Plant Physiol. (1991) 95, 1229–1236
0032-0889/91/95/1229/08/$01.00/0

Received for publication July 27, 1990
Accepted November 24, 1990

# Studies on the System Regulating Proton Movement across the Chloroplast Envelope[1]

## Effects of ATPase Inhibitors, $Mg^{2+}$, and an Amine Anesthetic on Stromal pH and Photosynthesis

**Jeanne S. Peters and Gerald A. Berkowitz***

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

### ABSTRACT

**Studies were undertaken to further characterize the spinach (*Spinacea oleracea*) chloroplast envelope system, which facilitates $H^+$ movement into and out of the stroma, and, hence, modulates photosynthetic activity by regulating stromal pH. It was demonstrated that high envelope-bound $Mg^{2+}$ causes stromal acidification and photosynthetic inhibition. High envelope-bound $Mg^{2+}$ was also found to necessitate the activity of a digitoxin- and oligomycin-sensitive ATPase for the maintenance of high stromal pH and photosynthesis in the illuminated chloroplast. In chloroplasts that had high envelope $Mg^{2+}$ and inhibited envelope ATPase activity, 2-(diethylamino)-*N*-(2,6-dimethylphenyl)acetamide was found to raise stromal pH and stimulate photosynthesis. 2-(Diethylamino)-*N*-(2,6-dimethylphenyl)acetamide is an amine anesthetic that is known to act as a monovalent cation channel blocker in mammalian systems. We postulate that the system regulating cation and $H^+$ fluxes across the plastid envelope includes a monovalent cation channel in the envelope, some degree of (envelope-bound $Mg^{2+}$ modulated) $H^+$ flux linked to monovalent cation antiport, and ATPase-dependent $H^+$ efflux.**

Upon illumination, the pH of the spinach chloroplast stroma rises by approximately 0.4 to 0.5 pH unit (11). This stromal alkalinization is a regulatory mechanism that facilitates optimal photosynthesis in the light (24), primarily due to the high pH optima of the photosynthetic carbon reduction cycle enzymes fructose 1,6-bisphosphatase and sedoheptulose 1,7-bisphosphatase (1, 8). For quite some time, the rise in stromal pH in the light has been primarily attributed to $H^+$ pumping into the thylakoid lumen (11, 24). However, optimal photosynthetic capacity, both of isolated chloroplasts (11) and *in situ* in illuminated leaves (17), is dependent on the maintenance of a transenvelope pH gradient between the stroma and the solution external to the chloroplast.

The chloroplast envelope is known to be at least somewhat permeable to protons; the transenvelope pH gradient readily breaks down in the dark (11). However, previous research focusing on the chloroplast envelope components that regulate $H^+$ fluxes, and therefore contribute to the regulation of photosynthesis, has been somewhat inconclusive. Demmig and Gimmler (6, 7), along with Huber and co-workers (13, 15), have presented evidence suggesting that $K^+$ and $H^+$ can readily be exchanged across the envelope, in either direction. Although net $K^+/H^+$ exchange is likely not stoichiometric (7), Huber and co-workers (13, 15) concluded that a $K^+/H^+$ antiporter facilitated this exchange. This hypothesized antiport mechanism was thought to result in either stromal acidification or alkalinization, depending on the extrachloroplastic monovalent cation concentration. However, in later studies, Demmig and Gimmler (6, 7) speculated that $H^+$ pumping into the thylakoid during illumination is not entirely charge-balanced, and the resultant membrane potential developed between the stroma and external solution (inside negative) drives $K^+$ and/or $H^+$ movement into the stroma. $K^+$ efflux was thought to result in $H^+$ influx due to changes in this membrane potential. They concluded, in contrast to Huber, that $K^+/H^+$ exchange across the envelope was not facilitated by specific intrinsic envelope proteins acting as "antiporters."

The studies of Huber and co-workers (13, 15) also suggested that an envelope ATPase may act as an $H^+$ pump. They postulated that under some conditions the activity of this ATPase can contribute to the maintenance of stromal alkalinization in the light. In a subsequent study, however, Robinson (19) concluded that $H^+$ fluxes across the chloroplast envelope are not influenced by stromal [ATP], and that an envelope ATPase does not contribute to $H^+$ efflux in the light.

Another significant component of the system regulating the stromal pH in the light, which was initially characterized by Huber (13, 15) and Gimmler (6), is the effect of extrachloroplastic $Mg^{2+}$ on $K^+$ and $H^+$ fluxes. These studies indicated that although external $Mg^{2+}$ did not readily penetrate the chloroplast envelope, millimolar external concentrations of unchelated $Mg^{2+}$ somehow caused stromal acidification. Huber and co-workers (13, 15) concluded that external $Mg^{2+}$ may be acting by "activating" the hypothesized $K^+/H^+$ antiporter which, in the absence of high external $K^+$, could cause stromal acidification. Subsequent work from this laboratory (22) indicated that the effect of external $Mg^{2+}$ on chloroplast

[1] New Jersey Agricultural Experiment Station, Publication No. 12149-3-89. This material is based upon work supported by the U.S. Department of Agriculture Competitive Research Grants Office under award No. 90-37262-5586.

metabolism is likely caused by an increase in the extent of chloroplast envelope-bound $Mg^{2+}$.

In the studies reported here, an attempt was made to further characterize the factors that influence the stromal pH of the illuminated chloroplast. Treatment effects on both stromal pH and photosynthesis were monitored with the objective of addressing some of the discrepancies in the pertinent literature. An attempt was made to develop a model of how $H^+$ movement across the chloroplast envelope is facilitated and how this regulatory system subsequently affects photosynthetic capacity.

## MATERIALS AND METHODS

### Plant Material

Spinach seeds (*Spinacia oleracea* var "Melody") were grown in 1:1 peat/vermiculite (three seeds per pot in 2000-$cm^3$ mix). Pots were watered twice weekly with water and once weekly with complete commercial (Peters) fertilizer. Plants for most experiments were grown in a growth chamber with a 10-h light (250 $\mu$mol/$m^2$/s) regime maintained at 21°C (day) 16°C (night). Exceptions are noted in the text; for some experiments, plants were grown in a new growth chamber that had a higher (480 $\mu$mol/$m^2$/s) light level at bench height. Fully expanded, nonsenescing leaves taken from 6- to 8-week-old plants were used for chloroplast isolation.

### Chloroplast Isolation

Deribbed leaves (8–10 g) were sliced into 50 mL of grind medium containing 0.33 M sorbitol, 50 mM Hepes-NaOH (pH 6.8), 2 mM $Na_2EDTA$, 1 mM $MnCl_2$, and 1 mM $MgCl_2$, and ground twice for 3 s at 25,000 rpm in a Virtis grinder. (As discussed by Sen Gupta and Berkowitz [22], this grind medium contains approximately 45 nM free EDTA and $Mg^{2+}$, and essentially no free $Mn^{2+}$.) The homogenate was filtered through four layers of cheesecloth and two layers of Miracloth, and then centrifuged (SS34 rotor and RC5B centrifuge; Sorvall, Wilmington, DE) for 50 s at 750*g*. The loose pellets were resuspended in a total of 15 mL grind medium. This resuspension step was used to "wash" the chloroplasts in solutions containing either free $Mg^{2+}$ or EDTA. As indicated in the text, 5 mM $MgCl_2$ or 5 mM $Na_2EDTA$ was added to the "standard" grind medium (*i.e.* above that already contained in the medium). For these wash treatments, the chloroplasts were incubated for 5 min, and then removed from the $Mg^{2+}$ or EDTA wash medium by centrifugation again at 750*g* for 50 s. The pellets were resuspended in standard grind medium, layered onto grind medium containing 40% Percoll and 0.2% BSA in 50-mL tubes, and centrifuged in a swinging bucket (HB4) rotor at 2000*g* for 1 min. The intact (>90%, according to the ferricyanide reduction assay described in ref. 2) chloroplasts contained in the pellet were resuspended in a small volume of standard grind medium. It should be noted that in previous work from this laboratory (22), the $Mg^{2+}$- and EDTA-wash treatments have been found, respectively, to increase and decrease the amount of $Mg^{2+}$ bound to the chloroplast envelope. The chloroplasts were kept at 4°C throughout the isolation procedure.

### Photosynthesis

$CO_2$-supported $O_2$ evolution of intact chloroplasts was measured with a Hansatech $O_2$ electrode (Decagon Inst., Seattle, WA) at 1500 $\mu$mol/$m^2$/s light and 25°C. Small (50 $\mu$L) aliquots of chloroplasts stored in grind medium were added to 1 mL final volume of reaction medium containing 0.33 M sorbitol, 5 mM $Na_2HCO_3$, 1000 units catalase, 50 mM Hepes-NaOH (pH 7.6), 2 mM $Na_2EDTA$, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 0.25 mM $KH_2PO_4$, and additions as noted in the text.

### Stromal pH

The silicone oil microcentrifugation method of Heldt (10) was used with modifications. Tubes (400 $\mu$L) were prepared in advance with a bottom layer of 20 $\mu$L 14% (v/v) $HClO_4$ overlayered by 100 $\mu$L of a silicone oil mixture with a ratio (by weight) of 0.1416:0.6917:0.1667 of 200 (2 centistoke):550:710 Dow Corning silicone oils. Chloroplasts were added to photosynthesis reaction medium, which had 0.25 mM [$^{14}$C]DMO[2] at 1 $\mu$Ci\mL. Then, 180 $\mu$L of this solution was layered onto the silicone oil in each microcentrifuge tube (five replicates/treatment). Tubes were incubated in a water bath for 5 min at 2200 $\mu$mol/$m^2$/s PAR and 25°C, and then transferred to a Beckman Microfuge B and centrifuged for 15 s. Control experiments indicated that the stromal pH assay was most precise (*i.e.* smallest SE) when all tubes for a given set of treatments in an experiment were centrifuged at the same time, the time for transfer of tubes from the water bath to the microfuge was kept under 1 s, and the tubes were illuminated during centrifugation by placing a 500-W floodlight over the Plexiglas lid of the Beckman Microfuge. These precautions were considered critical because of the small differences in stromal pH between the various treatments described in this report.

Chloroplast volume measurements, necessary for stromal pH calculations, were ascertained using [$^3$H]$H_2O$ and [$^{14}$C] sorbitol as described previously (21). These measurements were made on each chloroplast preparation after the DMO measurements were completed for a given experiment. For pH and volume measurements, label associated with chloroplasts pelleting into the $HClO_4$ layer was determined using liquid scintillation spectrophotometry as described previously (21).

Labeled sorbitol and [$^3$H]$H_2O$ were obtained from ICN (Irvine, CA), DMO was from Amersham (Chicago, IL), silicone oils were from William F. Nye Co. (New Bedford, MA), and Hepes was from Research Organics (Cleveland, OH). All other reagents were from Sigma (St. Louis, MO).

All experiments were repeated at least twice.

## RESULTS AND DISCUSSION

### Envelope-Bound $Mg^{2+}$, Stromal pH, and Photosynthesis Inhibition

Huber and Maury (13) noted that the presence of free $Mg^{2+}$ in the reaction medium caused stromal acidification, which

[2] Abbreviation: DMO, 5,5-dimethyl oxazolidine-2,4-dione; lidocaine, 2-(diethylamino)-*N*-(2,6-dimethylphenyl)acetamide.

**Table I.** *High Envelope-Bound $Mg^{2+}$ Effects on Photosynthesis and Stromal pH*

Envelope $Mg^{2+}$ was increased by washing plastids in grind medium with 5 mM free $Mg^{2+}$ as described in "Materials and Methods"; control chloroplasts were washed in standard grind medium. Values for photosynthesis are from single measurements. Stromal pH values represent the means of five replicates ± SE. Photosynthesis and stromal pH measurements were taken on the same chloroplast preparation in experiment 1. The second and third photosynthesis experiments were undertaken with different plastid preparations than the second and third pH experiments.

| Wash Treatment | Photosynthesis | Stromal pH |
|---|---|---|
| | $\mu mol\ O_2 \cdot mg\ Chl^{-1} \cdot h^{-1}$ | |
| Exp. 1 | | |
| Control | 113.8 | 8.01 ± 0.04 |
| 5 mM $Mg^{2+}$ | 67.2 | 7.91 ± 0.02 |
| Exp. 2[a] | | |
| Control | 174.5 | 7.67 ± 0.02 |
| 5 mM $Mg^{2+}$ | 90.0 | 7.57 ± 0.02 |
| Exp. 3 | | |
| Control | 108.0 | 7.65 ± 0.02 |
| 5 mM $Mg^{2+}$ | 73.5 | 7.55 ± 0.02 |

[a] Plants used for this experiment were grown at a higher light intensity (480 *versus* 250 $\mu mol/m^2/s$) than those used in photosynthesis experiments 1 and 3, hence the higher photosynthetic rates.

resulted in photosynthetic inhibition of isolated chloroplasts. Previous work in this laboratory (22) using chlorotetracycline fluorescence as a monitor of chloroplast envelope-bound $Mg^{2+}$ led to the speculation that it was not the presence of millimolar concentrations of unchelated $Mg^{2+}$ in the reaction medium, but rather a high extent of envelope-bound $Mg^{2+}$ that affected chloroplast metabolism. Data presented in Table I confirm this assertion. Incubation of intact plastids during the isolation protocol in solutions containing free $Mg^{2+}$, and then quantitative removal of the chloroplasts from these solutions, results in relatively high levels of envelope-bound $Mg^{2+}$ (22). As shown in Table I, this $Mg^{2+}$-wash treatment resulted in both stromal acidification and photosynthetic inhibition, mimicking the effect of high reaction medium $Mg^{2+}$ (15). The $Mg^{2+}$-induced photosynthetic inhibition has been previously shown to be reversed by high $K^+$ in the reaction medium (22). This effect of $K^+$ is likely not due to a displacement of $Mg^{2+}$ from binding sites on the chloroplast envelope (15, 22). It should be noted that in a recent study (4) external $Mg^{2+}$ was found to lower the pH of chloroplasts isolated from *Zea mays* mesophyll cells, but the effect of $Mg^{2+}$ on carbon metabolism in these chloroplasts was complex, and inconsistent with the effects reported here for $C_3$ chloroplasts.

### Envelope ATPase-Inhibitor Effects on Photosynthesis and Stromal pH

Maury *et al.* (15) found that oligomycin had no effect on ATPase activity in a chloroplast thylakoid membrane fraction, but inhibited the ATPase activity associated with the chloroplast envelope. In the same study, low concentrations of oligomycin were found to only inhibit photosynthesis when chloroplasts were incubated in solutions containing millimolar concentrations of unchelated $Mg^{2+}$. In our study, we extended this line of research by examining oligomycin effects on chloroplasts in the presence of high and low envelope-bound $Mg^{2+}$. As was expected, high envelope-bound $Mg^{2+}$ sensitized photosynthesis to oligomycin (Table II). With EDTA-washed (*i.e.* low envelope $Mg^{2+}$) chloroplasts, 20 $\mu g$/mL oligomycin did not inhibit photosynthesis. However, in two separate experiments, this concentration of oligomycin inhibited the photosynthetic capacity of $Mg^{2+}$-washed chloroplasts (Table II). Kromer *et al.* (14) found that similar concentrations of oligomycin had little to no effect on photosynthesis of isolated barley chloroplasts in the absence of high envelope-bound $Mg^{2+}$. They did not examine, however, the interaction between oligomycin and $Mg^{2+}$.

Further studies were undertaken with digitoxin, another inhibitor of membrane-bound ATPases. Although digitoxin has not been previously used with chloroplast preparations, it has been shown to inhibit the $Na^+/K^+$-ATPase of the cardiac cell membrane (20). The clinical use of digitoxin for medical purposes can result in reduced internal myocyte $K^+$ (12), and its effects on cardiac metabolism are modulated by external $K^+$ and $Mg^{2+}$ concentrations (12, 20). We know of no report in the literature offering convincing evidence that digitoxin (or any other cardiac glycoside analog) affects any plant ATPase.

As shown in Figure 1, the effects of digitoxin on chloroplast photosynthesis are similar to the effects of oligomycin as detailed in Table II. In the presence of increasing concentrations of digitoxin, photosynthetic capacity was maintained to a greater extent with EDTA-washed chloroplasts than with $Mg^{2+}$-washed chloroplasts (Fig. 1). For example, in the presence of 6.5 $\mu M$ digitoxin, photosynthesis in EDTA-washed and $Mg^{2+}$-washed chloroplasts was inhibited by 11 and 40%, respectively (Fig. 1).

Maury and co-workers (15) have previously speculated that

**Table II.** *Oligomycin Effects on Photosynthesis of Chloroplasts with High ($Mg^{2+}$-wash) and Low (EDTA-wash) Envelope-Bound $Mg^{2+}$*

| Wash Treatment | Oligomycin | Photosynthesis | |
|---|---|---|---|
| | | Rate | Change[a] |
| | $\mu g \cdot mL^{-1}$ | $\mu mol\ O_2 \cdot mg\ Chl^{-1} \cdot h^{-1}$ | % |
| Experiment 1 | | | |
| $Mg^{2+}$ | 0 | 60.8 | |
| | 20 | 38.9 | −36 |
| EDTA | 0 | 51.4 | |
| | 20 | 54.4 | +5.8 |
| Experiment 2[b] | | | |
| $Mg^{2+}$ | 0 | 185.3 | |
| | 20 | 148.3 | −20 |
| EDTA | 0 | 234.4 | |
| | 20 | 253.6 | +7.8 |

[a] For each wash treatment, the rate in the presence of 20 $\mu g$/mL oligomycin is compared with the rate in the absence of oligomycin. [b] Plants used for this experiment were grown at a higher light intensity (480 *versus* 250 $\mu mol/m^2/s$) than those used in experiment 1, hence the higher photosynthetic rates.

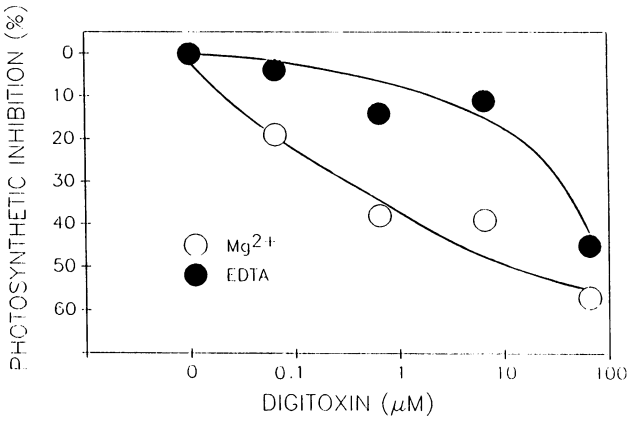


**Figure 1.** Digitoxin effects on photosynthesis of chloroplasts with high ($Mg^{2+}$-wash) and low (EDTA-wash) envelope-bound $Mg^{2+}$. The control rates of photosynthesis (*i.e.* in the absence of digitoxin) were 56.4 and 54.5 μmol $O_2$/mg Chl·h, respectively, for the EDTA-wash and $Mg^{2+}$-wash treatments. Each data point represents a single determination of photosynthesis.

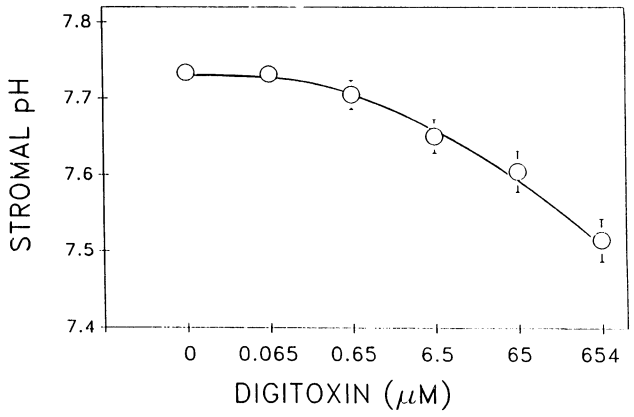


**Figure 2.** Effect of increasing reaction medium digitoxin concentration on stromal pH of $Mg^{2+}$-washed chloroplasts. Data are shown as the means of five replications ± the SE.

the oligomycin-sensitive envelope ATPase facilitated $H^+$ efflux from the stroma to the external medium, and that the inhibition of photosynthesis by oligomycin was due to the resultant stromal acidification. Stromal pH measurements in the presence of digitoxin and oligomycin seem to support their assertions.

In a number of experiments (Table III), oligomycin at a concentration that inhibited photosynthesis (Table II) was found to also cause stromal acidification in $Mg^{2+}$-washed chloroplasts. Although extent of stromal acidification due to oligomycin varied, the ATPase inhibitor did consistently cause some acidification in a number of chloroplast preparations (Table III). Maury *et al.* (15) also found the effect of oligomycin on stromal pH to be somewhat variable (see their Table III). Digitoxin was also found to cause stromal acidification (Fig. 2; also see Table VI), suggesting that oligomycin and digitoxin may have similar effects on chloroplast metabolism.

In this report (Fig. 1, Table II), digitoxin and oligomycin (at relatively low concentrations) were found to inhibit only photosynthesis of isolated chloroplasts when the plastids were $Mg^{2+}$-washed (*i.e.* they had a high level of envelope-bound $Mg^{2+}$). This photosynthetic inhibition occurred in concert with stromal acidification (Fig. 2, Tables III and VI). Also, the $Mg^{2+}$-wash treatment has been shown to cause stromal acidification and photosynthetic inhibition (Table I). Taken together, these data address a significant controversy in the pertinent literature. Huber and co-workers (13, 15) originally speculated that under certain conditions, the action of an envelope ATPase was important for the maintenance of high stromal pH and photosynthetic activity. In those studies, the chloroplasts that showed sensitivity to the envelope ATPase inhibitor likely had a high degree of envelope-bound $Mg^{2+}$. Robinson (19) concluded in a later study that the action of an envelope ATPase was not required for the maintenance of high stromal pH and maximal photosynthesis. However, in Robinson's study, the chloroplasts were not exposed to high external $Mg^{2+}$, and therefore did not have a high level of envelope-bound $Mg^{2+}$. In light of the work presented here, it is not surprising that Robinson came to a different conclusion than Huber about the physiological significance of the chloroplast envelope ATPase. We speculate from our results that high envelope-bound $Mg^{2+}$ causes $H^+$ influx, and it is only under this condition that the activity of the envelope ATPase is required to maintain high stromal pH and maximal photosynthetic activity.

**Table III.** *Stromal pH of $Mg^{2+}$-Washed Chloroplasts in the Presence of Oligomycin (20 μg/mL) and Lidocaine (21 μM)*

For all experiments, the data represent the mean of five replications and are shown ± SE.

| | Treatment | | | |
|---|---|---|---|---|
| | Control | Oligomycin | Lidocaine | Oligomycin + lidocaine |
| Exp. 1 | 7.85 ± 0.04 | 7.78 ± 0.02 | | |
| Exp. 2 | 8.09 ± 0.02 | 7.76 ± 0.02 | | |
| Exp. 3 | 8.09 ± 0.02 | 8.05 ± 0.02 | 8.11 ± 0.01 | 8.10 ± 0.01 |
| Exp. 4 | 7.96 ± 0.02 | 7.77 ± 0.02 | 7.93 ± 0.01 | 8.00 ± 0.02 |
| Exp. 5 | 7.57 ± 0.03 | 7.37 ± 0.06 | 7.56 ± 0.03 | 7.59 ± 0.07 |

## Reversing Stromal Acidification with an Amine Anesthetic

References to clinical studies in the medical literature (3) indicate that in patients suffering from digitoxin toxicity, reversing monovalent cation efflux across cardiac cell membranes by use of a local amine anesthetic can ameliorate the toxic effects of digitoxin. It is thought that therapeutic action of the local amine anesthetic lidocaine (at 4–21 μM in blood plasma) on cardiac cells exposed to toxic levels of digitoxin is facilitated by the blockage of $Na^+$ channels (23), although the amine anesthetic lidocaine has also been found to block $K^+$ channels (5). In this report, this amine anesthetic will be referred to as a monovalent cation channel blocker. In addition to its well-documented effect of blocking monovalent

cation fluxes across myocyte membranes, work showing that this anesthetic blocks divalent cation-activated $K^+$ channels in colon epithelial cells suggested its possible usefulness in our chloroplast system (5).

This monovalent cation channel blocker was found to partially alleviate the inhibition of photosynthesis in $Mg^{2+}$-washed chloroplasts caused by the ATPase inhibitors oligomycin and digitoxin. The pooled results from five separate experiments (Table IV) indicate that lidocaine nearly doubled the photosynthetic rate of $Mg^{2+}$-washed chloroplasts in the presence of oligomycin, reducing the photosynthetic inhibition from 63% down to 27%. During four separate experiments, lidocaine completely reversed the photosynthetic inhibition caused by digitoxin in $Mg^{2+}$-washed chloroplast (Table V).

In the study shown in Table V, the interaction of $NH_4^+$ and $K^+$ with digitoxin effects on photosynthesis was also investigated. As shown by Huber and co-workers (13, 15) and Demmig and Gimmler (6, 7), $K^+$ concentrations of 10 to 100 mM stimulate photosynthesis when chloroplasts are incubated in solutions containing several millimolar free $Mg^{2+}$. As shown in Table V, $K^+$ stimulated photosynthesis of $Mg^{2+}$-washed chloroplasts both in the presence and absence of digitoxin. These results further support our contention that the effects of the $Mg^{2+}$-wash treatment used in this study is similar to the inclusion of free $Mg^{2+}$ in the photosynthetic reaction medium as detailed in previous reports (6, 7, 13, 15). Another important result shown in Table V is the reversal of digitoxin inhibition of photosynthesis by $NH_4^+$. Maury *et al.* (15) have already convincingly demonstrated that the effects of oligomycin on chloroplast photosynthesis are mediated by the inhibitor's effects on a chloroplast envelope ATPase; oligomycin had no effect on the coupling factor ATPase of the thylakoid. No such analysis has been undertaken with regard to digitoxin. However, the data shown in Table V offer indirect evidence that suggests that digitoxin effects on chloroplast metabolism are also not mediated by an inhibition of coupling factor activity. At 1 mM concentrations and above, $NH_4^+$ has been shown to uncouple photophosphorylation and reduce ATP generation in the intact chloroplast (16). If photosynthesis was inhibited in the presence of digitoxin due to reduced coupling factor activity (and, hence, ATP generation), then the presence of an additional agent that can reduce ATP generation should further inhibit photosynthesis. However, $NH_4^+$ stimulated rather than inhibited photosynthesis in the presence of digitoxin (Table V), suggesting that the factor mediating digitoxin inhibition of photosynthesis was not reduced coupling factor activity. It was speculated that $Mg^{2+}$-wash, lidocaine, $K^+$, and $NH_4^+$ all may interact with digitoxin effects on photosynthesis by affecting the extent of digitoxin bound to the chloroplast envelope. Therefore, silicone oil microcentrifugation experiments were undertaken with chloroplasts incubated with [$^3$H]digitoxin in reaction media similar to those used in the experiments shown in Tables I and V (data not shown). These results indicated that none of the treatments altered the amount of digitoxin associated with the chloroplasts. Differential binding of digitoxin to the receptor site on the chloroplast envelope under the various treatments was discounted as the factor mediating their interaction with digitoxin effects on chloroplast metabolism.

Both $NH_4^+$ (9) and $K^+$ (15) have been shown previously to raise stromal pH. The results of the experiment shown in Table V, then, suggest that, like oligomycin (15), digitoxin may inhibit photosynthesis due to stromal acidification. $NH_4^+$, $K^+$, and lidocaine may stimulate photosynthesis in the presence of digitoxin by facilitating stromal alkalinization. This hypothesis was addressed in further experiments. Stromal pH measurements indicated that lidocaine reversed stromal acidification that was caused by oligomycin (Table III) and digitoxin (Table VI).

The pharmacological action of lidocaine on membrane-

**Table IV.** *Reversal of Oligomycin-Induced Photosynthetic Inhibition by Lidocaine in $Mg^{2+}$-Washed Chloroplasts*

The data represent the means ± SE ($n$ = 5) of five separate experiments, with two replications per experiment. Oligomycin and lidocaine were used at 20 μg/mL and 21 μM, respectively.

| Treatment | Photosynthesis | |
|---|---|---|
| | Rate | Change from control |
| | μmol $O_2$·mg $Chl^{-1}$·$h^{-1}$ | % |
| Control | 48.7 ± 4.0 | — |
| Oligomycin | 18.4 ± 3.3 | −62.2 |
| Lidocaine | 43.9 ± 2.4 | −9.8 |
| Oligomycin + lidocaine | 35.7 ± 3.9 | 26.6 |

**Table V.** *Effects of Digitoxin and Various Stromal Alkalinization Agents on Photosynthesis of $Mg^{2+}$-Washed Chloroplasts*

The concentrations of digitoxin, lidocaine, $NH_4$, and $K^+$ (both added as chloride salts) used for these studies were 6.5 μM, 21 μM, 0.75 mM, and 40 mM, respectively. The data represent the means ± SE ($n$ = 4) of four separate experiments, with two replications per experiment.

| Treatment | Photosynthesis | |
|---|---|---|
| | Rate | Change from control |
| | μmol $O_2$·mg $Chl^{-1}$·$h^{-1}$ | % |
| Control | 50.6 ± 3.4 | — |
| Digitoxin | 39.9 ± 1.8 | −21.1 |
| Digitoxin + $NH_4^+$ | 49.7 ± 5.1 | −1.7 |
| Digitoxin + $K^+$ | 62.2 ± 3.6 | +18.6 |
| Digitoxin + lidocaine | 53.0 ± 1.4 | +4.5 |
| $K^+$ | 61.7 ± 7.8 | +17.9 |

**Table VI.** *Effect of Digitoxin (6.5 μM) and Lidocaine (21 μM) on Str omal pH of $Mg^{2+}$-Washed Chloroplasts*

For all experiments, the data represent the mean of five replications and are shown ± SE.

| Treatment | Stromal pH | | |
|---|---|---|---|
| | Exp. 1 | Exp. 2 | Exp. 3 |
| Control | 8.09 ± 0.02 | 7.92 ± 0.00 | 7.57 ± 0.03 |
| Digitoxin | 8.03 ± 0.03 | 7.83 ± 0.03 | 7.47 ± 0.02 |
| Digitoxin + lidocaine | | 7.95 ± 0.11 | 7.63 ± 0.03 |
| Lidocaine | | 7.98 ± 0.02 | 7.56 ± 0.02 |

enclosed biological systems such as cells and organelles is known to be related to a blockage of monovalent cation channels. Therefore, the rise in stromal pH that occurs with oligomycin- and digitoxin-treated chloroplasts in the presence of lidocaine (Tables III and VI) likely occurs as a result of reduced $K^+$ and/or $Na^+$ efflux from the stroma specifically through an ion channel in the limiting membrane of the chloroplast envelope. This hypothesis should be viewed with caution, however, until further work is undertaken to confirm that the amine anesthetic affects monovalent cation fluxes across the chloroplast envelope. Nonetheless, the finding that lidocaine stimulates photosynthesis (Tables IV and V) and raises stromal pH (Tables III and VI) in $Mg^{2+}$-washed chloroplasts treated with oligomycin or digitoxin extends the work done previously on the system that modulates stromal pH by regulating monovalent cation and $H^+$ fluxes across the chloroplast envelope. Huber and co-workers (13, 15) initially presented a model of ion transport across the envelope. In this model, $K^+$ (and/or $Na^+$) efflux from the stroma was shown to occur through a specific "transporter site," and this cation efflux was thought to be linked (directly or indirectly) to $H^+$ influx. However, Demmig and Gimmler (7) challenged the notion that $K^+$ fluxes across the envelope were facilitated by a specific transporter site. The data presented here offer support for Huber's notion that $K^+$ efflux from the stroma is somehow coupled to $H^+$ influx, and occurs through a ($Mg^{2+}$-modulated) transporter site (*i.e.* ion channel).

Although both digitoxin and oligomycin were found to cause stromal acidification in a number of experiments (Fig. 2, Tables III and VI), the extent of stromal acidification caused by these agents was typically rather small (often less than 0.1 unit). As noted in "Materials and Methods," precautions were taken to maximize the precision of the pH assay. Nonetheless, in many experiments, the differences between treatments were only slightly greater than the variation among the treatment replicates. However, we believe that these pH differences, both the stromal acidification caused by the $Mg^{2+}$-wash, digitoxin, and oligomycin treatments, and the reversal of the oligomycin- and digitoxin-induced acidification by lidocaine, were directly linked to the various treatment effects on photosynthesis. Support for this contention can be found in previous studies, in which small reductions in the stromal pH of the illuminated chloroplast were found to directly cause substantial photosynthetic inhibition. For example, Maury *et al.* (15) determined that a 0.1 pH unit reduction could result in a 40% inhibition of photosynthesis. In the work of Werdan *et al.* (24), a similar extent of photosynthetic inhibition was linked to such small pH changes in the stroma.

Further support for this assertion is provided by the analysis presented in Figure 3. Previous research has shown that exposure of intact chloroplasts to millimolar concentrations of weak acids such as acetate causes stromal acidification, and the resultant inhibition of photosynthesis in the presence of acetate is caused only by these stromal pH changes (18, 24). Therefore, the effects of acetate on stromal pH and photosynthesis were used to "calibrate" the effects of the various treatments described in this report. As shown in Figure 3, a curve was developed to relate the effects of increasing reaction medium acetate on stromal pH and photosynthetic inhibition. As was found in previous studies (15, 18, 24), a curve devel-

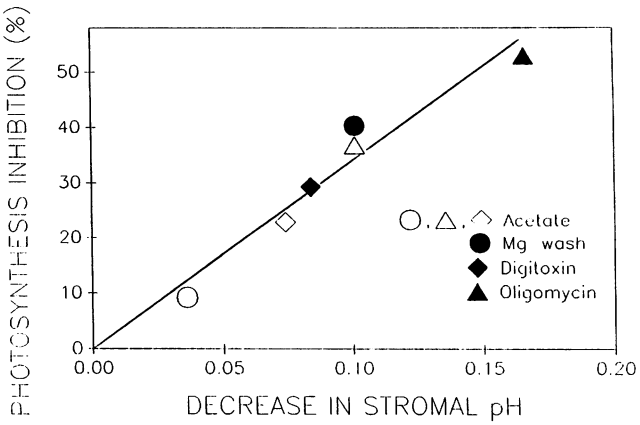


**Figure 3.** Relationship between treatment effects on stromal pH and inhibition of photosynthesis. The open symbols represent the extent of stromal acidification and photosynthetic inhibition that occurred in the presence of 5 mM (○), 10 mM (◇), and 15 mM (△) acetate added to the reaction medium as the sodium salt. Each of these data points was derived from two separate photosynthesis experiments and one stromal pH experiment. The control rates (*i.e.* in the absence of acetate) for the two photosynthesis experiments were 129.1 and 72.0 $\mu$mol $O_2$·mg $Chl^{-1}$·$h^{-1}$. For each acetate concentration, the photosynthetic inhibition shown is the average derived from the two experiments. For the stromal pH experiment, the stromal pH in the absence of acetate was 8.012 ± 0.005, and each value represents the mean of five replications. For the experiments with acetate, the chloroplasts were $Mg^{2+}$-washed during isolation. The data value shown for the effect of $Mg^{2+}$ (●) is derived from the data shown in Table I and is a comparison of chloroplast photosynthesis and stromal pH in $Mg^{2+}$-washed chloroplasts with measurements made on chloroplasts washed in standard grind medium. For this data point, the photosynthetic inhibition was derived from the mean of three separate experiments. The corresponding stromal pH change was derived from three separate experiments with five replications for each treatment in each experiment. For all experiments used to derive the oligomycin and digitoxin data points, $Mg^{2+}$-washed chloroplasts were used, and the concentrations of digitoxin and oligomycin were 6.5 $\mu$M and 20 $\mu$g/mL, respectively. For the data value representing the effects of digitoxin (◆), the photosynthetic inhibition was derived from the results of seven experiments; the five experiments from Figure 1 and Table V, along with two experiments not shown. In these last two experiments, the effect of digitoxin was compared with a control treatment in $Mg^{2+}$-washed chloroplasts; the control rates were 101 and 130, and the rates in the presence of digitoxin were 48 and 92 $\mu$mol $O_2$/mg Chl·h. The corresponding stromal pH change in the presence of digitoxin was calculated from the four experiments shown in Table VI and Figure 2. For oligomycin (▲), the value for photosynthetic inhibition was derived from the seven experiments shown in Tables II and IV, and the results from the five experiments shown in Table III were used to determine the effect on stromal pH.

oped from the acetate data indicates that stromal acidification of 0.1 pH unit causes about 35 to 40% inhibition of photosynthesis. Plotted along with these data are a compilation (from the various experiments described in this report) of $Mg^{2+}$-wash, digitoxin, and oligomycin treatment effects on photosynthesis and stromal pH. This analysis indicates that these treatments likely affected photosynthesis primarily due to their effects on stromal pH; the data values representing these treatments all generally fall on the line generated from the acetate data.

## CONCLUSION

The exposure of intact isolated chloroplasts to treatments such as $Mg^{2+}$-wash, digitoxin, oligomycin, and lidocaine affect photosynthesis and stromal pH. Data presented in this report suggest that this relationship may be causal. Although the treatment effects on photosynthesis and/or stromal pH were variable, and sometimes barely statistically significant, we believe that the extent of repetitions of the experiments reported here validates this assertion. When the data from the various experiments are pooled, as has been done in the analysis shown in Figure 3, a clear picture emerges of a class of treatments that all interact with mechanisms that modulate the stromal pH of the illuminated chloroplast. The results of previous work (6, 13, 15, 22) suggest that these mechanisms are likely a set of envelope-bound proteins that regulate $H^+$ and monovalent cation fluxes. Envelope-bound $Mg^{2+}$ "turns on" a transporter that causes $H^+$ influx (Table I) in the absence of high external $K^+$ (7, 15, 22; also see Table V). When an $H^+$ influx system is turned on by high envelope $Mg^{2+}$, the action of an envelope-bound ATPase is required to maintain high stromal pH in the light, and optimal photosynthesis (Figs. 2 and 3, Tables II–VI). The oligomycin- and digitoxin-sensitive envelope ATPase likely raises stromal pH by facilitating $H^+$ efflux, although this action may be an indirect result of the ATPase activity. Digitoxin mimicked the effect of oligomycin in our system, and digitoxin is only known to affect membrane-bound ATPases that facilitate $Na^+$ and $K^+$ exchange (20). Possibly, then, the envelope ATPase activity may be linked to $H^+$ efflux through the action of other, as yet unidentified, membrane components. The monovalent cation channel blocker lidocaine was found to prevent the stromal acidification that occurs when $Mg^{2+}$-washed chloroplasts are exposed to envelope ATPase inhibitors. Monovalent cation efflux specifically through an ion channel is likely linked (directly or indirectly) to $H^+$ influx. We speculate that lidocaine, acting independently from the ATPase inhibitors, acts to raise stromal pH through its effect on an as-yet-unidentified monovalent cation channel.

The data presented in this report support, in general terms, Huber's model of the chloroplast envelope system that modulates cation fluxes and stromal pH, and, hence, photosynthetic activity. However, our data suggest that this important regulatory system may be more complex (*i.e.* involve more individual components) than originally thought. In summary, the data presented here suggest that a complex system regulates $H^+$ fluxes across the chloroplast envelope. The substantial effect this system has on photosynthesis was also illuminated. However, with regard to the characterization of specific components of this system, the results of our experiments raise more questions than they resolve.

## LITERATURE CITED


1. **Baier D, Latzko E** (1975) Properties and regulation of C-1-fructose-1,6-biphosphatase from spinach chloroplasts. Biochim Biophys Acta **396:** 141–148
2. **Berkowitz GA, Gibbs M** (1982) Effect of osmotic stress on photosynthesis studied with the isolated spinach chloroplast. Generation and use of reducing power. Plant Physiol **70:** 1143–1148
3. **Bigger JT, Hoffman BF** (1980) Antiarrhythmic drugs. *In,* AG Gilman, LS Goodman, A Gilman, eds, The Pharmacological Basis of Therapeutics. Macmillan, New York, pp 761–792
4. **Boag S, Agostino A, Furbank RT, Hatch MD** (1989) Effects of external $Mg^{2+}$ and pH on the photosynthetic activities and stromal pH of intact chloroplasts isolated from $C_4$ plants. Aust J Plant Physiol **16:** 379–390
5. **Dawson DC** (1987) Properties of epithelial potassium channels. *In,* F Bronner, A Kleinzeller, eds, Potassium Transport: Physiology and Pathophysiology. Academic Press, New York, pp 41–71
6. **Demmig B, Gimmler H** (1979) Effect of divalent cations on cation fluxes across the chloroplast envelope and on photosynthesis of intact chloroplasts. Z Naturforsch **24C:** 233–241
7. **Demmig B, Gimmler H** (1983) Properties of the isolated intact chloroplast at cytoplasmic $K^+$ concentrations. I. Light-induced cation uptake into intact chloroplasts is driven by an electrical potential difference. Plant Physiol **73:** 169–174
8. **Flugge UI, Freisl M, Heldt HW** (1980) The mechanism of the control of carbon fixation by the pH in the chloroplast stroma studied with acid mediated proton transfer across the envelope. Planta **149:** 48–51
9. **Heath RL, Leech RM** (1978) The stimulation of $CO_2$-supported $O_2$ evolution in intact spinach chloroplasts by ammonium ion. Arch Biochem Biophys **190:** 221–226
10. **Heldt HW** (1980) Measurement of metabolite movement across the envelope and of the pH in the stroma and the thylakoid space in intact chloroplasts. Methods Enzymol **69:** 604–613
11. **Heldt HW, Werdan KW, Milovancev M, Geller G** (1973) Alkalization of the chloroplast stroma caused by light-dependent proton flux into the thylakoid space. Biochim Biophys Acta **314:** 224–241
12. **Hougen TJ, Lloyd BL, Smith TW** (1979) Effects of inotropic and arrhythmogenic digoxin doses and of digoxin-specific antibody on myocardial monovalent cation transport by digitalis. Circ Res **44:** 23–21
13. **Huber SC, Maury W** (1980) Effects of magnesium on intact chloroplasts. I. Evidence of activation of (sodium) potassium/proton exchange across the chloroplast envelope. Plant Physiol **65:** 350–354
14. **Kromer S, Stitt M, Heldt HW** (1988) Mitochondrial oxidative phosphorylation participating in photosynthetic metabolism of a leaf cell. FEBS Lett **226:** 352–356
15. **Maury WJ, Huber SC, Moreland DE** (1981) Effects of magnesium on intact chloroplasts. II. Cation specificity and involvement of the envelope ATPase in (sodium) potassium/proton exchange across the envelope. Plant Physiol **68:** 1257–1263
16. **McCarty RE** (1980) Delineation of the mechanism of ATP synthesis in chloroplasts: use of uncouplers, energy transfer inhibitors, and modifiers of coupling factor 1. Methods Enzymol **69:** 719–728
17. **Oja V, Laisk A, Heber U** (1986) Light-induced alkalinization of the chloroplast stroma *in vivo* as estimated from the $CO_2$ capacity of intact sunflower leaves. Biochim Biophys Acta **849:** 355–365
18. **Purczeld P, Chon CJ, Portis AR Jr, Heldt HW, Heber U** (1978) The mechanism of the control of carbon fixation by the pH in the chloroplast stroma. Studies with nitrite-mediated proton transfer across the envelope. Biochim Biophys Acta **501:** 488–498

19. **Robinson SP** (1985) The involvement of stromal ATP in maintaining the pH gradient across the chloroplast envelope in the light. Biochim Biophys Acta **806:** 187–194
20. **Schwartz A, Whitmer K, Grupp G, Grupp I, Adams RJ, Lee S-W** (1982) Mechanism of action of digitalis: is the Na, K-ATPase the pharmacological receptor? Ann NY Acad Sci **402:** 253–271
21. **Sen Gupta A, Berkowitz GA** (1988) Chloroplast osmotic adjustment and water stress effects on photosynthesis. Plant Physiol **88:** 200–206
22. **Sen Gupta A, Berkowitz GA** (1989) Development and use of chlorotetracycline fluorescence as a measurement assay of chloroplast envelope-bound $Mg^{2+}$. Plant Physiol **89:** 753–761
23. **Stoelting RK** (1987) Pharmacology and Physiology in Anesthetic Practice. J. Lippincott, Philadelphia, p 327
24. **Werdan K, Heldt HW, Milovancev M** (1975) The role of pH in the regulation of carbon fixation in the chloroplast stroma. Studies on $CO_2$ fixation in the light and dark. Biochim Biophys Acta **396:** 276–292