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EFFECT OF pH ON CHLOROPLAST PHOTOSYNTHESIS

INHIBITION OF O_2 EVOLUTION BY INORGANIC PHOSPHATE AND MAGNESIUM *

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Summary

1. The pH optimum of CO_2-dependent O_2 evolution by barley (*Hordeum vulgare* L.) chloroplasts was found to be between 7.8 and 8.2. The addition of 1 mM $MgCl_2$ in the dark inhibited O_2 evolution over the entire pH range tested and resulted in a much sharper pH profile centered around pH 8.2.

2. The pH optimum for O_2 evolution, in the presence and absence of 1 mM $MgCl_2$, was acid-shifted 0.3—0.4 pH units by 2 mM NH_4Cl. The pH optimum of O_2 evolution, with and without 1 mM $MgCl_2$, was base-shifted by 2 mM sodium acetate, approx. 0.5 pH units relative to the controls.

3. O_2 evolution in the presence of bicarbonate plus 3-phosphoglycerate or ribose-5-phosphate was considerably less sensitive to pH than CO_2-dependent O_2 evolution in the absence of substrate. With these substrates, both in the presence and absence of 1 mM $MgCl_2$, the pH optimum was broad and was centered around pH 7.8.

4. Inhibition of CO_2-dependent O_2 evolution by inorganic phosphate and magnesium increased as the pH of the reaction mixture was decreased below the optimum. Decreasing the pH from 8.2 to 7.6, reduced over 3-fold the concentration of inorganic phosphate required to inhibit O_2 evolution completely. For magnesium, a similar change in pH reduced the concentration required to inhibit O_2 evolution 50% approx. 5-fold. At pH 8.2, magnesium inhibition required inorganic phosphate. Magnesium was not required for inhibition of O_2

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Abbreviations: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Chl, chlorophyll.

evolution by inorganic phosphate, but increased the relative inhibition observed.

5. Illumination of intact barley chloroplasts increased the activity of NADP-glyceraldehyde-3-*P* dehydrogenase, phosphoribulokinase and fructose-1,6-diphosphatase. $MgCl_2$ and inorganic phosphate prevented this increase in enzyme activity at concentrations that completely inhibited CO_2-dependent O_2 evolution.

6. The results obtained suggest that magnesium inhibition of O_2 evolution may be caused by enhanced phosphate exchange across the chloroplast envelope.

Introduction

Techniques are currently available for the isolation of chloroplasts capable of high rates of photosynthesis in vitro. The assay conditions employed are extremely important and emphasize the potential for cytoplasmic control of chloroplast photosynthesis in vivo. The environment of the chloroplast in situ is difficult to define. In order to relate studies of chloroplast photosynthesis in vitro to the situation in vivo, an understanding of the interaction of pH and ion concentration is necessary. The influence of pH and the concentration of photosynthetic intermediates on the Warburg effect has been studied recently [1].

Under certain conditions, magnesium is very inhibitory to CO_2-dependent O_2 evolution by spinach (*Spinacea oleracea* L.) [2,3] and barley (*Hordeum vulgare* L.) [3] chloroplasts. Magnesium inhibition of chloroplast photosynthesis may have physiological significance because many cytoplasmic enzymes require this cation for activity. It therefore seemed important to determine the effect of other variables on magnesium inhibition of photosynthesis. Parallel trends were observed for inhibition of O_2 evolution by P_i and magnesium with varying pH and results were obtained that magnesium enhanced the inhibition of O_2 evolution by P_i. Because high concentrations of P_i (in the absence of magnesium) prevent the light-activation of certain photosynthetic enzymes, I propose that magnesium inhibits photosynthesis indirectly, by increasing P_i exchange across the chloroplast envelope.

Materials and Methods

Plant growth and chloroplast isolation. Barley (cv. Trophy) was grown and mesophyll protoplasts were isolated as previously described [4]. Intact chloroplasts were prepared by passing protoplasts through a 20-μm nylon net in a medium containing 0.3 M sorbitol, 10 mM $Na_4P_2O_7$, 5 mM $MgCl_2$ and 2 mM isoascorbate (pH 6.5). After centrifugation (200 $\times g$, 3 min), chloroplasts were resuspended in 0.3 M sorbitol, 10 mM HEPES/NaOH (pH 7.6), 1 mM $MgCl_2$, 1 mM $MnCl_2$ and 2 mM EDTA.

O_2 evolution. O_2 evolution was followed polarographically in 1.8-ml water-jacketed vessels (25°C). The basic reaction mixture contained 0.3 M sorbitol, 50 mM Tricine/NaOH (pH 8.2, except as indicated), 1 mM $MgCl_2$, 1 mM $MnCl_2$,

2 mM EDTA, 0.2 mM sodium phosphate (except as indicated), 6 mM $NaHCO_3$ and 15—30 μg Chl/ml. In tables and figures, the indicated concentration of magnesium represents the final net concentration (Mg^{2+} concentration minus EDTA concentration). Saturating illumination was provided by a 75 W floodlamp, giving 80 nE/cm^2 per s between 400 and 700 nm.

Light activation of Calvin cycle enzymes. Intact barley chloroplasts were incubated in the reaction mixture described above and other components as indicated. Aliquots, removed before and after 12 min illumination, were diluted with 5 volumes ice-cold water and stored on ice. Enzyme activity was followed spectrophotometrically at 340 nm. NADP-glyceraldehyde-3-*P* dehydrogenase and phosphoglycerokinase were assayed using the method of Latzko and Gibbs [5] and fructose-1,6-diphosphatase by the procedure of Kelley et al. [6]. Phosphoribulokinase was assayed in 100 mM Tricine/NaOH (pH 8.0), 10 mM $MgCl_2$, 2 mM ATP, 5 mM phospho*enol*pyruvate, 0.4 mM NADH, 0.5 mM ribose-5-phosphate, 6 I.U./ml pyruvate kinase, 9 I.U./ml lactate dehydrogenase, and 2 I.U./ml phosphoribuloisomerase. This assay was chosen because it does not require activity of other Calvin cycle enzymes. All enzymes and biochemicals were obtained from Sigma Chemical Co. *, St. Louis, Mo.

Results and Discussion

Effect of pH on O_2 evolution

The pH dependence of O_2 evolution by isolated spinach chloroplasts has been determined by several workers and is reported to have a slightly alkaline pH optimum. Werdan et al. [7] observed a broad optimum pH in the range of 7.8, whereas Avron and Gibbs [2] reported an optimum of 8.5. With intact barley chloroplasts, CO_2-dependent O_2 evolution reached a maximum between 7.8 and 8.2 (Fig. 1).

Magnesium, at millimolar levels, has been reported to inhibit photosynthesis by isolated chloroplasts [2,3]. At pH 8.2, 2—4 mM $MgCl_2$ was required to completely inhibit CO_2-dependent O_2 evolution by barley chloroplasts [3]. As shown in Fig. 1, the pH optimum of O_2 evolution in the presence of 1 mM $MgCl_2$ was sharper than that of the control and was shifted to a more alkaline pH. At the pH used in a previous study [3] of magnesium inhibition (pH 8.2), 1 mM $MgCl_2$ inhibited O_2 evolution approx. 50%, whereas at pH 7.6, complete inhibition was observed in the present study (Fig. 1).

In an effort to determine if external pH per se affects inhibition by magnesium, the pH dependence of O_2 evolution in the presence of 2 mM NH_4Cl was determined. As shown in Fig. 1, NH_4Cl (2 mM) lowered the pH optimum of O_2 evolution approx. 0.4 units without significantly affecting the rate of O_2 evolution at the pH optimum. Ammonia can be expected to diffuse across the envelope to the stroma where it would hydrolyze to form $NH_4^+ + OH^-$; hence, the stromal pH would increase. Werdan et al. [7] have demonstrated that the pH of the stroma varies with the pH of the medium and that Calvin cycle

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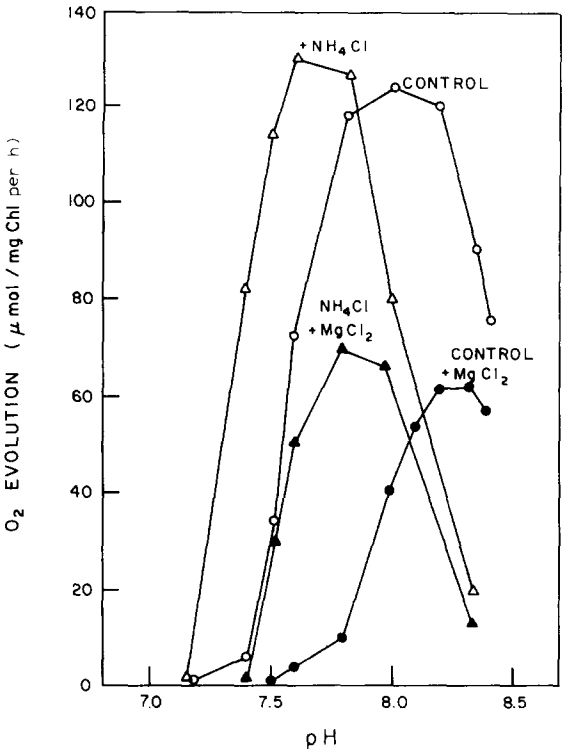

Fig. 1. Effect of pH and NH_4Cl (2 mM) on CO_2-dependent O_2 evolution by barley chloroplasts in the presence and absence of 1 mM $MgCl_2$.

activity is very sensitive to the stromal pH. At suboptimal pH, NH_4Cl would increase the stromal pH and, hence, stimulate O_2 evolution. At and above the pH optimum, NH_4Cl would be expected to inhibit O_2 evolution by increasing the pH of the stroma. Inhibition of O_2 evolution by NH_4Cl at the higher pH may also be caused in part by the increased concentration of NH_3 in solution, which may have an uncoupling effect. The expected trends were observed (Fig. 1). It is likely that suboptimal pH may be responsible in situations where stimulation of O_2 evolution by low concentrations of ammonia has been reported [8]. However, Werdan et al. [7] did not observe an increase in the stromal pH by low concentrations of methylamine. This is difficult to reconcile with the data of Fig. 1.

The above results suggest that O_2 evolution is dependent on the stromal pH (or pH gradient across the envelope) and not the external pH. NH_4Cl also acid-shifted the pH dependence of O_2 evolution in the presence of 1 mM $MgCl_2$ (Fig. 1). The data indicate that the increased inhibition by magnesium at low pH is related to the pH of the stroma (or the pH gradient across the envelope) and not the external pH. If magnesium inhibition were enhanced by low external pH per se, it would be expected that O_2 evolution in the presence of NH_4Cl plus $MgCl_2$ would be severely inhibited over the entire pH range.

The pH profile of CO_2-dependent O_2 evolution was base-shifted approx. 0.5 pH units relative to the control by 2 mM sodium acetate (Fig. 2). This

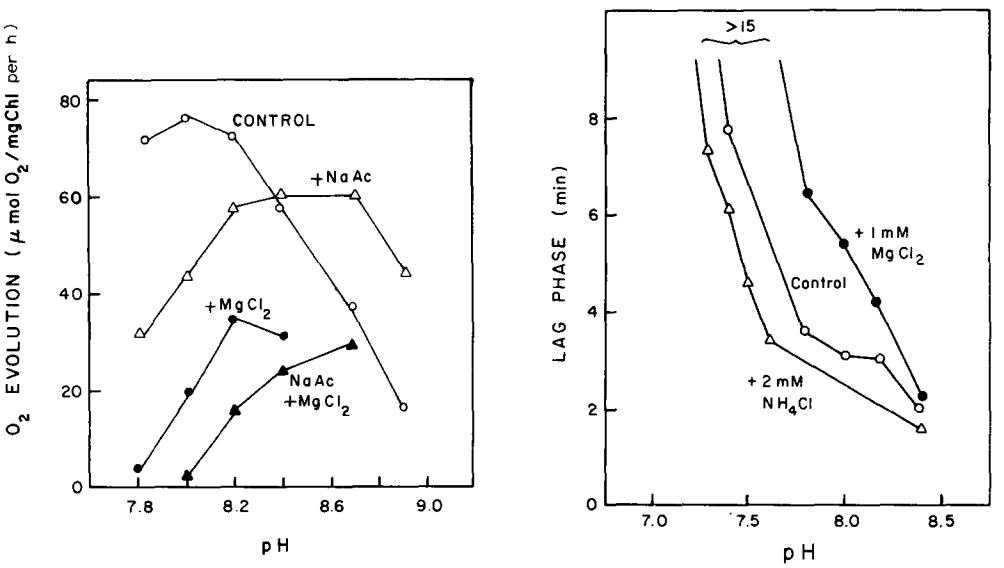

Fig. 2. Effect of pH and sodium acetate (2 mM) on O_2 evolution by barley chloroplasts in the presence and absence of 1 mM $MgCl_2$.

Fig. 3. Effect of pH on the lag phase of O_2 evolution by barley chloroplasts. As indicated, $MgCl_2$ (1 mM) and NH_4Cl (2 mM) were added in the dark before illumination.

would be expected because Werdan et al. [7] reported that acetate decreased the stromal pH. Hence, the pH optimum of O_2 evolution in the presence of acetate was higher than that of the control (Fig. 2). Acetate also base-shifted the pH profile of O_2 evolution in the presence of $MgCl_2$ (1 mM), which is consistent with the interpretation that magnesium inhibition is not affected by external pH per se.

When intact chloroplasts are illuminated with CO_2 as sole substrate, O_2 evolution begins gradually after an initial lag phase of several minutes [9]. The lag phase is thought to represent the time required for the autocatalytic increase of Calvin cycle intermediates. Whereas the rate of CO_2-dependent O_2 evolution had a defined optimum, the lag phase of O_2 evolution decreased as the pH of the medium was increased up to pH 8.4 (Fig. 3). In other experiments, lag phases greater than 10 min have been observed at lower pH values ($pH < 7.5$). Clearly the shape of the pH curve for O_2 evolution will vary with the duration of the experiment. Qualitatively similar trends were observed in the presence of $MgCl_2$ and NH_4Cl, although magnesium increased and ammonia decreased the lag phase (Fig. 3). The decreased lag phase by NH_4Cl indicates that the lag phase, similar to the maximum rate of O_2 evolution, is affected by the stromal pH (or the pH gradient) and not by external pH.

O_2 evolution at suboptimal pH was greatly increased by 3-phosphoglycerate or ribose-5-phosphate (Fig. 4). O_2 evolution in the presence of 3-phosphoglycerate at low pH is consistent with the data of Werdan et al. [7] that 3-phosphoglycerate-dependent O_2 evolution, in contrast to CO_2-dependent O_2 evolution, was not controlled by the pH of the stroma. However, increased activity at low pH by ribose-5-phosphate was unexpected because metabolism

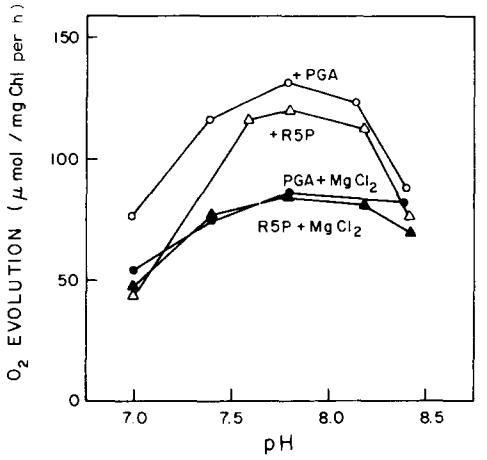

Fig. 4. Effect of pH and $MgCl_2$ (1 mM) on O_2 evolution by barley chloroplasts in the presence of 3-phosphoglycerate (PGA, 0.3 mM) or ribose-5-phosphate (R5P, 0.5 mM). As indicated, $MgCl_2$ and substrates were added in the dark before illumination.

of ribose-5-phosphate via the Calvin cycle involves the regenerative phase as well as the reductive phase of the cycle. The results may indicate that the regenerative phase is relatively insensitive to changes in the stromal pH or that substrates are affecting some other process. In contrast to O_2 evolution in the absence of substrates (Figs. 1 and 2), substantial O_2 evolution activity was observed with 3-phosphoglycerate or ribose-5-phosphate at low pH in the presence of $MgCl_2$ (1 mM) (Fig. 4). The data lend further support to the proposal that magnesium inhibition is not affected by external pH per se.

Effect of pH on inhibition of O_2 evolution by P_i and magnesium

The lag phase of O_2 evolution increased as the pH of the medium was decreased below pH 8.4 (Fig. 3). The results might be explained by an effect of pH on the phosphate translocator. Increasing concentrations of P_i have been shown to increase the lag phase of O_2 evolution and decrease the maximum rate of evolution [9]. Hence, at a given concentration of P_i, any factor that stimulated the activity of the phosphate translocator would be expected to increase the lag phase, and, ultimately decrease the rate of O_2 evolution. The results presented in Fig. 5 show that inhibition of O_2 evolution by P_i increased as the pH of the medium was decreased from pH 8.2 to 7.6. At the higher pH, approx. 1 mM P_i was required to inhibit O_2 evolution 50% as compared to 0.7 and 0.4 mM P_i at pH 7.9 and 7.6, respectively. The increased inhibition of O_2 evolution by P_i may explain the increased lag phase observed with decreasing pH (Fig. 3), and may also explain, at least partially, decreasing rates of O_2 evolution at suboptimal pH (Fig. 1). The proposal is supported by the observation that 3-phosphoglycerate and ribose-5-phosphate, which compete with P_i for transport on the phosphate translocator [10], extended the pH range, on the acid side, over which O_2 evolution was observed (Fig. 4). Previous work performed in the dark at 4°C [11] failed to detect an effect of pH on P_i transport with spinach chloroplasts. Studies of the phosphate translocator under

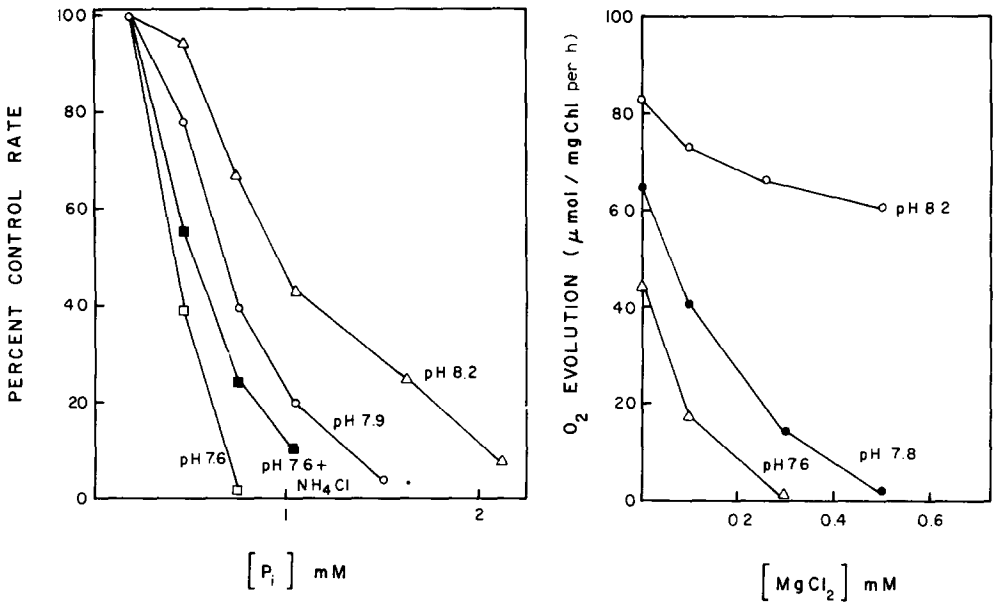

Fig. 5. Effect of pH on P_i inhibition of O_2 evolution by barley chloroplasts. As indicated, 2 mM NH_4Cl was added in the dark before illumination. Control rates, expressed as μmol O_2 evolved/mg Chl per h, were: pH 8.2, 94; pH 7.9, 95; pH 7.6, 64; pH 7.6 + NH_4Cl, 87.

Fig. 6. Effect of pH on magnesium inhibition of O_2 evolution by barley chloroplasts. The indicated concentration of $MgCl_2$ was added in the dark before illumination.

physiological conditions are currently underway to determine if pH directly affects the transport process.

The results presented in Fig. 1 suggest that inhibition of O_2 evolution by magnesium is also enhanced at suboptimal pH. At pH 8.2 (the pH used in previous studies of magnesium inhibition [3]), 0.5 mM $MgCl_2$ produced approx. 20% inhibition (Fig. 6) which was consistent with previous data [3]. However, 0.5 mM $MgCl_2$ completely inhibited O_2 evolution at pH 7.8, and 0.3 mM $MgCl_2$ produced complete inhibition at pH 7.6 (Fig. 6).

The parallel trends observed for inhibition of O_2 evolution by magnesium and P_i with variation in medium pH suggest that the two effects may be related. Magnesium enhanced the inhibition of O_2 evolution by P_i (Fig. 7). In the absence of free magnesium, O_2 evolution reached a maximum with 0.15 mM P_i and decreased with increasing concentrations of P_i (Fig. 7, 0 mM $MgCl_2$ curve). At pH 8.2, where P_i was least inhibitory to O_2 evolution (Fig. 6), magnesium inhibited O_2 evolution at high concentrations of P_i but stimulated O_2 evolution at rate-limiting P_i concentrations (Fig. 7). 1 mM magnesium decreased the optimum P_i concentration from approx. 0.15 to 0.04 mM (Fig. 7). Magnesium had no effect on O_2 evolution in the absence of added P_i, which was low and non-linear with time. These results are consistent with the proposal that magnesium enhanced P_i transport across the envelope and may explain the observation [3] that addition of $MgCl_2$ in the light to chloroplasts results in a reduced rate of O_2 evolution similar to addition of a higher concen-

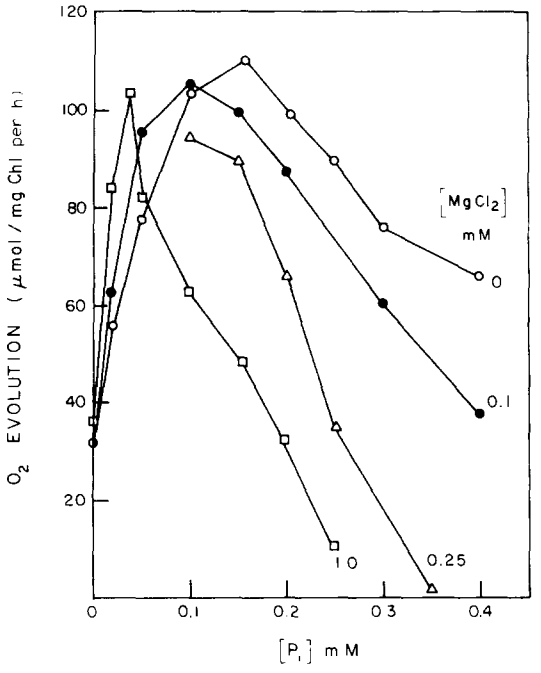

Fig. 7. Effect of magnesium on P_i dependence of O_2 evolution by barley chloroplasts. The indicated concentration of $MgCl_2$ was added in the dark.

tration of P_i. Lilley et al. [12] also observed magnesium inhibition of photosynthesis that was more pronounced at high concentrations of P_i and speculated that magnesium may influence the rate of exchange of external P_i for triose phosphates across the chloroplast envelope.

Effect of P_i and magnesium on the light-activation of certain Calvin cycle enzymes

The results do not explain, however, why 2 mM $MgCl_2$ (pH 8.2), completely inhibits O_2 evolution when added in the dark but produces only 30% inhibition when added in the light [3]. It has been proposed that magnesium inhibits O_2 evolution by preventing the light-activation of certain Calvin cycle enzymes [3]. The mechanism of inhibition remains unclear; however, a membrane interaction has been suggested because the chloroplast envelope is impermeable to divalent cations [13]. If magnesium acts indirectly by stimulating P_i transport across the evelope, high concentrations of P_i (in the absence of magnesium) should also prevent the light-activation of Calvin cycle enzymes.

Illumination of intact barley chloroplasts resulted in a 2—3-fold increase in activity of NADP-glyceraldehyde-3-*P* dehydrogenase and ribulose-5-*P* kinase, and a large increase in fructose-1,6-diphosphatase activity (Table I). Light activation of these enzymes has been demonstrated previously [3,14,15]. The increase in enzyme activity upon illumination was largely prevented by $MgCl_2$ (Table I and ref. 3). However, in the absence of free magnesium, 2 mM P_i also prevented the light-activation of enzyme activity. The data support the hypoth-

TABLE I

EFFECT OF P_i AND $MgCl_2$ ON THE LIGHT ACTIVATION OF PHOTOSYNTHETIC ENZYMES IN INTACT BARLEY CHLOROPLASTS

Chloroplast treatment	Enzyme (μmol product formed/mg Chl per h)			
	NADP-glyceraldehyde-3-*P* dehydrogenase	Ribulose-5-*P* kinase	Fructose diphosphatase	Phosphoglycerate kinase
Dark	188	128	<2	408
Light *	344	320	23	416
Light * + 2 mM P_i	136	116	7	396
Light * 4 mM $MgCl_2$	168	116	9	368

* 12 min illumination.

esis that an activation of P_i transport by magnesium may be responsible for the magnesium inhibition of enzyme activation and, hence, O_2 evolution. The inhibition of enzyme activation by high levels of P_i may also explain why P_i not only produces a lag extension, but also inhibitis the maximum rate of photosynthesis. As pointed out by Walker [16], if high P_i acted solely by draining triose phosphates from the chloroplast, rates of photosynthesis would eventually increase to the rates observed at low P_i.

Phosphoglycerate kinase is under energy charge control and is not light-activated per se [17]. Activity of phosphoglycerate kinase did not increase upon illumination of chloroplasts and was not inhibited by either P_i or $MgCl_2$ (Table I). The results suggest that magnesium and P_i specifically affect the Calvin cycle enzymes that are light-activated.

Concluding remarks

The results reported herein suggest that magnesium inhibition of O_2 evolution is indirect. Magnesium appears to enhance Pi exchange across the envelope which then prevents the light-activation of photosynthetic enzymes. The results may indicate that chloroplast substrate levels, or (substrate/P_i) ratios, regulate the light-activation process. The process of enzyme activation by light is known to involve photochemically produced reductant [6,14,18] and certain soluble stromal proteins [19]. It is possible that some step(s) in the process is modulated by substrate levels. Modulation of this type may represent a fine-tuning control mechanism to adjust enzyme activity to the level of substrates in order to maintain sufficient pool sizes.

The cytoplasm of a C_3 mesophyll cell contains many enzymes that require free magnesium for activity, e.g., the terminal enzymes involved in sucrose synthesis, UDPglucose pyrophosphorylase [20] and sucrose-phosphase synthetase [21], and the glycolytic enzymes. Thus, the chloroplast in situ must function in the presence of free magnesium. Whether magnesium inhibits photosynthesis will depend on the concentration of metabolites, the pH of the cytoplasm, and the concentration of P_i. The results also suggest that the export of photosynthetic intermediates into the cytoplasm (for sucrose synthesis [16,22]) will be controlled not only by the concentration of P_i, but also by the pH of the cytoplasm and the concentration of magnesium.

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