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ACTIVATION OF CO_2 FIXATION IN ISOLATED SPINACH CHLOROPLASTS

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SUMMARY

1. The effect of the Mg^{2+} concentration on the CO_2 fixation activity *in situ* in isolated and intact spinach chloroplasts upon suspension in hypotonic medium was examined. CO_2 fixation in the dark was activated 25–100 fold by 20 mM Mg^{2+} in the presence of added ATP *plus* either ribulose 5-phosphate or ribose 5-phosphate. 20 mM Mg^{2+}-stimulated fixation only 2–3 fold in the presence of the substrate of fixation, ribulose 1,5-diphosphate. The highest Mg^{2+}-stimulated rate of fixation in the dark observed with chloroplasts was 480 μmoles CO_2 fixed per mg chlorophyll per h.

2. The concentration of bicarbonate at half of the maximal velocity (apparent K_m) during the Mg^{2+}-stimulated fixation of CO_2 was 0.4 mM in the presence of ATP *plus* ribose 5-phosphate and 0.6 mM with ribulose 1,5-diphosphate.

3. Dithioerythritol or light enhanced Mg^{2+}-stimulated CO_2 fixation 1–3 fold in the presence of ATP *plus* ribose 5-phosphate but not ribulose 1,5-diphosphate.

4. These results indicate that Mg^{2+} fluxes in the stroma of the chloroplast could control the activity of the phosphoribulokinase with a lesser effect on the ribulosediphosphate carboxylase. An increase in Mg^{2+} of 6–10 mM in the stroma region of the chloroplast would be enough to activate CO_2 fixation during photosynthesis.

INTRODUCTION

Intact chloroplasts, isolated from spinach leaves, are capable of high rates of light-dependent $^{14}CO_2$ fixation, with the formation of radio-labeled sugar phosphates, glycolic acid, amino acids, and polysaccharides[1]. When light is excluded, CO_2 fixation soon stops. Kinetic labeling studies showed that except for a small fixation of CO_2 during the first 3 min of darkness, no further fixation of CO_2 could be induced in the dark even when the substrate levels of ribulose 1,5-diphosphate (Ribul-1,5-P_2) were increased[2]. Addition of ATP or of ATP and ribose 5-phosphate (Rib-5-*P*) in the dark stimulated the continued formation of Ribul-1,5-P_2, as shown by subsequent radiochromatographic analysis of aliquot samples of the chloroplast suspensions. Even though concentrations of Ribul-1,5-P_2 present in the dark were similar to those during the light period, when photosynthesis was high, little CO_2 fixation in the dark could be observed. It appeared that lack of substrate was not the reason that the car-

Abbreviations: Rib-5-*P*, ribose 5-phosphate; Ribul-5-*P*, ribulose 5-phosphate; Ribul-1,5-P_2, ribulose 1,5-diphosphate; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid.

boxylation reaction of photosynthesis in isolated chloroplasts was inactivated in the dark, but that it was activated in light by some control mechanism not involving substrate.

However, JOHNSON AND BRUFF[3] have induced high rates of CO_2 fixation with spinach chloroplasts in the dark. After the addition of ATP and Rib-5-*P*, they observed very high CO_2 fixation rates up to 330 μmoles/mg chlorophyll per h. Most likely, this rate indicates the upper capability of CO_2 fixation of the plastid. By comparison, photosynthetic rates with detached spinach leaves have been measured at 245 μmoles CO_2 fixed per mg chlorophyll per h[1].

Activation by light of the carboxylation reaction in chloroplasts has been proposed to result from light-driven ion fluxes which increase the local concentration of Mg^{2+} around the ribulosediphosphate carboxylase in the stroma region[2]. For activation, the direction of the light-driven movement of Mg^{2+} would be from the chlorophyll-containing grana lamallae into the stroma of the chloroplast. In the dark, Mg^{2+} would return to the grana lamallae and deactivate CO_2 fixation. This is plausible as light energy does drive ion movement across chloroplast grana membranes into the stroma. With spinach chloroplasts, DILLEY AND VERNON[4] measured a light-dependent efflux of Mg^{2+} of 0.30 and K^+ of 0.22 equiv/mole chlorophyll, balanced by an influx or uptake of protons of the same magnitude. NOBEL has also measured similar light-driven effluxes of Mg^{2+} and K^+ and influx of H^+ across spinach and pea chloroplast membranes[5,6].

The metabolic steps involved in the carboxylative phase of the photosynthetic carbon reduction cycle include the following reactions. Rib-5-*P* is converted to Ribul-5-*P* by ribosephosphate isomerase (EC 5.3.1.6) and then to Ribul-1,5-P_2 by ATP and phosphoribulokinase (EC 2.7.1.19). Carboxylation of Ribul-1,5-P_2 with $^{14}CO_2$ by ribulosediphosphate carboxylase (3-phospho-D-glycerate carboxy-lyase (dimerizing), EC 4.1.1.39) gives 3-phospho-D-[1-^{14}C]glycerate. Both phosphoribulokinase and ribulosediphosphate carboxylase require Mg^{2+} for full activity[7,8].

In these experiments, the effect of added Mg^{2+} and dithioerythritol has been investigated on the activation *in situ* of CO_2 fixation in the dark with isolated spinach chloroplasts suspended in a hypotonic medium. The substrate for carboxylation, Ribul-1,5-P_2, was either added directly or generated from ATP *plus* Rib-5-*P* or ATP *plus* Ribul-5-*P*.

METHODS AND MATERIALS

Preparation of chloroplasts

Chloroplasts were isolated from leaves of spinach (*Spinacia oleracea* L., var. Viroflay) grown in an environmental growth chamber at 20° with a 11-h day and 16° night. The method is essentially as described previously[1] with some modifications. The washed leaves were chopped with razor blades in 0.05 M 2-(*N*-morpholino)ethanesulfonic acid (MES) adjusted with NaOH to pH 6.5 in a sorbitol–salt solution containing 0.33 M sorbitol, 2 mM $NaNO_3$, 2 mM EDTA (dipotassium salt), 1 mM $MgCl_2$, 0.5 mM K_2HPO_4, 1 mM $MnCl_2$ and 2 mM sodium isoascorbate. The slurry was poured and pressed through nylon cloth to remove cell debris, filtered through "Nitex" nylon monofilament screen cloth (mesh opening, 25 μ) to remove whole cells, and centrifuged at 500 $\times$ *g* for 90 sec. The chloroplasts were suspended and stored in an ice bath in

the sorbitol–salt solution described above, *plus* 0.05 M *N*-2-hydroxyethylpiperazine *N'*-2-ethanesulfonic acid (HEPES) adjusted to pH 6.7 with NaOH.

Assay system for CO_2 fixation

CO_2 fixation was carried out at 20° in round-bottom flasks placed in the shaking apparatus described previously[1]. For light-dependent CO_2 fixation, Solution C[1] was modified to contain 0.025 M HEPES, adjusted to pH 7.8 with NaOH, and 2 mM $Na_4P_2O_7 \cdot 10\ H_2O$ in the sorbitol–salt solution above. For CO_2 fixation in the dark (investigated in flasks covered with black masking tape), sorbitol was inhibitory and most of the salts used in the sorbitol–salt solution were not needed and their addition had no effect. This solution contained 0.025 M HEPES, adjusted to pH 7.8 with NaOH, 4 mM dithioerythritol, 20 mM $MgCl_2$ (unless otherwise specified), and either 4 mM ATP *plus* 2 mM Rib-5-*P*, 4 mM ATP *plus* 2 mM Ribul-5-*P* or 0.7 mM Ribul-1,5-P_2 without ATP. ATP, Rib-5-*P*, Ribul-5-*P*, Ribul-1,5-P_2 and dithioerythritol were obtained from Sigma Chemical Co., St. Louis.

To 450 μl of the assay solution in each flask, 25 μl of 0.15 M $NaH^{14}CO_3$ containing 35 μC of ^{14}C were added. CO_2 fixation was started by addition of 25 μl of the chloroplast suspension (containing about 40 μg of chlorophyll). At various times, (usually 1, 3, 5 and 7 min), 50 μl samples were removed with a Hamilton microliter syringe and squirted into 200 μl of methanol to stop any further reaction. Total carbon fixed and chlorophyll content were determined as described previously[1].

RESULTS

Activation of CO_2 fixation by Mg^{2+}

CO_2 fixation in the dark can be greatly activated by adding Mg^{2+} to isolated spinach chloroplasts (Table I). In Expt. 1, Table I, with ATP *plus* Rib-5-*P*, the increase of Mg^{2+} from 1 to 20 mM increased carboxylation more than 100 fold to 480 μmoles CO_2 fixed per mg chlorophyll per h (highest observed). Similarly in Expt. 2, increasing the concentration of Mg^{2+} activated CO_2 fixation more than 25 fold with ATP *plus* Rib-5-*P* or ATP *plus* Ribul-5-*P*. Yet in both Expts. 1 and 2, in the presence of Ribul-1,5-P_2, Mg^{2+} stimulated CO_2 fixation only 2–3 fold and the maximum observed rates were less than those in the presence of ATP *plus* Rib-5-*P* or Ribul-5-*P*.

The presence of 0.33 M sorbitol reduced the activating effect of Mg^{2+} (Table I). Sorbitol maintains the integrity of the limiting outer chloroplast membrane and apparently restricts the diffusion of exogenous Mg^{2+} to the enzyme system. Ribul-1,5-P_2 does not cross the outer membrane but ATP and Rib-5-*P* can, as has been shown by their exchange in and out during photosynthetic CO_2 fixation[9,10], but their passage could be somewhat restricted. This may account for a further part of the limited stimulation by Mg^{2+} in 0.33 M sorbitol as shown in Table I. The outer membrane as a permeability barrier was easily overcome in hypotonic media where sorbitol was omitted. After incubation of the chloroplasts for 1 min in the sorbitol-free assay medium, the maximal rates of Mg^{2+}-stimulated CO_2 fixation were observed.

The assay solution (Solution C) used for photosynthetic CO_2 fixation with intact spinach chloroplasts, contained 0.33 M sorbitol and 1 mM Mg^{2+}: greater concentrations of Mg^{2+} are known to inhibit the light-dependent activity[1]. Addition of ATP *plus*

TABLE I

EFFECT OF Mg^{2+} ON ACTIVATION OF CO_2 FIXATION WITH ISOLATED SPINACH CHLOROPLASTS

The assay media in the dark contained 0.025 M HEPES (pH 7.8), 4 mM dithioerythritol, and 7.5 mM $NaH^{14}CO_3$ (9.4 μC/μmole). Additions where indicated: 4 mM ATP, 2 mM Rib-5-*P*, 0.7 mM Ribul-1,5-P_2, 2 mM Ribul-5-*P*, 0.33 M sorbitol. The reaction was started by addition of chloroplasts having 21 μg chlorophyll (Expt. 1) or 17 μg chlorophyll (Expt. 2) in a volume of 0.5 ml. Samples were taken at 1, 3, 6 and 10 min and the maximum rate determined. For comparison, the photosynthetic rate in Solution C (see MATERIALS AND METHODS) which contains 1 mM Mg^{2+}, is also included.

Expt. No.		*Additions*	*Rate of CO_2 fixation (μmoles CO_2 fixed per mg chlorophyll per h)*	
			20 mM Mg^{2+}	*1 mM Mg^{2+}*
1	Dark	—	12	4
		ATP + Rib-5-*P*	480	3
		Ribul-1,5-P_2	224	130
		Sorbitol	6	5
		Sorbitol + ATP + Rib-5-*P*	32	—
		Sorbitol + Ribul-1,5-P_2	26	—
	Light	Solution C	—	273
		Solution C + ATP + Rib-5-*P*	—	281
2	Dark	ATP + Rib-5-*P*	128	5
		ATP + Ribul-5-*P*	123	4
		Ribul-1,5-P_2	73	27
	Light	Solution C	—	73

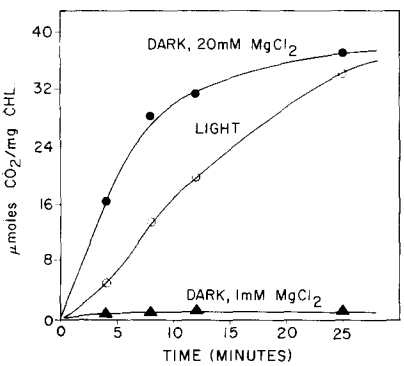


Fig. 1. Comparison of light-dependent CO_2 fixation to CO_2 fixation in the dark stimulated by $MgCl_2$. Fixation in the dark had 4 mM ATP, 2 mM Rib-5-*P* and either 1 mM or 20 mM $MgCl_2$. The reactions were started by addition of chloroplasts having 27 μg chlorophyll. At 25 min, the amount of CO_2 fixed in the dark with 20 mM $MgCl_2$ was 95 % of the amount of Rib-5-*P* added.

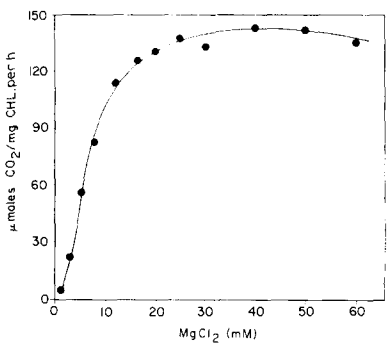


Fig. 2. Dependence of CO_2 fixation on Mg^{2+} concentration in the presence of ATP and Rib-5-*P*. Chloroplasts containing 22 μg chlorophyll were added to flasks containing 4 mM ATP and 2 mM Rib-5-*P* in the dark to start fixation.

Rib-5-*P* to Solution C only slightly enhanced photosynthetic CO_2 fixation (Table I).

The amount of CO_2 fixed as a function of time during photosynthesis in the light or in the dark with 1 and 20 mM Mg^{2+} is further compared in Fig. 1. In this experiment the initial rate of photosynthesis was 134 μmoles CO_2 fixed per mg chlorophyll per h. The corresponding rate with ATP *plus* Rib-5-*P* and 20 mM Mg^{2+} in the dark was 240

μmoles per mg chlorophyll per h; and with 1 mM Mg^{2+} only 4. Although the spinach leaf material was grown under controlled conditions, considerable variations were noted among different chloroplast preparations in their ability to fix CO_2. However, with the same chloroplast preparation, the rate of fixation as induced by Mg^{2+} in the dark was usually greater than the photosynthetic rate.

Effect of Mg^{2+} on the CO_2 fixation rate

Between 20 and 30 mM Mg^{2+} was required for maximum stimulation of CO_2 fixation with ATP *plus* Rib-5-*P* (Fig. 2). Half-maximal activity was obtained with 6 mM Mg^{2+}. As previously indicated with Ribul-1,5-P_2 (Table I), Mg^{2+} stimulated about 2 fold (Fig. 3), and the rates at low Mg^{2+} were already quite high. Possible reasons for the differences between the two assay systems in their response to Mg^{2+} will be discussed later.

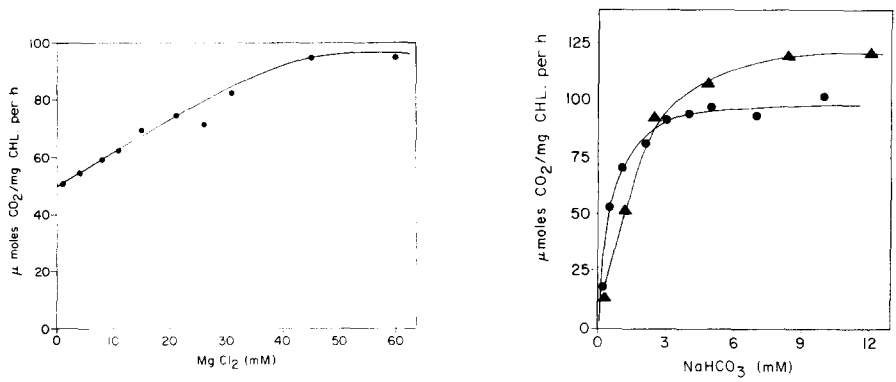


Fig. 3. Dependence of CO_2 fixation on Mg^{2+} concentration in the presence of Ribul-1,5-P_2. Chloroplasts containing 18 μg chlorophyll were added to start fixation in the dark to flasks containing 0.7 mM Ribul-1,5-P_2.

Fig. 4. Rate of CO_2 fixation in the dark as a function of bicarbonate concentration with 20 mM $MgCl_2$, 4 mM ATP and 2 mM Rib-5-*P*. The bicarbonate concentration at half-maximal velocity (apparent K_m) with chloroplasts (28 μg chlorophyll) prepared and assayed with a minimal of physical agitation (●) was 0.4 mM. Chloroplasts from another preparation (27 μg chlorophyll) were homogenized 1 min with a loose-fitting Teflon pestle tissue grinder (▲) had an apparent K_m of 1.5 mM bicarbonate.

Effect of bicarbonate on the CO_2 fixation rate

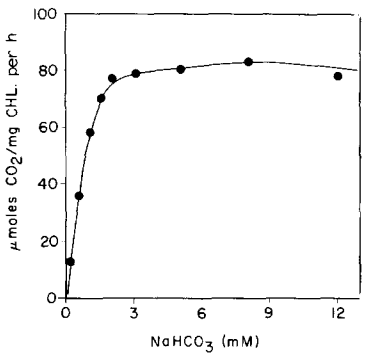
The dependence of CO_2 fixation in the dark on bicarbonate concentration in the presence of ATP and Rib-5-*P* is shown in Fig. 4. Chloroplasts which were prepared and assayed with a minimum of physical agitation, showed an apparent K_m (equal to the bicarbonate concentration at half-maximal velocity) of 0.4 mM $NaHCO_3$. After grinding another preparation for 1 min in a glass homogenizer with a loose-fitting teflon plunger, the K_m rose to 1.5 mM. K_m values to 5 mM have been seen after further physical disruption of the chloroplasts.

Fig. 5 shows a similar dependence on bicarbonate for dark fixation with Ribul-1,5-P_2. The apparent K_m was 0.6 mM $NaHCO_3$. Physical agitation of the chloroplasts in a glass homogenizer again caused a similar increase in the K_m (data

not shown). The substrate, Ribul-1,5-P_2 was inhibitory above 0.7 mM; an observation previously reported with the purified carboxylase[8,11].

Effect of Mg^{2+} on the pH optimum for CO_2 fixation

The pH optimum for activity of the isolated and purified ribulosediphosphate carboxylase depends on the Mg^{2+} concentration [12,13]. BASSHAM *et al.*[12] reported that raising the Mg^{2+} level with the purified enzyme from 1.8 mM to 45 mM dropped the pH optimum from 8.5 to 7.7. However, with chloroplast preparations in the dark, the pH optimum was shifted only slightly by changes in Mg^{2+} concentration (Fig. 6). At 1 mM $MgCl_2$, the pH optimum was 8.2; at 5 mM $MgCl_2$, 8.0; and at 18 mM $MgCl_2$, 7.8.



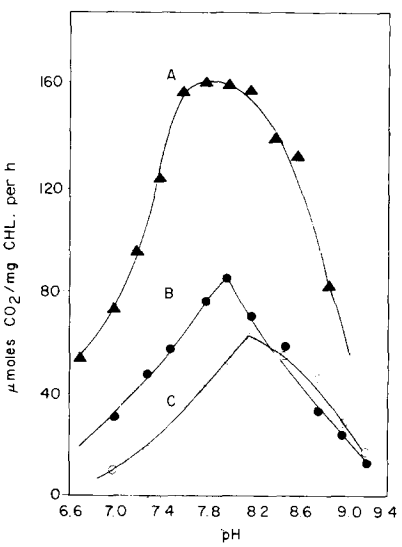


Fig. 5. Rate of CO_2 fixation in the dark as a function of bicarbonate concentration with 20 mM $MgCl_2$ and 0.7 mM Ribul-1,5-P_2. The apparent K_m with the chloroplasts (38 µg chlorophyll) was 0.6 mM bicarbonate.

Fig. 6. Effect of Mg^{2+} concentration on the pH optimum for CO_2 fixation in the dark. 4 mM ATP, 2 mM Rib-5-*P* and chloroplasts with 21 µg chlorophyll were used. A, 18 mM $MgCl_2$; B, 5 mM $MgCl_2$; and C, 1 mM $MgCl_2$. The ordinate in C expanded 10 ×, *i.e.* actual value at pH 8.2 is 6 µmoles CO_2 fixed per mg chlorophyll per h.

Activation by other cations

Other cations can also stimulate a limited amount of CO_2 fixation. With 4 mM Mg^{2+}, addition of K^+ gave a 3-fold stimulation (Table II). At 20 mM Mg^{2+}, added K^+ had no effect. Substitution of Mg^{2+} with Ca^{2+} or Mn^{2+}, both optimal at 10 mM, stimulated the fixation only slightly.

Stimulation by dithioerythritol and light

In the presence of ATP and Rib-5-*P*, CO_2 fixation with 20 mM Mg^{2+} could be further stimulated by dithioerythritol and by light (Table III). Typically, as shown in this experiment, light stimulated the rate of CO_2 fixation about 1.4 fold over the rate in the dark. Dithioerythritol in the dark stimulated about 2.4 fold. The effect of one was not mutually exclusive of the other as together they stimulated about 2.6 fold, which is not significantly different than that of dithioerythritol alone.

TABLE II

CATION STIMULATION OF CO_2 FIXATION WITH ATP AND Rib-5-*P*

Chloroplasts (35 μg chlorophyll) were suspended in the dark in 0.5 ml of 0.025 M HEPES (pH 7.8), 4 mM dithioerythritol, 4 mM ATP, and 2 mM Rib-5-*P*.

Additions		*Rate* (μmoles $^{14}CO_2$ *fixed per mg chlorophyll per h*)
Mg^{2+} (*mM*)	*Other ions* (*mM*)	
—	—	2
4	—	10
4	20 (K^+)	33
4	40 (K^+)	38
20	—	155
20	20 (K^+)	149
20	40 (K^+)	149
10	—	83
—	10 (Ca^{2+})	13
—	10 (Mn^{2+})	13

TABLE III

EFFECT OF LIGHT ON CO_2 FIXATION WITH CHLOROPLASTS IN HYPOTONIC MEDIUM IN THE PRESENCE OF ATP *plus* Rib-5-*P*

In the presence of 4 mM ATP and 2 mM Rib-5-*P*, CO_2 fixation with spinach chloroplasts was assayed in the light and dark, with and without added Mg^{2+}. The reaction was started by addition of 25 μl of chloroplasts having 8.7 μg chlorophyll and samples were taken at 4 min. Total volume: 250 μl. Other assay conditions as described in MATERIALS AND METHODS. Additions where indicated: 4 mM dithioerythritol, 1 mM NADPH, 1 mM $NADP^+$. N_2 flushing of flasks: 5 min before addition of bicarbonate, dithioerythritol and chloroplasts. Light intensity: 2300 ft candles, white light. The rate, averaged from duplicate samples, is given in μmoles CO_2 fixed per mg chlorophyll per h.

Additions	*Rate of CO_2 fixation*			
	Light		*Dark*	
	20 mM Mg^{2+}	*0.1 mM Mg^{2+}*	*20 mM Mg^{2+}*	*0.1 mM Mg^{2+}*
None	64.1	13.7	44.7	2.7
+ dithioerythritol	115.5	11.5	106.6	3.9
+ NADPH	37.9	15.2	21.0	2.2
+ dithioerythritol + NADPH	88.1	13.1	83.5	3.8
+ $NADP^+$	51.8	12.1	49.4	2.7
+ N_2 flushed + dithioerythritol	115.1	15.0	97.4	3.8

The addition of NADPH inhibited fixation, both in the light and in the dark. $NADP^+$ inhibited the stimulation by light, showing little effect in the dark. A set of flasks containing the assay medium were flushed with N_2 and CO_2 fixation assayed under N_2. This was done because the presence of O_2 has been reported to inhibit the stimulation by light of the phosphoribulokinase[14]. In the presence of dithioerythritol, the replacement of air by N_2 had no effect on the rate of CO_2 fixation.

In contrast, with low Mg^{2+} concentrations (0.1 mM) and in the presence of ATP and Rib-5-*P*, the low rate of CO_2 fixation was stimulated only by light (Table III).

TABLE IV

EFFECT OF ADDITIONS ON CO_2 FIXATION WITH CHLOROPLASTS IN THE PRESENCE OF Ribul-1,5-P_2

CO_2 fixation with chloroplasts was assayed in the presence of 0.7 mM Ribul-1,5-P_2 in light and dark, with and without added Mg^{2+}. Chloroplasts having 9.6 μg chlorophyll started the reaction with samples taken at 1.5 min. Other conditions were the same as described in Table III. The rate, averaged from duplicate samples, is given in μmoles CO_2 fixed per mg chlorophyll per h.

Additions	*Rate of CO_2 fixation*			
	Light		*Dark*	
	20 mM Mg^{2+}	*0.1 mM Mg^{2+}*	*20 mM Mg^{2+}*	*0.1 mM Mg^{2+}*
None	44.8	10.8	41.0	10.4
+ dithioerythritol	44.0	10.2	42.0	10.4
+ NADPH	30.4	8.4	35.4	7.8
+ dithioerythritol + NADPH	25.0	7.4	26.8	5.2
+ $NADP^+$	35.6	9.2	41.8	9.0
+ N_2 flushing	44.8	11.6	45.6	10.8

Somewhat different results were noted in the presence of Ribul-1,5-P_2 (Table IV). In this case, dithioerythritol and light had little effect on CO_2 fixation. Again NADPH inhibited as did $NADP^+$. N_2 flushing had no effect.

Effects of various metabolites on CO_2 fixation

It has previously been reported that addition of 5 mM concentrations of NADP, ATP, ADP, or AMP to intact spinach chloroplasts had little effect on photosynthetic CO_2 fixation[15]. However, in the dark it was observed that 5 mM ADP inhibited 50 % the Mg^{2+}-stimulated fixation of CO_2 in the presence of 2 mM ATP *plus* 1 mM Rib-5-*P*, but had no effect in the presence of 0.7 mM Ribul-1,5-P_2. AMP, orthophosphate and 3-phospho-D-glycerate at 3 mM did not affect either assay.

The products labeled during Mg^{2+}-stimulated $^{14}CO_2$ fixation, as determined by paper chromatography and radioautography[1], were primarily 3-phospho-D-glycerate with less than 5 % of the label in 2-phospho-D-glycerate, phosphoenolpyruvate and aspartate.

DISCUSSION

Effect of Mg^{2+} on chloroplast CO_2 fixation activity

CO_2 fixation in isolated spinach chloroplasts in low osmotic media (no sorbitol) could be greatly stimulated in the dark by addition of 20 mM Mg^{2+} if ATP *plus* Rib-5-*P* or Ribul-5-*P* were present to generate Ribul-1,5-P_2. The rates were usually higher than those demonstrated during photosynthesis with chloroplasts.

Ribulosediphosphate carboxylase in the chloroplasts appeared to have considerable activity at low Mg^{2+} (1 mM) concentrations, and this activity was increased 2–4 fold at 20–30 mM Mg^{2+}. However, there was very little CO_2 fixation at low Mg^{2+} concentrations in the presence of ATP *plus* Rib-5-*P*, where the isomerase, kinase, and carboxylase were involved, or in the presence of ATP *plus* Ribul-5-*P*, where the kinase and carboxylase were involved. With the latter two assays, an increase in the Mg^{2+} concentration gave a 25–100-fold increase in fixation, indicating that at low Mg^{2+}

concentrations, the phosphoribulokinase was inoperative and the increase in Mg^{2+} activated this enzyme.

Chloroplasts contain a large proportion of the intracellular magnesium in plant leaves[16]. We have measured the concentration of nonchlorophyll Mg^{2+} with spinach chloroplast pellets centrifuged at 2400 $\times$ g and found it to be between 18 and 20 mM. It is not known with spinach chloroplasts how much of the Mg^{2+} is unbound and thus available for the light-driven ion movements, but NOBEL[6] has calculated the presence of 16 mM Mg^{2+} in the osmotically responding space of isolated pea chloroplasts.

High affinity for bicarbonate

Mg^{2+}-stimulated CO_2 fixation with either ATP *plus* Rib-5-*P* or Ribul-1,5-P_2 had K_m values for bicarbonate of 0.4 mM and 0.6 mM respectively. These values are similar to the K_m for bicarbonate of photosynthetic CO_2 fixation with isolated spinach chloroplasts (0.6 mM)[1], indicating that the high affinity for bicarbonate observed in these experiments resembles that during photosynthesis. Much higher K_m values for bicarbonate have been reported after purification of the ribulosediphosphate carboxylase, with values ranging between 11 and 22 mM[8,11,13,17]. SUGIYAMA *et al.*[13] found that addition of 20 mM Mg^{2+} lowered the K_m from 20 to 5.6 mM bicarbonate and BASSHAM *et al.*[12], with 45 mM Mg^{2+}, observed a K_m of 1.8 mM bicarbonate. We have found that physical disruption of the chloroplast raises the K_m for bicarbonate, approaching that for the isolated enzyme. It appears that some factor or orientation of the local environment of the carboxylation enzyme gives it a greater affinity for CO_2 when the chloroplast is intact and the same factor may allow for the activation of the kinase and stimulation of the carboxylase by Mg^{2+}.

Difference in the CO_2 fixation rates

It is not clear why dark CO_2 fixation in the presence of added Ribul-1,5-P_2 should exhibit lower rates than CO_2 fixation in the presence of ATP *plus* Rib-5-*P* or ATP *plus* Ribul-5-*P*. The difference may be due to the inability of the added Ribul-1,5-P_2 to diffuse to the active site(s) of the carboxylation enzyme in the chloroplast. It has been observed that Ribul-1,5-P_2 does not diffuse through the outer limiting membrane of intact spinach chloroplasts[10], but this explanation is not applicable in these experiments because CO_2 fixation was run under low osmotic conditions without sorbitol; a condition which ruptures the outer membrane. Complete rupture of the chloroplast outer membrane by preincubation for several minutes under low osmotic conditions with 20 mM Mg^{2+} before addition of ATP *plus* Rib-5-*P* or Ribul-1,5-P_2 still had no effect on the difference in rates between the two assay systems.

It may be that the phosphoribulokinase and ribulosediphosphate carboxylase (and possibly the ribosephosphate isomerase) exist together as a complex; aggregated together in some manner in the presence of Mg^{2+} so as to stabilize an active conformation. Attempts to isolate such a multienzyme complex have been unfruitful[18]. Although, when ribulosediphosphate carboxylase was first isolated as "Fraction 1 protein", it contained amounts of the other two enzymes[19,20], but the carboxylase has since been purified[11,21,22]. Recently, CRIDDLE[18] has tried without success to identify a soluble molecular complex of the isomerase and kinase using sedimentation studies on chloroplast extracts prepared with a minimum of physical agitation. With crude

chloroplast extracts, the major portion of the isomerase and kinase enzymes were associated with particles of a very high sedimentation coefficient (> 25 S), suggesting that if the complex exists, it is insoluble. The three enzymes may well be organized about a membrane structure such as the lamellae[22], which would explain why physical disruption of the chloroplast raised the K_m for bicarbonate and reduced the stimulation by Mg^{2+} for CO_2 fixation.

Stimulation by dithioerythritol and light

Both dithioerythritol and light enhanced CO_2 fixation in the presence of ATP and Rib-5-*P* with 20 mM Mg^{2+}. If Ribul-1,5-P_2 was supplied directly, there was no enhancement with dithioerythritol or light. It appears that the phosphoribulokinase was stimulated by light and dithioerythritol in addition to the activating effect by Mg^{2+}. In contrast at low Mg^{2+} concentrations, dithioerythritol does not stimulate fixation and the small amount of stimulation by light may reflect a slight activation *in vivo* of CO_2 fixation during photosynthesis before complete breakage of the chloroplast outer envelope (Table III). Recently, LATZKO *et al.*[14] similarly reported that phosphoribulokinase can be activated up to 4 fold by light with intact spinach chloroplasts or by dithiothreitol with broken chloroplasts after gassing with N_2. Their enzyme assay media contained 10 mM Mg^{2+}. Gassing with N_2 had no observable effect in our assays.

As proposed by LATZKO *et al*[14], and indicated by our observations, the activation of phosphoribulokinase by dithioerythritol and by light may involve similar mechanisms as the two effects were not additive. The photochemistry could be reducing an endogenous dithiol compound or another compound which subsequently reduces essential thiol groups with stimulation of the phosphoribulokinase. The drop in the light-activating effect caused by $NADP^+$ could be due to its reduction to NADPH by the photosynthetic electron transport system at the expense of phosphoribulokinase activation.

These experiments indicate that in spinach chloroplasts, the phosphoribulokinase is much more responsive to both light and Mg^{2+} activation than is the ribulosediphosphate carboxylase. This conclusion appears to disagree with earlier kinetic observations of changes in the amount of ^{14}C label in the intermediate pools which suggested that the ribulosediphosphate carboxylase was the enzymic step controlled by the action of light during photosynthesis with intact spinach chloroplasts[2,23] or with *Chlorella pyrenoidosa*[24]. Although the levels of the pentose monophosphates did not appear to change much when the light was turned off[24], it does not follow that the phosphoribulokinase is, therefore, not under control in the intact organism. The pentose monophosphates are quickly equilibrated by transketolase to 3-phosphoglyceraldehyde, fructose 6-phosphate and sedoheptulose 7-phosphate. As well, it is possible that the activity of the ribulosediphosphate carboxylase may be controlled by other mechanisms not apparent in this investigation.

Interpretation of these findings

For activation of CO_2 fixation in plant chloroplasts, the following is proposed. Magnesium and potassium are both stored in the dark in the thylakoids. In the light they are released from the chlorophyll-containing thylakoids into the stroma as a result of the uptake of protons[4]. This flux of magnesium to the stroma greatly

activates the phosphoribulokinase and stimulates 2–4 fold the ribulosediphosphate carboxylase activity, resulting in the activation in light of the carboxylation reaction. From Figs. 1 and 2, the approximate increase of Mg^{2+} needed in the chloroplast stroma, calculated to give the observed rate of photosynthetic CO_2 fixation, is about 6–10 mM. In the presence of increased Mg^{2+} concentrations, light would further stimulate CO_2 fixation by further stimulating the phosphoribulokinase activity.

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