Chl·h*		*%*
1	0	0.5	0	365	28	92.3
	30	0.5	0	428	36	91.6
	0	0	0.05	455	32	93.0
	30	0	0.05	327	24	92.7
2	0	0.5	0	698	72	89.7
	30	0.5	0	697	61	91.2
	0	0	0.20	551	64	88.4
	30	0	0.20	526	40	92.4

FIG. 4. Loss of Mg^{2+} from chloroplasts incubated with EDTA, then re-uptake when excess Mg^{2+} is added back. Conditions as in Figures 1–3, with 0.22 mM EDTA present. Aliquots were taken for analysis of stroma Mg at the times shown. At 33 min (arrow) $MgCl_2$ was added to a final concentration of 1.22 mM, or 1.0 mM free Mg^{2+}. The initial rate of loss was 2.88 μg-atoms Mg^{2+}/mg Chl/h; initial rate of uptake was approximately 11.4 μg-atoms/mg Chl/h. The zero-time level of stroma Mg^{2+} was 1.67 μg-atoms/mg Chl.
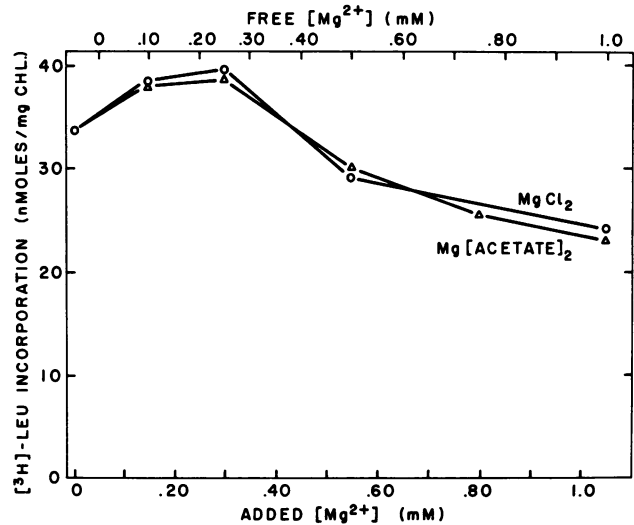

FIG. 5. Requirements for Mg^{2+} in light-driven protein synthesis. Isolated chloroplasts were incubated for light-driven protein synthesis under the conditions specified earlier (1). Incubation was for 20 min at 700 to 900 μE/m²·s, at 27°C with continuous shaking. EDTA was present in all tubes at 0.050 mM, and $MgCl_2$ (O) or Mg[ACETATE]₂ (△) was added at the concentrations shown on the lower abscissa. The upper abscissa shows the concentrations of free Mg^{2+} after subtracting the amount chelated by EDTA.

content of the stroma. In another, the EDTA wash was put in after the incubation period but before lysis; and again it had no appreciable effect on the results.

Permeability to Mg^{2+} was also shown by its reentry into depleted chloroplasts. A time-course for loss, then re-uptake, is shown in Figure 4. The rate of reentry was considerably faster than that of loss, perhaps due to a higher concentration gradient of free, diffusible Mg^{2+} from outside to in during uptake than during loss. The amount entering appeared to saturate, in several experiments, at a level 10 to 15% higher than that found in the zero-time samples, even when the external Mg^{2+} was as high as 10 mM. Entry of Ca^{2+} ions at appreciable rates (at 4°C) was observed earlier (4) using similar chloroplasts.

Leakage of Mg^{2+} in the absence of added ATP or Mg^{2+}, and its prevention by low concentrations of external Mg^{2+}, suggested that light-driven protein synthesis might have a requirement for

these low concentrations. In previous work the external Mg^{2+} concentration effect on translation had been tested only over a high concentration range, not in the region between 0 and 1.0 mM (3, 6). Stimulation of leucine incorporation by 0.1 to 0.3 mM free Mg^{2+} was indeed observed (Fig. 5). While the stimulation by added $MgCl_2$ (15 to 25% in several experiments) was not as great as in experiments with ATP-driven translation (often up to 100% [7]), the early onset of inhibition by excess Mg^{2+} may have decreased the height of the peak. In this experiment inhibition was apparent by 0.5 mM $MgCl_2$; in others it did not occur until 1 mM free Mg^{2+}.

To see if the inhibition by excess Mg^{2+} might be due to action of the K^+/H^+ antiporter of the envelope which is dependent on external Mg^{2+} (5, 10, 11), protein synthesis in the light was measured at three different concentrations of $MgCl_2$ (Fig. 6). It
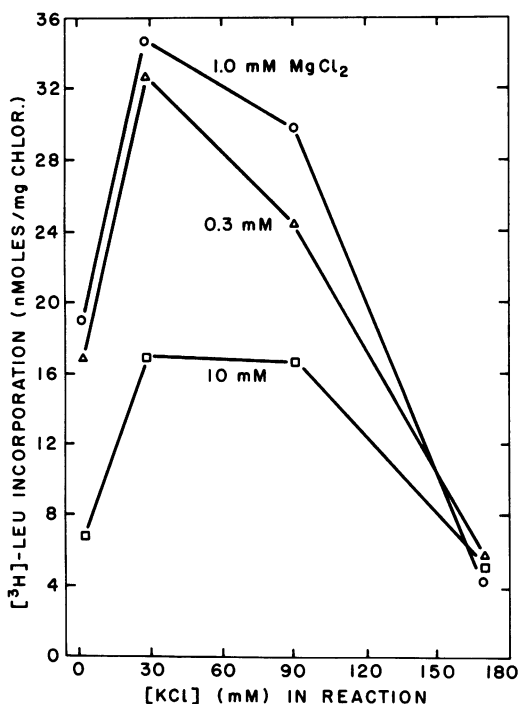
FIG. 6. Potassium effect on light-driven protein synthesis at three levels of $MgCl_2$. Chloroplasts were incubated for light-driven protein synthesis as in Figure 4, with 0.3 (△), 1.0 (○), or 10.0 (□) mM $MgCl_2$ added. The lower K^+ concentration (1.5 mM) represented the amount brought in with the chloroplasts (*i.e.* their resuspension medium); in this case the Hepes in the incubation medium was neutralized with NaOH not KOH. The 30 mM K^+ point represents the usual neutralization of the incubation medium Hepes with KOH. Higher concentrations were achieved by adding KCl, and in each case the sorbitol concentration was lowered to keep the osmotic strength constant.

is apparent that neither raising nor lowering the K^+ concentration was able to relieve the inhibition due to too much Mg^{2+}. In this experiment the sorbitol concentration was lowered as extra KCl was added to keep the osmotic strength of the medium constant, since this might otherwise have been a factor (5) in inhibition by excess KCl.

DISCUSSION

Gimmler *et al.* (8) in a brief note reported that spinach chloroplast envelopes were virtually impermeable to $^{28}Mg^{2+}$, with maximal flux rates no more than 0.1 μg-atoms/mg Chl·h. Based entirely on this report, it has often been assumed that Mg^{2+} will not leak out of or enter isolated, intact chloroplasts (5, 10, 12, 15, 17-19), and the divalent cation ionophore A23187 is added to induce such loss (5, 12, 17, 19). In none of these papers was the endogenous permeability of envelopes to Mg^{2+} tested further. A brief counter-claim (13) that $^{28}Mg^{2+}$ penetrates spinach chloroplast envelopes as rapidly without A23187 as with the ionophore present has been ignored until now.

Although quoting Gimmler *et al.* on the issue of envelope permeability to Mg^{2+}, two figures in the paper by Miginiac-Maslow and Hoarau (15) show very significant rates and amounts of Mg^{2+} loss from intact spinach chloroplasts in the absence of A23187, and the loss was said to be prevented by 0.4 mM external Mg^{2+}. Most of their paper had to do with internal concentrations and movements of Mg^{2+} as aided by A23187 and EDTA, and they do not discuss endogenous permeability or the percentage of the total stroma Mg^{2+} which can leak out without A23187.

Our current work supports and extends the observation of Miginiac-Maslow and Hoarau (15). With young pea plant chlo-

roplasts, initial rates of loss are found to be from 1.0 to 4.5 μg-atoms Mg^{2+}/mg Chl·h, or 10 to 45 times faster than those estimated by Gimmler *et al.* (8). Restoration of Mg^{2+} to the stroma of depleted chloroplasts is faster still (Fig. 4). The loss of Mg^{2+} from these chloroplasts is not due to breakage, as shown by (a) distribution of incubated plastids when sedimenting through silicone oil layers, (b) retention of protein and A_{260} absorbing materials to an equal extent + or − Mg^{2+}, and (c) retention of impermeability to ferricyanide. In addition, it is highly unlikely that reabsorption of Mg^{2+} would be seen following depletion, as in Figure 4, if the plastids had ruptured during the Mg^{2+} loss period.

The difference between the results of Gimmler *et al.* (8) and others, and Miginiac-Maslow and Hoarau (15) and ourselves, might be due to differing envelope permeability characteristics resulting from physiological state of the plants, growth conditions, cultivar, etc. We saw slower and considerably less efflux of Mg^{2+} from older pea plant plastids, using the same methods throughout. But spinach chloroplasts are probably ordinarily permeable to Mg^{2+} as seen in our limited experience (Fig. 3), in the report of Lendzian (13), and as deduced from the figures in the paper of Miginiac-Maslow and Hoarau (15). Also in the paper by Portis (17) Figure 1 shows control chloroplasts (lacking A23187), assayed after 5 min of incubation, demonstrating a clear response of stroma Mg^{2+} to levels of the cation in the medium. That figure is very similar to our Figure 3 except for its lack of a zero-time control. The response to medium Mg^{2+} was explained (17) by an *ad hoc* assumption of electrostatic association of Mg^{2+} with the envelopes, but this hypothesis was not tested further. It seems much more likely that in those experiments as well as in the present ones, spinach chloroplasts suspended in a medium containing sorbitol as an osmoticum, and low levels of K^+, lost or regained Mg^{2+} through the envelope.

In addition to genetic or physiological variations, a further source for differing results between publications might be in the temperature used during incubation. Permeability of membranes is likely to rise with temperature, and when experiments are performed significantly below 27°C, the Mg^{2+} loss might be slower. This possibility remains to be evaluated.

Gimmler *et al.* (8) did not report the concentration of Mg^{2+} used during a 2-h preloading period with ^{28}Mg. If it had been quite low, perhaps most of the mobile Mg^{2+} was lost from the plastids at that time. Then, since efflux rates must depend on the concentration differences as well, the net loss of $^{28}Mg^{2+}$ could have been very low from the already depleted chloroplasts. Whether the differing results are due to differences in the chloroplasts or to different methods, it is at least clear that it should no longer be taken for granted that isolated chloroplasts are impermeable to Mg^{2+} ions.

A number of unresolved questions remain about Mg^{2+} permeation through chloroplast envelopes. The mechanism of this movement, whether through pores, or via a carrier, etc. is unknown. The nature of the counter-ion is unknown; whether Mg^{2+} movement is balanced by H^+ or some other cation moving in the opposite direction, or by symport with some anion such as Cl^-. Another question is whether permeability does indeed vary with growth conditions (young *versus* old pea plants, for instance) or even with the nature of the suspending medium (compare Figs. 1 and 2 in Ref. 17).

The concentration of external free Mg^{2+} needed to just prevent Mg^{2+} loss is not an accurate indication of the free Mg^{2+} concentration in the stroma because membrane potentials across the envelope, or gradients of pH or of other ions may strongly affect the equilibrium concentrations. No efforts were made to estimate the true concentration of free Mg^{2+} in the stroma in the current work, but they are probably not very different from the 1 to 4 mM estimated by Portis (17) or by Kaiser *et al.* (12) for spinach

chloroplasts. The stroma Mg^{2+} that we measured included amounts complexed with metabolites, proteins or even membranes, extracted by the 1 mM EDTA used in the lysis buffer. Since up to 75% of this total pool can leak out during incubation, a large proportion of the bound cation must be in a reversible equilibrium with the free Mg^{2+}. The fact that incubation of chloroplasts in higher levels of external Mg^{2+} did not raise the stroma content to much beyond that present at zero-time, indicates saturation of the internal binding sites.

The large amount of stromal Mg^{2+} that can leak out may actually have a significant effect on the incubation medium. It is not unusual, in our experience, for chloroplasts to lose 0.5 to 1.0 μg-atoms Mg^{2+}/mg Chl. If the incubation medium has chloroplasts at 0.1 mg Chl/ml, the leakage will bring the medium concentration to 0.05 to 0.10 mM. Without external chelators, these concentrations will begin to have an effect in slowing down further leakage, which would therefore be partially self-limiting. This could be one reason for concentration-dependent effects on chloroplast enzymatic activities.

Loss of considerable Mg^{2+} from the stroma may be a sufficient reason for the inhibition of protein synthesis by excess ATP noted earlier (7). Consistent with this, the major effect of excess ATP (7) and of EDTA, in other experiments not shown, was to shorten the time-course for leucine incorporation. Presumably protein synthesis stopped once the internal Mg^{2+} dropped below a critical threshold level. However, other effects of Mg^{2+} are not ruled out yet. It is possible that Mg^{2+} facilitates entry of ATP into chloroplasts. This would explain why adding sufficient Mg^{2+} usually has a greater effect on ATP-driven protein synthesis (7) than on light-driven translation (Fig. 5). In a recent study Piazza and Gibbs (16) found 19% more rapid exchange of internal [^{14}C] ADP for external Mg-ATP than for ATP alone, and much bigger effects of Mg^{2+} on penetration rates of external ADP and AMP, using *Sedum* chloroplasts. A third possibility, difficult to evaluate quantitatively, is that ATP entering the stroma by itself might be inhibitory because it complexes stromal Mg^{2+}. Entry of Mg-ATP would leave the stroma Mg^{2+} bound to its usual sites, some of them probably necessary for protein synthesis. Finally, if (as with A23187) loss of Mg^{2+} is balanced by entry of H^{+}, acidification of the stroma due to this process might also slow down the rate of protein synthesis. This effect would be less severe in light-driven protein synthesis, where the stroma pH becomes more alkaline due to activities of the thylakoids, than in dark, ATP-driven protein synthesis.

It is not clear how disruptive the loss of 25 to 75% of the Mg^{2+} content is to stroma metabolism. Certainly the loss correlates with inhibition of protein synthesis. However, light-driven synthesis of RNA proceeded undiminished for 1 h, with EDTA in the medium, no Mg^{2+} and assuredly considerable loss of Mg^{2+} from these isolated chloroplasts (H. Doremus, unpublished results). In a large fraction of the work on CO$_2$ fixation by isolated chloroplasts, no free Mg^{2+} has been supplied. In one instance excess EDTA was added to chelate any divalent cations present, and still there was only a slight inhibition of CO$_2$ fixation (2). The loss of still further Mg^{2+} induced by A23187 does, finally,

inhibit CO$_2$ fixation (5, 17–19). These results suggest that there are pools of Mg^{2+} bound with varying strength and having different significance for stroma biochemistry. Our pea chloroplasts are prepared on Percoll gradients, and seem to have larger amounts of Mg^{2+} (1.0 to 1.7 μg-atoms/mg Chl) than do chloroplasts from spinach (Fig. 3) or older pea plants, which have 0.4 to 0.7 μg-atoms/mg Chl. Certainly with the chloroplasts having more abundant Mg, and to varying degrees with those having less, the most loosely bound Mg^{2+} can leak out to the medium easily, disrupting protein synthesis but not CO$_2$ fixation. Further, ionophore-induced loss of Mg^{2+} is needed to deplete the pool sufficiently to interfere with photosynthesis.

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